Title
Elucidation of the interconversion and metabolism of prednisone and prednisolone using in situ perfused leporine organs

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Elucidation of the Interconversion and Metabolism of Prednisone and Prednisolone using *in situ* Perfused Leporine Organs

by

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DISSERTATION

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Dedication

To my husband, Ahvie,

whose support, humor and love across the miles

made this project so enjoyable.

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List of Abbreviations

Superscripts and subscripts:

1	prednisone
2	prednisolone
10	irreversible elimination of prednisone
12	reductive conversion of prednisone to prednisolone
20	irreversible elimination of prednisolone
21	oxidative conversion of prednisolone to prednisone
D	drug administered
H	hepatic
M	metabolite administered
P	pulmonary
PO	prednisone
POH	prednisolone
pt	based on the parallel tube model
R	renal
U1	urine concentration of prednisone
U2	urine concentration of prednisolone
ws	based on the well stirred model

Abbreviations:

A	absorbance
A_{final}	final absorbance
$A_{initial}$	initial absorbance

ATP	adenosine triphosphate
AUC	area under the concentration-time curve
AUMC	area under the first moment of the concentration-time curve
CAP	binding capacity of corticosteroid binding globulin for prednisolone
CBG	corticosteroid binding globulin
C	concentration of drug
$C_{arterial}$	arterial drug concentration
C_{in}	drug concentration in entering perfusate
CL	clearance: intrinsic, apparent or organ, dependent upon subscripts and context
CL_{app}	apparent systemic clearance
CL_{CR}	creatinine clearance
CL_{int}	intrinsic clearance
CL_{organ}	organ clearance
CL_R	apparent renal clearance
CL_U	unbound apparent systemic clearance
CL_{ur}	apparent urinary clearance
C_{max}	maximum plasma concentration
C_{out}	drug concentration in exiting perfusate
cpm	counts per minute
C_{portal}	portal perfusate drug concentration
Css	steady state concentration of drug in plasma or perfusate,
C_U	unbound concentration of drug
C_{urine}	concentration of drug in urine
C_{venous}	venous perfusate drug concentration
dpm	disintegrations per minute
E_{exp}	experimentally determined extraction ratio

E	extraction ratio
E_{ideal}	extraction ratio determined under conditions of ideal flow rate
F	bioavailability
fu	unbound fraction of drug
GFR	glomerular filtration rate
11β-HSD	11β-hydroxysteroid dehydrogenase
11β-OH	11β-hydroxyl group
IV	intravenous
k	rate constant for elimination
k_a	rate constant for absorption
K_{ALB}	affinity constant for binding of POH to albumin
K_d	dissociation constant for the binding of prednisolone
K_m	Michaelis constant
K_{M21}	concentration at which half maximal rate, υ21, is achieved in
MRT	mean residence time
NADP	triphosphopyridine nucleotide
NADPH	reduced triphosphopyridine nucleotide
P_{ALB}	concentration of albumin
P_{max}	maximum concentration of prednisone achievable
PO	prednisone
PO/POH	ratio of areas under the curves or drug concentrations of
POH	prednisolone
Q	flow rate of perfusate
Q_{UR}	urine flow rate
S	binding parameter related to drug binding to albumin
$t_{1/2}$	half-life
T_m	rate of maximal transport

t_{max}	time of maximal plasma drug concentration
UW	University of Wisconsin preservation solution
υ_{21}	maximal velocity of oxidative conversion reaction, as modeled within a perfused organ; analogous to V_{max} in Michaelis kinetics
V	apparent volume of perfused organ
V_{ss}	steady state volume of distribution

Abstract

Elucidation of the Interconversion and Elimination of Prednisone and Prednisolone using *In Situ* Perfused Leporine Organs

The pharmacokinetics of prednisone (PO) and prednisolone (POH) is nonlinear in humans and demonstrates increases in apparent systemic clearance with dose administered, as well as apparently saturable formation of PO from POH. It is hypothesized that these nonlinearities may be associated with the organ-specific disposition of the compounds. Using organ perfusion techniques and pharmacokinetic principles, the objectives of these experiments were to characterize the organ-specific extraction, interconversion and elimination of PO and POH in the *in situ* perfused rabbit liver, kidney and lung. This knowledge would permit determination of the contributors to nonlinear *in vivo* disposition, thereby promoting optimized utilization of these compounds for treatment of one-organ disease. Organ extraction was constant and not related to dose, even when concentrations were up to 100 times those experienced *in vivo*. Extraction by the rabbit lung and kidney was low; the liver highly extracted PO and the hepatic extraction of POH was intermediate, demonstrating the magnitude of importance of the liver versus the other organs examined. The liver appeared to convert PO to POH unidirectionally. The kidney and lung demonstrated bidirectional conversion in addition to apparently saturable formation of PO from POH. Renal excretion was low and urine-flow dependent. Conclusions from this work demonstrate that the three organs of the rabbit differentially metabolize PO and POH. *In toto*, the nonlinear plasma protain binding of POH, nonlinearities in renal clearance and the observations of saturable oxidative conversion of the lung and kidney and the unidirectional hepatic conversion all contribute to the nonlinearities observed *in vivo*.

Chapter 1

Background and Pharmacology of the Glucocorticoids

Introduction

The investigation of the role of adrenal cortical hormones began several decades ago. In 1855, Addison first described the clinical manifestations of atrophy of the suprarenal (adrenal) glands (Addison, 1855), observing impaired appetite, low blood pressure, extreme muscular weakness, gastrointestinal upset and skin discoloration. In 1932, Cushing (1932) observed the effects of excessive adrenal excretion as weight gain distributed over the trunk, head and neck, acne, back pain, hirsutism, proximal muscle weakness and purple straie over the abdomen. Addison's and Cushing's diseases are named in recognition of the seminal contributions of these scientists. Hench's observation (1949) that arthritic patients who became pregnant or jaundiced experienced remission of their disease evoked immediate scientific interest and initiated the investigation of the adrenal cortical hormones as therapeutic agents. In 1949, Hench was the first to administer beef adrenal extract for therapeutic purposes. Reichstein and Kendall identified corticosterone in 1937 (De Fremery et al., 1937) and the partial synthesis of cortisol was achieved in 1946 (Sarett, 1946). The Nobel Prize in Medicine was awarded to Reichstein, Kendall and Hensch in 1950 for their discoveries and the advancement of endocrinology.

The glucocorticoids are a class of steroid hormones produced by the adrenal cortex upon stimulation by adrenocorticotropic hormone (ACTH) from the anterior pituitary; ultimate control is centrally mediated by hypothalmic secretion of corticotropin-releasing factor. The glucocorticoids are responsible for modifying and controlling carbohydrate, protein, fat, and purine metabolism. They are classified as glucocorticoids and/or mineralocorticoids because of their effects on glucose and electrolyte metabolism, respectively. These hormones cause definite changes in cellular metabolism in response to environmental stimuli, with ultimate control exerted by the central nervous system. Experimentally, it has been observed that adrenalectomized mammals can survive mineralocorticoid loss with electrolyte supplementation, but death occurs despite glucose replacement in the absence of glucocorticoids (Haynes et al., 1985). In humans, the most active endogenous glucocorticoid is cortisol (hydrocortisone), although cortisone, corticosterone, 11-dehydrocorticosterone, and 11-desoxycorticosterone have been documented to exert biologic activity (Haynes et al., 1985). In animals, the major endogenous glucocorticoid is either cortisol or corticosterone: dogs and monkeys secrete mainly cortisol, while rats, rabbits, and mice synthesize the latter (Heftmann, 1970a).

The adrenal glands, lying just above the kidneys and weighing about 4 to 5 grams (James, 1979), are fed by multiple arteries. Only the lung receives more blood flow per unit weight of tissue (Neville and O'Hare, 1982). The adrenal cortex secretes aldosterone, cortisol, cortisone, corticosterone and other minor corticosteroids. None of these natural compounds possesses pure mineralo- or glucocorticoid effects: aldosterone is a mineralocorticoid with weak glucocorticoid effects; the others exhibit mixed effects also, with glucocorticoid properties prevailing. In humans, the adrenal glands secrete daily 20 to 30 mg cortisol, 2 to 4 mg corticosterone, and 20 to 200 μg aldosterone (Makin, 1984).

Circulating and local concentrations of cortisol are regulated via complex coordination of biochemical and physiologic mechanisms: a) glucocorticoid production and release demonstrates circadian patterns; secretion can also occur on demand in stressful

situations; b) enzymatic inactivation occurs in nearly all organs; c) excretion of unchanged hormone occurs in the kidney; d) interconversion of inactive (cortisone) and pharmacologically active (cortisol) steroid occurs in all organs; and e) regulation of unbound concentrations is accomplished by a combination of nonspecific, nonsaturable binding (albumin) and by specific or tight, saturable binding (corticosteroid binding globulin). The integrated result of the specific binding processes is that at low (physiologic) concentrations, little of the cortisol is free (<10%), while at high concentrations, as in stress or during therapy, an increasing fraction of the hormone is free (up to 50%). The proportion of molecules that actually reaches the active site is small; most of the endogenous output is involved in maintaining proper gradients.

Glucocorticoids are used therapeutically predominately as anti-inflammatory and immunosuppressive agents. The adverse effects of these drugs commonly produce a wide range of morbidity and can indirectly lead to fatal complications, particularly in those patients receiving chronic therapies. Short term treatment (ca. two weeks) though, is rarely harmful, even at doses 10 to 100 times physiologic (Haynes et al., 1985). Synthetic analogues were designed in the 1950's and 60's to minimize cortisol's mineralocorticoid effects (and thereby some side effects), and to enhance its glucocorticoid potency. Of particular interest in the work described here are the compounds prednisone and prednisolone. They have remained the most commonly prescribed glucocorticoids for more than thirty years.

Chemistry

The structures of prednisone and prednisolone are presented in Figure 1.1, together with the structures of their respective endogenous analogues, cortisone and cortisol. The structures of two other commonly prescribed glucocorticoids, methylprednisolone and dexamethasone, are also shown. The single structural difference between the first two pairs is a double bond between carbons 1 and 2. Due to their great similarity, these

compounds exhibit many similar characteristics with respect to metabolic pathways, distribution patterns and plasma protein binding profiles. Methlyprednisolone and dexamethasone possess additional functional groups. Animals were the original source of

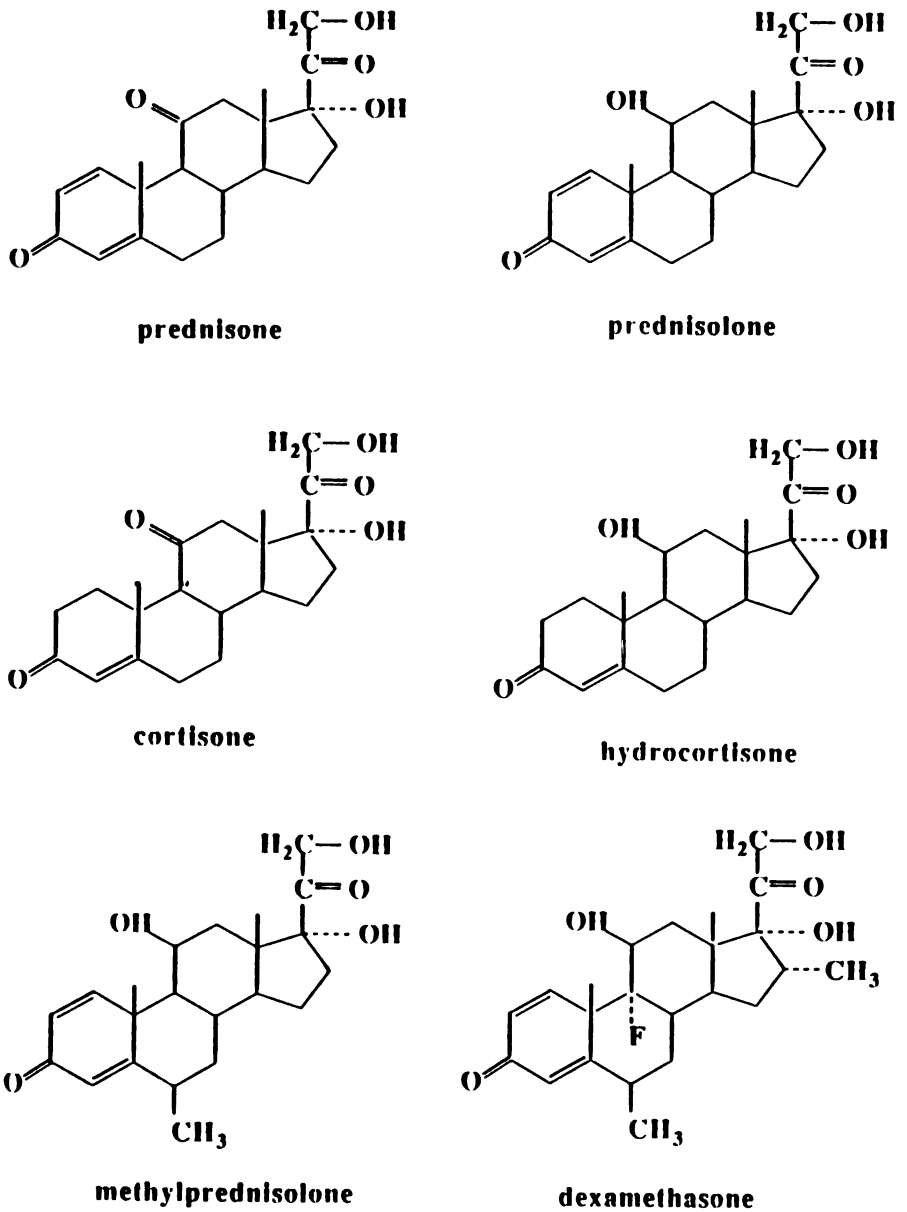

Figure 1.1. Structure of prednisone, prednisolone and some endogenous and synthetic analogues.

glucocorticoids (bovine adrenals); today the Mexican yam, *Dioscorea composita*, is the primary source of the steroid nucleus for the production of steroid analogues (Fullerton, 1982).

The chemical name for prednisone (PO) is 17α,21-dihydropregna-1,4-diene-3,11,20-trione; that of prednisolone (POH) is 11β,17α,21-trihydroxypregna-1,4-diene-3,20-dione. Figure 1.2 depicts a three dimensional representation of cortisol. It is apparent here that steroid molecules are relatively flat, allowing them to easily lie between phospholipid chains within a membrane bilayer, for instance, where they lend fluidity to the structure. Glucocorticoids possess 21 carbons; their numbering is depicted in Figure 1.2. Carbons 18, 19, 20, and 21 lie on the β or upper side of the steroid backbone; the under side of the molecule is termed the α side in this depiction.

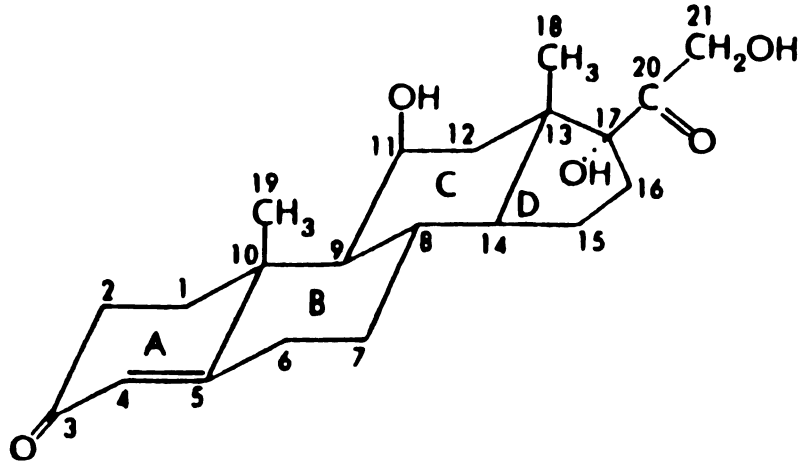

Figure 1.2. Three dimensional structure of cortisol.

The requirements for glucocorticoid activity are hydroxy groups at positions 11β and 21, a double bond linking carbons 4 and 5, and keto functions in positions 3 and 20. A hydroxy group at the 17α-position enhances glucocorticoid activity, but is not essential.

It has been possible to separate mineralo- and glucocorticoid activities, but it appears that glucocorticoid activity is chemically inseparable from immunosuppressive activity (Haynes et al., 1985).

Structural changes to the steroid backbone affect: a) absorption, b) distribution, c) metabolism (clearance, half-life) and d) receptor affinity as summarized below after Wolff (1984). No other class of medicinal agents possesses such extensive documentation of their structure-activity relationship as the steroid hormones, particularly the glucocorticoids.

a) To decrease the time to onset of action of an orally administered glucocorticoid, absorption is facilitated by increased aqueous solubility; this is the objective of developing steroid esters with ionizable groups. To enhance percutaneous absorption, hydrophobic substituents are added to the basic structure, such as triamcinolone acetonide.

b) The distribution of a drug is expanded when a greater proportion of it is unbound to plasma proteins. The affinity of steroids for albumin decreases with the addition of polar substituents; the specific binding protein, corticosteroid binding globulin (CBG, transcortin), has a greater affinity for glucocorticoids than does albumin; adding any substituents to the cortisol nucleus decreases binding, e.g., dexamethasone, Δ^1, 9α-fluoro, 16α-methylcortisol shows minimal binding to CBG. Prednisolone is the only synthetic glucocorticoid to demonstrate binding to CBG; it possesses an additional double bond in the A ring but no other alterations.

c) The potency of a glucocorticoid can be associated with the time the drug spends in plasma (Wolff, 1984). Therefore, any substituent that inhibits metabolism at the primary sites of inactivation can be correlated with an increased pharmacologic effect. These sites are at the A ring, C-11, C-20, C-6, or the two carbon side chain.

d) Receptor affinity is another determinant of pharmacologic potency. According to Wolff, steroid-receptor binding forces are mainly hydrophobic in nature, and the displacement of water molecules is the principal driving force for their uniting (Wolff et al., 1978). Hence, receptor affinity is enhanced by the addition of hydrophobic substituents to the molecule.

Metabolic interconversion

With the advent of more sensitive analytical methodologies, it has become apparent that some drugs reversibly interconvert *in vivo* between two chemical entities. This process may or may not be enzymatically mediated. The two chemical forms may be enantiomers, reduced and oxidized forms, or yet another combination. Often only one of the moieties is pharmacologically active. This implies that the inactive moiety can serve as a reservoir of active drug, to be converted when *in vivo* conditions are appropriate. It has been known for some years that several classes of steroid hormones interconvert, such as the estrogens, androgens, and the glucocorticoids. Other drugs which interconvert are dapsone (Gelber et al., 1971), clofibrate (Meffin et al., 1983), sulindac (Duggan et al., 1980), and carboxylic acid non-steroidal antiinflammatory agents (Verbeek, 1982).

Both endogenous and synthetic glucocorticoids are interconverted at the C-11 position, between a ketone and a β-alcohol (oxidation-reduction). This process requires catalysis by the purported enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) (Osinski, 1960). Both the 11β-hydroxy and and the 11-oxo forms are extremely stable and unreactive; the 11α-hydroxy, which is not found in nature, is normally reactive (Bush, 1956). This metabolic interconversion is important because the 11β-hydroxyl group conveys glucocorticoid activity. This chemistry yields a circulating pool of inactive steroid (11-keto) which is available for reduction to the active moiety in any organ of the body.

11β-HSD has been studied *in vitro* by several laboratories and the variation in results between laboratories is great (Monder and Shackleton, 1984). Despite conflicting results, several generalizations can be made. It appears that activity differs between species, between organs, and with developmental stage and age (Ghraf et al., 1975; Monder and Shackleton, 1984). Although interconversion has been identified in many tissues, the significance of a specific organ's contribution to total body interconversion of the glucocorticoids is limited. The liver is most active (Jenkins, 1966); the kidney, lung

and the placenta possess the next greatest 11β-HSD activity *in vitro* (Koerner, 1966). The lung possesses less activity, but because this organ receives total cardiac output, its significance *in vivo* may be much greater than predicted from microsomal preparations.

One important consideration of *in vitro* experimental design when studying 11β-HSD is the effect of tissue disruption. O'Hare (1973) and Bernal and coworkers (1982) have observed that monolayer culture compared to damaged monolayer cells produce very different results. Oxidation of dehydrocorticosterone to corticosterone was increased 250-fold in the deliberately damaged preparation. The implications of this observation are very significant when extrapolating *in vitro* work to the *in vivo* situation.

Biochemical characterization of the enzyme would aid in understanding and predicting the complex coordination of organ-specific interconversion processes. Three laboratories have reported unsuccessful attempts to isolate and purify the enzyme. Hurlock and Talalay (1959) used acetone precipitation followed by detergent solubilization, but their product precipitated upon centrifugation. Bush et al. (1968) added Sephadex chromatography and phospholipase A treatment to the protocol; their product was no more active than their starting microsomes. More recently, Lakshmi and Monder (1985a) succeeded in isolating a more pure product with the use of synthetic detergents. However, the ratio of oxidative to reductive capacity varied significantly with detergent type and concentration, yielding multiple apparently unique protein products.

11β-Hydroxysteroid dehydrogenase is a NADPH-linked dehydrogenase and a microsomal protein (Hurlock and Talalay, 1959). It was initially hypothesized to be a single, bidirectional enzyme, performing both the reduction of 11-keto and oxidation of 11β-hydroxyl groups (Osinski, 1960). With mounting evidence against this simple theory, several hypotheses have developed with which to rationalize the divergent behavior of 11β-HSD in various tissues at various stages of development. Nicholas and Lugg (1982) proposed that altered ratios of NADP to NADPH in the tissues accounts for the activities. Changes in pH have been implicated by Deckx and de Moor as well (1966). A family of

diverse yet related enzymes has also been proposed to account for the age-dependent behavior (Bernal et al., 1980). These three hypothesized patterns are not likely in that they involve dramatic microenvironmental changes or complex genetic control. An unsupported but possible rationale is that a compound (probably endogenous) selectively inhibits one direction of the reaction during growth and development (Murphy, 1979); this compound has yet to be identified. Bush and Mahesh (1959) proposed a multi-enzyme hypothesis which suggested that the enzyme responsible for oxidation of 11β-OH is distinct from the 11-keto reductive protein. Lakshmi and Monder (1984) have modified this hypothesis and propose that the protein is more likely an enzyme complex, composed of two or more independent yet coordinated subunits. In this theory, activity in either direction is regulated by the local phospholipid environment. The primary evidence for the enzyme complex hypothesis is: a) detergent solubilization and exposure to phospholipases result in an increase in the oxidative activity (loss of latency), but no increase in the reductive activity (Lakshmi and Monder, 1985a); b) the two activities are unequally stable toward temperature increases, and the reductive direction alone undergoes a phase transition (Lakshmi and Monder, 1985b); c) when tissue structure is maintained, oxidation prevails in explanted lung tissue, but when cells differentiate freely in culture, reduction is expressed to a greater extent (Abramovitz et al., 1982); and d) clinical evidence in humans suggests extremely low activities in either the reductive or the oxidative direction with excessive excretion of the synthesized member of the pair, as would be expected in the case of a unidirectional enzymatic deficiency (Monder et al., 1986; Phillipou and Higgins, 1985).

Irreversible biotransformation

The study of the metabolism of the glucocorticoids is complicated by the numerous pathways available to the compounds, although the synthetic glucocorticoids utilize fewer pathways than do their endogenous counterparts. As a general rule, the metabolic profile of the endogenous glucocorticoids can be extrapolated to the synthetic congeners. (This is

fortunate, in that little direct experimentation has been conducted with the synthetic analogues.) The products of metabolic biotransformation are distinct from intermediates in steroid hormone biosynthesis; active hormone is the result of only one process: synthesis from cholesterol.

Urinary profiles and *in vitro* tissue preparations (minces, homogenates, and microsomes) have supplied much of the present knowledge regarding steroid biochemistry. Most of this work was performed by endocrinologists in the 1950's and 1960's; analytical methodology was limited to thin layer chromatography and detection limits were significantly higher than at present. Nevertheless, few modern contributions utilizing current techniques have modified our understanding of steroid biochemistry beyond that achieved two decades ago.

The concensus of numerous *in vitro* studies performed in a variety of species is that: a) sufficient differences exist in enzymatic capacities of tissues from different animals to question the validity of extrapolation to other species (Meigs and Engel, 1961). Some of these differences may be a function of methodology, in that different laboratories commonly report different results for the same preparation (Monder and Shackleton, 1984). b) Steroids are not chemically altered within the body, only by enzymatic catalysis. Once formed, the metabolites too are stable in biologic fluids. c) The steroid metabolizing enzymes are very specific stereochemically yet are not necessarily selective, in that a single enzyme may hydroxylate stereospecifically at several locations on the steroid molecule (Miyabo et al., 1973). d) Multiple phase I metabolic steps may be involved in the biotransformation of a single molecule before its elimination (Miyabo et al., 1973). e) The liver possesses the greatest capacity to biotransform these drugs (Mahesh and Ulrich, 1959; Roberts and Szego, 1955), although many other organs also express steroid metabolizing enzymes. Unlike for 11β-HSD (interconversion), there is no evidence to suggest that tissue disruption results in incongruous metabolic profiles; the biotransformation enzymes are apparently stable to membrane disruption.

Approximately 90% of an administered dose of any of the glucocorticoids depicted in Figure 1.1 is recovered within 24 to 48 hours in human urine as unchanged drug and metabolites (Sandbergand Slaunwhite, 1957); little is excreted into the feces (Samuels and West, 1952). Biliary excretion of cortisol is quite low in humans, representing 1-5%, relative to other steroid hormones such as estrogens (31-42%) or androgens (12-25%) (Sandberg and Slaunwhite, 1956). The synthetic analogues are excreted unchanged into the urine to a significantly greater extent than is cortisol; this is particularly true at times when concentrations are high, such as after intravenous bolus administration (Sandberg and Slaunwhite, 1957). The existence of saturable renal tubular reabsorption has been debated in humans and animals, *in vivo* and in perfused kidney preparations (Blair et al., 1963; Schedl et al., 1959; Burstein et al., 1964; Beisel et al., 1964; Lindholm, 1973; Frankeet al., 1982; Boonayathap and Marotta, 1974); most evidence suggests that apparent saturation can be explained as an experimental artifact or is not a significant pathway if saturation does occur.

The primary inactivation pathway of cortisol, cortisone and corticosterone is reduction of the double bond in the A ring by Δ^4-reductase; 3β-hydroxysteroid dehydrogenase (3β-HSD) subsequently reduces the 3-keto group to 3β-OH and conjugation follows (Heftmann, 1970a). This series of reactions probably does not occur to a great extent for Δ^1-steroids because the additional double bond prevents reduction of the A ring (Gray et al., 1956; Tomkins, 1956). Consequently, there is significantly reduced conjugation of the synthetic analogues (Sandberg and Slaunwhite, 1957). Additionally, the half-lives of the synthetic compounds are extended considerably (clearance is reduced) relative to cortisol.

The primary route of inactivation of synthetic glucocorticoids (Δ^1) occurs at the opposite end of the molecule with reduction of the 20-keto group to 20β-OH (Vermeulen, 1959a). Hydroxylation may occur at the 6β and 16β positions. Conjugation may occur at the 17α-OH, 21-OH, 16β-OH or 20β-OH, most commonly with glucuronic or sulfuric

acids (Musey et al., 1979). The C-21 alcohol may be oxidized to an aldehyde or to an acid (Monder and Bradlow, 1977). Side chain cleavage may occur resulting in the loss of C-21 (Caspi, 1958). The 17α-OH group may be hydrolyzed, as well (Payne and Singer, 1979). Several of these phase I reactions may occur to the same molecule before excretion (Miyabo et al., 1973).

The most metabolically active organ toward irreversible elimination of the glucocorticoids in all species tested is the liver (Mahesh and Ulrich, 1959; Roberts and Szego, 1955). The most active extra-hepatic organ is the kidney (Jenkins, 1966). The lung, adrenals or gonads have been identified as the next most active, depending upon the species, enzyme in question, or laboratory of study (Koerner, 1966; Jenkins, 1966). It is believed that ring A reduction by Δ^4-reductase occurs only in the liver since hepatectomized animals do not produce ring A-reduced products (Gold, 1961; Berliner et al., 1958).

Steroid dehydrogenases are the most important enzymes for metabolism of the Δ^1-glucocorticoids; an enzyme has been identified for all positions in which a keto and secondary alcohol might interconvert (Heftmann, 1970b). In general, these enzymes act stereospecifically, position-specifically, and perform reversibly. Yet one enzyme may reduce or oxidize at more than one specific position on the steroid. 20β-Hydroxysteroid dehydrogenase is primarily responsible for inactivation of the synthetic (Δ^1) glucocorticoids and has been demonstrated in all tissues of the dog except the brain (Miyabo et al., 1973). It appears to require substrates with a 17α-OH substituent (Recknagel, 1957). Controversy exists as to whether 20α-OH metabolites are formed *in vivo* (Vermeulen, 1959a; Gray et al., 1956; Vermeulen, 1959b); enzymology principles propose that if 20α-metabolites are formed, a unique, stereospecific enzyme performs the reaction, i.e., 20α-hydroxysteroid dehydrogenase. Other dehydrogenases have been described above: 3α-HSD and 11β-HSD.

Hydroxylases play a much less important role in steroid biotransformation than the dehydrogenases. The former are P_{450} enzymes, or mixed function oxidases which act

irreversibly and require atmospheric oxygen. Oxidation (hydroxylation) of the molecule may occur at several positions, but most commonly at 6β or 16α. 6β-Hydroxylation accounts for approximately 10% of an administered dose of prednisolone or prednisone (Frey and Frey, 1983). Recently, interest has grown in the quantitation of urinary 6β-OH cortisol as an indicator of oxidative enzyme induction (Park, 1981).

Transferases perform conjugation reactions at many positions of the steroid molecule. The most common type of conjugate is formed as an ester with alcohols present on the rings. Most common are glucuronides, sulfates, and sulfoglucuronides; less common are methyl, acetyl, and glutathione conjugates. Greatest activity of the glucuronyltransferases is found in the liver, kidney and the intestinal wall (Musey et al., 1979). Sulfotransferases have activity almost exclusively in the liver (Payne and Singer, 1979).

Pharmacology

Steroid hormones have aided in elucidating gene expression as well as the integration of cellular functions since the 1960's. The glucocorticoids act by altering the rate of RNA synthesis; this can be in a stimulatory or inhibitory fashion. Briefly, the proposed mechanism of action of the glucocorticoids (Thompson and Lippman, 1974) is free (unbound) steroid enters the cell and binds to a cytoplasmic receptor (glucocorticoid receptors are widely distributed in mammalian tissue [Ballard et al., 1974]). Cellular influx may be either via simple diffusion or by some facilitated transport processes (Ballard, 1979). The conformation of the steroid-protein unit is altered, whereby chromatin or DNA binding sites may be exposed. Migration to the nucleus follows, and interactions with chromatin occur. The synthesis rates of some mRNA's and tRNA's are affected in this process. Most, although not all pharmacologic effects can be explained on the basis of this genomic interaction; research examining the details of the receptor-steroid interaction within the nucleus continues.

In vivo, glucocorticoids circulate as free and bound (albumin or transcortin) forms, the significance of which has been addressed by many laboratories. Several theories have developed, the most popular of which is that only unbound drug is available to exert activity or to be metabolized. In 1959, Sandberg and Slaunwhite proposed that glucocorticoid bound to corticosteroid binding globulin was both inactive and could not be metabolized. The inclusion of CBG with cortisol inhibited glycogen deposition in mouse liver (Slaunwhite et al., 1962). CBG also prevented the conversion of cortisol to reduced cortisol (A-ring reduction) in rat liver microsomes (Sandberg and Slaunwhite, 1963). Lewis et al. (1971) correlated albumin plasma concentrations in humans with corticosteroid side effects; they proposed that albumin-bound steroid is unavailable to exert its effects *in vivo* . Most experimentation supports the hypothesis that only the unbound steroid fraction is active (Mendel et al., 1989 and Hillier, 1969).

The active bound-drug hypothesis also has some supporters. Siiteri and co-workers (1982) have developed a theory wherein binding to specific globulins serves a physiologic function, in that the steroid-protein complex is the active species which attaches to the target cell membrame and is then internalized.

A third theory is a hybrid of the unbound and bound theories; it states that non-specifically bound drug is available for interactions but specifically-bound drug is tightly held and not available for activity. Partridge and Mietus (1979) investigating the transport of several steroid hormones across the anesthetized rat blood brain barrier observed that while globulin-bound steroid was not available for diffusion, albumin did not exert an inhibitory effect on the transport of these compounds into the brain.

Indications

The approved clinical uses of the glucocorticoids are multiple, as might be expected for synthetic hormones. Most common approved uses are presented in Table 1.1. A

Table 1.1. Approved Indications for Prednisone *

Endocrine disorders
- primary or secondary adrenocortical insufficiency
- adrenal hyperplasia

Rheumatic disorders
- rheumatoid arthritis
- anklyosing spondylitis
- post-traumatic osteoarthritis

Collagen diseases
- systemic lupus erythematosus
- polymyositis

Dermatologic diseases
- pemphigus
- Stevens-Johnson syndrome
- exfoliative dermatitis
- severe psoriasis

Allergic states (for conditions intractable to conventional treatment)
- bronchial asthma
- contact dermatitis
- serum sickness
- drug hypersensitivity

Ophthalmic diseases
- herpes zoster ophthalmicus
- allergic conjunctivitis
- optic neuritis

Respiratory diseases
- fulminating tuberculosis
- aspiration pneumonitis

Gastrointestinal diseases
- regional enteritis
- ulcerative colitis

Hematologic disorders
- idiopathic thrombocytopenia purpura
- autoimmune hemolytic anemia

Neoplastic diseases
- adult leukemias
- adult lymphomas
- acute childhood leukemia

Nervous system
- acute exacerbations of multiple sclerosis

* abstracted from Deltasone® package insert

number of nonlabelled uses are also often employed. Prevention of transplant graft rejection and treatment of autoimmune disorders are of particular importance.

Despite more than three decades of wide-spread use, the dosing of glucocorticoids remains empirical, except in the case of replacement therapy (Haynes et al., 1985). Clinicians are cautioned to continually monitor patients for toxicities and to reduce dosage as needed. This effort is made more difficult by the nonlinear disposition of the compounds (cortisol, prednisone) and the lack of therapeutic endpoints (Frey et al., 1985). It will not be possible to more optimally prescribe these compounds until the pharmacokinetics and pharmacodynamics of these agents are correlated.

Adverse reactions attributed to the glucocorticoids can be viewed as the undesired physiologic effects. They are predictable yet difficult to eliminate or even minimize. It has been possible to separate mineralo- from glucocorticoid effects. The most frequently observed adverse reactions are listed in Table 1.2. The most dangerous complications of glucocorticoid use are secondary adrenal insufficiency and infectious complications secondary to prolonged immunosuppression. Adrenal insuffuciency is induced by continued exogenous administration of glucocorticoids. The adrenal cortex and pituitary cease production of corticosteroids and corticotropic factors, respectively. When therapy is discontinued, full endocrine function may not return; the condition can be fatal. For this reason therapies of more than several weeks duration and/or of moderate to high doses require tapering prior to complete cessation.

Table 1.2. Adverse Reactions to Prednisone *

Fluid/Electrolyte Disturbances
- sodium & water retention
- hypokalemia
- hypertension

Musculoskeletal
- muscle weakness & wasting
- osteoporosis
- vertebral compression fractures

Gastrointestinal
- peptic ulcer

Dermatologic
- impaired wound healing
- thin fragile skin

Metabolic
- negative nitrogen balance

Neurologic
- headache
- vertigo

Ophthalmic
- cataracts
- glaucoma

Endocrine
- menstrual irregularities
- Cushingoid state
- suppression of growth in children
- secondary adrenocortical and pituitary unresponsiveness
- latent diabetes mellitus

Miscellaneous
- allergic, anaphylactic, or hypersensitivity reactions

* abstracted from Deltasone® package insert

Chapter 2

Absorption and Disposition of Prednisone and Prednisolone

Introduction

The disposition of the glucocorticoids is complicated by multiple factors *in vivo*. The compounds are interconverted between pharmacologically active and inactive species, confounding the kinetic analysis of their disposition. The body expresses differential organ metabolism toward these compounds; all body organs contribute to the interconversion and most to the irreversible elimination of the glucocorticoids; numerous elimination pathways exist and are found throughout the organs of the body to various extents. Endogenous glucocorticoids, unless suppressed, compete with exogenous compounds for metabolic biotransformation pathways and for binding to corticosteroid binding globulin (e.g., cortisol and prednisolone). Saturable biotransformation pathways potentially exist; these may be involved with interconversion, irreversible metabolism or renal excretion. Prednisolone and cortisol exhibit saturable binding to corticosteroid binding globulin (CBG, transcortin). Albumin is the second significant binding protein, but in this case, binding is linear. The binding characteristics result in an increasing free fraction of drug with dose. Each of these potentially complicating features is addressed in this chapter.

The earliest *in vivo* studies with the glucocorticoids were simply designed: oral administration of radiolabelled drug followed by scintillation counting of urine samples (Caspi and Pechet, 1959a; Vermeulen, 1959a; Sandberg and Slaunwhite, 1957). With the

development of sensitive radioimmunoassay and competitive protein binding techniques in the 1970's, physiologic concentrations of glucocorticoids could be measured in blood. Nonlinearities in the disposition of cortisol and prednisolone were first documented using these analytical methods by Meikle et al., (1975); Loo et al., (1978); and Pickup et al., (1977). Dose-dependent changes in clearance, volume and/or half-life were revealed. These technologies were not without error, since it has been well documented that both radioimmunoassay as well as competitive protein binding assays do not effectively distinguish between endogenous and synthetic glucocorticoids (Gambertoglio et al., 1980b). General characteristics of these protocols which make these results of limited use at this time are: a) half-life was commonly the only parameter described or measured; b) non-steady state studies were usually performed; c) linear compartmental modelling techniques were commonly used to derive parameters, yielding time-averaged values; d) unbound glucocorticoid concentrations were not determined even though saturable binding to CBG results in an increasing free fraction with dose.

Identification of an animal model for glucocorticoid disposition would aid in the examination of the complex metabolic and excretory processes. As proposed by Dedrick and Bischoff (1980), the selected species need not demonstrate the same disposition profile as humans, but merely similar characteristics. To date, the pharmacokinetics of the glucocorticoids have been studied in the following animal models: mice, rats, guinea pigs, rabbits, dogs, monkeys and pigs (Manin et al., 1983; Eriksson, 1971; Boudinot and Jusko, 1986; Frey et al., 1988; Frey et al., 1980; El Dareer et al., 1977; Rocci and Jusko, 1981; Pepe and Albrecht, 1985). Some of the recognized differences which occur between these species are: the primary circulating glucocorticoid (cortisol vs. corticosterone), existence and capacity of metabolic biotransformation pathways, organ specific disposition, the magnitude of the contribution of biliary and renal excretory pathways, and the concentration and/or affinity of corticosteroid binding globulin for the glucocorticoids.

In addition to cross-species investigations, studies with multiple glucocorticoids can lead to further understanding of the nonlinearities expressed by prednisone and prednisolone. The *in vivo* disposition of cortisol, cortisone, corticosterone, prednisone, prednisolone, methylprednisolone and dexamethasone have been studied most extensively. Studies in both humans and animals have been performed using intravenous bolus doses, steady state infusions, and oral dosage forms of the glucocorticoids. These routes provide information on presystemic metabolism and each will be examined individually. Studies abound which solely determine the values of pharmacokinetic parameters; it is not the purpose of this discussion to address parameter values as such studies are reviewed elsewhere (Gambertoglio et al., 1980a; Gustavson and Benet, 1985). The effect of disease states and drug interactions are also reviewed in these references. This chapter addresses studies which examine the dose- or route-dependencies (bioavailability) of the kinetics of the glucocorticoids.

Plasma protein binding

It is a major assumption of most pharmacokinetic and pharmacodynamic theories that only the free or unbound glucocorticoid can partition through plasma membranes to reach sites of metabolic transformation or receptors (Slaunwhite et al., 1962; Westphal, 1983). Therefore results based on total drug cannot truly reflect events at the molecular level. It is essential that both *in vivo* and *in vitro* experiments include the determination of unbound concentration if they are to contribute to the understanding of kinetic and dynamic processes.

Several methods exist with which to determine the unbound fraction of drugs in biologic fluids. The major techniques have been thoroughly reviewed (Chignell, 1983). Most studies of the binding of glucocorticoids have been performed using equilibrium dialysis (Sophianopoulos et al., 1978); theoretically, ultrafiltration and equilibrium dialysis are equivalent (Sophianopoulos et al., 1978).

The binding of corticosteroids to plasma proteins has been reviewed (Gustavson and Benet, 1985). As previously mentioned, corticosteroids bind primarily to two proteins in plasma: corticosteroid binding globulin (CBG, transcortin) and albumin. Alpha$_1$-acid glycoprotein (Milsap et al., 1983) contributes insignificantly (< 3%) and is not considered in this discussion.

Two independent groups discovered CBG (transcortin) at approximately the same time (Sandberg and Slaunwhite, 1958; Daughaday, 1957), hence the two common names for the protein. CBG migrates electrophoretically as an α-globulin. It is present in human serum at 0.71 μM (0.037 g/l) and is a 52 K protein. The affinity of prednisolone for CBG approximately 3×10^7/M (Pugeat et al., 1981). Albumin's molecular weight is 69 K and it is present at concentrations of 550 μM (38 g/l), greatly exceeding that of CBG; the affinity constant of albumin for prednisolone, about 6×10^3/M (Pugeat et al., 1981), is orders of magnitude less than that of CBG.

CBG predominates as the major binding protein at low or physiologic glucocorticoid concentrations (Ballard, 1979) but exhibits saturability at concentrations above 100 ng/ml of cortisol or prednisolone (Rocci et al., 1980). Above 1000 ng/ml, albumin is the major binding protein. Consequently, at physiologic concentrations little of the steroid is unbound; with increasing concentrations, greater quantities are free. This system is well-integrated with the glucocorticoid biosynthetic system, in that when plasma glucocorticoid concentrations rise above normal physiologic concentrations, this mechanism allows more of the hormone to be free to exert activity, as well as to be eliminated (metabolized).

Prednisolone is the only synthetic glucocorticoid known to bind to corticosteroid binding globulin (Wolff, 1983; Pugeat et al., 1981). It appears that the addition of any substituent to the steroid nucleus results in diminished affinity for CBG. The additional double bond in the A ring of prednisolone (Δ^1-cortisol) has no effect on binding (the affinity constants for prednisolone and cortisol are equivalent). Yet methylprednisolone

(6α-methyl, Δ^1-cortisol) and dexamethasone (9α-fluoro, 16α-methyl, Δ^1-cortisol) demonstrate linear binding and are not bound to CBG. Some controversy exists, but most investigators agree that 11-keto compounds such as cortisone and prednisone do not bind to CBG. It is known that glucocorticoid metabolites and conjugates (compounds more polar than the parent) bind only to albumin (Wolff, 1983).

Dose-dependent clearance

It is widely recognized that the glucocorticoids prednisone (PO) and prednisolone (POH) display nonlinear pharmacokinetics in humans and animals. A comprehensive review of human studies is presented by Gustavson and Benet (1985). Stated most simply, over the therapeutic range, clearance increases with dose. Traditionally, nonlinearities in disposition are related to saturation of enzymatic processes, resulting in a *decreasing* clearance with dose. The observation of increased apparent clearance associated with prednisolone indicates that processes other than enzymatic saturation are probably involved.

Pickup et al. (1977) were the first to document the nonlinear kinetics of prednisolone in humans; the nonlinear pharmacokinetics of prednisone was first demonstrated by Rose et al. (1980) following oral drug administration. Legler et al. (1981) proved the dose-dependent apparent clearance of both total and unbound POH in steady state studies; both POH and PO exhibit nonlinear renal clearance (Rose et al., 1980). The nonlinear kinetics of PO and POH have been attributed to one or more of the following factors: saturable plasma protein binding of prednisolone to corticosteroid binding globulin, nonlinear renal clearance and saturable metabolic pathways of biotransformation. The contribution of each of these processes will be addressed, as one or more may be in effect in humans.

Clearance definitions

At the start, it is necessary to explain the concept of apparent clearance, which is defined as the clearance measured after administration of a drug. This parameter is not necessarily related to any specific metabolic processes. It is defined in one of two ways:

$$CL_{app} = \text{Dose} / \text{AUC} \quad (1)$$

$$CL_{app} = \text{Rate of infusion} / C_{ss} \quad (2)$$

where AUC is the area under the plasma concentration versus time profile and C_{ss} is the steady state concentration achieved after infusion of the drug to steady-state. The clearances of PO and POH are referred to as *apparent* because the contribution of interconversion is not considered in the definitions. Any drug which is formed or consumed reversibly in the interconversion process is not measured. It can then be understood that the apparent clearance is always lower than the *true* clearance of compounds which interconvert.

The method of calculation of interconversion and elimination clearances was adapted from theoretical work in the field of endocrinology several decades ago. Mann and Gurpide (1966) developed calculations of rates of reversible transfer of drugs between physiologic pools. This deviated from the previous methods which produced results which were dependent on the specific model employed. Duggan and coworkers (1980) adapted this physiologic approach to pharmacokinetics in describing the tissue disposition of sulindac. In 1981, Wagner et al. used these relationships to discriminate between models of interconversion and to calculate the clearances of PO and POH after administration to humans. The derived definitions for the apparent clearances of prednisone, $CL_{1,app}$, and of prednisolone, $CL_{2,app}$, modeled parsimoniously as in Fig 2.1 are:

$$CL_{1,app} = CL_{10} + CL_{12}\left(\frac{CL_{20}}{CL_{20}+CL_{21}}\right) \quad (3)$$

$$CL_{2,app} = CL_{20} + CL_{21}\left(\frac{CL_{10}}{CL_{10}+CL_{12}}\right) \quad (4)$$

The values of the individual clearance terms on the right-hand sides of equations 3 and 4 can only be determined after the administration of both compounds and the measurement of both compounds after administration of each. The values are calculated using dose and AUC (or infusion rate and C_{ss}) measurements; these relationships, as derived by Hwang et al. (1981), are presented in Table 2.1. These equations will be used in Chapters 9 and 10.

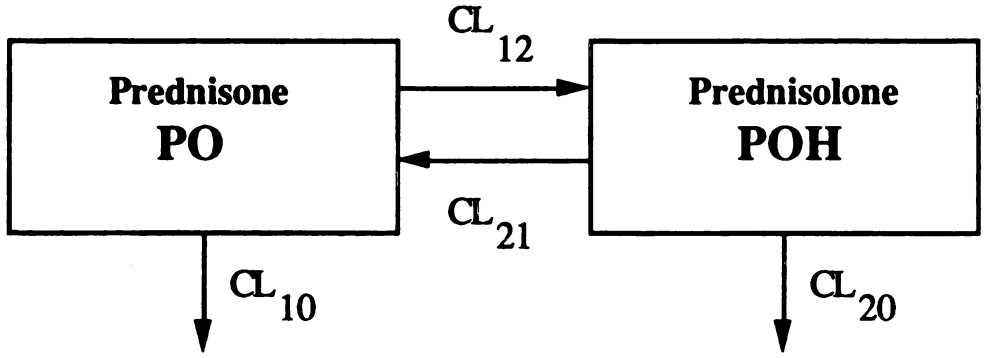

Figure 2.1. Model of the interconversion and elimination of prednisone and prednisolone in isolated perfused rabbit organs. Clearance terms represent intrinsic clearance.

CL_{10} = irreversible elimination of prednisone
CL_{12} = reductive conversion of prednisone to prednisolone
CL_{20} = irreversible elimination of prednisolone
CL_{21} = oxidative conversion of prednisolone to prednisone

Table 2.1 Calculation of fundamental clearance values*

$$CL_{10} = \frac{Dose^{D} \cdot AUC^{M}_{POH} - Dose^{M} \cdot AUC^{D}_{POH}}{AUC^{D}_{PO} \cdot AUC^{M}_{POH} - AUC^{D}_{POH} \cdot AUC^{M}_{PO}}$$

$$CL_{20} = \frac{Dose^{M} \cdot AUC^{D}_{PO} - Dose^{D} \cdot AUC^{M}_{POH}}{AUC^{D}_{PO} \cdot AUC^{M}_{POH} - AUC^{D}_{POH} \cdot AUC^{M}_{PO}}$$

$$CL_{12} = \frac{Dose^{M} \cdot AUC^{D}_{POH}}{AUC^{D}_{PO} \cdot AUC^{M}_{POH} - AUC^{D}_{POH} \cdot AUC^{M}_{PO}}$$

$$CL_{21} = \frac{Dose^{D} \cdot AUC^{M}_{PO}}{AUC^{D}_{PO} \cdot AUC^{M}_{POH} - AUC^{D}_{POH} \cdot AUC^{M}_{PO}}$$

They are derived from the simultaneous solution of these four mass balance expressions:

$$CL^{D}_{10} \cdot AUC^{D}_{PO} + CL^{D}_{20} \cdot AUC^{D}_{POH} = Dose^{D}$$

$$CL^{M}_{10} \cdot AUC^{M}_{PO} + CL^{M}_{20} \cdot AUC^{M}_{POH} = Dose^{M}$$

$$CL^{D}_{12} \cdot AUC^{D}_{PO} = (CL^{D}_{21} + CL^{D}_{20}) \cdot AUC^{D}_{POH}$$

$$CL^{M}_{21} \cdot AUC^{M}_{POH} = (CL^{M}_{12} + CL^{M}_{10}) \cdot AUC^{M}_{PO}$$

* from Hwang et al.(1981)
* see table of abbreviations in introduction of thesis

Animals express kinetic profiles of PO and POH disposition which vary from that of humans. Rabbits express a similar pattern of increased apparent clearance with dose (Unadkat and Rowland, 1985; Rocci and Jusko, 1981). Dogs and rats demonstrate a dose-dependent decrease in clearance, opposite that observed in humans (Frey et al., 1980; Boudinot and Jusko, 1980). However, species differences in metabolism, binding, as well as the dosage size studied contribute significantly to these observations and do not necessarily imply that the general disposition of these compounds differs between species.

Methylprednisolone does not exhibit dose-dependent clearance despite sharing most metabolic pathways with POH (Szefler et al., 1986). The structural difference between the compounds is a 6α-methyl group which prevents binding to CBG and conveys linear plasma protein binding (Ebling et al., 1986). Similar observations have been made for dexamethasone (Rohdewald et al. 1987). Together, these lead to the hypothesis that the dose-dependent clearance of prednisolone may be a function of nonlinear plasma protein binding.

Time-averaged clearance determinations

Kinetic studies utilizing bolus injections and oral administration are most commonly encountered in the literature due to their relative ease of performance. Steady state intravenous studies require special equipment (tubing, pumps and intravenous solutions) and necessitate several hours of cooperation by the subjects.

Bolus or oral administration studies utilize equation 1 to define clearance. Plasma concentrations continually change after a bolus injection or upon absorption from an oral dosage form. Since clearances are concentration-dependent, the values calculated in these studies are time-averaged. Most reports have defined the clearance of PO and POH in this manner. Yet despite the values being time-averaged, an increase in apparent systemic clearance of total drug is usually observed if the dose range is at least two-fold.

The dose-dependent kinetics of prednisolone were first documented in the late 1970's. Meikle et al. (1975) dosed 10 and 40 mg prednisolone phosphate as bolus injections. Clearance, volume and half-life increased with dose, but only increases in apparent volume were statistically significant.

Pickup et al. (1977) administered 0.15 and 0.3 mg/kg intravenous bolus doses of POH as the phosphate ester. Apparent systemic clearance increased from 0.09 to 0.12 L/hr per kg. This group was the first to observe a significant clearance nonlinearity and proposed that an increasing free fraction was consequently available for metabolism and the cause of the increase in apparent clearance.

Loo et al. (1978) administered two oral doses of prednisolone, 10 (0.134 mg/kg) and 20 mg (0.268 mg/kg) in alcohol solutions to eight healthy subjects. The dose normalized prednisolone AUC for the 20 mg dose was 78% that of the 10 mg dose; the higher dose prednisone AUC was 88% that of the lower dose. The apparent clearance of POH increased from 0.10 to 0.13 L/hr per kg between the two doses. Changes in protein binding were proposed to account for the observation.

Tanner et al. (1979) observed dose-dependent clearance across an intravenous POH dosage range of 20 to 100 mg. They documented no change with dose in the unbound fraction of POH or in the half-life, concluding that the observed increase in volume of distribution was the cause of the nonlinearities in the AUC of POH. This group observed linear binding of prednisolone, which was probably determined at relatively high concentrations of POH where binding approaches linearity.

Rose et al. (1981) administered both oral prednisone (5, 20, and 50 mg tablets) and intravenous prednisolone phosphate (5, 20, and 40 mg bolus doses). They observed dose-dependencies for both drugs. The apparent clearance of PO increased from 572 to 2271 ml/min per 1.73 m^2 over the 10-fold dose range. The apparent clearance of POH increased from 111 to 194 ml/min per 1.73 m^2 over an 8-fold dose range. The unbound clearances were relatively constant with dose; the unbound fraction of prednisolone increased greatly

over this range and the unbound fraction of PO was constant. Rose and coworkers (1981) concluded that protein binding only partially accounted for the nonlinearities and another process, perhaps metabolic, could be involved.

In animals, Boudinot and Jusko (1986) examined the dose dependency of POH in normal and adrenalectomized rats. The intravenous doses were quite high, 5 and 50 mg/kg; these doses were required to detect drug in the small plasma volumes used. They observed a 2-fold drop in the apparent clearance of POH (total, unbound, and CBG-free) over the 10-fold dose range. The binding of POH was nonlinear, but this observation would predict that clearance increase with dose, or the opposite of what was observed. They concluded that saturation of metabolism and saturation of uptake into metabolic sites at the high doses used could have contributed to the decreased clearance.

Derendorf et al. (1985) administered extremely high and moderate doses (1200 and 75 mg) of prednisolone hemisuccinate to humans; they observed a *decreased* apparent clearance of POH with increasing dose. This observation was surprising and was opposite to that observed in humans to date. This result was attributed to saturation of metabolic pathways at the very high dose employed, as in the case of the adrenalectomized rats, above.

The only group to observe no change in apparent clearance was Al-Habet and Rogers (1980). They administered bolus doses of 16, 32, 48, and 64 mg prednisolone phosphate. Apparent clearance, volume, and half-life all exhibited little change; these results have not been not supported by any other laboratory.

Steady state clearance determinations

Where kinetics are linear, bolus injection studies are as valid as steady state infusion studies but when nonlinearities exist, the two methods produce inequivalent results (Frey et al., 1982). At the cellular level, this difference may be envisioned as a concentration gradient existing between plasma and metabolic sites after bolus administration, particularly

immediately after injection when concentrations are very high and decrease rapidly. Hence, the dose-dependency of the kinetics of POH predicted by intravenous bolus studies remained to be proven with steady state investigations. Equation 2 was used to determine clearance at steady state. When nonlinearities in pharmacokinetic parameters exist, studies performed at steady state offer the greatest information because calculated parameters are exact for a specific steady state concentration, and not time- and/or concentration-averaged. Legler et al. (1982) proved the dose-dependent apparent clearance of prednisolone in healthy human volunteers with the infusion of two doses of the phosphate ester, 5.5 and 64 μg/hr per kg. The drug was infused to steady state by the combination of a short, rapid infusion, followed by a sustained, lower infusion rate, during which time plasma measurements were made. Since the steady state concentrations of POH only increased 4.8-fold when the infusion rate was increased 11.6-fold, the apparent clearance of total POH increased by a factor of 2.5.

The unbound clearance (CL_U) also increased slightly but significantly in eight of the ten subjects studied by Legler et al. (1982), increasing an average of 29%. This observation was quite unexpected. Whereas changes in the apparent clearance of total drug may be explained by binding changes, the observed increase in unbound clearance implies that some saturable metabolic processes may be involved. Of the groups that have measured the binding of POH, this study was the only one to report a change in the *unbound* clearance of POH.

Frey et al. (1980) performed a similar study in dogs, infusing multiple doses of prednisolone, ranging from 0.427 to 26.1 μg/min per kg, producing a wide range of steady state concentrations. They observed a slight *decrease* in the apparent systemic clearance of both total and unbound drug with dose. Apparent clearance of total drug dropped from 10.9 to 6.5 ml/min per kg when the concentration increased from 39 to 4014 ng/ml total drug. In nine of the ten animals, the unbound clearance also decreased an average of 29%.

The authors associated the observations with saturation of metabolic pathways, specifically the oxidative conversion of POH to PO.

The steady state clearance of prednisolone phosphate and of prednisone succinate in rabbits was examined in a multiple infusion protocol by Unadkat and Rowland (1985). Over a range of 0.2 to 2.0 μg/min/kg, concentrations of POH ranged from 100 to 600 ng/ml. The clearance of POH increased from a range of 2.54 to 4.08 at low rates to 6.08 to 10.98 ml/min per kg at high rates of infusion. They observed a small increase in the apparent CL_U of POH at low concentrations, but not at higher concentrations.

Khalafallah and Jusko (1984) observed no change in the apparent steady state clearance of POH in rabbits. The POH steady state concentrations achieved ranged from 360 to 4500 ng/ml in this study. Recall that clearance increased in the rabbit studies of Unadkat and Rowland, where POH concentrations reached only 400 ng/ml. Perhaps linearity was observed by Khalafallah and Jusko because the concentrations examined were beyond the range of saturable binding in the rabbit. Rocci et al. (1980) have observed the nonlinear binding of POH in the rabbit to occur between 100 and 700 ng/ml.

Interconversion

Prednisone and prednisolone are interconverted at position 11 by the putative enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD). This enzyme is responsible for the formation of the majority of cortisone found *in vivo*, as only twenty percent of circulating cortisone in humans is secreted; 80% is the result of oxidative conversion of cortisol at extra-adrenal sites (Srivastava et al., 1973). Interconversion is a conservative mechanism in that it slows the elimination of the 11β-hydroxyl form of the drug (cortisol). Unfortunately, interconversion complicates the calculation and definition of classic pharmacokinetic parameters.

Interpretation of studies

Interconversion has been examined in two ways, by the comparison of areas under the curve after a single dose and of steady state concentrations of the interconverted species. Steady state investigations of interconversion are superior to single dose measures since the latter requires the assumption that clearance remains constant over the concentration range examined. With the steady state concentration method of evaluating interconversion, it is not necessary to assume that clearances are linear (including the two fundamental clearances defined in Figure 2.1); nonlinearities do not violate model assumptions and multiple concentrations may be compared. Therefore the single dose and steady state methods theoretically may differ in conclusions regarding interconversion because of the time-dependency of the clearance in the former method.

Most investigations of PO-POH interconversion have involved only prednisolone administration. It is significant to note that the area and concentration measurements obtained are dependent upon which drug is administered, PO or POH (Ferry and Wagner, 1988). Prednisolone is often regarded as the standard by virtue of its activity. Issues surrounding the choice of which drug to consider as the standard for bioavailability assessment are further addressed in Chapter 9.

Studies of interconversion

Legler and coworkers (1981) infused two doses of prednisolone phosphate to steady state in humans, at 5.5 and 64 μg/hr per kg. A maximum PO concentration of 52 ng/ml was achieved. When the infusion rate of POH was increased 12-fold, the PO steady state concentration increased only 2-fold while that of POH increased 5-fold; this implied that perhaps the formation of PO from POH is saturable.

Frey et al. (1980) infused prednisone and prednisolone to dogs in similar steady state studies and observed nonlinear interconversion as well. A 100-fold increase in POH steady state concentration resulted in only an 11-fold increase in PO concentration. They

suggested that saturation of 11β-hydroxysteroid dehydrogenase was a probable contributor.

In both of these studies, the oxidative conversion of POH to PO was modeled by Michaelis-Menten kinetics and enzyme parameters for the dog and human were calculated. A term analogous to the Michaelis constant, K_m (the POH concentration at which half the maximum PO concentration is achieved), was calculated. The values for dogs and humans were 658 and 199 ng/ml, respectively. The traditional V_{max} was redefined as the maximal concentration of PO achievable, P_{max}; it was 63 and 52 ng/ml for the dog and human, respectively.

Frey et al. (1984) also examined the conversion of POH to PO in renal transplant patients receiving treatment for acute rejection. Seven mg/kg equivalents of prednisolone were administered as oral prednisone, intravenous prednisolone phosphate and intravenous prednisolone tetrahydrophthalate on three occasions. The resultant PO area was about 1/30 that of the POH area, much less that that observed with 0.2 or 0.8 mg/kg doses. The authors acknowledged that this patient group was not expected to perform as normal healthy subjects because other drugs were also administered. The observation of an apparent maximal PO concentration achievable from this high prednisolone dose is significant and consistent with previous findings.

The ratio of total PO to POH was observed to remain constant by Unadkat and Rowland in multiple infusions to rabbits (1985). In contrast, Khalafallah and Jusko (1984) observed a decreasing ratio with infusion rate, concluding that 11β-HSD oxidation to PO is saturable. Conflicting observations within the same species are not as surprising as one might believe. First, Unadkat and Rowland administered PO; Khalafallah and Jusko administered POH. Such a difference in protocol is recognized to yield conflicting results (Hwang et al., 1981). Secondly, the steady state concentrations achieved in each laboratory differed by about an order of magnitude (400 vs. 4500 ng/ml). Perhaps the

nonlinearity observed by Khalafallah and Jusko was observed due to the higher doses and larger concentration range examined.

In humans, Rose et al. (1981) observed a variable PO to POH ratio between intravenous prednisolone and oral prednisone administration (dosage range 5 to 50 mg). Suspecting that saturable binding of POH to corticosteroid binding globulin might be responsible, unbound concentrations of PO and POH were compared; the ratio of POH to PO still increased with dose. The interconversion ratio of POH to PO varied with time and dose, but averaged 4 to 10-fold in favor of the reduced POH. Hence, they concluded that a metabolic process might be responsible for this observation.

Boudinot and Jusko (1986) examined the dose dependency of the PO/POH ratio in normal and adrenalectomized rats. Over the dosage range of 5 to 50 mg/kg, they observed a two-fold decrease in the AUC ratio of PO to POH. They proposed that saturation of 11β-HSD might have contributed to this observation. Additionally, they recognized the potential for dose-dependency of transport as well as metabolism in this high concentration range.

It appears that regardless of dosage range, study design or species, prednisone and prednisolone concentrations or areas do not demonstrate a linear relationship. One potential explanation is the saturation of the enzymatic conversion of POH to PO. Another contribution may be associated with the saturable protein binding of prednisolone.

Oral administration (Bioavailability)

Many investigations of the bioavailability of prednisone and prednisolone have been performed due to the suspicion and documentation of the poor bioavailability of prednisone tablet formulations, as reviewed by Gambertoglio et al. (1980a). These results are of interest because presystemic metabolism of prednisone and prednisolone includes a large hepatic component.

Intravenous forms of prednisolone are available as phosphate and hemisuccinate esters; prednisone is not marketed in parenteral form in the United States. The development of steroid esters evolved due to the extremely low aqueous solubilities of PO and POH. Solubilities of PO and POH are increased about 100-fold in alcohol, permitting the development of a cosolvent solution of the drug for parenteral administration; such a formulation is used in human studies with intravenous prednisone and prednisolone as presented in Chapters 9 and 10.

As described previously, steady state clearances are determined following intravenous infusion of the drug of interest. More studies have examined the clearance of POH than of PO; steady state investigations of POH clearance have been performed using one of the esters of prednisolone, where it has been assumed that the prodrug esters are hydrolyzed completely and quickly by plasma esterases (Rose et al., 1980; Legler et al., 1982). Few studies have tested this assumption, particularly in humans. In fact, the hydrolysis of several steroid esters have been shown to be incomplete and/or delayed in rabbits and humans (Unadkat and Rowland, 1985; Ebling et al., 1985; Frey et al., 1985). The significance of this finding will be discussed in detail in Chapter 9.

Special considerations for interconverted drugs

Ferry and coworkers (1988) have recently addressed a series of questions concerned with pharmacokinetic calculations for drugs which interconvert. Many issues have arisen that have not required consideration until recently. For example: what is the meaning of bioavailability for a compound which interconverts? which compound should serve as the reference standard? are drug or metabolite or both concentration ratios compared? do definitions change if one or both compounds possess activity? At the present time, bioavailability studies are performed without special consideration for interconversion.

Hwang et al. (1981) have proposed methods for determining the bioavailability of compounds which undergo interconversion. A compilation of cases is presented in which renal, nonrenal and systemic (apparent) clearances independently remain constant or change with dose. The theory is based on both urine and plasma sampling and administration of both compounds individually. This treatment of the subject is utilized in analyzing the human data in Chapter 9.

Traditional bioavailability studies of both prednisone and prednisolone compare steady state concentrations or areas after single doses only of prednisolone (Gambertoglio et al., 1980a); this has been rationalized on the basis of only POH possessing pharmacologic activity. Therefore, in prednisone bioavailability tests, it is concentrations of the metabolite, POH, which are measured and compared. In this way, prednisone is treated like a prodrug: PO is a latent source of POH. It differs from a prodrug, however, in that the drug, POH in this case, can form the prodrug, PO, *in vivo*, and this conversion continues as long as either drug is in the body.

Another important issue is which drug to administer as the standard, since different comparisons result in different bioavailability estimates. For instance, Ferry et al. (1988) showed that the bioavailability estimate obtained was dependent on the reference compound; a 30% variation was observed between methods when intravenous PO or POH were used as the reference compound for the bioavailability determination of PO tablets.

Bioavailability studies

Ferry and coworkers (1988) administered 10 mg doses of oral tablets of PO and POH, and intravenous doses of prednisolone phosphate, and non-esterified PO in an ethanol:water (1:1) solution. They observed approximate bioequivalence of PO and POH tablets, based upon comparisons of POH areas. The absolute bioavailabilities of the tablets were dependent on the intravenous dosage form chosen as the reference. Prednisolone was equivalently available from both tablets when intravenous POH was the reference and POH

areas were compared. When PO was measured from the two tablets, the two intravenous treatments yielded different bioavailability estimates; use of intravenous PO as the standard resulted in 70% availability whereas use of intravenous POH resulted in availability greater than unity.

A few bioavailability studies have been performed in animals. Colburn et al. (1976) administered 5 mg tablets of PO and POH to beagle dogs. The combined areas of both dosage forms were equal between treatments, but the POH area was greater after POH administration by 8 to 13% than after PO administration. This observation of drug-dependent results is similar to that observed in humans.

In the past, inequivalency of prednisone and prednisolone tablets had been reported (Gambertoglio et al., 1980a). These problems have been eliminated in current formulations. In general, the bioavailability of prednisone and prednisolone tablets ranges between 0.77 and 0.99, is independent of dose and is a function of the formulation (Gustavson and Benet, 1985).

Urinary excretion

Glucocorticoid urinary excretion was the first type of pharmacokinetic study conducted in humans. This method of sample collection is noninvasive and sample size is large. Advancements in analytical chemistry made plasma analyses possible over the next decades and the reliance on urinary studies decreased. Today, urinary data are being reexamined for drugs with special renal transport processes or nonlinear renal excretory processes.

It is possible to predict some of the major routes of renal elimination of the glucocorticoids with knowledge of their physico-chemical characteristics. The glucocorticoids circulate in two forms: unbound and bound to plasma proteins. The unbound form of the compound is filtered at the glomerulus. The lipophilic nature of the glucocorticoids suggests that the compound is passively reabsorbed in the renal tubule.

Due to their nonpolar, lipophilic nature, these compounds are expected to be excreted in a urine flow-dependent manner. There is no information available on the existence of tubular steroid secretory processes.

Study assumptions

The examination of renal elimination processes *in vivo* involves multiple assumptions. The earliest examinations of renal excretory processes were performed using the intact subject or animal; where plasma concentrations were measured, it was assumed that antecubital vein blood reflected concentrations in arteries, i.e., the renal artery. This assumption, based on there being no metabolism by muscle, has not been verified to date.

A similar assumption must be made when examining the kidney in isolation. In perfused kidney studies, it is assumed that renal arterial concentrations do not differ significantly from those within the two capillary networks of the nephron. Little is known of specific renal metabolism of the glucocorticoids but this assumption may be incorrect. Micropuncture techniques theoretically minimize this error by measuring local concentrations, but have been infrequently performed with these drugs.

Studies in intact animals as well as isolated organs have defined our present knowledge of renal steroid disposition. Very little is known about species differences in renal excretory and metabolic events. Only general concepts can be extrapolated to the human.

Renal clearance and *in vivo* studies

Most studies of renal glucocorticoid disposition have been performed with endogenous glucocorticoids, particularly cortisol. We assume that both PO-POH and cortisone-cortisol pairs exhibit similar urinary excretion patterns; no evidence of a difference in filtration or reabsorption has been suggested. Additionally, their binding characteristics are nearly indistinguishable. Renal metabolism of the glucocorticoids has

not been studied to date, with the exception of a few homogenate or microsomal analyses. It is known that the only biotransformation pathway that differs between the two sets of compounds, reduction of the A ring, occurs exclusively in the liver (Gold, 1961). Hence, it is probably reasonable to assume that the renal handling of cortisone and cortisol reflects that of PO and POH. Several studies of renal elimination processes have been performed to test whether corticosteroids exhibit dose-dependent excretion. Cumulative evidence supports saturation only at extremely high doses, much beyond those encountered in conventional therapies.

Lloyd (1952) observed a direct correlation between urine flow (volume) and excretion rate of the endogenous glucocorticoids in humans. These observations were made in both healthy normal subjects and adrenal insufficiency patients undergoing replacement therapy. The relationship between urine flow and steroid excretion was not apparent for 17-ketosteroids. No hypotheses were presented as to the causes of this observation.

Passive reabsorption and filtration were proposed by Beisel et al. (1964) to be the mechanism of renal handling of cortisol. Increased infusion rates of cortisol resulted in decreased binding, increased filtration, and linear increases in the excretion and reabsorption of cortisol. There was no apparent transport maximum (T_m) for cortisol concentrations up to 1200 ng/ml.

Blair et al. (1963) documented in humans that 17-hydroxycorticosteroids (cortisol, cortisone, and some of their metabolites) were excreted less readily than inulin. Thus, they concluded that glomerular filtration and tubular reabsorption must have been involved. (They did not consider the effect of binding to plasma proteins, which could have also explained these results.) The glucuronides, on the other hand, were excreted at rates greater than the glomerular filtration rate (GFR), implying the involvement of secretion in conjugate excretion.

The results of Lindholm (1973) support the passive reabsorption process in humans as well. Cortisol was infused and a close linear correlation was observed between the excretion of cortisol and its unbound concentration. A similar correlation was observed with GFR and tubular reabsorption of cortisol. There was no significant relationship between plasma concentration and renal clearance, however.

In contrast, Schedl et al. (1959) measured total and filterable cortisol as well as GFR and urinary cortisol, concluding that 80-90% of cortisol is reabsorbed. This group proposed the involvement of active reabsorption processes, which operate near the T_m at physiologic concentrations of the glucocorticoids. Their conclusions were based on hypothetical calculations of the amount of glucocorticoid the kidney should excrete daily versus measured excretion. No further support for apparent saturation within the physiologic concentration range of the steroids has been published.

The contribution of renal clearance to systemic clearance was analyzed by Rose et al. (1981). They observed after oral PO and intravenous POH administration (dosage range 5 to 50 mg) that 2-5% of either compound was excreted as PO and 11-24% was excreted into human urine as POH. The apparent renal clearances were nonlinear and not related to protein binding. No consistent trend with dose was observed and inter-subject variability was great.

The results in animals are similar to those of humans. Burstein et al. (1964) observed great variability in glucocorticoid excretion between and within guinea pigs. They observed no correlation between urine flow and corticosteroid excretion; other groups produced conflicting results: Lazo-Wasem and Hier (1958) found a significant correlation between urine flow and 17-hydroxycorticosteroid excretion in guinea pigs. Binding determinations were not performed in either study.

In the dog, Boonayathap and Marotta (1974b) found evidence for a T_m of cortisol. They claimed that a T_m had not been found in earlier studies because the concentrations had only been varied within a narrow range. They employed free cortisol concentrations up to

1200 ng/ml, and observed a T_m at 9 μg/min for unbound cortisol. This is much beyond concentrations experienced physiologically (unbound cortisol ~20 ng/ml).

Isolated kidney preparations

In many ways, the isolated kidney experiment is the preferred method of study, as arterial concentrations and flow rates are known. Therefore, results from such experiments may be more reliable that those obtained by investigation *in vivo*. The perfused rat kidney (Rocci et al., 1981) exhibited bidirectional interconversion of PO and POH. Initial concentrations began at 1000 ng/ml and about half of the perfused drug was metabolized irreversibly by the end of the experiment (recirculating system). POH urinary excretion was less than 7% in all cases and not related to dose. Rocci and coworkers (1981) concluded that filtration and passive reabsorption regulated steroid excretion in the rat.

The half-life of ^{14}C-cortisol in the perfused rat kidney was estimated to be about 10 to 15 minutes (Reach et al., 1977). Initial concentrations of cortisol were near physiologic values, 80 ng/ml; after 120 minutes of recirculation, 77% of the radioactivity remained in the perfusate and 12% was present in the urine. Cortisol was not apparently glucuronidated by the rat kidney and urinary excretion was found to be diuresis-dependent.

Szefler et al. (1980) perfused rat kidneys with prednisone and prednisolone and observed nonlinearities. The half-life of disappearance of PO from the perfusate increased from 5 to 30 minutes after initial concentrations of 100 to 1000 ng/ml; that of POH increased from 25 to 78 minutes. The steroids were eliminated primarily as metabolites. Urine flow rate and steroid perfusate concentrations were important determinants in the urinary excretion of both PO and POH.

Boonayathap and Marotta (1974b) performed stop flow studies in the dog kidney, at concentrations ranging from 100 to 4000 ng/ml. In this technique, the ureteral catheter was occluded for 8 minutes; immediately upon release, a series of small volumes of urine were collected in series so as to measure the secretion and/or reabsorption activity of

specific sites within the nephron. They observed that cortisol was absorbed primarily along the distal tubules and T_m was achieved at about 14 μg/min; the glucuronide did not appear to exhibit saturable transport.

The most elegant studies of the renal excretion of cortisol have been performed by Franke et al. (1982), using micropuncture as well as isolated perfusion techniques of the rat kidney. A linear correlation was found between fractional sodium and cortisol reabsorptions (the respective clearances divided by the inulin clearance); this suggested that the reabsorption of cortisol was dependent upon the glomerular filtration rate. Cortisol reabsorption in the proximal tubule and Loop of Henle demonstrated a dependence on urine flow rate. Net renal excretion processes appeared to be linear; the total nephron, including the collecting duct, seemed to be able to compensate for changes in cortisol reabsorption in the proximal portions of the nephron. No saturable transport mechanisms were evident within the concentration range of 9 to 375 ng/ml Thus, Franke and coworkers (1982) concluded that there was no evidence for a specific cortisol transport system in the rat kidney.

The general conclusion from all isolated renal studies with glucocorticoids is that in most species, excretion is apparently linear at physiologic through moderate therapeutic doses; glomerular filtration, passive reabsorption and urine flow rate are probable contributors to glucocorticoid disposition. Renal metabolism of the glucocorticoids occurs and at high doses of steroid, a transport maximum may become apparent.

Development hypothesis and experimental approach

Chapter 1 addressed the steroid metabolizing enzymes which act on cortisol, cortisone, corticosterone, prednisolone and prednisone. This chapter has reviewed the disposition of the glucocorticoids *in vivo*, with particular emphasis on PO and POH. It is evident that many factors other than enzymatic biotransformation modulate the disposition

of these interconverted species. The nonlinear kinetics of PO and POH may be caused by a number of interrelated factors and specific results may be a function of study design.

It is necessary to separately examine the three potential contributors to the nonlinear *in vivo* disposition of these glucocorticoids: plasma protein binding, metabolic pathways and renal excretory pathways. Additionally, organ-specific contributions to elimination must be determined since there are great differences in interconversion and irreversible elimination exhibited by individual organs. Endogenous steroids should be removed to minimize the effects of competition for binding or metabolic sites. It would be useful to eliminate nonlinearities in protein binding, as calculations and experimental work are greatly simplified.

The rabbit was chosen as the model species due to its similarity to humans with respect to clearance changes, binding profiles, renal excretion and metabolism. The selection of perfused organ systems allows for the examination of the contributions of individual organs to total body disposition. Complications surrounding nonlinear binding can be eliminated by the omission of CBG from the perfusion medium. If red blood cells are included, physiologic flow rates can be used and the results are more useful in clearance calculations. Endogenous glucocorticoids can be removed by the substitution of synthetic perfusate for autologous or homologous blood.

Results from individual organ examination must not be construed to be exactly representative of the *in vivo* condition. Particularly, neuronal innervation, lymph drainage and the effects of organ byproducts and metabolites on the function of other organs are not considered. However, much useful information can be obtained which is presently lacking or controversial.

Chapter 3

Analytical Methodology

Quantitation of glucocorticoids

The glucocorticoids have been measured in biologic fluids by various methods since their large scale introduction in the 1950's. The earliest methods used scintillation counting whereby drug and metabolites were viewed nondiscriminately. Functional group assays such as the Porter-Silber reaction were used (Sandberg and Slaunwhite, 1957), but were by definition nonspecific. Later, thin layer chromatography and fluorescence were developed for the separation of metabolites of various polarities from the parent compounds (Jenkins, 1966). Quantitation was difficult with these methods, and specificity was uncertain. Early gas chromatographic methods (Bacon, 1969) were not sensitive enough to detect plasma concentrations of endogenous glucocorticoids. Competitive protein binding and radioimmunoassays were then utilized for quantitation (Pegg and Keane, 1969; English et al., 1974; Colburn and Buller, 1973). These two methods were not specific in their separation, as endogenous glucocorticoids were indistinguishable from their synthetic analogues. Gas chromatography-mass spectrophotometry (GC-MS) was next developed for specific quantification (Matin and Amos, 1976) but the major deterrents to its use were availability and cost of the instrumentation. In the late 1970's, a relatively affordable technique with high specificity was developed: high performance liquid chromatography (HPLC). A normal phase HPLC assay could resolve 10 ng of steroid and differentiate

endogenous from Δ^1-glucocorticoids easily. Several methods were developed with only subtle differences (Frey et al., 1979; Loo et al., 1977; Rose and Jusko, 1979). In recent years, reverse phase systems have been published which appear to perform as well as normal phase systems (Cheng et al., 1988).

Prednisone and prednisolone in plasma and perfusate

The method of measurement of prednisone (PO) and prednisolone (POH) in plasma and perfusate is adapted from Frey et al. (1979), with a clean up procedure (Stewart et al., 1982) utilizing disposable solid phase extraction tubes (Chem Elut, E 1003, Analytichem, Harbor City, CA).

The hardware was composed of an automatic sample injector (WISP, model 710B, Waters, Milford, MA), a pump (Beckman, model 112, San Ramon, CA), an ultraviolet detector (Perkin Elmer, model LC-15, San Jose, CA) and an integrator (Hewlett-Packard, model 3390A, Palo Alto, CA). The column was silica, 5 μm, 25 cm x 4.6 mm I.D. (Beckman, Ultrasphere SI, Fullerton, CA). All steroids were purchased from Sigma (St. Louis, MO); solvents were HPLC grade Fisher products (Santa Clara, CA).

Extraction: To each tube, one of the following was added: 1.0 ml plasma sample (or less with water, q.s. 1 ml), 100 μl standard solution plus 1 ml blank plasma (preparation to follow) or 1 ml blank plasma or blank perfusate (preparation to follow). To each tube, 100 μl internal standard solution (dexamethasone) was added, followed by an alkalinization step to aid in the removal of acidic contaminants using one ml 0.1 N NaOH. The solution was vortexed then poured over Chem Elut tubes and allowed to adsorb for five minutes. Five ml methylene chloride was added to the empty test tubes to rinse them and poured over the Chem Elut tubes, twice. When all solvent appeared to have passed through the tubes, the eluate was evaporated to dryness under nitrogen gas. To quicken the evaporation step, the samples can be heated in an N-Evap (Organomation Associates,

South Berlin, MA) water bath to about 30 °C without degradation. The dried samples were reconstituted with 200 µl of this solution:

97 ml ethylene chloride
2 ml methanol
1 ml tetrahydrofuran
0.05 ml glacial acetic acid.

Ethylene chloride was substituted for methylene chloride in this reconstitution mixture to minimize evaporation of the sample solvent prior to injection by the auto-sampler. Fifty µl was injected onto the HPLC column and the peak areas were integrated. A standard curve was created as concentration versus peak area ratio. One standard curve was required for each 20 to 30 samples.

Blank plasma preparation: Plasma drawn from healthy human volunteers was treated with activated charcoal to remove endogenous glucocorticoids, as adapted from Chen (1967). One unit of freshly drawn plasma was placed in a semipermeable membrane tube (Spectra/Por *2, American Scientific Products, McGaw Park, IL) with ends secured and suspended in a saline solution. To the saline was added 60 g powdered activated charcoal (Fluka-Garantie, Buchs, Switzerland). The 4-liter flask was kept in a refrigerated room and the charcoal suspension was continuously circulated by a magnetic stirrer. The solution and charcoal were changed after 24 hours. The procedure required 40 to 50 hours for completion. This time was determined by testing the plasma every five hours after 40 hours. The sample was extracted and analyzed as above. When no corticosterone, cortisone or cortisol peaks were evident, the plasma had been dialyzed enough to use as a blank.

Blank perfusate preparation: Bovine serum albumin (4.5 g, Sigma) and glucose (100 mg) were placed in 100 ml cold Krebs-Heinseleit buffer (see below) and refrigerated overnight. The solution was filtered by gravity through Whatman no. 1 paper (Whatman,

Maidstone, England). The pH was brought to 7.4 with sodium bicarbonate. Ten ml aliquots were frozen and replaced weekly.

Krebs-Heinseleit buffer: The following solutions were prepared and combined in this manner:

electrolyte	amount (g)	water (ml)	volume (ml)
$MgSO_4$	9.55	250	3.8
NaCl	90.0	500	38.3
KH_2PO_4	10.6	500	7.8
KCl	5.75	500	31.2
$NaHCO_3$	14.0	1000	150
$CaCl_2$	6.10	500	22.7

(These solutions are stable for three months at room temperature.) About 500 ml of double distilled water was added to a 1000 ml volumetric flask; the first five electrolyte solutions above were added in their prescribed volume and swirled to mix. While swirling, the $CaCl_2$ solution was slowly added; some cloudiness appeared temporarily but disappeared within a few seconds. The volume was brought to 1000 ml with water and adjusted to pH 7.4 with saturated $NaHCO_3$ in 0.9% NaCl. Cloudiness of the solution could be attributed to precipitated calcium phosphate and the solution was discarded. The use of fresh $NaHCO_3$ solution appeared to prevent this occurrence. The solution was stored tightly sealed to prevent loss of CO_2. The buffer was stable in the refrigerator for one month or as long as no precipitate or cloudiness is visible.

Note: the precipitated calcium phosphate can be dissolved by bubbling CO_2 in or adding a small volume of dry ice to the flask; this must be performed within minutes of precipitation for effectiveness. The resultant pH of the solution is in the range of 6.8 to 7.0 and must be adjusted accordingly.

Dexamethasone internal standard solution (10 mg/L): Dexamethasone (Sigma), 5.0 mg, was accurately weighed and dissolved in 500 ml methanol. The solution was stored refrigerated or in the freezer and replaced periodically, corresponding with new standard curves.

Glucocorticoid standard solution preparation: About 20 mg each of prednisone, prednisolone, cortisol and cortisone (all obtained from Sigma) were very accurately weighed, combined and dissolved in 20.0 ml methanol. This super stock solution (approximately 1 mg/ml) was used in sequential dilution steps.

These stock solutions were prepared as follows:

Super Stock Volume (ml)	Water Volume (ml)	Resultant Concentration (ng/100 µl)	Stock
1	9	10,000	X
1	99	1,000	Y
1	999	100	Z

These standard solutions were then prepared from the above stock solutions as follows:

Stock Solution	Stock Volume (ml)	Water Volume (ml)	Resultant Concentration (ng/100 µl)	Standard
X	2	8	2,000	A
X	1	9	1,000	B
Y	5	5	500	C
Y	2	8	200	D
Y	1	9	100	E
Z	5	5	50	F
Z	2	8	20	G
Z	1	9	10	H

The solutions were stored at -20 °C and were stable indefinitely, but were replaced every 12 months or with the start of a new project.

The mobile phase used in this analysis was prepared by combining the following:

960 ml methylene chloride
30 ml methanol
10 ml tetrahydrofuran
0.5 ml glacial acetic acid.

The solution was filtered through a Millipore glass funnel filter (Millipore, Bedford, MA) with the aid of a vacuum using Whatman paper number 1 or 2 (Whatman). It was stirred and degassed with the vacuum for 10 minutes, ensuring that the methanol did not begin to boil (evaporate). The mobile phase container was tightly sealed as two of the components are volatile and water contamination is detrimental to chromatographic results. The mobile phase was recirculated for either 100 to 150 injections or three days, then replaced. Flow rates was varied from 1.2 to 2.0 ml/min to obtain optimal resolution and retention times. With column use, the steroid retention times decreased and were lengthened by reduction of the methanol from 3.0% sequentially, by about 0.2%, as needed. The sensitivity was set at 0.004 A.U.F.S. (absorption units full scale) and detection was performed at 254 nm. Linearity was observed through 3000 ng/ml and the detection limit of the assay was 20 ng PO and POH in plasma or perfusate.

A sample chromatogram of human plasma at the completion of a six hour infusion of 40 mg prednisone to subject 4 is presented in Figure 3.1. Table 3.1 gives the interday and intraday variabilities for the determination of prednisone and prednisolone in plasma. The low concentration of prednisone (50 ng) displayed the greatest intra- and interday variabilities: 13.2 and 11 % C.V.; the elution of lipophilic compounds may have interfered with the PO peak because of its early elution. The higher prednisone concentration (500 ng/ml) and the two prednisolone concentrations demonstrated <10% C.V. Perfusate samples performed at least as well as plasma as they possessed even cleaner baselines (statistics not performed).

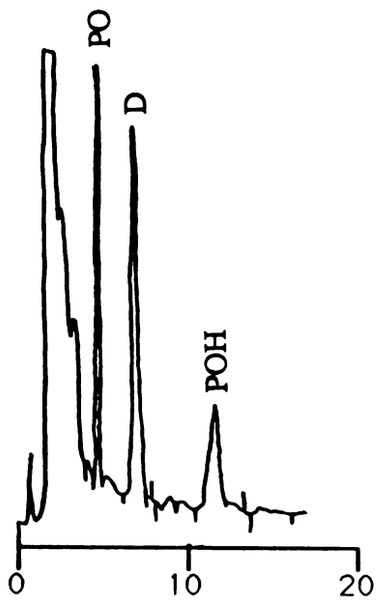

Figure 3.1. Chromatogram of plasma at completion of a six-hour infusion of prednisone, 40 mg total dose, to human subject 4. Key: prednisone, PO; dexamethasone, D; prednisolone, POH.

Table 3.1. Interday and intraday variability and precision for prednisone and prednisolone concentration determinations in spiked plasma samples.

	Intraday		Interday	
Standard Concentration (ng/ml)	Concentration (ng/ml)	C.V. (%)	Concentration (ng/ml)	C.V. (%)
Prednisone				
50	50.6 ± 5.6	11.0	49.7 ± 6.6	13.2
500	510 ± 44	8.6	481 ± 47	9.8
Prednisolone				
50	50.9 ± 2.7	5.4	48.6 ± 4.9	10.0
500	506 ± 30	5.9	519 ± 43	8.3

n = 6
abstracted from L.E.Gustavson thesis
plasma samples were spiked and stored at -20 °C until analysis
interday determinations were made over a six week period

Prednisone and prednisolone in urine

The urinary assay of prednisone, prednisolone and their endogenous analogues was adapted from Frey et al. (1979). Urine traditionally yields more interfering peaks than does plasma and there are a great variety of acidic products which yield a large chromatographic front. Therefore, an additional alkalinization and drying stage was added to the plasma extraction procedure; sensitivity was reduced to 0.008 A.U.F.S for human urine analyses, versus the plasma. Kidney perfusion urine did not require the additional extraction step or reduced sensitivity and, in essence, was treated like plasma.

As urine cannot be dialyzed to remove endogenous interfering substances such as glucocorticoids, some investigators use human urine and subtract the peak areas obtained from the endogenous glucocorticoids in standard samples. The use of human urine is satisfactory if endogenous glucocorticoids are not being measured and no interfering peaks occur near PO and POH. Other methods dilute human urine so as to minimize the contribution of the interfering peaks. In our case, water was substituted for blank human urine to assure consistency and a clean baseline; this is because volunteer subjects' blank urine gave quite noisy and highly variable baselines. Urine replacement solution (described below) was used for blank perfusate urine.

Extraction: It was necessary to dilute the urine samples 1/2 to 1/10 for extraction to ensure that sample readings fell within the range of the standard curve. The plasma extraction steps through the Chem Elut extraction were repeated. To the methylene chloride extract, one ml 0.1 N NaOH was added and mixed by inversion for 15 minutes. The aqueous (top) layer was aspirated after centrifugation (Beckman, model TJ-6, Palo Alto, CA). One g Na_2SO_4 was placed in clean tubes and the organic solution was transferred into these tubes and shaken well. After sitting for one hour, the solution was decanted and evaporated as above. An equivalent but separate set of standard solutions was used. A

sample chromatogram of human urine is presented in Figure 3.2, obtained from subject 4 at the completion of a six hour infusion of 20 mg prednisolone. Inter- and intraday variabilities for this assay are presented in Table 3.2. As with plasma, PO demonstrated the greatest variability, undoubtedly associated with late front peaks in urine. Linearity was observed through 3000 ng and the sensitivity of the urine assay is 25 ng PO and POH, less than that of plasma (10 ng).

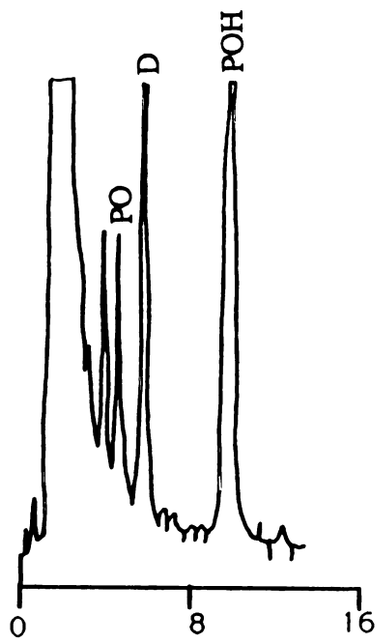

Figure 3.2. Chromatogram of human urine collected between four and six hours at completion of a six hour infusion of prednisolone, 20 mg total dose, to human subject 4. Key: cortisone, CO; prednisone, PO; dexamethasone, D; prednisolone, POH.

Table 3.2 Interday and Intraday variability and precision for prednisone and prednisolone concentration determinations in urine.

	Intraday		Interday	
Standard Concentration (ng/ml)	Concentration (ng/ml)	C.V. (%)	Concentration (ng/ml)	C.V. (%)
Prednisone				
64	75 ± 10	13.3	84 ± 9.6	11.0
320	316 ± 51	16.1	330 ± 32	9.8
2400	2504 ± 58	2.3	2515 ± 54	2.1
Prednisolone				
65	53 ± 8	6.9	58 ± 3.6	6.2
325	312 ± 40	5.9	344 ± 28	8.1
2438	2439 ± 133	5.4	2514 ± 58	7.4

n = 6
urine samples were spiked and stored at -20 °C until analysis
interday determinations were made over a six week period

Prednisone and prednisolone in tissue incubation samples

Incubation experiments were performed with initial concentrations of PO and POH of about 1000 ng/ml. The tissue preparations were either minces, homogenates or microsomes of rabbit or human organs. The samples were clean from an analytical perspective, so it was only necessary to extract the glucocorticoid; tissue debris precipitated in the organic solvent (methylene chloride). There were no significant binding proteins present in the incubated preparations, so a simple extraction method was employed, adapted from Lakshmi and Monder (1985). Methylene chloride was substituted for ethyl acetate to leave behind the more polar metabolites, or preferentially extract the prednisone and prednisolone. Additionally, an internal standard was incorporated and an HPLC assay was used, rather than column chromatography.

Extraction procedure: The tubes for the samples were prepared in advance, to contain two ml methylene chloride and 1000 ng dexamethasone. At the designated sampling times, the one ml sample was withdrawn from the incubation mixture and added to the tube. The tube was vortexed for 10 seconds to stop the enzymatic reaction, then centrifuged (2000 g x 10 min) to separate organic, aqueous, and tissue fractions. The top aqueous layer was aspirated, with partial removal of the tissue layer. Next, the methylene chloride solution was transferred to a dry tube leaving behind biologic tissue and residual water. The sample was evaporated under nitrogen; it was capped and frozen at -20 °C until analyzed.

Standard curves for Incubation experiments: To each tube, one of the following was added: 100 µl of standard solution, containing 20 to 1000 ng each of PO and POH, 100 µl dexamethasone internal standard solution (10 mg/L) or 1 ml water. Two ml methylene chloride was added and the solution was vortexed for 10 minutes at 2000 g. The top aqueous layer was aspirated. The methylene chloride layer was then transferred to a clean tube with care to leave behind any residual water. Evaporation under nitrogen was performed until the samples were completely dry, as even small quantities of water have deleterious effects on chromatographic quality and baseline stability. The samples were then capped and stored at -20 °C until analyzed. The samples were removed from the freezer about six hours before analysis and uncapped, which allowed any resultant condensation to evaporate before reconstitution of the samples. One standard curve was incorporated for each 20 to 30 incubation samples.

Figure 3.3 is a chromatogram of a sample from incubation with rabbit liver homogenates to measure prednisone reduction. The late peak corresponds to a polar metabolite of prednisone or prednisolone, potentially a 20β-hydroxylated product. The variability of this assay is presented in Table 3.3. The low coefficient of variation obtained with perfusate samples demonstrates the effect of plasma components on assay sensitivity,

as 10 ng of PO and POH was the limit of detection in the perfusate system, compared to 20 ng for the plasma assay. Percentage recoveries of 1000 ng of PO and POH were 96.4 ± 3.6 and 90.5 ± 6.2, respectively.

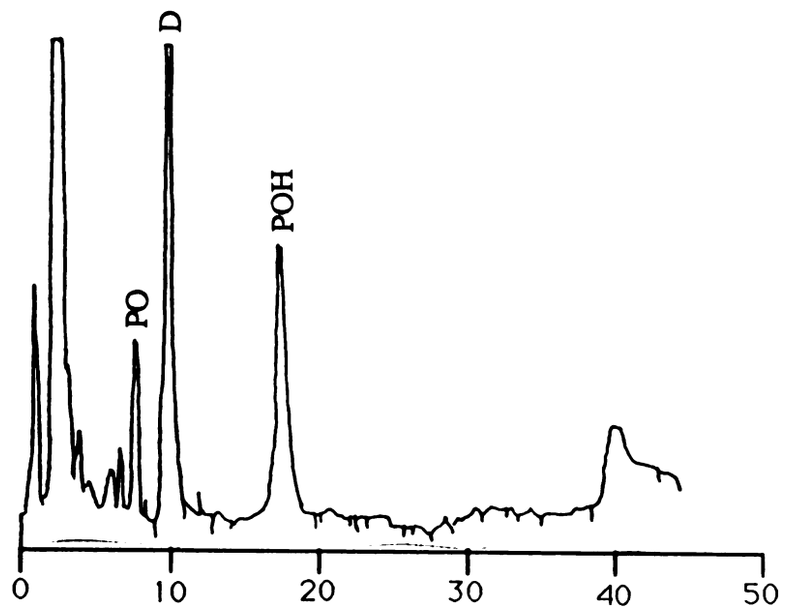

Figure 3.3. Chromatogram of an incubation sample of prednisone with rabbit liver homogenates. Sample was obtained 10 minutes after the addition of 1000 ng/ml prednisone. Key: prednisone, PO; dexamethasone, D; prednisolone, POH.

Table 3.3 Intraday and interday variability and precision for prednisone and prednisolone determinations in incubation samples

Standard Concentration (ng/ml)	Intraday Concentration (ng/ml)	Intraday C.V. (%)	Interday Concentration (ng/ml)	Interday C.V. (%)
Prednisone				
50	51.5 ± 0.8	1.5	52.6 ± 1.2	2.3
200	187.6 ± 5.7	3.1	184.1 ± 7.7	4.2
1000	1063 ± 14.5	1.3	1078 ± 42.0	3.9
Prednisolone				
50	50.1 ± 0.8	1.5	50.6 ± 1.2	2.3
200	185.0 ± 7.7	4.2	189 ± 9.3	4.9
1000	1042 ± 19.3	1.9	1057 ± 29.6	2.8

n = 6
phosphate buffer was spiked and stored at -20 °C until analysis
interday determinations were made over a four week period

Prednisolone phosphate in plasma

When a prodrug is administered, its disappearance must be measured or assumptions regarding its elimination must be made. Prednisolone disodium phosphate is often administered as it is the most rapidly hydrolyzed parenteral form of POH available. There is no published assay for the measurement of prednisolone phosphate in plasma. Prednisolone phosphate and its degradation products have been measured in pharmaceutical formulations (Dijkstra and Dekker, 1982); prednisolone acetate (Carlin et al., 1988) and hemisuccinate (Derendorf et al., 1985) have been quantitated in plasma.

In the development of this assay, several internal standard solutions were examined. Hydrocortisone phosphate was indistinguishable from prednisolone phosphate; dexamethasone phosphate demonstrated a very long retention time under these conditions. (The prednisolone and hydrocortisone phosphates were graciously supplied by Merck

Sharp and Dohme Laboratories, West Point, PA.) A series of steroids was examined, but none were found to be appropriate: neutral steroids were found to yield very long retention times, approaching one hour for diethylstilbestrol, spironolactone, progesterone and equilin. Other steroids gave retention times which interfered with that of prednisolone phosphate: triamcinolone and 6α-methylprednisolone. Commonly used drugs were examined and found to be better potential internal standards: acetaminophen and salicylic acid. Salicylic acid, 2-hydroxybenzoic acid, was selected to serve as the internal standard.

Saliclyic acid internal standard solution: About 1 mg salicylic acid was accurately weighed and dissolved in 100 ml methanol, yielding a resultant concentration of 10 μg/ml. The solution was stored at room temperature, tightly sealed and was stable for several months.

Prednisolone phosphate standard solutions: About 10.0 mg prednisolone phosphate was accurately weighed and dissolved in 1.00 ml water. This super stock solution of the phosphate ester provided about 1.0 mg/ml. The super stock solution was subsequently diluted to make stock solutions by combining 1.0 ml super stock and 5.0 ml water, yielding a concentration of about 167 μg/ml prednisolone phosphate.

The standard solutions were prepared as follows:

Stock Volume (ml)	Water Volume (ml)	Resultant Concentration (μg/100 μl)	Standard
2	12	2.4	Q
2	20	1.5	R
1	13	1.2	S
1	25	0.64	T
1	40	0.41	U
0.5	40	0.21	V
0.5	80	0.10	W

Extraction: Sample tubes were prepared to contain one of each of these: one ml of plasma sample, one ml blank plasma + 100 µl standard solution, or one ml blank plasma. One hundred µl salicylic acid internal standard solution was added, followed by one ml 0.1 N NaOH and then vortexed. Three ml hexane was added and the samples were shaken for 10 minutes. After centrifugation, the organic layer was discarded. This step removed a great portion of the neutral steroids present in the sample and reduced the size of the chromatographic front. Next, one ml 1.0 N HCl was added and the samples were vortexed, followed by three ml ethyl acetate. They were then shaken for ten minutes, centrifuged and the organic layer was separated and reserved. The three ml ethyl acetate wash was repeated and the organic extracts were combined then evaporated under nitrogen gas. The phosphate ester is not heat stable, so the extracts were evaporated at room temperature. The samples were reconstituted in 200 µl 20% methanol and 50 to 200 µl was injected.

HPLC conditions: The mobile phaseconsisted of the following:

215 ml acetonitrile
15 ml tetrahydrofuran
1.5 ml phosphoric acid 85%
q.s. 1000 ml water

The solution was filtered and degassed for ten minutes. A Waters µBondapack column, C18, 39 mm I.D. x 30 cm (Milford, MA) was used with a flow rate of 1.7 ml/min, detection at 254 nm, and sensitivity at 0.005 AUFS.

A chromatogram of a plasma sample from a subject taken five minutes after a short intravenous infusion of 31.7 mg prednisolone phosphate is presented in Figure 3.4. Assay sensitivity was 0.2 µg/ml; inter- and intraday variabilities are displayed in Table 3.4. Coefficients of variation were below 10% for 0.678 and 3.41 µg/ml samples.

Figure 3.4. Chromatogram of prednisolone phosphate in plasma of subject 6 taken five minutes after a short intravenous infusion of 31.7 mg prednisolone phosphate. Key: prednisolone phosphate, PP; salicylic acid, SA.

Table 3.4 Inter- and intraday variability and precision for prednisolone phosphate concentration determinations in plasma samples.

Prednisolone Phosphate Concentration (μg/ml)	Intraday Concentration (μg/ml)	C.V. (%)	Interday Concentration (μg/ml)	C.V. (%)
0.678	.633 ± .06	9.5	.659 ± .051	7.8
3.41	3.52 ± .10	2.7	3.58 ± .17	4.6

n = 6
plasma samples were spiked and stored at -20 °C until analysis
interday determinations were made over a three week period

interday determinations were made over a three week period

Determination of unbound fraction of glucocorticoid

The physiologically active fraction of drug is believed to be that which is not bound to plasma proteins. Measurement of this fraction is accomplished in many ways and has most frequently been performed by equilibrium dialysis (Sophianopoulos et al., 1978). Other methods include ultrafiltration, gel filtration, ultracentrifugation, gel electrophoresis, ammonium sulfate precipitation and charcoal adsorption. None of these techniques is ideal, as each possesses advantages and disadvantages. It is now recognized that dilution of drug (Behm and Wagner, 1979) and volume shifts (Tozer et al., 1983) are potential sources of error inherent to equilibrium dialysis.

Equilibrium dialysis was used for the measurement of the unbound concentration of prednisone and prednisolone in human plasma samples and rabbit perfusate samples. Tritiated steroid was used to accurately measure the free drug at very low concentrations, where POH demonstrates nonlinear binding characteristics due to saturation of corticosteroid binding globulin. The sensitivity of the HPLC assays was not high enough to accurately estimate the unbound concentration of drug in the low nanogram range.

Equilibrium dialysis

Equilibrium dialysis is the technique most often used to study the binding of drugs to proteins (Chignell, 1983). Estimates of binding parameters are a function of the method of calculation (Tozer et al., 1983). The determination of the unbound concentration of drug by equilibrium dialysis is not as simple as measuring the concentration of drug on the buffer side of the membrane. Two sources of error occur: firstly, diffusion of drug into the buffer compartment, as described by Behm and Wagner (1979), results in drug loss from the plasma side of the chamber. Secondly, osmotic fluid shifts increase the volume of the plasma compartment (Tozer et al., 1983), diluting the binding proteins.

Methodology

The binding of POH begins to show nonlinearities at about 100 ng/ml. Since prednisolone may have an unbound fraction as low as 0.05, radiolabelled drug was used to quantify the steroid. Tritiated POH and PO were obtained from Amersham (Arlington Heights, IL) as [2,4,6,7-^{3}H]prednisolone and [1,2(n)-^{3}H]prednisone, specific activity 1 mCi/ml. Their synthesis involved reduction of a cortisol or cortisone derivative with tritium gas, followed by dehydrogenation of the A ring; the product was then purified by HPLC and verified by thin layer chromatography (silica gel with various organic cosolvent systems) and/or HPLC (similar to the system described above for the quantitation of PO and POH in plasma).

Estimation of the radioactive purity of ^{3}H-PO and ^{3}H-POH: The specifications of these products are presented in Table 3.5. A verification of these values is required periodically to assure that chemical decomposition has not occurred, which would then necessitate HPLC separation and purification. A solution of 97% methylene chloride, 3% methanol and 0.05% glacial acetic acid (Fisher, HPLC or reagent grade quality) was added to a rectangular glass chromatography development chamber (Fisher) to a depth of about five mm. A large piece of filter paper (Whatman, no. 1, Maidstone, England) was cut to extend from the base of the chamber to one cm from the top and taped in place. The chamber was equilibrated.

Five µl of the commercial solution in toluene:ethanol (1:1) was pipeted into a scintillation vial and the solvent evaporated. The residue was dissolved in 1 ml methanol to result in a concentration of 10^7 dpm/ml. Ten µl was then applied to one band of a silica gel plate (LK5DF linear-K silica gel, Whatman, Maidstone, England). The spot was dried and then placed in the development chamber, with the silica side facing the saturated filter

paper. The plate was removed when the solvent front migrated to within 1 cm of the top and dried on the countertop, then placed in an oven (80 to 100 °C) for 5 minutes. The silica was then scraped from the plate with a scalpel blade in 1 cm^2 sections; the scrapings were placed into a scintillation vial with 10 ml Aquasol and counted for 5 minutes. The percentage radioactivity associated with each fraction was quantitated and the radioactive purity of the compound was determined. Deviation of more than 5 % from the labelled purity necessitated purification by HPLC.

Table 3.5 Tritiated prednisone and prednisolone: technical information

Prednisone

[1,2(n)-^{3}H]prednisone

specific activity:	34.2 Ci/mmol
	95.1 mCi/mg
radioactive concentration:	1 mCi/ml
radiochemical purity:	97%

Prednisolone

[2,4,6,7-^{3}H]prednisolone

specific activity:	68.8 Ci/mmol
	191 mCi/mg
radioactive concentration:	1 mCi/ml
radiochemical purity:	98%

^{3}H-PO and ^{3}H-POH buffer: The quantity of label needed for experiments was determined in this manner: the minimum cpm (counts per minute) required for each sample (100 to 150 µl) was 1000. The minimum free fraction encountered would be 0.05, so 2 x 10^4 cpm per sample were needed. Counting efficiency was assumed to be 50%, so 4 x 10^4 dpm (disintegrations per minute) were needed for each sample. If the minimal sample volume was 100 µl, the concentration of the label in buffer must be 4 x 10^5 dpm/ml. Twenty µl of the commercial solution was evaporated under nitrogen and the residue dissolved in 110 ml phosphate buffer. This buffer solution of labelled drug is stable for three months.

Phosphate buffer (0.13 M, pH 7.4): These stock solutions were prepared in water: Na_2HPO_4, 1.85 g in 100ml, and KH_2PO_4, 1.77 g in 100 ml. They were stable at room temperature for several months. They were combined in the ratio of 4:1 and refrigerated; the buffer was stable for several months, as well, or as long as the pH remained within 0.1 pH unit of 7.4.

Equilibrium dialysis procedure: The plasma samples and phosphate buffer were first brought to room temperature. The Spectra/Por*2 dialysis tubing (American Scientific Products) was soaked in water for 10 minutes to soften the tubing, then in 50% ethanol solution to remove residual fats which might impede diffusion, followed by a water rinse. The tubing was then stored refrigerated in phosphate buffer. Equilibrium dialysis cells, 0.5 ml capacity, were made by the Laboratory for Research and Development of the University of California San Francisco (similar to Fisher item number 08-666-15). A single layer of tubing was spread over one half of the dialysis cell; matched halves were secured with screws. One side of the chamber was filled with 300 µl ^{3}H-buffer using a syringe and needle (1 ml tuberculin with 20 ga needle, 1 1/2 inches long, Becton Dickinson,

Rutherford, NJ); the other side received 300 µl plasma. The compartments were then capped with screws to prevent evaporation of CO_2. A shaking water bath (GCA Corporation, Precision Scientific Group, Chicago, IL) was used to equilibrate the cells for 7 to 8 hours at 37 °C. With a syringe and needle, 150 µl of buffer was then withdrawn and added to a scintillation vial containing 10 ml Aquasol (New England Nuclear Research Products, Boston). The samples were shaken and counted for 5 minutes (Beckman model LS 1801, Beckman Instruments, Palo Alto, CA).

Corrections and calculations

Behm and Wagner (1979) were the first to document the dilutional loss of drug from the plasma compartment during dialysis. They demonstrated that the unbound fraction of drug determined by equilibrium dialysis did not correspond to the unbound fraction of drug present in the original plasma sample. Unbound drug enters the buffer compartment, reducing the unbound concentration and altering equilibrium; consequently bound drug becomes unbound to replace it and achieve a new equilibrium.

Osmotic shifts occur in equilibrium dialysis cells as well, due to the presence of plasma proteins, water and electrolytes from the buffer which cross into the plasma compartment until osmotic equilibrium is achieved. Tozer et al. (1983) determined that volume shifts during a 16-hour equilibrium dialysis of prednisolone in human plasma samples averaged 31% (1983). The degree of binding and the relative volumes of the two compartments contribute to both the effective dilution of drug and the magnitude of the volume shift. Calculations must be performed to correct for these effects. The methods employed in the analysis of this data corrected for both dilutional drug loss and volume shifts.

Calculation of unbound fraction: The unbound concentrations of prednisolone present in the original plasma samples are calculated according to the method of Tozer et al.

(1983). When initial plasma and buffer volumes are the same in equilibrium dialysis, calculations are greatly simplified. The concentration of unbound prednisolone, C_U, and the bound concentration of drug after dilution of proteins, C_{BZ}, can be defined in terms of the buffer dpm before (D_B) and after (D_{BZ}) dialysis:

$$C_U = (C_T + C^*) \cdot \frac{D_{BZ}}{D_B} \tag{1}$$

$$C_{BZ} = C_T + C^* - 2\,C_U \tag{2}$$

where C_T is the total prednisolone present in the plasma sample (measured by HPLC) and C^* is the concentration in the buffer at the start of dialysis. Bound versus unbound prednisolone concentrations are modeled using a nonlinear regression program and weighting of $1/Y_{obs}^2$ to provide increased influence to points within the low concentration range. The following expression is fit to the data:

$$C_B = \frac{CAP \cdot C_U}{\frac{1}{K_d} + C_U} + S_{POH} \cdot C_U \tag{3}$$

where CAP is the binding capacity of CBG for POH, K_d is the affinity constant for the drug to CBG and S_{POH} is the slope factor for prednisolone. S_{POH} is defined as:

$$S_{POH} = n \cdot P_{ALB} \cdot K_{ALB} \tag{4}$$

where n equals the number of POH binding sites on albumin (assumed to be 1), P_{ALB} is the concentration of albumin as determined by the Sigma assay to follow, and K_{ALB} is the affinity constant for the binding of POH to albumin.

The unbound and bound concentrations of all plasma samples can be determined from the following relationships:

$$C_U = \frac{C_T - X + \sqrt{(C_T - X)^2 + Y \cdot C_T}}{Z} \tag{5}$$

$$C_B = C_T - C_U \tag{6}$$

where X, Y, and Z are defined as follows:

$$X = \frac{S_{POH}}{K_d} + CAP + \frac{1}{K_d} \tag{7}$$

$$Y = \frac{4\,(S_{POH} + 1)}{K_d} \tag{8}$$

$$Z = 2(S_{POH} + 1) \tag{9}$$

These unbound concentrations were used in the calculation of unbound pharmacokinetic parameters in the human studies and in the rabbit organ perfusion experiments. Figure 3.5 shows binding data of subject 9 who participated in the prednisolone pharmacokinetic study presented in Chapter 9; nonlinear regression of the results of the above calculations was performed so as to obtain the binding parameters CAP, K_d and S_{POH}. This allowed the calculation of unbound prednisolone concentrations in all plasma samples. Figure 3.6

is the result of prednisone and prednisolone binding determinations in all rabbit kidney perfusion experiments, where albumin was the sole binding protein and therefore, the fraction unbound of both PO and POH remained constant across a large concentration range.

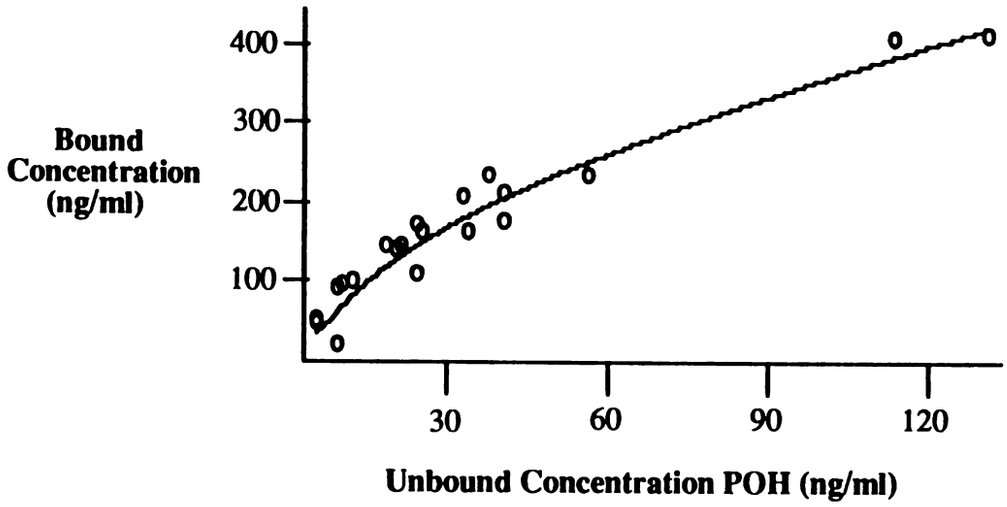

Figure 3.5. Plot of the relationship between the bound and unbound concentrations of prednisolone determined by equilibrium dialysis in human subject 9 (equation 3); the subject received five treatments of prednisone and prednisolone (see Chapter 9).

Protein measurement

Two protein measurements were made in conjunction with these experiments: albumin in plasma and perfusate samples and total protein present in organ microsomal preparations. In the first case, it was important to measure human albumin so as to calculate binding parameters; bovine albumin was quantified in perfusate samples to verify protein content. Microsomal protein concentration determinations were necessary so as to normalize the metabolic rates in the incubation experiments.

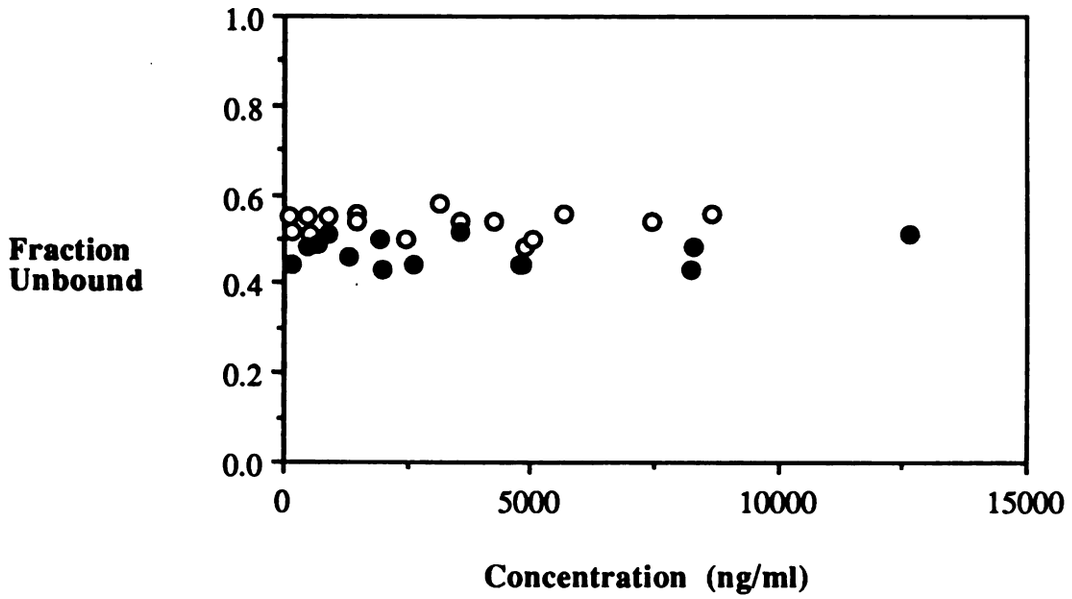

Figure 3.6. Linear protein binding of prednisone (solid circles) and prednisolone (open circles) in rabbit kidney perfusate samples (equation 6; see Chapter 7).

Albumin quantification

The albumin present in plasma and perfusate samples was quantitated using a commercially available colorimetric assay. The Sigma Diagnostics kit 631 (Sigma, St. Louis, MO) method measures the binding of albumin to bromocresol green (BCG); a blue green color is produced upon binding which obeys Beer's law at concentrations near the physiologic range.

Albumin standard solution preparation: Standards available from Sigma are human albumin at 2.0, 4.0, 6.0, 8.0 and 10.0 g/dl. Each of these was diluted as follows:

Standard Concentration (g/dl)	Volume (ml)	Water Volume (ml)	Resultant Concentration (g/dl)
2.0	0.5	0.5	1.0
2.0	1.0	0.0	2.0
6.0	0.5	0.5	3.0
4.0	1.0	0.0	4.0
10.0	0.5	0.5	5.0
6.0	1.0	0.0	6.0

Method: The absorbance reading of the spectrophotometer (Coleman 55, LC-55, Perkin Elmer, Norwalk, CT) was zeroed at 628 nm with water. Into test tubes was pipeted 1.0 ml Albumin Reagent (bromocresol green, 0.3 mmol/L). Next, 0.01 ml of either sample, water, or diluted protein standard was added to each tube. The contents were mixed by gentle inversion for 10 seconds. The absorbance was read at 628 nm, after 1 minute or less. (The same time interval was allowed for all samples.) A standard curve was prepared from 0 to 6 g/dl, and sample concentrations were determined from the regression line. For each ten samples, one standard curve was prepared. A typical standard curve is presented in Figure 3.7. Linearity was observed up to 10.0 g/dl albumin. According to the test guidelines, albumin is stable in frozen plasma for months (-20 °C) and mild hemolysis does not affect measurements.

Total protein

The determination of the total protein present in microsomal preparations was accomplished using the familiar Lowry method (Lowry, 1951). The chemical basis of this method is the interaction of alkaline copper with the phenolic groups of a protein, resulting in a measurable color change.

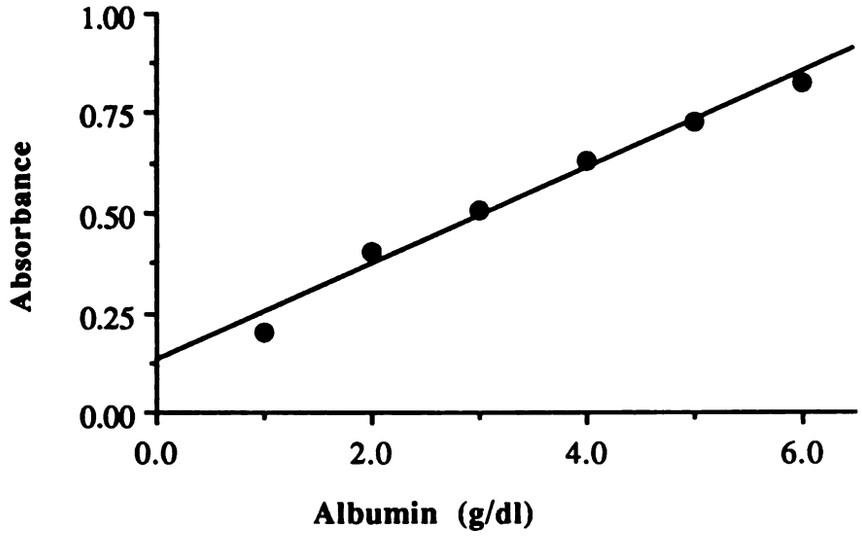

Figure 3.7. Albumin standard curve from Sigma albumin assay.
The regression line is:
Absorbance = 0.12887 + 0.11994 (Albumin, g/dl); $r^2 = 0.982$.

Protein stock solution preparation: Each of the following stock solutions was prepared in water: 2% w/v Na_2CO_3 (1 liter),1% v/w $CuSO_4.5H_2O$ (50 ml), and 2.3% w/v sodium tartrate · $2H_2O$ (50 ml). They were stable for several months. The solutions were combined in the proportion 100: 1: 1 immediately prior to use. The following solutions were also required: 2N NaOH (100 ml), 1 N Folin reagent (freshly diluted 2N Folin reagent [Sigma]), and 2 mg/ml bovine albumin fraction V stock (prepared earlier and frozen at -20 °C until use).

Standard solutions: The albumin standard solutions were prepared as follows on the day of analysis:

Albumin Stock (µl)	Water (µl)	Protein Concentration (µg/ml)	Standard
0	500	0	0
2.5	498	10	A
5.0	495	20	B
12.5	488	50	C
25	475	100	D
50	450	200	E
125	375	500	F
250	250	1000	G

Method: Duplicates of standards and triplicates of samples were prepared. Microsomal samples were diluted about 100-fold so that the concentrations fell within the range of the standard curve. The sample or standard, 0.1 ml each, was combined with 0.1 ml 2 N NaOH and hydrolyzed at 100 °C for 10 minutes. The samples were cooled to room temperature then 1 ml of the three component alkaline mixture was added. The samples stood at room temperature for 10 minutes. Folin reagent, 0.1 ml, was added and the samples were vortexed then stood at room temperature for 30 to 60 minutes. The absorbance at 750 nm was read. Regression of the standard curve yielded sample concentrations. A representative standard curve is presented in Figure 3.8; the plot of absorbance versus concentration was linear through about 1000 µg/ml.

Urea measurement

The synthetic function of a perfused organ, as presented by Hems et al. (1966), suffers early when a tissue deteriorates. Gluconeogenesis of the liver demands the maximal energy, requiring 6 moles of ATP per mole of glucose formed; urea is close behind at 4 moles of ATP. Because glucose was included in perfusate as the carbohydrate energy source, urea synthesis was measured as an indicator of hepatic viability. Ornithine

and ammonia (as the hydrochloride) were added to the perfusate as they are mammalian urea precursors.

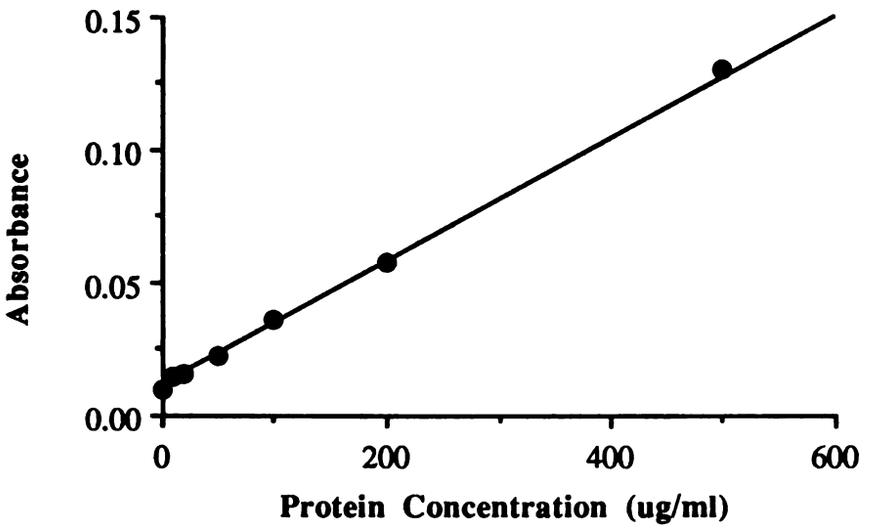

Figure 3.8. Standard curve for total protein from the Lowry protein assay. Bovine serum albumin solutions composed the standard curve. The regression line is:
Absorbance = $1.1334^{-2} + 2.3712^{-4}$ (protein, μg/ml); $r^2 = 1.000$.

The quantitation of blood urea nitrogen is primarily performed by one of two methods. The first is the reaction of urea with diacetyl monoxime; the second is urease hydrolysis with liberation of ammonia. The first method is used here, as employed in Sigma Diagnostics kit 535, Blood Urea Nitrogen (BUN). A pink chromogen is produced; according to Beer's law, the urea concentration of the sample is directly proportional to the intensity of the absorption by the chromogen, within a limited concentration range.

The assay can be performed on serum, plasma or whole blood. A protein precipitation step is recommended by Sigma for *highly* icteric or hemolyzed samples. The

rabbit liver perfusion samples were centrifuged and the red cells discarded. Some samples were slightly hemolyzed due to fragility of the aged red blood cells (none were icteric); to ensure consistency of the method, all perfusion samples, regardless of condition, were treated with the protein precipitation step.

Preparation of urea standard solutions: Benzoic acid solution, 0.1%, was combined with Sigma Urea Nitrogen Standard (150 mg/dl) as follows:

Urea Standard (ml)	Benzoic Acid Solution (ml)	Final Urea Concentration (mg/dl)	Standard
0.0	5.0	0	0
0.5	4.5	15	A
1.0	4.0	30	B
1.5	3.5	45	C
2.0	3.0	60	D
2.5	2.5	75	E

The solutions were stable for three months, refrigerated.

Protein precipitation step: 1.8 ml cold 3% trichloroacetic acid (TCA) and 0.2 ml sample or standard were mixed in a test tube. (Only the number of samples which can be read in 20 minutes were prepared at one time.) The tubes were shaken well for 15 seconds and stood for 5 minutes to allow precipitation, then centrifuged to obtain clear supernatant.

Method: Seven parts BUN Acid Reagent (sulfuric acid, 1.8 mol/L) were premixed with five parts BUN Color Reagent (diacetyl monoxime 0.18% w/v); each sample required 4.8 ml of this mixture. To the blank tube was added 0.2 ml 3% TCA; to the standards and sample tubes, 0.2 ml of clear supernatant was added. To all tubes, 4.8 ml of the above mixture was added. All tubes were simultaneously placed in a boiling water bath for 10.0 minutes, then quickly removed and placed in cold tap water for 3 to 5 minutes to stop the reaction. The absorbances of samples and standards were read versus the blank as reference at 515 nm within 20 minutes. A standard curve was prepared as concentration

versus absorbance of the standards; a representative curve is presented in Figure 3.9. Linearity was observed up to 60 mg/dl with this experimental method. Hence, if sample concentrations exceed 60 mg/dl, they were diluted and assayed again.

Urea synthesis rate

The urea synthesis rate was calculated from the slope of a plot of the cumulative amount of urea present in the perfusate versus time.

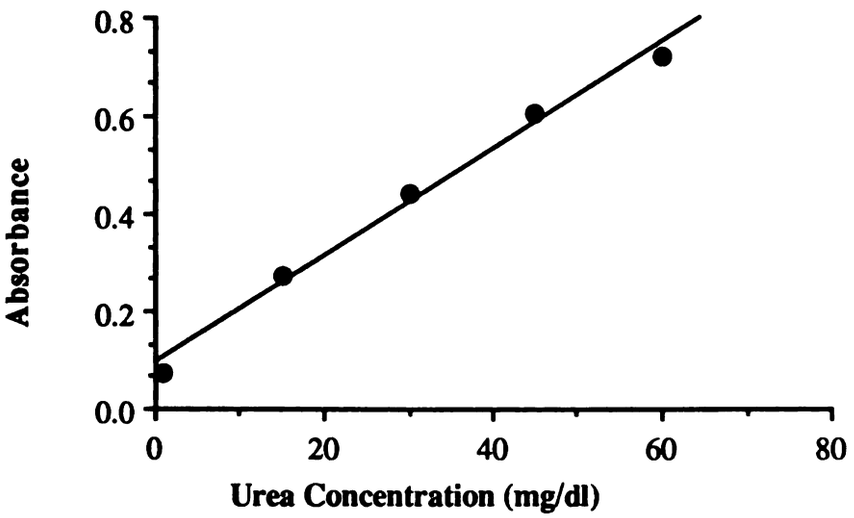

Figure 3.9. Standard curve from the Sigma blood urea nitrogen assay.
The regression line is:
Absorbance = 9.4014^{-2} + 1.0953^{-2} (urea, mg/dl); $r^2 = 0.989$.

Lactate and pyruvate measurement

Lactate and pyruvate are widely used as measures of oxygenation status of tissues; they can be an indicator of the severity of circulatory failure. Increased lactate is the cause of metabolic acidosis; pyruvate rises in liver disease, congestive heart failure, and diabetes. In this case, it is used to monitor the oxygenation status of the perfused liver.

Lactate dehydrogenase catalyzes this reversible reaction *in vivo* and is used in this assay method:

Pyruvate + NADH ↔ Lactate + NAD

Both lactate and pyruvate can be measured in terms of the formation or disappearance of NADH, which exhibits high absorption at 340 nm. In the presence of excess NADH, substantially all of the pyruvate is converted to lactate and the diminished absorbance at 340 nm is due to the oxidation of NADH to NAD. This change in absorption is reflective of the pyruvate concentration present in the original sample. Conversely, lactate is measured in the presence of excess NAD; pyruvate is formed and trapped with hydrazine. The increase in absorption from initial conditions is due to NADH formation and reflects the original lactate concentration.

Sigma Diagnostics kits 726-UV and 826-UV were used. Lactate and pyruvate concentrations change quickly after specimen collection; to minimize this occurrence, it is necessary to stabilize the samples. The only way to preserve pyruvate is to immediately precipitate blood proteins and skip centrifugation and separation steps.

Stabilization of lactate and pyruvate: Two ml of perfusate was collected and *immediately* added to a test tube containing 4 ml cold 8% perchloric acid and shaken vigorously for 30 seconds. The tube remained in an ice bath for 5 minutes and was then centrifuged for 10 minutes at 1500 to 2000 g. If the supernatant was not clear, the sample was spun again. Pyruvate is stable in this solution for 1 month refrigerated; lactate is stable for 1 week.

Pyruvate determination: For each 4 tests perfomed, 2.2 ml TRIZMA Base solution (TRIZMA 1.5 mol/L) was pipetted into a NADH vial (1 mg/vial). Two ml supernatant from the stabilization procedure above was placed in a test tube and 0.5 ml TRIZMA Base solution was added. The solution was mixed by inversion. Then 0.5 ml NADH solution was added and inverted several times then transferred to a cuvet. The absorbance of the sample was read versus water at 340 nm: this was the initial absorbance.

Quickly, 0.05 ml lactate dehygrogenase solution (>1000 U/ml crystalline suspension of lactate dehydrogenase from beef heart) was added to the same cuvette mixture and the cuvette inverted several times. After 2 to 5 minutes, the absorbance was read: this was the final absorbance. The sample was read again after 5 minutes to be sure that the reaction was complete. The calculation of pyruvate was performed simply from measured absorbances (A):

$$\text{pyruvate (mg/dl)} = (A_{initial} - A_{final}) \times 6.37$$

It was necessary to assure linearity of the system to use this simple calculation method so a calibration curve for the Perkin Elmer model LC-55 spectrophotometer was prepared (Figure 3.10). It can be seen that linearity was observed up to 3 mg/dl.

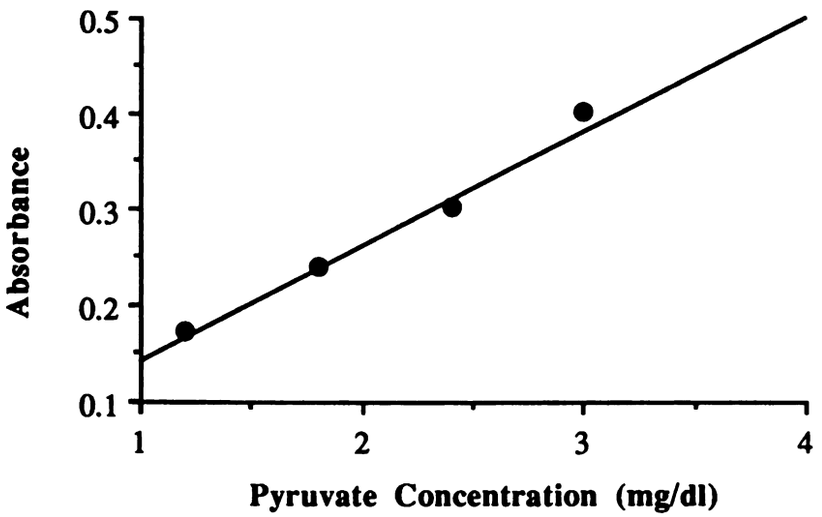

Figure 3.10. Standard curve from the Sigma pyruvate assay.
The regression line is:
Absorbance = 1.4900^{-2} + 0.12600 (pyruvate, mg/dl); $r^2 = 0.988$.

Lactate determination: The number of NAD vials to be reconstituted was determined as:

$$\frac{\text{number of samples} + 1}{2}$$

To each of the NAD vials (10 mg/vial), was added 2.0 ml glycine buffer (glycine 0.6, mol/L and hydrazine, 0.5 mol/L) , 4.0 ml water, and 0.1 ml lactate dehydrogenase. The vials were inverted several times then all of the solutions were combined in a test tube and mixed well. 2.8 ml of this mixture was pipetted into each test tube. 0.2 ml 8% perchloric acid was added to the blank; 0.2 ml clear supernatant from stabilization procedure, above, was added to the samples. The test tubes were incubated at 37 °C for about 30 minutes. The absorbance was read for the next two minutes; if the values increased more than 0.002 per minute, another 15 minute incubation was performed.
The lactate concentration was determined as follows:

$$\text{lactate (mg/dl)} = A \times 65.1$$

The linearity of this system was documented over the concentration range of 0 to 50 mg/dl lactate (Figure 3.11).

Lactate/pyruvate ratio

The lactate/pyruvate ratio is as stated, the ratio of concentrations. It is an important measure of the state of oxygenation of tissues. Schimassek (1962) determined that the isolated perfused rat liver requires 30 minutes after surgery to recover from its anaerobic respiratory state. Ratios which deviate substantially from this value are indicative of abnormal oxygenation. Higher ratios signify excessive oxygen delivery; lower ratios indicate inadequate oxygenation.

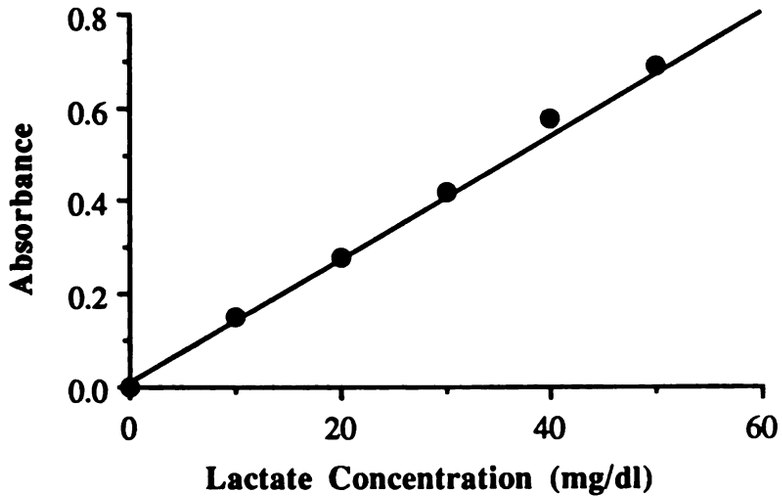

Figure 3.11. Standard curve from the Sigma lactate assay.
The regression line is:
Absorbance = $6.5238^{-3} + 1.3846^{-2}$ (lactate, mg/dl); $r^2 = 0.999$.

Creatinine measurement

The clinical significance of blood creatinine concentrations is as an index of renal function, as high creatinine plasma concentrations are indicative of renal disease. The creatinine clearance is a measure of the glomerular filtration rate and is used in this manner with rabbit kidney perfusions. Both perfusate and urine creatinine concentrations were measured.

When creatinine is treated with alkaline picrate, a yellow/orange color is formed; this reaction is not specific for creatinine, so an additional step is added. Under acidic conditions, the creatinine color fades faster than do the interfering substances' colors. The difference in color at 500 nm before and after acidification is proportional to creatinine concentration. Beer's law is obeyed over a 10-fold concentration range.

Plasma (perfusate) samples are run without dilution; urine usually requires a 10 to 15-fold dilution for use in this assay.

Creatinine standard solution preparation: Sigma creatinine standard solution, 15 mg/dl, was used to prepare this standard curve daily:

Creatinine Standard (ml)	Water (ml)	Creatinine Concentration (mg/dl)	Standard
0.0	0.30	0.0	0
0.05	0.25	2.5	A
0.10	0.20	5.0	B
0.15	0.15	7.5	C
0.20	0.10	10.0	D

Method: To each test tube was added 0.3 ml sample or 0.3 ml standard; the blank received 0.3 ml water. Three ml Alkaline Picrate solution was then added to all tubes; they were mixed and stood at room temperature for 8 to 12 minutes. The samples were transferred to cuvettes and the absorbance was read at 500 nm versus the blank as the reference; this was the initial absorbance.

To the same tubes, 0.1 ml Acid reagent (sulfuric and acetic acids)was added and mixed immediately by inversion for 30 seconds. These samples stood at room temperature for 5 minutes. The absorbance was then read at 500 nm versus the blank as the reference; this was the final absorbance.

A standard curve was prepared and sample concentrations were determined from the regression line. A representative standard curve is presented in Figure 3.12, encompassing 0 to 10 mg/dl creatinine. For samples above 10 mg/dl, the samples were diluted and assayed again as this region demonstrated nonlinearity with respect to Beer's law.

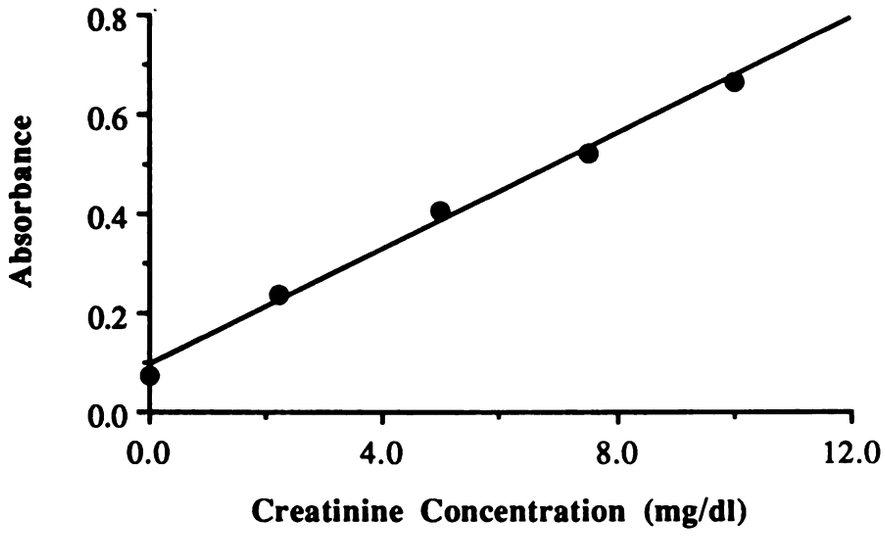

Figure 3.12. Standard curve from the Sigma creatinine assay.
The regression line is:
Absorbance = 9.6100^{-2} + 5.7591^{-2} (creatinine, mg/dl); r^2 = 0.994.

Creatinine clearance

The creatinine clearance was calculated utilizing both urinary and plasma measurements. From steady state data, the creatinine clearance is defined as:

$$CL_{cr} = \frac{\text{excretion rate}}{\text{steady state concentration}}$$

Chapter 4

Disrupted Membrane Studies

Disrupted membrane studies

The current understanding of glucocorticoid disposition is derived primarily from extensive studies with these compounds in the 1950's and 1960's; recent work did not significantly impact on this knowledge until the studies of O'Hare in 1973. In the investigation of corticosterone interconversion, O'Hare reported that disruption of cellular membranes significantly altered metabolic activity relative to that of undamaged tissue. Cultured rat adrenocortical cells were used to study the oxidation of corticosterone (11β-OH) to its 11-keto form (11-dehydrocorticosterone). Oxidation increased 250-fold in the damaged monolayer culture, relative to the undamaged culture. The cause of this observation was not determined. Subcellular delocalization of enzyme or cofactors as well as leakage from the cells were proposed as potential explanations. These results were substantiated by Nicholas and Lugg (1982) in rat lung preparations. Cortisol was poorly oxidized in the perfused rat lung, but lung homogenates and microsomes readily oxidized cortisol to cortisone. Bernal et al. (1982) documented similar results with human decidua: intact tissue demonstrated predominantly reduction, but homogenized tissue performed oppositely, primarily oxidizing cortisol to cortisone.

The results of this previous work impact on all *in vitro* steroid metabolism studies in that most of these investigations were made with tissue homogenates or microsomes.

11β-Hydroxysteroid dehydrogenase (11β-HSD) is one of a family of enzymes which oxidize steroids, including 20β-hydroxysteroid dehydrogenase (20β-HSD) and 3β-hydroxysteroid dehydrogenase (3β-HSD); these proteins could be similarly affected by cellular disruption.

The objectives of this work were multiple: the primary goal was to determine whether tissue disruption and/or subcellular fractionation of rabbit and human tissue affects glucocorticoid metabolism. The studies up to this time have yield contradictory results. The results of these experiments would dictate whether measurements of metabolic rates derived from *in vitro* studies can be applied to subsequent physiologic pharmacokinetic modeling. The relative importance of each organ studied in oxidative interconversion, reductive interconversion and elimination was to be determined. Lastly, these experiments tested the hypothesis that steroid metabolism is species-dependent. The rabbit was chosen as a model of human glucocorticoid disposition based upon *in vivo* studies (see Chapter 2); as the rabbit was not often used in studies of glucocorticoid metabolism, few references characterizing *in vitro* leporine metabolism are available.

Rationale and significance

Data obtained from *in vitro* experiments have been successfully used to model *in vivo* drug disposition for a number of drugs. Estimates of metabolic constants can be obtained from a variety of *in vitro* experimental systems; if the same results can be obtained from simple incubation studies, there is no need to perform technically difficult perfusion experiments. Dedrick and coworkers (1971) measured deamination rates of cytarabine in homogenates of human liver, kidney and heart. They subsequently incorporated the resultant Michaelis constants for these three organs as well as urinary excretion data into a physiological pharmacokinetic model and successfully predicted plasma concentrations of cytarabine in humans after a single bolus dose. Other attempts have been less successful: Wiersma and Roth (1980) incorporated enzyme kinetic parameters derived from rat liver

and lung homogenates to predict hepatic and pulmonary extraction in perfused organs. The predictions for the perfused liver were successful; pulmonary extraction was considerably underestimated.

Intuitively, intact organ studies would seem to be superior to disrupted organ preparations because capillaries remain intact, specialized reabsorptive or secretory processes function in an organized fashion, and local concentration gradients are maintained. Yet organ perfusion studies do not necessarily reflect organ function *in vivo*, as the direct or indirect effects of other organs are not considered, lymph drainage is generally impaired and neuronal innervation is compromised. However, of all of the *in vitro* preparations which may be studied, the enzymatic microenvironment is generally well conserved in the perfused organ.

This series of experiments examines the interconversion and elimination of the glucocorticoids prednisone and prednisolone in organ minces, homogenates and microsomes from rabbit liver, kidney, lung and skeletal muscle and from human liver and kidney. These results will be compared with results of prefusion studies of the same organs to determine the effects of membrane disruption on the metabolism of prednisone and prednisolone.

Organ preparations

The experiments with rabbit organs utilize tissues obtained from two sources. Rabbits were decapitated without anesthesia or were exanguinated using the same anesthetic regimen as in the perfusion experiments, i.e., ketamine/xylazine. Neither ketamine nor xylazine have been shown to affect the activity of the dehydrogenases, the major metabolic enzymes of the glucocorticoids. Ketamine is an enzyme inducer (Wright, 1982), but requires administration for several days for its effects to become evident; ketamine has also been shown to inhibit the uptake of serotonin in perfused rat lungs (Martin et al., 1989). Neither of these effects are significant for the experiments with the

glucocorticoids. Physiologically, ketamine increases cardiac output resulting in increased regional perfusion of nearly all organs (Idvall et al., 1980). This effect is advantageous in organ perfusion experiments, as it is desirable to minimize pre-perfusion anoxia. Xylazine, administered adjunctively, increases the half-life of ketamine by decreasing the rate of production of the primary metabolite of ketamine, norketamine (Waterman, 1983). As for the case of ketamine, the biochemical effect of xylazine is not significant for glucocorticoid metabolism. To test the effects of anesthetics on metabolism, microsomes prepared from unanesthetized/decapitated and anesthetized/exanguinated animals were incubated and compared. No significant differences in rates of interconversion or the elimination of prednisone and prednisolone were found (data not shown).

Human livers and kidneys were surgically removed from the donors without anesthesia, but with the administration of pancuronium bromide, heparin, phentolamine and mannitol in some cases (see Chapter 6 for details of acquisition of human organs experimentation). No information regarding the effects of these compounds on glucocorticoid metabolism or distribution could be found.

Minces and homogenates of rabbit and human organs were always incubated immediately following preparation. The human specimens had been stored on ice for up to 20 hours before use; the human minces and homogenates were then prepared and incubated.

Microsomes of human or rabbit liver, kidney or skeletal muscle were prepared from fresh tissue. Only the rabbit lung microsomes were prepared from a mixture of fresh and frozen tissue; this is because a large amount of lung tissue was required to achieve useable quantities of microsomes. To minimize the number of animals used, lungs were obtained from donor laboratories, frozen and used within two weeks. The preparation and incubation of all microsomes were performed on separate days, due to the length of the ultracentrifugation procedures; microsomes were frozen and used within a reasonable time (one day to two weeks).

Minces

The animal was sacrificed by decapitation and its blood was drained for a few seconds. The abdomen and chest were quickly accessed, organs of interest were removed and immediately dropped into ice-cold sucrose buffer (see below). The tissue was cut into small pieces with scissors over the next few minutes. Small portions of diced organ were transferred to a glass or ceramic board which had been cooled over a bed of ice. The tissue was minced with two razor blades until the pieces were approximately one to two mm square. This preparation was stored over ice and used as soon as possible to assure maximal enzymatic activity.

Sucrose buffer (0.25 M): The following was prepared and stored refrigerated:

85 g sucrose
water q.s. 1000 ml.

Homogenates

The tissue was prepared as described above to the point of being cut into small pieces with scissors. Approximately ten g of tissue was then homogenized (Waring blender, Waring, New York, NY) with two volumes of buffer until most of the chunks of tissue were dispersed. Further homogenization followed with a teflon pestle and glass tube (Wheaton Instruments, Millville, NJ) for one minute with continuous suspension in a beaker of ice. The preparation was stored over ice and incubated as soon as possible to assure maximal activity.

Microsomes

The homogenization procedure was followed as described above, with preparation of large quantities of the product. The first centrifugation step separated nuclei and debris (Beckman TJ-6 centrifuge, Palo Alto, CA): 600 g for 10 minutes at 3 °C; the pellet was

discarded. The supernatant was centrifuged (Beckman TJ-6) at 10,000 g for 20 minutes at 3 °C; the pellet contained mitochondria and was discarded. The supernatant was resuspended (Wheaton homogenizer) and centrifuged (Beckman L8-M ultracentrifuge; Beckman TI-35 rotor) at 100,000 g for 60 minutes at 3 °C; the cytosol supernatant was discarded. The pellet was resuspended in two volumes of buffer and centrifuged at 100,000 g for 60 minutes at 3 °C; the first microsomal wash supernatant was discarded. The pellet was homogenized in two volumes of sucrose buffer and centrifuged at 100,000 g for 60 minutes at 3 °C; the second microsomal wash supernatant was discarded. The pellet was resuspend in a small volume of sucrose buffer, divided into small portions and stored at -80 °C.

Incubation conditions

Phosphate buffer (0.13 M, pH 7.4): Each of the following were dissolved in 1000 ml water:

18.46 g Na_2HPO_4
17.68 g KH_2PO_4

These solutions were combined in a 4:1 ratio; the buffer was stable for one month, refrigerated, or as long as the pH remained within 0.1 pH unit of the original.

Methanol solution of steroids: One mg of PO or POH was weighed and dissolved in 100 ml methanol; when refrigerated, stability was assured for at least one year.

NADPH and NADP solutions (5 mM): 10.3 mg NADPH or 8.7 mg NADP were dissolved in 2.0 ml water and stored refrigerated. The cofactors are expensive and their stability in water is undetermined, so minimal quantities were prepared weekly as required for experiments.

The incubation medium initially contained 2.7 pM steroid (1000 ng/ml) and 250 pM cofactor in phosphate buffer. For each sample, one ml phosphate buffer, 100 µl steroid solution and 50 µl cofactor solution were combined. The shaking water bath was warmed to 37 °C (GCA Corporation, Precision Scientific Group, Chicago, IL) and the steroid-cofactor-buffer solution was preincubated for at least ten minutes prior to addition of tissue.

At time zero, the measured volume or mass of tissue was added to the mixture, the vial capped, shaken, and placed in the water bath. At the individual sampling times the incubation mixture was shaken and a one ml sample was withdrawn; the sample was immediately added to methylene chloride to stop the reaction. These collection tubes were prepared in advance to contain two ml methylene chloride and 1000 ng dexamethasone (in 100 µl methanol solution; internal standard for HPLC assay). The samples were vortexed and then centrifuged (2000 g x 10 minutes); the organic phase was subsequently separated and evaporated, or stored in the refrigerator overnight and separated and evaporated the next day (see Chapter 3).

Effect of tissue disruption on interconversion

The effect of tissue disruption on glucocorticoid interconversion and elimination was examined by the comparison of organ minces, homogenates and microsomes. Of these three, the mince retained the greatest original architecture, followed by the homogenate, then the microsomes. Hence, if the enzymes which interconvert or eliminate prednisone and prednisolone were dependent on cellular integrity, the three preparations would be expected to demonstrate different results.

The oxidation and reduction rates were determined with data collected up to about ten minutes. In this manner, most of the substrate remained in the incubate such that enzyme capacity was rate-limiting. The stability of prednisone and prednisolone in buffer was determined under incubation conditions; the concentrations of both PO and POH remained constant for 180 minutes (data not shown).

The peak concentration of product in some liver incubations was measured at the first sampling time (one minute); in this case, an estimate of the rate of formation was obtained with the assumption that formation to that point was linear. Additionally, it was assumed that none of the product had been eliminated.

Experiments were performed on at least two separate occasions, in an effort to improve but not necessarily optimize experimental conditions with respect to the amount of tissue added to the incubation medium. The results presented here represent data obtained from the last experiment and are based upon a single determination at each sampling point.

Concurrent interconversion and irreversible elimination result in a complex metabolic scheme. Fortunately, reductive and oxidative interconversion capacities can be examined separately due to the cofactor requirement: the reduction of prednisone requires NADPH and the oxidation of prednisolone, NADP. The cofactor was added in nearly 100-fold excess, to assure no cofactor limitation. Rates of conversion should therefore have been limited only by enzyme concentrations (or capacities).

All three tissue preparations were obtained from the same animal with the exception of the lung, which required tissue from several animals for the preparation of microsomes. Hence, biological variation can be ruled out as a contributor to differences among preparations. Incubation of the mince and homogenate were performed simultaneously; microsomal incubations were performed within a few days.

It is difficult to normalize the reaction rates of minces, homogenates and microsomes such that they might be compared. Graphically, the rates in these preparations were normalized to gram of tissue or, in the case of microsomes, to gram-equivalent of tissue (the mg microsomal protein which resulted from one g of tissue); microsomal rates were also normalized to mg protein. Tables 4.1 and 4.2 present initial rates of oxidation and reduction, respectively. In the determination of the effect of tissue disruption on metabolic activity, the exact rate was not as useful as the relative rate, as presented in

Tables 4.3 and 4.4. Here, the conversion rate of minces was arbitrarily set at one; homogenate and microsomal activities were calculated relative to the value of the mince.

The homogenates possessed the greatest oxidative activity in five of the six species-organs examined. With one exception, the microsomes possessed intermediate and the minces the lowest oxidative activity when normalized per g or gram-equivalent of tissue. Homogenization apparently promoted oxidation; subcellular fractionation resulted in increased activity relative to minces but did not exceed that of homogenates.

For reductive interconversion, a different pattern was observed: with one exception, homogenates were again the most active, this time followed by minces; microsomes possessed the lowest activity. Subcellular fractionation appeared to be associated with a decrease in reductive interconversion capacity.

Table 4.1 Apparent rates of oxidation of various organ preparations.

organ	mince (nmol/min) per g	homogenate (nmol/min) per g	microsomes (nmol/min) per g-equiv	microsomes (nmol/min) per mg prot
rabbit liver	>0.44	>3.0	>1.9	>0.95
human liver	>0.70	>1.5	>2.0	>1.0
rabbit kidney	0.018	.11	0.046	0.046
human kidney	0.0052	0.021	0.0006	0.0006
rabbit lung	0.0015	0.61	0.009	0.044
rabbit muscle	0.0051	0.015	0.012	0.12

Table 4.2 Apparent rates of reduction of various organ preparations.

organ	mince (nmol/min) per g	homogenate (nmol/min) per g	microsomes (nmol/min) per g-equiv	microsomes (nmol/min) per mg prot
rabbit liver	>0.425	>0.27	>0.12	>0.06
human liver	0.067	0.8	0.038	0.02
rabbit kidney	0	.004	0	0
human kidney	0.0044	0.019	0	0
rabbit lung	0.0044	0.7	0.0007	0.004
rabbit muscle	0.0021	0.014	0.0001	0.001

Table 4.3 Relative rates of oxidation of various organ preparations.

organ	mince	homogenate	microsomes
rabbit liver	1.0	6.8	4.3
human liver	1.0	2.1	2.9
rabbit kidney	1.0	6.1	2.6
human kidney	1.0	4.0	0.1
rabbit lung	1.0	120	6.0
rabbit muscle	1.0	2.9	2.4

Table 4.4 Relative rates of reduction of various organ preparations.

organ	mince	homogenate	microsomes
rabbit liver	1.0	0.6	0.3
human liver	1.0	11.9	0.6
rabbit kidney	*	* 0.004	*
human kidney	1.0	4.3	0.0
rabbit lung	1.0	160	0.2
rabbit muscle	1.0	6.7	0.5

* indicates value which could not be compared to zero;
units are nmol/min per g or g-equivalent of tissue

These observations corroborate the discrepant results obtained between laboratories. The results of these studies of the oxidation of POH to PO confirm the result of O'Hare (1973) wherein damaged cells (e.g., homogenates) demonstrated significantly greater activity than undamaged cells (analogous to minces). The enzymatic capacity of 11β-HSD could have been directly enhanced by homogenization or subcellular fractionation. On the other hand, a major elimination route for the product, PO, could have been destroyed in homogenization or subcellular fractionation. Homogenates generally demonstrated greater reductive activities than minces, as well, an issue that O'Hare did not address.

Another potential explanation for enhanced apparent oxidative and reductive activity in the homogenates is that the elimination of the compounds was in some way reduced in the homogenate preparation, perhaps by damage of one of the enzymes involved. As the minces and homogenates were prepared simultaneously from the same tissue, biologic variation cannot account for this observation.

It is also possible that upon tissue disruption by homogenization, diffusion of the drug into intracellular sites was no longer rate-limiting. While not all cell walls are broken in homogenized preparations, most of the connective tissue and macro structure is disrupted. In minces, cells on the surface of the 1 to 2 mm^2 piece of tissue have immediate access to drug and cofactor but cells within the mass require diffusion of both drug and cofactor to the metabolic site. The cofactors, triphosphopyridine nucleotide ($NADPH^+$) and reduced triphosphopyridine nucleotide (NADP), could also present diffusional problems.

Microsomal activities relative to minces demonstrated increased oxidation and reduced reduction in all six species-organs examined. Lakshmi and Monder (1985b) experimentally modified rat liver microsomes using detergents, phospholipases and elevated temperature. They observed that all of these manipulations resulted in increased oxidative activity of 11β-HSD and unchanged or diminished reductive activity. They hypothesized that 11β-HSD is a multi-enzyme complex and that in its natural configuration, reductive activity is maximally expressed but oxidative activity is not; membrane disruption releases the latency of the oxidation portion of the protein complex, perhaps by permitting greater exposure to the environment. The results for greater degrees of membrane disruption obtained here are compatible with this theory. In another publication, Lakshmi and Monder (1985a) observed that the ratio of oxidative to reductive activity was dependent upon the detergent used in the isolation of the protein. Such evidence also supports the theory that 11β-HSD is a multi-enzyme complex, composed of two or more separate but associated proteins which perform oxidation and reduction. Perhaps membrane disruption exposes more of one protein or in some other way activates the protein(s) responsible for oxidation and/or reduction.

Effect of tissue disruption on irreversible loss of drug

Tissue disruption affected not only the interconversion of PO and POH, but the irreversible elimination of these compounds as well. Cumulative loss was analyzed in the NADPH-containing (reductive) system, as this cofactor is required for 20β-hydroxylation, the major elimination pathway of these compounds (Miyabo et al., 1973; Jenkins, 1966). The contribution of other elimination pathways (P_{450} hydroxylation, hydrolysis, or cleavage, for instance) was examined in the oxidative system where 20β-hydroxylation occurs minimally (NADPH is replaced by NADP). The difference between the two results yields insights into 20β-hydroxylation, specifically.

The initial rates of elimination of PO and POH in the presence of NADPH are presented in Table 4.5; the relative rates are presented in Table 4.6. It can be seen that the mince was most active followed by the homogenates, then the microsomes.

Table 4.5. Apparent rates of elimination in various organ preparations.

organ	mince (nmol/min) per g	homogenate (nmol/min) per g	microsomes (nmol/min) per g-equiv	microsomes (nmol/min) per mg prot
rabbit liver	0.026	0.02	0.0022	0.0011
human liver	0.11	0.11	0.0044	0.0022
rabbit kidney	0.0125	.0041	0	0
human kidney	0.0121	0.0062	0	0
rabbit lung	0.00006	0	0	0
rabbit muscle	0.0012	0.00064	0	0

Table 4.6. Relative rates of elimination in various organ preparations.

organ	mince (nmol/min/g) per g	homogenate (nmol/min) per g	microsomes (nmol/min) per g-equiv
rabbit liver	1.0	.77	.11
human liver	1.0	1.0	.04
rabbit kidney	1.0	.33	.0
human kidney	1.0	.51	.0
rabbit lung	1.0	.0	.0
rabbit muscle	1.0	.53	.0

The fraction of originally administered drug remaining as either PO or POH by the end of a 180-minute incubation period was determined for all tissue preparations; Figure 4.1 documents the fraction of drug remaining as PO and POH. Subcellular fractionation of the liver of the rabbit and human significantly reduced the elimination of the glucocorticoids; microsomal incubates retained nearly all of the drug as PO or POH, whereas minces and homogenates demonstrated various degrees of loss. This was especially apparent in the rabbit liver: minces retained about 30%, homogenates about 50% and microsomes about 80% of the drug after 180 minutes. The human liver differed from the rabbit with homogenates, where homogenates and microsomes performed similarly.

The kidney of the human differed from that of the rabbit with respect to mince activity; whereas the rabbit kidney mince eliminated about 80% of the drug, the human kidney mince did not eliminate any drug. Yet human and rabbit kidney homogenates eliminated similar fractions of drug. This observation is difficult to explain as both the mince and homogenates were derived from the same organ and were prepared and incubated in parallel.

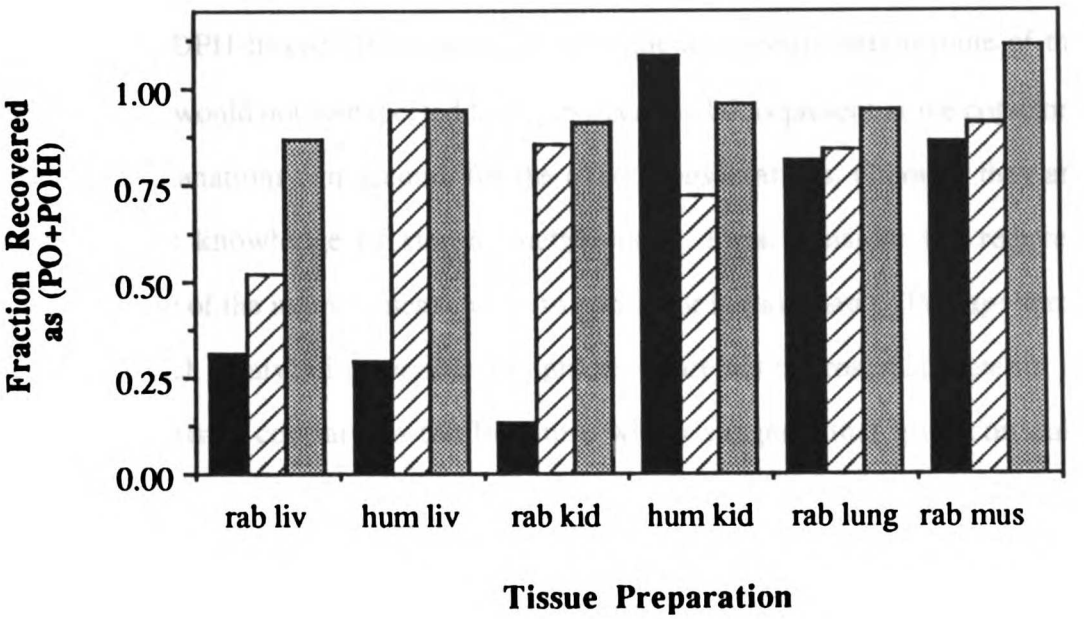

Figure 4.1 Irreversible drug loss with various tissue preparations in the presence of NADPH. Fraction of drug recovered as either prednisone or prednisolone after incubation of 1000 ng/ml PO for 180 minutes; minces, black; homogenates, hatched; microsomes, grey.

The elimination activities of the lung and muscle of the rabbit were low, and all three preparations expressed minimal but measurable elimination capacities. The lung and muscle were insensitive to disruption of tissue by homogenization or subcellular fractionation but the kidney and liver were sensitive to it; this may be associated with the generally low elimination capacity of the pulmonary and muscle tissues. In general, the liver and kidney demonstrated reduced elimination capacity as tissue was progressively disrupted or fractionated. This is suggestive of the requirement of a particular membrane orientation for maximal elimination activity.

Consider next the fraction remaining in the presence of NADP, Figure 4.2. This incubation was performed exactly as the experiments in Figure 4.1, except for the change in cofactor. The relative activities of the various preparations were unchanged between the two experiments, and progressive disruption diminished elimination. All six species-

organs demonstrated the same relative activities, independent of the cofactor. This was unexpected, as NADPH-linked 20β-hydroxylation is the major elimination route of the glucocorticoids and would not be expected to occur when NADP is present as the cofactor.

Several explanations can account for the overall observations, although they are contrary to current knowledge of steroid metabolism. First, consider the reduced elimination capacity of the microsomes relative to minces or homogenates. Perhaps some of the enzymes which catalyze irreversible loss processes are not microsomal proteins; as discussed above, this is contrary to the literature which suggests that glucocorticoid elimination and interconversion enzymes are almost exclusively microsomal (Mahesh and Ulrich, 1959; Koerner, 1966).

The stability of the microsomal enzymes must be considered: perhaps some of the enzymes which eliminate drug were inactivated in the preparation procedure or in the -80 °C storage period. The stability of one batch of frozen rabbit liver microsomes was examined to determine whether oxidative, reductive, or elimination activity deteriorated or increased upon storage at -80 °C. Activity after one day, one month, and three months remained unchanged (data not shown). The same was true for lungs which were homogenized freshly or after two days' freezer storage. 20β-Hydroxysteroid dehydrogenase is stable enough to be purchased commercially (Boehringer Mannheim, Mannheim, W. Germany). A P_{450} enzyme (6β-hydroxylase) eliminates about 10% of prednisone and prednisolone at position C-6 through β-hydroxylation; this class of microsomal enzymes are generally stable to freezing. It appears that cold storage did not have a significant effect on these observations.

Stability of 11β-HSD toward heat was examined by Koerner (1969); rat liver miocrosomal 11β-HSD was incubated for 10 minutes at 45 °C at pH 10 in the presence of NADP without loss of activity. Hence the pH 7.4, 37 °C conditions used here would not be expected to inactivate the enzyme.

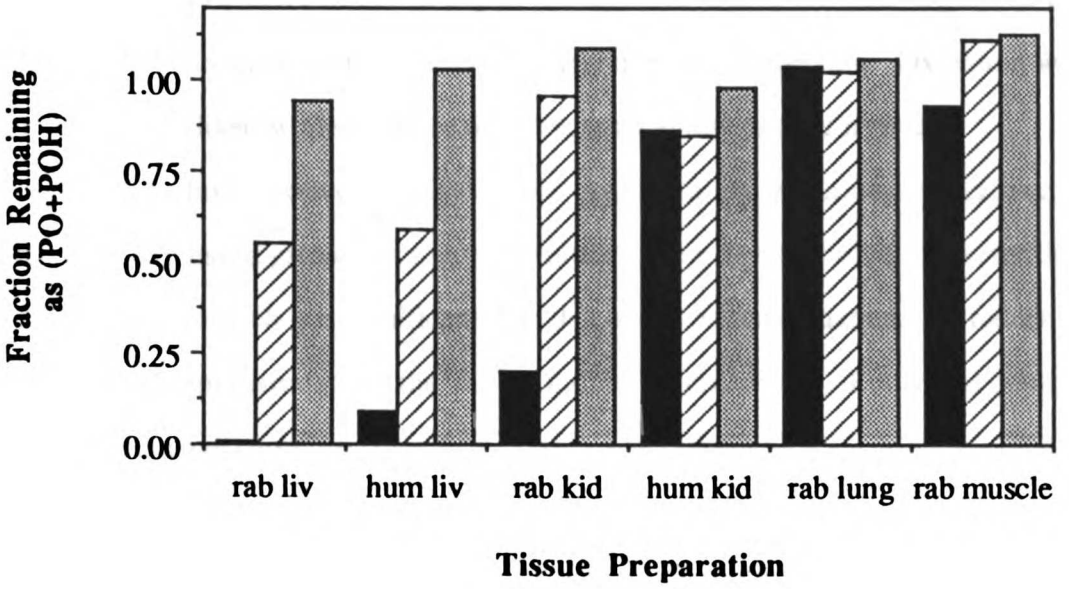

Figure 4.2 Irreversible drug loss with various tissue preparations in the presence of NADP. Fraction of drug recovered as either prednisone or prednisolone after incubation of 1000 ng/ml POH for 180 minutes; minces, black; homogenates, hatched; microsomes, grey.

One additional consideration which has not been fully examined at this time is the possible preferential elimination of either PO or POH. Mahesh and Ulrich (1959) observed that various human kidney preparations preferentially reduced cortisone, over cortisol, at position 20. Additionally, cortisone was shown to be metabolized by 20β-HSD 15-fold faster than was cortisol (Kawamura et al., 1980). Greater elimination capacity was demonstrated when POH was incubated (Figure 4.2), so this explanation is not helpful.

Organ-specific metabolism

The organ-specific metabolism of the glucocorticoids prednisone and prednisolone was analyzed by comparison of the % conversion (both oxidation and reduction) between

organ microsomes of the human and of the rabbit. Figure 4.3 depicts a plot of the % oxidative conversion of POH to PO in organ microsomes where the four organs of the rabbit are compared; results are plotted as % conversion per mg protein. Oxidation was extremely high in the liver, while the three other organs had low relative activities.

Figure 4.4 shows conversion by rabbit organs in the reduction experiments. Again, the liver expressed greatest activity; the other organs demonstrated low activities, but the difference was much less significant than that observed for oxidation. No reductive activity could be demonstrated in the rabbit kidney microsomes. Lung and muscle possessed low reductive capacity.

Figure 4.5 and 4.6 compare oxidation and reduction, respectively, in the human liver and kidney. For both reactions, the liver was much more active than the kidney. Again, the difference was more apparent in the oxidation experiments than in the reduction experiments.

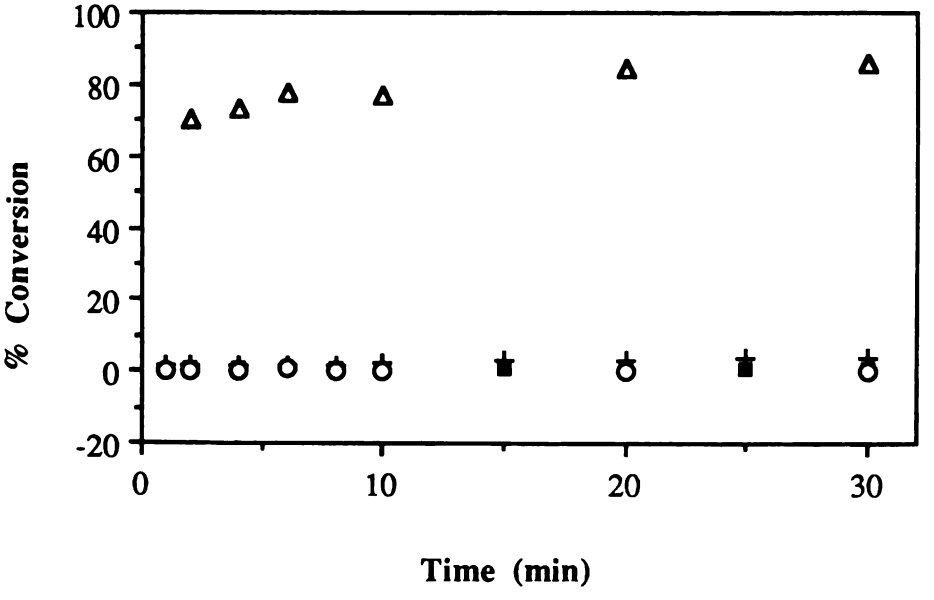

Figure 4.3. Percent of prednisolone oxidized to prednisone during incubation with rabbit organ microsomes, normalized to mg protein. Liver, triangle; kidney, plus symbol; lung, solid square; muscle, open circle.

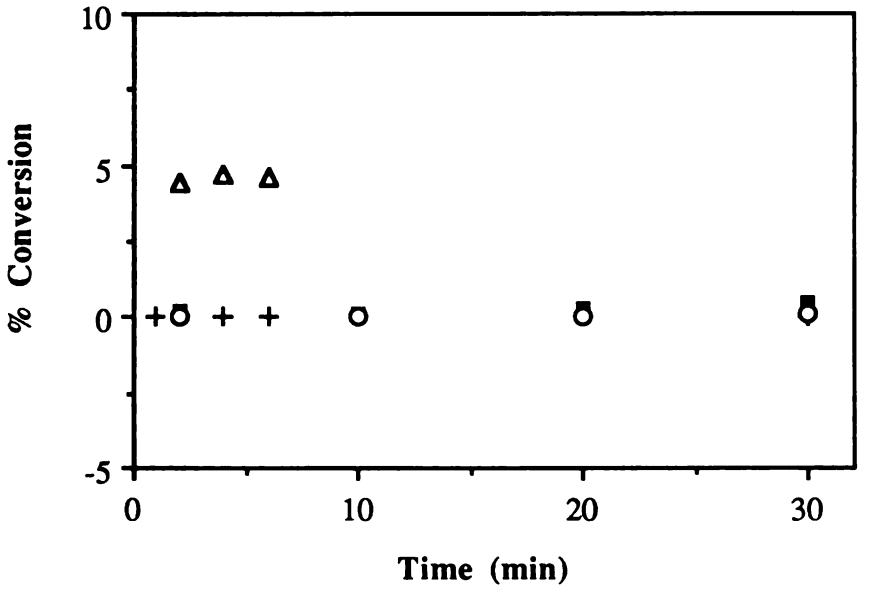

Figure 4.4. Percent of prednisone reduced to prednisolone during incubation with rabbit organ microsomes. Liver, triangle; kidney, plus symbol; lung, solid square; muscle, open circle.

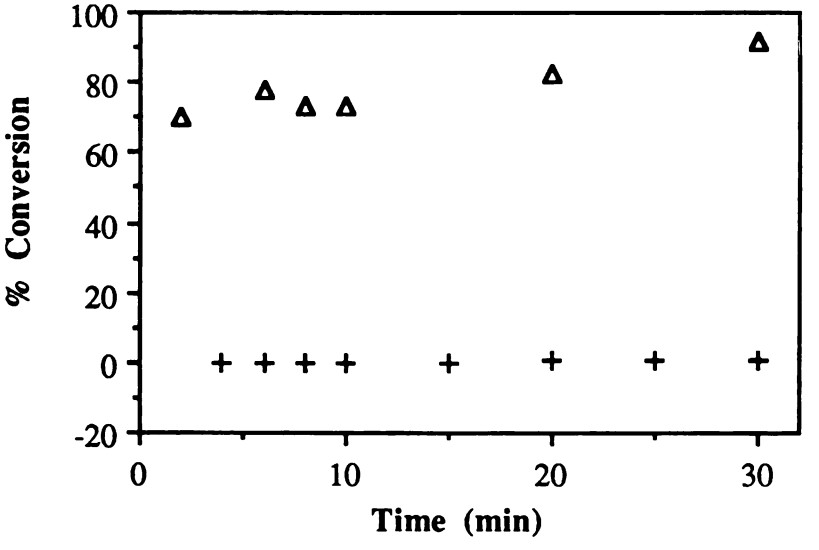

Figure 4.5. Percent of prednisolone oxidized to prednisone during incubation with human organ microsomes. Liver, triangle; kidney, plus symbol.

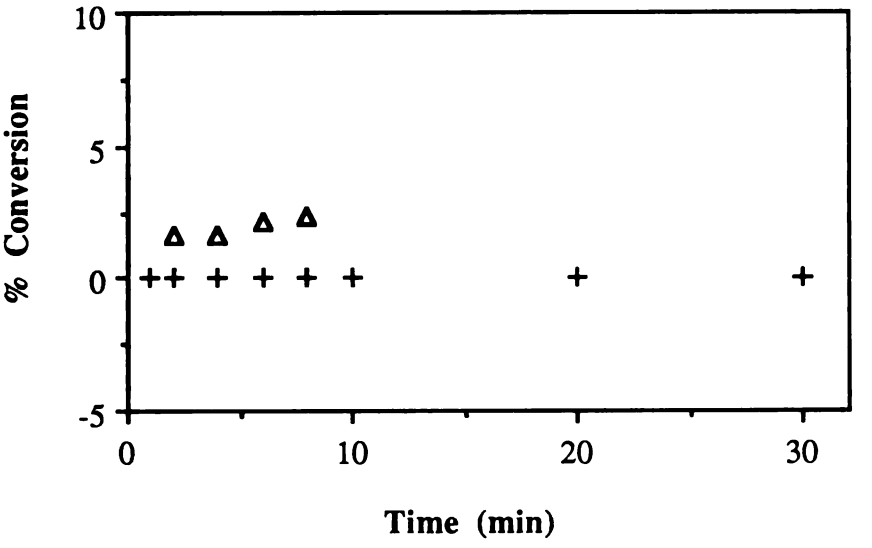

Figure 4.6. Percent of prednisone reduced to prednisolone during incubation with human organ microsomes; liver, triangle; kidney, plus symbol.

Species comparisons

The literature states that there are great species differences in the metabolism or general disposition of the glucocorticoids, as studied *in vitro* (Monder and Shackelton, 1984): the livers of the rat, mouse, rabbit, guinea pig and human oxidize cortisol to cortisone but the livers of pigs, sheep and cows do not appear to perform this reaction. Experiments with organ microsomes were performed for up to 30 minutes with the conditions described above. Figures 4.7 and 4.8 compare hepatic microsomal oxidation and reduction, respectively, in rabbits and humans. The time course of conversion of POH to PO was very similar for both species; both livers were extremely oxidative. The plots of comparative reduction are very similar between species as well, but the % converted was significantly less than for oxidation.

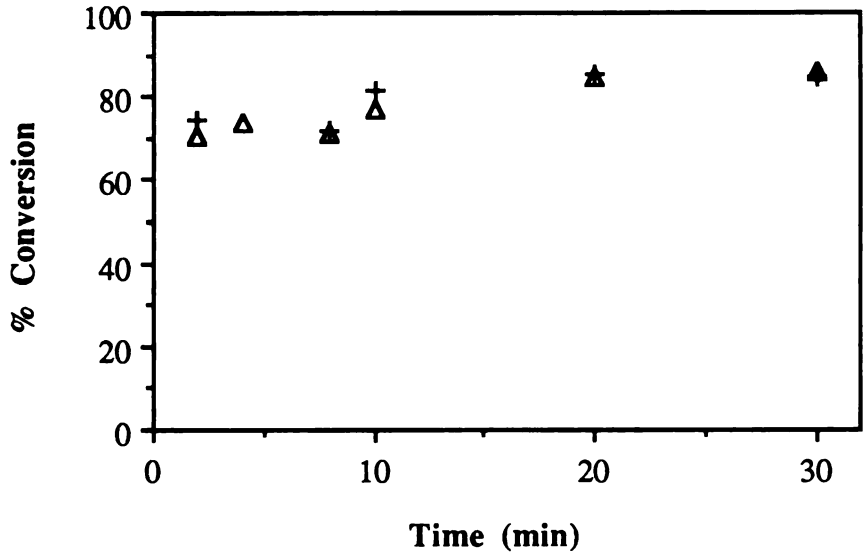

Figure 4.7. Percent of prednisolone oxidized to prednisone during incubation with liver microsomes; rabbit liver, triangle; human liver, plus symbol.

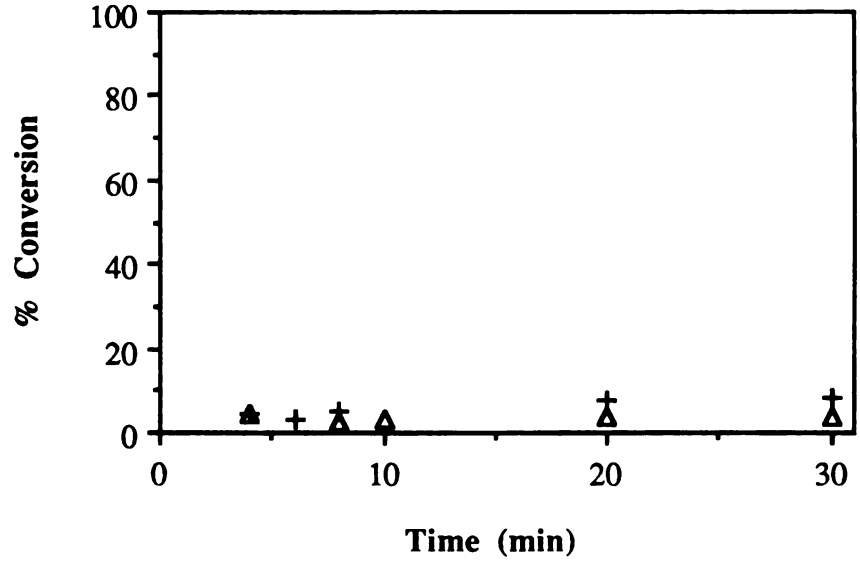

Figure 4.8. Percent of prednisone reduced to prednisolone during incubation with liver microsomes; rabbit liver, triangle; human liver, plus symbol.

Figure 4.9 compares renal oxidation in human and rabbit organ microsomes. The two species demonstrated great similarities in renal metabolism: oxidative activity was low but significant; the rabbit kidney appeared to oxidize POH at a faster rate than did the human, but both rates were low. Reductive activity was not demonstrated by the kidney of either the rabbit or the human (not shown).

The observation of similarity between the rabbit and human in interconversion capacities was unexpected. Microsomal liver and kidney preparations were compared; any differences were relatively small.

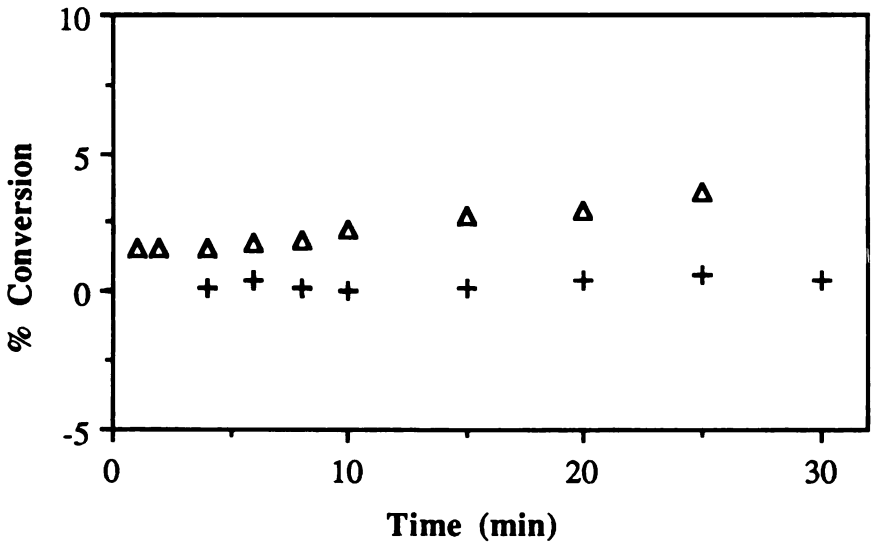

Figure 4.9. Percent of prednisolone oxidized to prednisone during incubation with kidney microsomes; rabbit kidney, triangle; human kidney, plus symbol.

Significance and conclusions

This series of experiments was performed to determine whether disruption of tissue architecture substantially affects glucocorticoid metabolism. It appeared that homogenates interconvert PO and POH at rates greater than those of minces. Microsomes demonstrated greater oxidative and lesser reductive activity than minces. These results are compatible with the hypothesis that the system which interconverts PO and POH, (11β-HSD), consists of multiple enzymes or a multi-enzyme complex, (Bush and Mahesh, 1959). These enzymes may be dependent on an anchor membrane for position and exposure to cofactors and substrates. Upon homogenization or subcellular fractionation, oxidative and reductive capacities would be modified.

Capacity for irreversible elimination of PO and POH appeared to be reduced with progressive disruption of tissue architecture. It was generally observed that microsomes possessed the lowest elimination capacity and minces the greatest. Elimination capacity was significantly affected by tissue damage only in the liver and kidney, the organs with substantial elimination capacities. Elimination capacity was apparently independent of cofactor requirements, as incubation with NADP or NADPH produced similar results.

The liver, kidney, lung, and muscle biotransformed the glucocorticoids differently. The liver demonstrated great bidirectional interconversion capacity; the kidney was predominantly oxidative, as was the muscle; the lung was primarily reductive.

Species differences toward interconversion and elimination were studied with microsomes; very small differences in the liver and kidney of the human and the rabbit were observed. This is in contrast to literature reports of significant species differences in metabolism of the glucocorticoids.

Chapter 5

In Situ Leporine Liver Perfusions

Introduction

The liver is the most metabolically active organ for the glucocorticoids in both phase I and phase II reactions (Roberts and Szego, 1955). Endogenous glucocorticoids are inactivated primarily by A-ring reduction which has been documented only in the liver (Berliner, 1958); the synthetic glucocorticoids are inactivated by 20β-hydroxylation which occurs in multiple organs but the greatest contribution is hepatic. Conjugation of endogenous glucocorticoids (glucuronidation and sulfation) occurs primarily in the liver (Gold, 1961).

We have hypothesized that the unusual disposition profiles of PO and POH cannot be clarified further using only human or whole animal investigations. Individual organ studies are necessary to elucidate the complexities of this system. The present work was undertaken to explicitly define the hepatic metabolism of prednisone and prednisolone in the rabbit liver, independent of the interpretational problems introduced by saturable protein binding.

Selection of the perfused rabbit liver model

The New Zealand White rabbit model of glucocorticoid disposition is a highly representative model of human synthetic glucocorticoid disposition (Rocci and Jusko,

1981). In both rabbits and humans, the plasma protein binding of prednisolone is nonlinear, as is apparent systemic clearance of total drug. Renal clearance is limited (ca. 30%) , and prednisolone predominates over prednisone by at least five to one (Khalafallah and Jusko, 1984; Ferry and Wagner, 1988).

Perfused organ methodologies were chosen over technically easier *in vitro* experimental systems such as microsomal or homogenate preparations because of the observation by M. J. O'Hare (1973) that disrupted tissue preparations produced metabolic profiles which differed greatly from those in which tissue architecture was maintained. Corticosterone (11β-OH) was oxidized to its 11-keto form (11-dehydrocorticosterone) to a 250-fold greater extent in homogenates and damaged monolayer culture preparations when compared to the undamaged monolayer culture. Similar work by Bernal et al. (1982) and Nicholas and Lugg (1982) have substantiated this observation. Chapter 4 outlines the results of incubation studies in this laboratory with organ minces, homogenates and microsomes; it was observed that the oxidative interconversion rate of human and rabbit liver homogenates and microsomes greatly exceeded that of the mince. The enzyme(s) which performs this reaction is responsible for the interconversion of cortisol/cortisone and prednisolone/prednisone. This observation, when considered with reference to a great number of previously performed *in vitro* experiments, suggests that conclusions regarding organ specific metabolic capacity toward the glucocorticoids could be misleading.

Hepatocytes were not studied because they have been shown to function less well than the perfused organ (Blom et al.,1982): the transport of dibromosulfophthalein (organic anion), tubocurarine (organic cation) and ouabain (neutral compound) was compared in isolated hepatocytes, the perfused liver and the liver *in vivo* in rats. Transport functions were well maintained in the perfused liver and hepatocytes but secretory functions were slower in hepatocytes when compared to the other two systems.

In the perfused rabbit liver system, the liver's metabolic contribution is determined in isolation. This segregation is not reflective of true events *in vivo*; nervous innervation

and lymph drainage are compromised with suturing and cutting of vasculature. Regardless, this system is the closest to that *in vivo* of all the commonly employed *in vitro* systems, as architecture and anatomic spacing are maintained and membranous structures remain intact.

The effect of saturable binding is eliminated by the omission of corticosteroid binding globulin from the perfusate. This is not reflective of physiologic conditions, but all calculations are made based upon unbound drug concentrations, so the effect of binding to CBG is not important unless the protein directly exerts some physiologic effect on glucocorticoid disposition.

Methodology

Most published surgical procedures for the perfused liver are similar; other elements of the perfusion may differ substantially. An comprehensive review by Ross (1972) is a useful technical and procedural reference. A review by Pang (1984) presents a conceptual understanding and survey of modern applications of the preparation for drug toxicity studies.

The liver was first perfused in 1855 (Ross et al., 1972); great advances have been made since that time in methodology with particular regard to equipment. The great majority of liver perfusion literature addresses the rat liver but most of the details can be adapted to the livers of other species rather easily. The ease of adaptation of the liver perfusion method does not apply to other organ systems such as the kidney. The method of liver perfusion by Miller et al. (1951), with slight modifications, is referenced most frequently. Mortimore (1961) first perfused the liver *in situ* and Schimassek (1963) successfully substituted a synthetic medium for blood. We chose to allow the liver to remain *in situ*, as it functions equivalently to those perfused *ex vivo* (Ross, 1972) and because of the risk associated with handling its weakly supported lobes.

Our liver perfusion method incorporated red blood cells so that physiologic flow rates could be used. In the absence of a highly efficient oxygen carrier, flow rates must be greatly elevated to achieve adequate oxygenation. A recirculating design was chosen due to the limited availability of red cells.

Steady state experiments were performed so as to simplify calculations involving differential equations and to avoid concern with potential concentration gradients. About ten minutes was required to achieve steady state conditions with the drug administration routine described below. Sampling was performed for the subsequent 20 to 25 minutes, then a new steady state concentration was targeted. Over the 150 minute perfusion period, it is possible to achieve four randomized concentrations from one liver preparation (30 to 35 minutes at each concentration). Fifteen rabbit livers were perfused; it was necessary to perform additional PO perfusions (nine versus six) to obtain sufficient POH data in these experiments. The concentrations encompassed a 66-fold range (Tables 5.1 and 5.2). Three to five measurements were made at each steady state concentration, then averaged. Due to the viability acceptance standards (described in the viability section, below), not all concentration measurements were included in the calculations. Eighteen prednisolone and 32 prednisone steady state concentrations were examined in total.

Two control experiments were performed in the absence of drug to obtain estimates of the pressure-flow relationship, urea synthesis rate, lactate-pyruvate ratio and to ascertain whether the presence of drug affected viability.

The two experiments performed to distinguish between the well stirred and parallel tube models for hepatic disposition of PO were similar to those described by Pang and Rowland (1977b), modified for a recirculating system. Briefly, perfusate flow rates were varied randomly, above and below the "ideal" hepatic flow rate (Tables 5.1 and 5.2). Several times during the experiment, the flow rate was returned to the "ideal" rate to assure consistent liver function. Two measurements were made at each flow rate, and averaged.

Table 5.1.
Rabbit Liver Perfusions: Prednisone Experiments.

Total Steady State Concentration Prednisone (ng/ml)	Unbound Steady State Concentration Prednisone (ng/ml)	Total Steady State Concentration Prednisolone (ng/ml)	Unbound Steady State Concentration Prednisolone (ng/ml)	Extraction Ratio	Apparent Hepatic Clearance (ml/min)	Apparent Hepatic Clearance (ml/min/kg)
8120	4385	3157	1705	0.99	32.7	14.9
3277	1770	1176	635	0.97	32.0	14.5
1101	595	533	288	0.93	30.7	14.0
460	248	177	96	0.94	31.0	14.1
768	415	316	171	1.00	32.0	13.9
819	442	294	159	0.95	30.4	13.2
457	247	267	144	0.90	29.7	12.9
479	259	171	92	0.92	30.4	13.2
1153	507	501	261	0.93	32.6	18.1
1601	704	620	322	0.90	29.7	16.5
1373	604	742	386	0.90	29.7	16.5
1191	536	694	368	0.96	31.7	15.1
1875	844	721	382	0.97	32.0	15.2
4230	1904	508	269	0.98	32.3	15.4
2046	921	955	506	0.92	30.4	14.5
289	130	243	124	1.00	29.8	15.7
889	400	540	275	1.00	31.0	16.3
425	191	662	338	1.00	31.0	16.3
225	101	493	251	1.00	31.0	16.3
1059	477	901	478	0.95	28.3	17.7
775	349	1101	584	0.91	28.2	17.6
837	335	306	153	0.99	29.7	15.6
443	177	292	146	0.99	30.7	16.2
569	228	385	193	0.99	31.2	16.4
533	213	403	202	0.97	29.9	15.7
6786	2511	1123	595	0.95	28.8	13.1
495	183	1260	668	0.97	30.1	13.7
1763	652	865	458	0.97	30.1	13.7
531	196	562	298	0.94	29.8	13.5
1708	734	727	393	0.98	29.4	14.0
11143	4791	7073	3819	0.95	28.5	13.6
5565	2393	1873	1011	0.94	28.2	13.4
			mean:	**0.96**	**30.4**	**15.0**
			S.D.	**0.03**	**1.2**	**1.5**

Table 5.2
Rabbit Liver Perfusions: Prednisolone Experiments

Total Steady State Concentration Prednisolone (ng/ml)	Unbound Steady State Concentration Prednisolone (ng/ml)	Extraction Ratio	Apparent Hepatic Clearance (ml/min)	Apparent Hepatic Clearance (ml/min/kg)
2723	1470	0.56	17.9	11.9
1260	680	0.51	17.9	11.9
448	242	0.42	15.4	10.3
607	285	0.52	17.1	10.1
1502	706	0.54	18.4	10.8
2727	1282	0.50	17.0	10.0
4687	2203	0.45	13.5	7.9
369	221	0.41	11.9	7.4
225	135	0.53	15.9	9.9
181	109	0.43	12.9	8.1
6008	3184	0.48	15.8	10.5
2489	1319	0.47	15.8	10.5
1737	816	0.48	16.6	9.8
8602	4043	0.49	17.6	10.4
11997	5639	0.48	16.6	9.8
4855	2622	0.46	16.9	8.5
2009	1085	0.50	18.9	9.5
356	192	0.53	19.4	9.7
	mean	**0.49**	**16.4**	**9.8**
	S.D.	**0.04**	**2.0**	**1.2**

Experimental conditions

The perfusate was prepared as follows: to Krebs-Heinseleit bicarbonate buffer (1932) (see Table 5.3), pH 7.4, 100 mg% dextrose was added. Bovine serum albumin fraction V, 70 mM (4.5%), and urea precursors l-ornithine, 10 mM, and ammonium chloride, 2.7 mM, were also added. (All chemicals were obtained from Sigma, St. Louis, MO.) The solution was then filtered (Whatman, No.1, Maidstone, England). Washed human red blood cells were added to obtain a hematocrit of 0.25. Type O-positive or O-negative red blood cells, from recently outdated blood, were obtained from the hospital blood bank (University of California Hospitals), as either washed or packed cells. The cells were washed one to three additional times as described by Pang (1984). The buffer-protein solution was prepared one to three days prior to use. The red cells were added to this solution one day in advance. The total volume used in the perfusion experiments was 200 ml.

The apparatus was composed of the following elements, with experimental conditions specified. Within a 250 ml glass reservoir the perfusate was continuously stirred magnetically (mini model 200, VWR, San Francisco, CA). A roller ball pump (Masterflex, model N-07553-20, Cole Parmer, Chicago, IL) delivered non-pulsatile flow. A blood transfusion filter (SQ40S, Pall, Fajardo, PR) trapped clumps of red cells, but permitted single cells to pass. An oxygenation chamber was created from a glass jar, continuously perfused with carbogen, 95% 0_2 / 5% CO_2. Perfusate flowed through five meters of coiled thin walled medical grade silicon tubing, 0.058" ID x 0.077" OD (Scientific Products, McGaw Park, IL); the pO_2 was maintained at or above 50. The path of flow then traversed an in-line temperature probe, an in-line pH electrode (Cole Parmer, Chicago, IL), a manometer (W.A.Baum, New York, NY), a flow meter (Gilmont, Great Neck, NY), and a bubble trap. The artificial vasculature was composed of Tygon tubing, 1/8" ID x 3/16" OD (Fisher, Pittsburg, PA). Sampling ports (Argyle, St. Louis, MO) were placed in-line prior to entrance into and upon exit from the liver. Perfusate exiting the

Table 5.3

Composition of Krebs-Heinseleit physiologic buffer.

Electrolyte	**stock concentration**	**ml per L buffer**
$NaHCO_3$	13 g / l	161.7
$CaCl_2$	6.1 g / 500 ml	22.7
KCl	5.75 g / 500 ml	31.2
NaCl	90 g / 500 ml	38.8
$MgSO_4$	9.55 g / 250 ml	3.8
KH_2PO_4	10.55 g / 500 ml	7.8
water		q.s. 1000

Ion	**buffer concentration (mmol/l)**	***in vivo* concentration (mmol/l)**
HCO_3^-	25	21-30
CL^-	124	100-106
SO_4^{-2}	1.2	0.25-0.4
PO_4^{-3}	1.2	1-1.4
Na^+	143	136-145
K^+	6.0	3.5-5.3
Mg^{+2}	1.2	0.7-1.2
Ca^{+3}	2.5	2.1-2.6

animal carcass returned to the reservoir for recirculation. All devices and the intact animal remained inside a two-tiered plexiglass chamber (Air Control Inc., Huntingdon Valley, PA). One and one half ml of perfusate was taken from the in and out sampling ports at specified times; the samples were centrifuged, separated and the red cell-free perfusate was frozen until the time of analysis (within one week). The thermostatically controlled chamber temperature remained near 37 °C; the pH of the perfusate was continuously monitored and manually adjusted to remain between 7.35 and 7.45 by the addition of 0.5 N HCl or saturated $NaHCO_3$ (both prepared with 0.9% NaCl) as needed. The flow was adjusted to maintain perfusate pressure at about 20 mmHg, and ranged between 30 and 40 ml per minute. At the start of an experiment, a bolus dose of PO or POH (Sigma, St. Louis, MO) dissolved in a minimal volume of methanol was added to the reservoir to achieve a specific concentration. To maintain this concentration at steady state, it was necessary to infuse drug into the system. Knowing the extraction of the drug from preliminary experiments, together with the flow rate of the perfusate and the perfusate volume, it was possible to predict the infusion rate of drug needed to maintain steady state conditions in such organ perfusion experiments. This quantity was continuously infused (Harvard infusion pump, Model 975, Mills, MA), at a rate and concentration which maintained the steady state concentration of drug and replaced the volume of perfusate removed in sampling.

The analytical methods used are documented in Chapter 3. A normal phase high performance liquid chromatographic (HPLC) assay was used for the detection and quantitation of prednisone and prednisolone in perfusate samples. Equilibrium dialysis was used to measure the free fractions of prednisone and prednisolone present in the perfusate samples. Enzymatic methods were used to quantify urea, lactate and pyruvate concentrations present in perfusate samples.

Surgical techniques

Nineteen New Zealand White rabbits, of both sexes, 1.5 to 2.3 kg were used (Nitabell, Hayward, CA). Experiments consistently were begun between 8 a.m. and 10 a.m. Each animal was anesthetized intramuscularly with 50 mg/kg ketamine hydrochloride (Vetalar, Parke-Davis, Morris Plains, NJ) and 10 mg/kg xylazine hydrochloride (Rugby, Rockville Center, NY); fractional doses were administered subsequently if needed. Diazepam (Elkins Sinn, Cherry Hill, NJ), 0.5 mg/kg, was injected into the ear vein immediately prior to initiation of surgery to prevent hyper-reflexia. The rabbit's abdomen was shaved and a midline abdominal incision was made. Silk suture was placed around the portal vein in three positions to secure the cannula. The proximal most suture also encircled the hepatic artery. The portal vein was then cannulated with a 14- or 16-gauge teflon catheter (Angiocath, Deseret, Sandy, UT) and secured, also ligating the hepatic artery. Initiation of perfusate flow was begun at this time with about 100 ml of an oxygenated solution of 1% bovine serum albumin (Sigma) and 10% human red blood cells in normal saline, to clear rabbit blood from the liver. After about three minutes, the experimental perfusate (described above) was substituted. The chest was opened along the sternum, and three sutures were placed around the vena cava. This vessel was cannulated with a 14-gauge teflon cannula (Angiocath) and collection of the effluent began. The vena cava was then ligated immediately above the right kidney. The animal died of respiratory failure under full anesthesia. Complete isolation of the hepatic circulation was achieved and the liver remained *in situ*.

Viability tests

Several indexes of viability were monitored in this experimental preparation. First, the synthetic capacity of the liver was evaluated by the rate of synthesis of urea in the presence of its precursors. It is believed that synthetic functions are amongst the first to show evidence of deterioration in an organ (Hems et al., 1966). For preparations in which

urea synthesis was not adequate or did not continue to increase at the same rate with time, experimental data were not included in calculations; hence experiments vary in the number of steady state concentrations achieved.

The constancy of the extraction ratio with time is a nonspecific indicator of viability (Pang, 1984), as is the stability of the pressure-flow relationship. Decreased extraction would be expected in the case of reduced viability; the flow required to maintain constant pressure would vary, most likely decreasing if the liver was unhealthy.

The lactate/pyruvate ratio is measured as an indicator of oxygenation status. Lactate would increase in the case of excessive oxygenation, increasing the ratio; pyruvate would increase in the case of insufficient supply, decreasing the ratio. Recall that the interconversion of PO and POH is an oxidation/reduction reaction and that it is important to verify the oxygenation status.

The commonly referenced viability indicators of bile flow rate and oxygenation consumption were not measured. According to Brauer (1963), even in the presence of extensive tissue ischemia and damage, bile flow can remain unchanged. Oxygen consumption is known to increase when synthesis reactions are promoted by the presence of precursors (Hems et al., 1966).

Modeling intra-hepatic metabolic processes

The *in vivo* pharmacokinetic modeling of the disposition of the glucocorticoids prednisone (PO) and prednisolone (POH) has traditionally been performed using the classic multi-exponential and noncompartmental methods. While some useful information has been gained from these analyses, problems have become evident as well. The assumptions involved in multi-exponential or compartmental fits (Pickup et al., 1977 and many others) are violated by the interconversion of PO and POH, and by the apparently nonlinear clearance of the compounds. In the case of noncompartmental analysis (Loo et al., 1978; Rose et al., 1981), interconversion is permitted but dose-dependent clearances result in

time-averaged parameters. More recently, we have carried out steady state experiments designed to circumvent the nonlinear aspect of disposition (Legler et al., 1982; Frey et al., 1981; Chapter 10). However, a major problem with these *in vivo* studies is that the results of modeling probably have little relationship to molecular events.

Physiologic modeling of an individual organs' contributions to total body disposition is probably the most appropriate method of modeling this complicated system. This procedure allows for differential organ metabolism, interconversion and nonlinearities, and more closely predicts molecular metabolic events.

Model assumptions

The pharmacokinetic model presented in Figure 5.1 is chosen for its parsimony. It is a model of the interconversion and elimination of unbound PO and POH at steady state, without distributional compartments. The perfusion rate limitation was assumed, implying there was no barrier to transport of drug from perfusate into hepatocytes. This assumption is supported by the facts that these steroids are lipophilic (octanol water partition coefficients are approximately 40), relatively small (molecular weights = 360 and 361), and nonpolar.

Experiments were performed at steady state; in this manner, the rates of elimination and presentation were equal; the differential equations were simplified, and the parameters were solved explicitly. Additionally, potentially nonlinear parameters were determined at individual steady state concentrations.

It was assumed that only unbound drug was free to traverse the hepatocyte membrane to distribute or to gain access to sites of enzymatic biotransformation. Unbound prednisone and prednisolone concentrations were used in all calculations as measured by equilibrium dialysis. The result is that clearance parameters represent intrinsic clearance values.

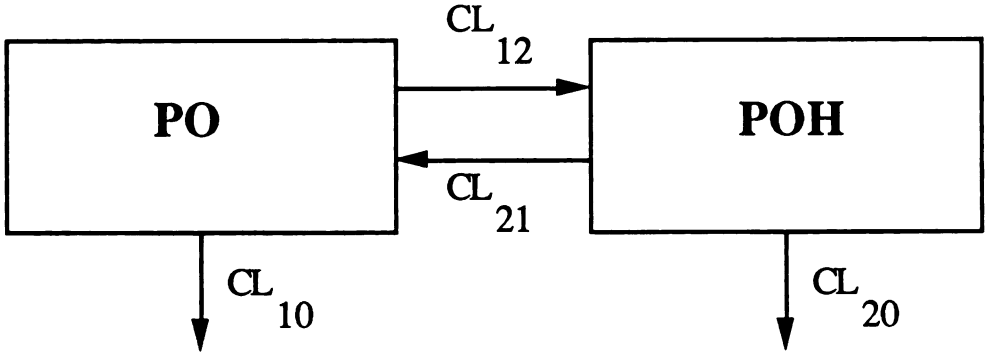

Figure 5.1. Model of the interconversion and elimination of prednisone and prednisolone in the perfused rabbit liver. (Clearance terms represent intrinsic clearance.)

CL_{10} = irreversible elimination of prednisone
CL_{12} = reductive interconversion of prednisone
CL_{20} = irreversible elimination of prednisolone
CL_{21} = reductive interconversion of prednisolone

Calculation of intrinsic clearance

Applying mass balance, the disposition of PO and POH across the liver can be described by:

$$V_H \frac{dC^{POH}}{dt} = Q_H\left(C_{in}^{POH} - C_{out}^{POH}\right) + CL_{12}\,C_H^{PO} - CL_{21}\,C_H^{POH} - CL_{20}\,C_H^{POH} \quad (1)$$

$$V_H \frac{dC^{PO}}{dt} = Q_H\left(C_{in}^{PO} - C_{out}^{PO}\right) + CL_{21}\,C_H^{POH} - CL_{12}\,C_H^{PO} - CL_{10}\,C_H^{PO} \quad (2)$$

where Q_H is the hepatic perfusion rate; entering and exiting unbound concentrations of POH and PO are designated C_{in} and C_{out} respectively, while V_H and C_H are the volume of

the liver and hepatic concentration of each species; the clearance terms represent the processes depicted in Figure 5.1. Since all studies were performed under steady state conditions, equations 1 and 2 were set equal to zero.

The liver concentrations of drug, C_H^{POH} and C_H^{PO}, are not measurable and must be estimated by a particular model of intra-organ disposition. Several models exist, but here the well stirred and parallel tube models were compared as they represent the boundary conditions (Roberts and Rowland, 1986). The hepatocyte concentration for the well stirred model (ws) is:

$$C_{H,ws} = C_{out} \tag{3}$$

while for the parallel tube model (pt), the concentration is:

$$C_{H,pt} = [\ (C_{in} - C_{out}) / \ln (C_{in} - C_{out})\ ] \tag{4}$$

The values for the concentration term were substituted into the corresponding physiologic model differential equations, allowing the solution of intrinsic clearance values. Based on the results of the discrimination experiments (described below), either the $C_{H,ws}$ or the $C_{H,pt}$ were used throughout the calculations.

Consider first experiments in which POH is infused. Since no PO was detectable in the perfusate, it was assumed that $CL_{21} = 0$; the C_H^{PO} values were also zero, and equation 1 becomes:

$$V_H \frac{dC^{POH}}{dt} = Q_H\left(C_{in}^{POH} - C_{out}^{POH}\right) - CL_{20}C_H^{POH} \tag{5}$$

equation 2 has no corollary in POH experiments since PO is not detectable. Three of the five parameters were measured: Q_H, C_{in}^{POH}, C_{out}^{POH}. The fourth parameter, C_H^{POH}, was predicted for the two boundary models of organ disposition. Thus CL_{20} was obtained algebraically, for each perfused steady state concentration of POH, for each of the two models.

Prednisone hepatic perfusion disposition is defined by equations 1 and 2, as well. Again, $CL_{21} = 0$, so that the following two equations were sufficient to represent the metabolic events:

$$V_H \frac{dC^{POH}}{dt} = Q_H\left(C_{in}^{POH} - C_{out}^{POH}\right) + CL_{12}C_H^{PO} - CL_{20}C_H^{POH} \qquad (6)$$

$$V_H \frac{dC^{PO}}{dt} = Q_H\left(C_{in}^{PO} - C_{out}^{PO}\right) - CL_{12}C_H^{PO} - CL_{10}C_H^{PO} \qquad (7)$$

As stated above, at steady state the left hand sides of equations 5 to 7 equal 0. Equation 6 was first solved numerically. Q_H, C_{in}, and C_{out} were measured; C_H^{PO} and C_H^{POH} were predicted for the well stirred and parallel tube models using equations 4 and 5. Using the average CL_{20} value calculated from POH perfusions with equation 5 (see Tables 5.4 and 5.5), CL_{12} of equation 6 was solved for various concentrations of POH. This exact (not averaged) CL_{12} value was then used in equation 7 to solve for individual CL_{10} values.

Distinguishing between models

The purpose of obtaining a model to describe organ perfusion data is to be able to predict the effects of physiologic and/or biochemical changes on the disposition of a drug across the organ. Within an organ perfusion system, the disposition of a compound can be described by several models which predict tissue concentrations of drug. It is possible to discriminate between models of hepatic disposition of a highly extracted drug by altering

Table 5.4
Intrinsic clearance estimates using the well stirred model.

POH ng/ml	CL_{20} ml/min	PO ng/ml	CL_{12} ml/min	CL_{10} ml/min
2723	42	6786	181	422
1260	32	4905	330	612
448	26	1763	542	374
607	26	531	489	-72
1502	39	837	1234	680
2727	32	443	841	801
4687	19	569	1041	1303
369	21	533	935	522
225	40	1153	216	60
181	24	1601	147	72
6008	24	1373	176	-14
2489	28	1191	510	521
1737	36	1875	386	265
8602	41	4230	269	166
11997	31	2046	265	383
4855	31	1708	538	599
2009	37	11143	124	167
356	41	5565	180	47
		289	386	898
mean	**32**	889	557	880
S.D.	**7.4**	425	896	289
		225	876	199
		1059	557	16
		775	477	-166
		768	1269	1859
		819	452	1054
		574	179	240
		479	144	198
		8120	745	1382
		3277	478	1060
		1101	105	123
		460	152	203
		mean	**490**	**473**
		S.D.	**332**	**475**

Table 5.5
Intrinsic clearance estimates using the parallel tube model.

POH ng/ml	CL_{20} ml/min	PO ng/ml	CL_{12} ml/min	CL_{10} ml/min
2723	27	6786	20	74
1260	22	4905	26	80
448	19	1763	46	59
607	24	531	69	22
1502	26	837	68	58
2727	23	443	70	51
4687	16	569	60	72
369	15	533	96	35
225	26	1153	34	30
181	18	1601	29	39
6008	18	1373	40	13
2489	20	1191	44	90
1737	25	1875	34	50
8602	27	4230	21	18
11997	23	2046	43	102
4855	22	1708	36	73
2009	26	11143	14	31
356	27	5565	22	19
		289	25	88
mean	**22**	889	32	87
S.D.	**3.9**	425	66	54
		225	91	46
		1059	60	29
		775	80	16
		768	40	109
		819	34	95
		574	26	60
		479	23	57
		8120	32	102
		3277	29	96
		1101	21	40
		460	23	58
		mean	**42**	**58**
		S.D.	**22**	**28**

any of the three determinants of organ clearance: organ blood flow, fraction unbound, or intrinsic clearance of unbound drug (Pang and Rowland, 1977b). Due to the ease of performance within our experimental system, we chose to study the effects of perfusate flow rate changes. Whereas the extraction of a compound shows little change (values are large and fractional changes are difficult to detect), the availability (1 - extraction) of a compound is quite sensitive to flow changes. The high extraction ratio drug (0.96) prednisone was used to examine the effect of perfusate flow on hepatic availability.

Experiments similar to those of Pang and Rowland (1977a) were designed with slight modification. The combination of high leporine hepatic flow rate, protein costs, and availability of red blood cells necessitated the use of recirculating methodology rather than that of single pass. In a recirculating system, because of varied extraction with flow, steady state concentrations (C_{in}) are not consistently maintained. This can be corrected by normalizing C_{out} to C_{in}. This ratio defines the availability at a particular flow rate, equivalent to one minus extraction ratio (1 - E). The availability at any flow rate was normalized to the availability at the ideal flow rate: $(1 - E_{exp}) / (1 - E_{ideal})$. Two determinations were made at each flow rate and averaged.

For the model predictions of the effects of flow changes on availability, the intrinsic clearance (CL_{int}) at the ideal flow rate (that which produced a pressure of 20 mmHg) was determined for both the well stirred and the parallel tube models from equations 8 and 9 in this fashion: extraction (E_{ws} and E_{pt}) was determined from C_{in} and C_{out} concentrations at the ideal flow; fu (fraction unbound) equaled one in all of the calculations because unbound drug concentrations were used throughout, and flow (Q_H) was measured. This allowed for the solution of CL_{int} for each model.

$$E_{ws} = \frac{fu\ CL_{int}}{Q_H + fu\ CL_{int}} \qquad (8)$$

$$E_{pt} = 1 - e^{-\left(\frac{fu\ CL_{int}}{Q_H}\right)} \qquad (9)$$

For varied flow rate predictions of availability, the intrinsic clearance term was assumed to be independent of flow. Predicted extractions at different experimental flow rates were calculated from equations 8 and 9.

Results

Viability

The viability of the *in situ* perfused rabbit liver was demonstrated in several ways. The urea synthesis rate was measured as the amount of urea formed, normalized to 100 grams of liver, over 150 minutes of perfusion. Figure 5.2 depicts representative time dependent production of urea in an experimental and a control perfusion (no steroid present in perfusate). Also included are cumulative values determined by Rothschild et al. (1968) in the *in situ* perfused liver of rabbits and by Hems et al. (1966) in the isolated perfused rat liver. Experiments were included only if urea synthesis continued at a constant rate over time, as demonstrated in the figure.

The synthesis of urea is a high energy process; four moles of ATP are required for the synthesis of one mole of urea. Although several values of the rate of urea synthesis are published for rat liver perfusions, few are available for the rabbit. Rothschild et al. (1968) have examined the rate of urea synthesis in the *in situ* perfused rabbit liver, reporting the production of 91 mg urea/100 g of liver/150 minute perfusion period, with no exogenous precursors. The livers in the present experiments were provided with the urea precursors ammonium chloride and ornithine; urea was synthesized at about 13 times the above rate (Figure 5.2). In two experiments in which no precursors were supplied, the rabbit livers

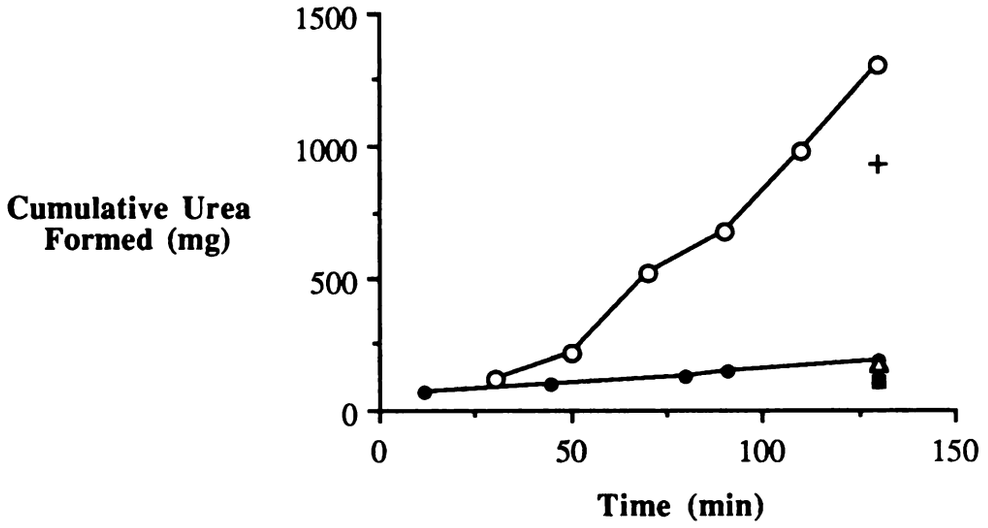

Figure 5.2. The urea synthesis rate in experimental (drug and precursors added, open circle) and control (no drug or precursors added, solid circle) rabbit liver perfusions. The amount of urea formed was normalized to 100 grams of liver, over 150 minutes of perfusion. Also included are cumulative values determined by Rothschild et al. (1968, solid square, open triangle) in the *in situ* perfused liver of rabbits and by Hems et al. (1966, plus symbol) in the isolated perfused rat liver.

appeared to synthesize urea at a higher rate than rat livers, although analytical methodologies differed and species comparisons may not be appropriate. Rat livers supplied with precursors produced 881 and the rabbit livers 1275 mg urea/100 g liver/150 min. Since our values, under similar experimental conditions, were at least equal to those reported in rabbits, and were greater than literature values in rats, we concluded that adequate energy forming processes occurred in the present experiments.

The stability of the extraction ratio of prednisone and prednisolone over time is another indicator of liver viability. Literature generally indicates good hepatic function for 120 to 180 minutes in the perfused liver (Ross, 1972). As can be seen in Figure 5.3, the extraction of both PO and POH remained constant for up to 150 minutes.

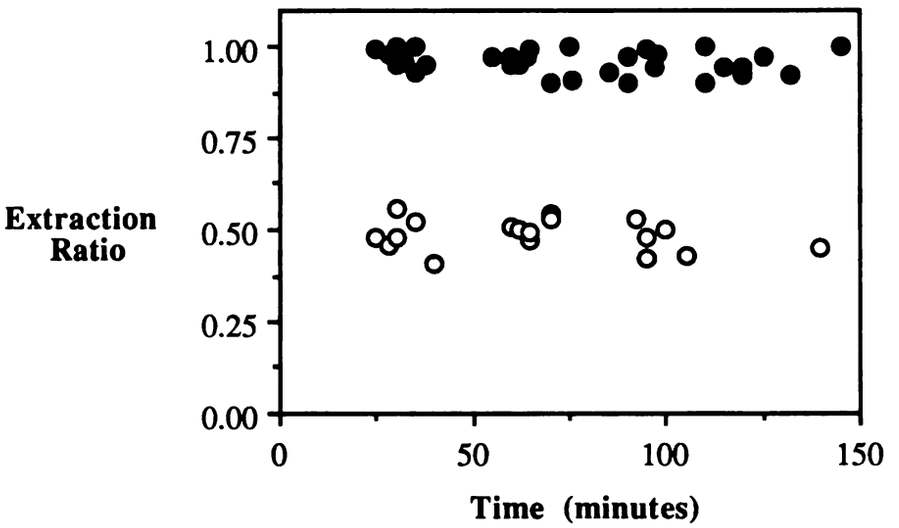

Figure 5.3. Extraction ratios of prednisone (solid circles) and prednisolone (open circles) by the perfused rabbit liver as a function of time.

The pressure-flow relationship remained very stable over the experimental period. These viability measurements for a representative rabbit liver perfusion are presented in Table 5.6. Flow changes ranged from 0 to 2 ml/min over the 150 minute experiment so as to maintain constant perfusion pressure, i.e. changes of only 0 to 6% of flow were necessary.

Table 5.6
Viability test measurements for a representative rabbit liver perfusion.

	Time (minutes)				
Viability Test	**30**	**60**	**90**	**120**	**150**
Lactate/Pyruvate Ratio	44.6	27.6	21.5	27.5	16.3
Perfusion Flow Rate (ml/min)	37	35	37	37	36
Perfusion Pressure (mmHg)	20	20	20	20	20

The lactate/pyruvate ratio is an indicator of the adequacy of tissue oxygenation; this ratio was measured every thirty minutes in these experiments. Schimassek (1962) examined the capacity of the perfused rat liver to regulate perfusate concentrations of lactate and pyruvate. He observed that the liver remained in an anaerobic state for 30 minutes after surgery; with adequate flow, it maintained lactate/pyruvate ratios equal to those found *in vivo* for 150 minutes. The lactate-pyruvate ratio in experimental and control perfusions performed here ranged from 44 to 16 (Table 5.6). The experiments of Rothschild et al. (1968) produced a ratio of 40, decreasing to 9 over time. We concluded that adequate oxygenation was achieved and that insufficient oxygenation did not contribute to the heavily reductive directionality of the rabbit liver toward the glucocorticoids.

Based upon multiple indicators of viability and liver function, we assumed that the perfused rabbit livers were viable and that these *in situ* experiments were a good approximation of the *in vivo* condition.

Hepatic extraction

The results from the fifteen perfusions performed are presented in Tables 5.1 and 5.2. Steady state concentrations were used to calculate hepatic extraction:

$$E_H = (C_{in} - C_{out}) / C_{in} \qquad (10)$$

where $C_{in} = C_{portal}$ and $C_{out} = C_{venous}$. Variability was quite small (C.V. = 8% for POH and 3% for PO perfusions) considering the concentration range encompassed, as well as the large number of animals involved.

Prednisone extraction approached unity (E_H =0.96) and can be classified as a high (hepatic) extraction ratio compound. This distinction implies that only the rate of delivery limits extraction. The extraction of prednisolone averaged 0.49, making it an intermediate

extraction drug; its hepatic removal was therefore dependent upon flow, binding, and intrinsic clearance.

An unexpected result of the liver perfusion studies was the apparent linearity of the extraction processes (Figure 5.4). This relationship was maintained through the 66-fold concentration range of 200 to 12,000 ng/ml steroid.

The liver and kidney purportedly exert primary influence over the disposition of the glucocorticoids, therefore evidence of nonlinear processes would be expected in these organs specifically. Systemic nonlinearities observed by us and others in whole animal studies may be the result of renal or yet another organ's contribution to the process of interconversion or biotransformation of one or both compounds.

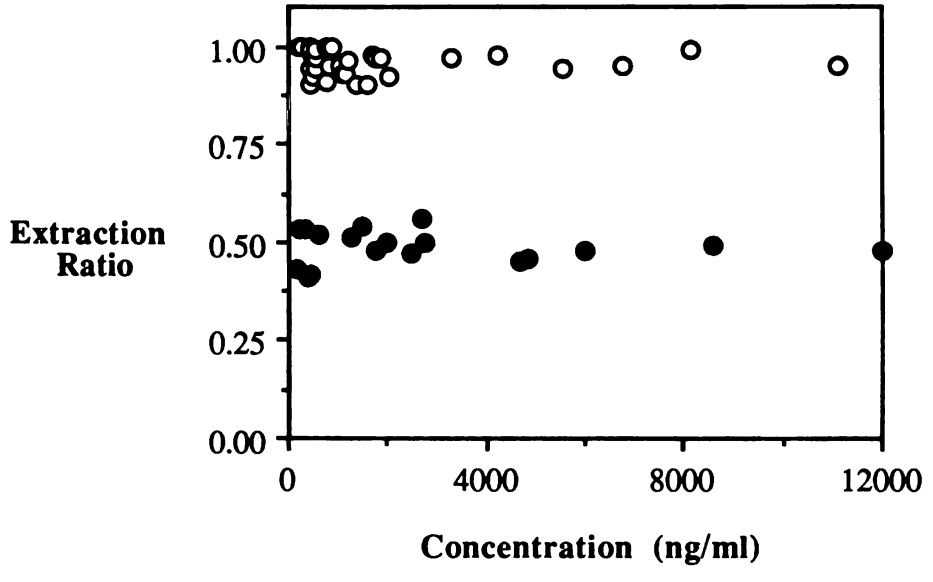

Figure 5.4 Extraction ratios of prednisone (open circles) and prednisolone (solid circles) by the perfused rabbit liver as a function of perfusate drug concentration.

Apparent hepatic clearance

Organ clearance, in this case hepatic, is defined as the volume of blood passing through the liver which is completely cleared of drug per unit time. Hepatic clearance (CL_H) is the product of hepatic blood flow (Q_H) and hepatic extraction (E_H), where the extraction ratio is defined as above.

$$CL_H = Q_H \cdot E_H \qquad (11)$$

The hepatic blood flow in rabbits of this weight was 30 to 40 ml/min. Table 5.1 shows that the apparent hepatic clearance of prednisone approached hepatic perfusate flow at 30.4 ml/min, due to its nearly complete extraction. The clearance of prednisolone was significantly less, 16.4 ml/min (Table 5.2).

Intrinsic clearance

For perfusion of two drugs which interconvert, a minimum of two differential equations each, or four total, describe the hepatic disposition of the drugs. Because the rabbit liver was found to be overwhelmingly reductive in these experiments, the absence of prednisone concentrations during prednisolone perfusions allows deletion of one equation in addition to the assumption that $CL_{21} = 0$. With three simultaneous equations, it is possible to obtain exact solutions for three parameters. The fundamental intrinsic clearance values of Figure 5.1 are presented in Tables 5.4 and 5.5. The well stirred model values will be discussed first.

The well stirred model CL_{20} values were quite constant within the 66-fold concentration range (181 to 11997 ng/ml); they averaged 32 ml/min and ranged from 19 to 42. The prednisone parameters displayed greater variability and three negative values were calculated for CL_{10}. CL_{12} ranged 12-fold from 105 to 1269 ml/min; CL_{10} exhibited great variation, -166 to 1859 ml/min. Despite large standard deviations, the CL_{12} and CL_{10}

values were approximately equal, with means of 490 ± 332 and 473 ± 475 ml/min, respectively. (All values of CL_{10} were included in the calculation of the mean CL_{10} value.)

The parallel tube model predictions for CL_{20}, which averaged 22 ± 3.9 ml/min, were of similar magnitude to that of the previous model. Similarly, these values exhibited little variability and a small standard deviation. In contrast, the magnitude of the prednisone intrinsic clearance parameters were significantly less than the well stirred model values. CL_{12} ranged 7-fold from 14 to 96 ml/min with a mean of 42 ± 22 ml/min; the range of CL_{10} values was similar, 13 to 102, averaging 58 ± 28 ml/min. For this model, $CL_{10} > CL_{12}$ but the difference was not significant due to the great variability.

Potential concentration or time dependencies were evaluated for the six clearance terms. The parameter with the greatest variation in values was selected for the statistical challenge (CL_{10}, well stirred model). As documented in Figures 5.5 and 5.6, despite great variability, a wide concentration range and nine individual animals, no significant correlation was observed with either concentration, $r^2 = 0.040$, or time, $r^2 = 0.028$.

For both models, some similarities were apparent: the irreversible elimination of POH (CL_{20}) was less than perfusate flow (35 ml/min) and the interconversion and irreversible loss clearances of PO (CL_{12} and CL_{10}, respectively) were approximately equivalent when it was assumed that $CL_{21} = 0$. The well stirred and parallel tube model estimates of these prednisone parameters differed by an order of magnitude, due to the high extraction of PO and the subsequent concentration differential across the liver. The well stirred model estimates of CL_{12} and CL_{10} were each about 500 ml/min; those of the parallel tube were about 50 ml/min.

The formation rate of POH from PO in mg/hr per kg versus steady state PO concentration is presented in Figure 5.7, This calculation was performed to document the apparent linearity of CL_{12}, despite its variability. The formation rate of POH from PO was calculated as the product of the concentration of formed prednisolone in the perfusate and the hepatic clearance of prednisone.

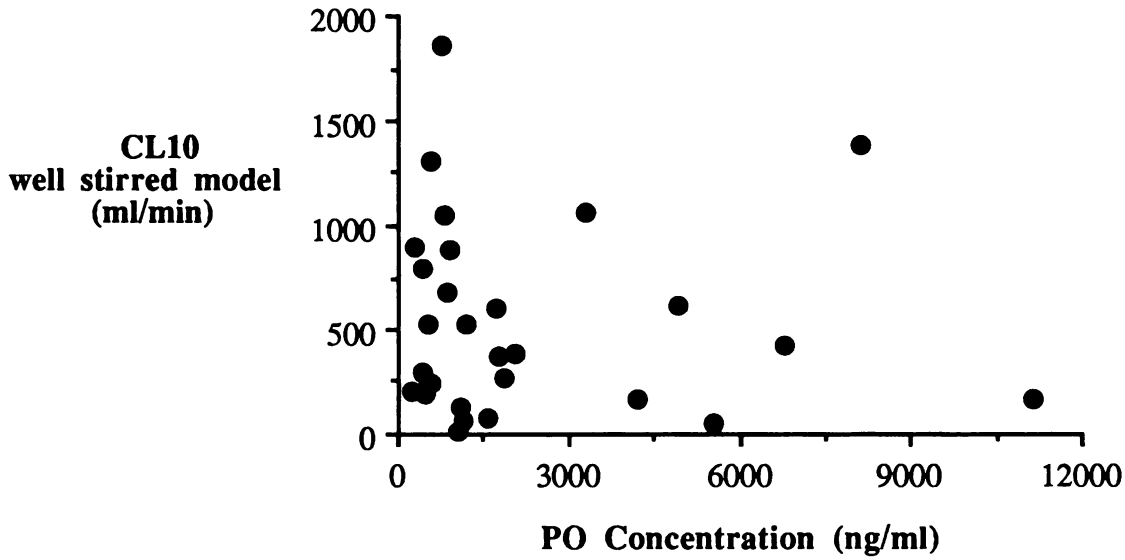

Figure 5.5. Relationship of $CL_{10,ws}$ to the steady state perfusate concentration of prednisone.

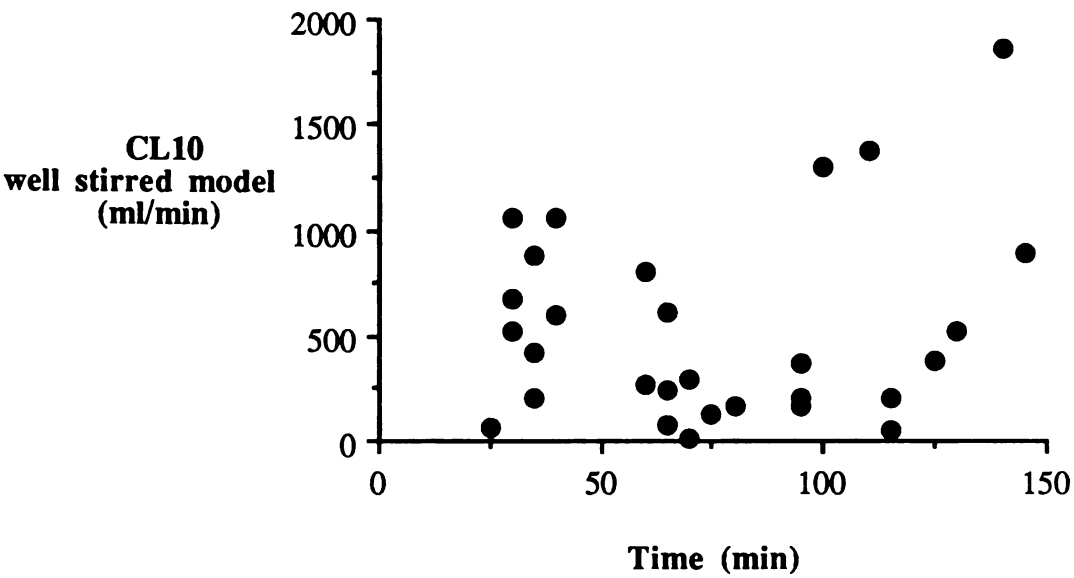

Figure 5.6. Relationship of $CL_{10,ws}$ to perfusion time.

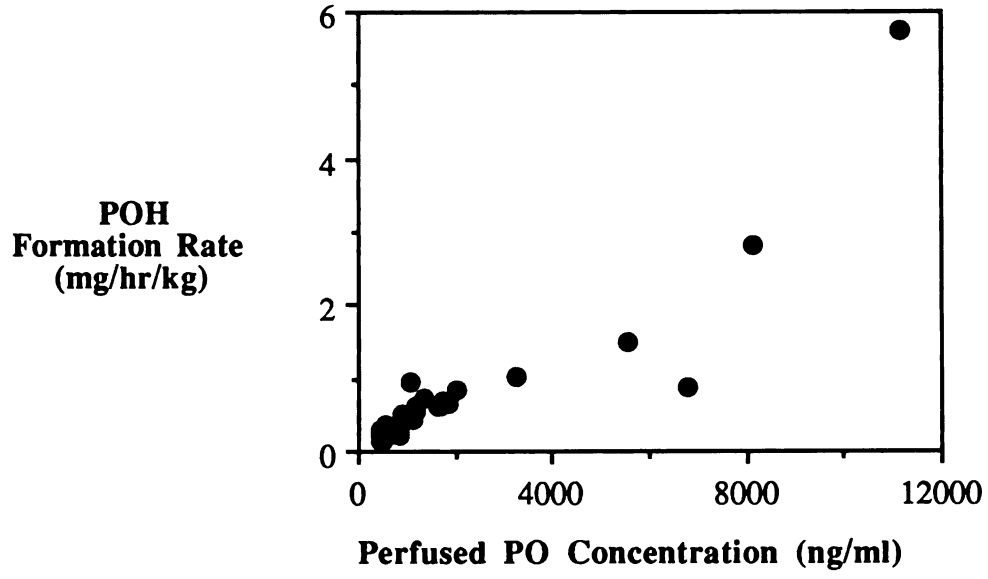

Figure 5.7 Formation rate of prednisolone from prednisone in rabbit liver perfusion experiments performed at steady state.

Distinguishing between models

For the calculation of hepatic intrinsic clearance parameters, it was necessary to estimate or predict hepatocyte concentrations of the drugs. The well stirred and parallel tube models comprise the boundary estimates of predicted tissue concentrations (Roberts and Rowland, 1986). The former assumes that the entire liver is in equilibrium, implying that intrahepatic drug concentrations, C_H, are equivalent to those exiting the liver, C_{out}. The parallel tube model assumes that drug concentration is reduced exponentially as the perfusate travels through the liver, or that the average C_H is a function of both C_{in} and C_{out} and is of intermediate value.

The selection of the well stirred or parallel tube model was made by examination of the change in out-flowing concentration, with changing perfusate flow. Figures 5.8 and 5.9 are plots of the theoretical effects of flow changes on extraction (or availability) for the

well stirred and parallel tube models. The two models deviate as flow is increased beyond ideal (to the right of the point of intersection of the two lines). The availability predicted by the parallel tube model demonstrates an exponential dependence on flow (equation 9). Well stirred model availability is linearly related to flow (equation 8). The effect of flow dynamics on the availability of the high extraction ratio compound prednisone, was determined in two experiments represented in Tables 5.7 and 5.8. The experiment presented in Table 5.8 encompassed a wider range of flows than did the former. Portal (C_{in}) and venous (C_{out}) averaged concentrations are presented with perfusate flow rate in the order performed experimentally. C_{out} concentrations were normalized to C_{in} to yield the availability. Attempts to regulate the C_{in} concentrations were somewhat successful, although it can be seen in Table 5.8 that C_{in} values varied extensively. Figures 5.8 and 5.9 are plots of the ratio of availability at the new flow rate to that at the ideal flow rate (that which yielded 20 mmHg pressure). This ratio demonstrated little variability, despite a six-fold range in flow rate, as is characteristic of the well stirred model. Both experiments

Table 5.7. Altered hepatic flow experiment as depicted in Figure 5.8.*

Flow (ml/min)	C in (ng/ml)	C out (ng/ml)	Availability	Normalized Availability
40	1637	116	0.071	1.039
30	1580	85	0.054	0.789
44	1128	95	0.084	1.235
25	2044	114	0.056	0.818
40	1405	92	0.065	0.960
46	1100	96	0.087	1.280
21	2440	103	0.042	0.619

*normalized availability is defined as the availability at any flow rate divided by the average availability at a flow rate of 40 ml/min.

Table 5.8. Altered hepatic flow experiment as depicted in Figure 5.9.*

Flow (ml/min)	Cin (ng/ml)	Cout (ng/ml)	Availability	Normalized Availability
30	1603	48	0.030	0.962
58	1112	58	0.052	1.677
28	1527	44	0.029	0.926
12	2040	42	0.021	0.662
24	1855	66	0.036	1.143
46	1517	90	0.059	1.906
63	3698	157	0.042	1.364
30	2415	78	0.032	1.037
72	2158	162	0.075	2.412

*normalized availability is defined as the availability at any flow rate divided by the average availability at a flow rate of 30 ml/min.

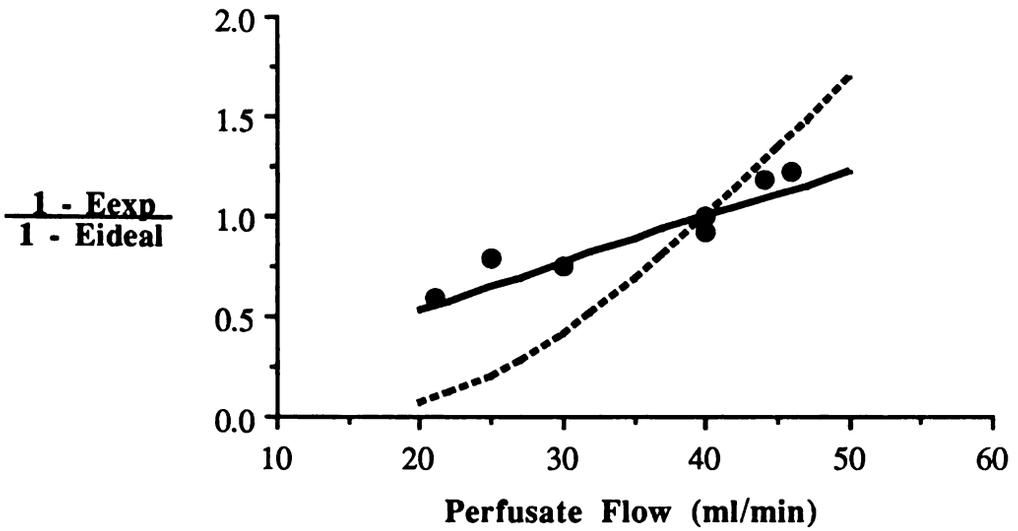

Figure 5.8. Theoretical and experimental effects of flow changes on the extraction of prednisone in the perfused rabbit liver for the well stirred (solid line) and parallel tube (broken line) models from experiment 1; experimental data, solid circles.

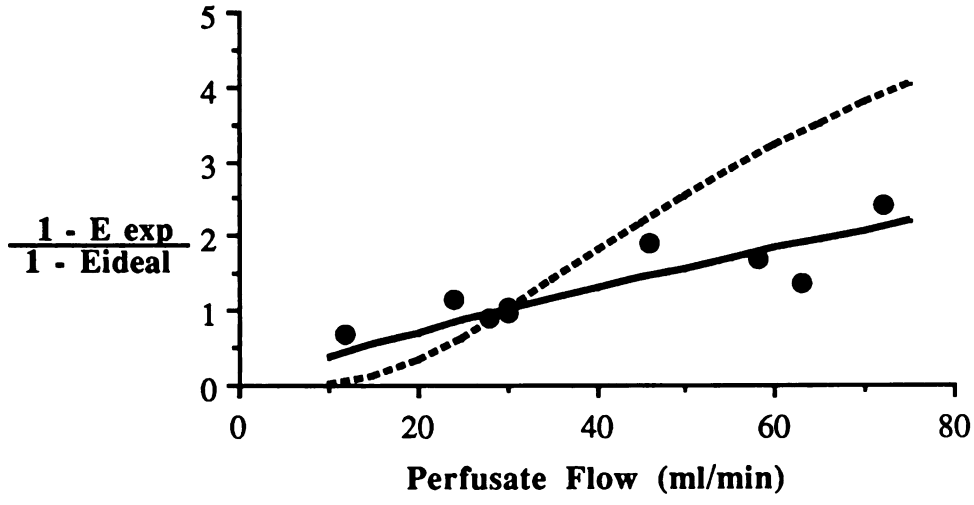

Figure 5.9. Theoretical and experimental effects of flow changes on the extraction of prednisone in the perfused rabbit liver for the well stirred (solid line) and parallel tube (broken line) models from experiment 2; experimental data, solid circles.

clearly demonstrated the superiority of the well stirred model over the parallel tube model. This pattern was followed at flow rates both above and below the ideal hepatic perfusate flow in two rabbit liver preparations.

Discussion

Little attention has been given to glucocorticoid disposition in the perfused liver. The kidney has been perfused to examine the potential existence of saturable elimination processes; the role of glucocorticoids in lung maturation has been studied by several groups with lung perfusions in several species. The liver is recognized as the predominant steroid metabolizing organ, but homogenates and microsomes were utilized for the studies which resulted in this observation. Three liver perfusion studies with glucocorticoids have been published: Berliner et al. (1962), Axelrod et al. (1955) and Hechter et al. (1957). Each of

these measured total radiolabelled compound in perfusate and bile, very nonspecific analyses. Additionally, only the work by Berliner et al. was performed with reasonable (low) doses of steroid, which might permit extrapolation of results to the intact animal.

The results from experiments in humans and animals has suggested that a saturable process(es) may be involved in the disposition of PO and POH; and there may be a maximal concentration of PO achievable in some species (Frey et al., 1981), including humans (Legler et al., 1982). Therefore, the result of linear extraction in these rabbit liver perfusion studies over a 66-fold concentration range (Figure 5.4) was unexpected. The same observation has been made in our laboratory in the perfused rat liver (Table 5.9). The majority of therapeutic uses of these glucocorticoids result in concentrations of less than 1000 ng/ml total drug. Hence, the concentrations examined in these perfusion studies easily encompassed the therapeutic concentration range.

At prednisolone concentrations of nearly 12,000 ng/ml, no PO was measurable. Assuming half of the POH was converted to PO (~6,000 ng/ml), and 4% escaped extraction, a 240 ng/ml PO concentration would result; this is about 10-fold higher than our detection limit and should have been measurable if metabolism occurred in discrete steps.

The absence of prednisone in the perfusate upon prednisolone perfusion of the rabbit liver can be explained by several hypotheses. The first is that the liver is extremely reductive in its interconversion of PO and POH, such that little or no net PO is formed from POH. The literature supports this observation (Murphy, 1981; Koerner, 1966). In terms of our model, $CL_{12} >> CL_{12}$, and taken to its extreme, $CL_{21} = 0$. In such a case, both oxidation and reduction occur, although one process was much less significant than the other.

A second explanation for the apparent lack of PO is that the irreversible elimination of PO occurs to a very great extent. That is, upon POH perfusion, any PO which is formed is immediately eliminated before it can escape the liver or can be back converted to POH: $CL_{10} >> CL_{20}$. The major elimination route of both PO and POH, 20β-

Table 5.9
Isolated Perfused Rat Liver Experiments

Prednisone perfusions			**Prednisolone perfusions**	
Total Steady State Prednisone Concentration (ng/ml)	Extraction Ratio	Prednisone: Prednisolone Concentration Ratio	Total Steady State Prednisolone Concentration (ng/ml)	Extraction Ratio
752	0.94	0.32	668	0.65
1314	0.94	0.42	1038	0.56
648	0.97	0.20	672	0.54
1190	0.93	0.59	961	0.60
325	0.93	0.37	374	0.64
2030	0.97	0.20	279	0.80
782	0.97	0.45	685	0.61
1854	0.96	0.31		
685	0.96	0.41		
mean	**0.95**	**0.36**	mean	**0.63**
S.D.	**0.02**	**0.12**	S.D.	**0.09**

hydroxylation, occurs for both compounds (Miyabo et al., 1973); whether it occurs at a greater rate for PO than POH is unknown. A variant of this theory is nonuniform enzyme distribution, resulting in an apparently unidirectional process across the whole liver.

A third hypothesis involves differential extraction of prednisone (including back conversion to prednisolone), dependent upon whether PO was formed or presented: the extraction of PO may exceed 99% when prednisone is formed intracellularly, but when presented extracellularly, as in perfusate, diffusion may be rate limiting and only 96% extraction is seen. Consequently, PO is formed from POH intracellularly, but is not measurable in perfusate because it is converted back to POH or irreversibly eliminated.

Of the above proposals, the first is most supported by biochemical experimentation. The assumption that $CL_{21} = 0$ is probably incorrect, because liver preparations incubated *in vitro* demonstrated the ability to convert POH to PO. CL_{21} is probably much smaller than CL_{12} or CL_{10}, so the influence of such an assumption on the calculation of these parameters should be small. Estimation of the true value of CL_{21}, the oxidative conversion of POH to PO, in the perfused liver of the rabbit was not possible, as no prednisone was measurable in the perfusate after prednisolone perfusion. The same observation of the absence of PO has been made in our laboratory in rat liver POH perfusions (Table 5.9). It is highly possible that CL_{21} has a non zero value and may even be saturable in the rabbit liver. In fact, multiple laboratories have documented the ability of the liver of nearly all species to perform bidirectional conversion *in vitro*, by the oxidation to 11-keto and reduction to 11β-OH of structural analogues of PO and POH (Koerner, 1966; Hurlock and Talalay, 1959). Whereas conversion of POH to PO is not apparent in the rabbit liver, the calculated CL_{12} is probably representative of the net interconversion process, (CL_{12} - CL_{21}). Studies with inhibitors or isotopically labelled compound would be necessary to examine this process further. *In vitro* studies of hepatic microsomes, homogenates and slices have shown that the liver of many species can oxidize POH to PO or cortisol to cortisone (Hurlock and Talalay, 1959; Chapter 4); therefore, CL_{21} has a nonzero value *in vitro*.

An important factor to consider in assessing the linearity of these processes is the proteins present in the perfusate. Rabbits, like humans, display nonlinear binding of POH to corticosteroid binding globulin (Rocci et al., 1980); it has been theorized that the observed nonlinearities in the pharmacokinetics of POH can be attributed to this binding (Pickup et al., 1977). Experimentally, these livers were cleared of rabbit blood at the start of the perfusion. The binding of both PO and POH to perfusate proteins is linear (data not shown), due to the omission of corticosteroid binding globulin from the perfusate. Hence, the effect of saturable plasma protein binding on the hepatic extraction of PO and POH

cannot be assessed using this methodology; experiments must be performed with human and rabbit blood, which both contain the specific binding protein.

The numerical solution of the three intrinsic clearance parameters was accomplished in the following manner: an average value of CL_{20} across all concentrations was obtained from the POH perfusion experiments using equation 5; this value was then substituted into PO perfusion equations first to estimate CL_{12} (equation 6) which was then used to estimate CL_{10} (equation 7). Variability was greatest with CL_{10}, as three negative values resulted. These occurred in three different preparations, all at intermediate concentrations. These negative values may be meaningful: the well stirred model may not perfectly predict and summarize hepatocyte drug disposition. Additionally, the assumption that $CL_{21} = 0$ may not be valid. But no negative values were obtained for the parallel tube model where this assumption was used.

Discrimination between the two models of hepatic disposition was accomplished by examining the effect of increasing and decreasing perfusate flow on the availability of the highly extracted PO. The well stirred model was clearly superior to the parallel tube model (Figures 5.8 and 5.9). The hepatic disposition of several other drugs is also more appropriately predicted by the well stirred than the parallel tube model (Pang and Rowland, 1977a; Ahmad et al., 1983; Keiding and Chiarantini, 1978; Keiding and Steiness, 1984). Physiologic or anatomic support for this model of hepatic disposition has been presented by Brauer (1963), wherein the hepatic vascular tree can be visualized as a convoluted path with multiple shunts and poor laminar flow. Brauer postulates that reversed-direction flow is even possible in some sinusoids. A reversible metabolic process may be envisaged in a similar manner, as the reaction proceeding forward, and occasionally backward. Certainly, it is easier to rationalize the fit of the well stirred model over the more restrictive, unidirectional parallel tube model. In actuality, the best prediction of the hepatic disposition of PO may result from the distributed (Forker and Luxon, 1978) or the dispersion models (Roberts and Rowland, 1986), which both lie somewhere between the extremes of the

model predictions made here. For interconverted compounds, whether the mechanism be chemical or enzymatic, the well stirred model may be at first approximation a better predictor of hepatic disposition than the parallel tube model. Obviously, this is very dependent on the details of the interconversion mechanism, which include the rate and extent of interconversion within an organ, both independently and relative to elimination pathways. Figure 5.10 describes prednisone and prednisolone disposition in the liver; CL_{21} was assumed to equal zero and was therefore omitted from the figure; well stirred model calculations of the three remaining intrinsic clearances are presented.

Similar profiles of exiting drug were produced by perfusion of either prednisone or prednisolone. Upon perfusion, about half of either drug was recovered as POH and little or none was recovered as PO. A dose of PO or of POH yielded similar plasma profiles *in vivo*. Following an oral dose of PO, extraction was nearly complete and little PO was measurable; about half of the PO perfusion dose was recovered as POH (Figure 5.11). The formation rate of POH from PO in the perfused rabbit liver was graphically depicted in Figure 5.6. It is evident that despite variability and a large concentration range, the formation of POH was nearly constant with respect to substrate concentration. Following the same dose of POH administered orally, extraction was 0.49, such that half of the POH was recovered; no PO was detected.

The interconversion and metabolism of the glucocorticoids differ among species, organs, and with age or developmental stage (Monder and Shackleton, 1984). For this reason, leporine hepatic results cannot be directly extrapolated to human hepatic processes. However, conceptual understanding gained from rabbit experiments may be applied to human experimental data because in this way, hypotheses may be created to explain the complex *in vivo* metabolic profile and apparently nonlinear pharmacokinetics of prednisone and prednisolone in humans.

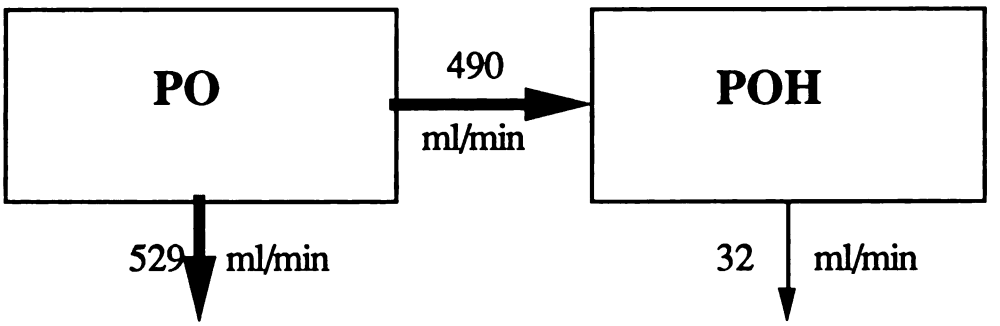

Figure 5.10. Model of the interconversion and elimination of prednisone and prednisolone in the perfused rabbit liver. Intrinsic clearance values were calculated using the well stirred model predictions of hepatic disposition as defined in Figure 5.1.

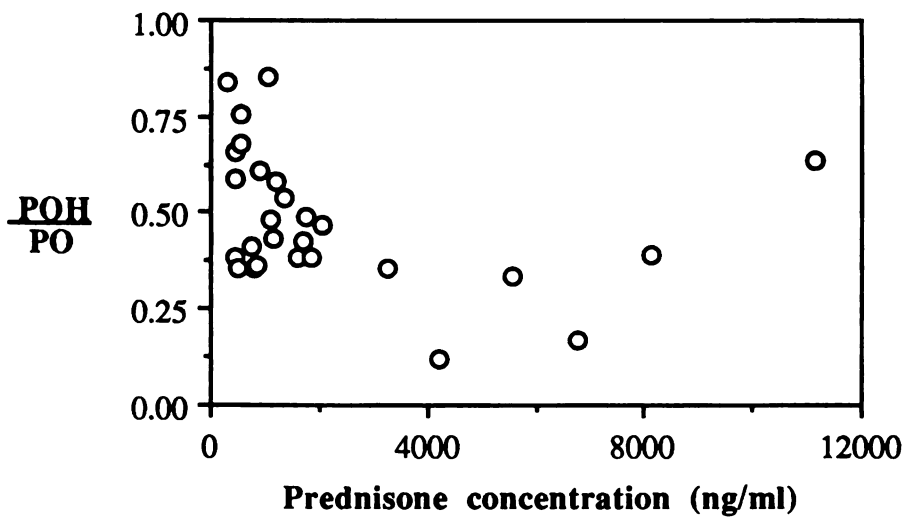

Figure 5.11. Ratio of formed prednisolone to perfused prednisone for perfusion of the rabbit liver.

Conclusions drawn from modeling must be termed *apparent* until specific experiments verify the observation: it *appears* that the rabbit liver does not produce prednisone from prednisolone. This assertion is particularly relevant for any reversible metabolic system because of its inherent complexities. The organ perfusion experiments described here yield *apparent* hepatic and intrinsic clearance values (Tables 5.1, 5.2, 5.4 and 5.5).

Conclusions

Pharmacokinetic studies performed in multiple species, including humans and rabbits, have documented nonlinearities in the apparent clearance of both PO and POH, and some investigators have suggested that a saturable process may be involved in the oxidation of POH to PO. Under conditions which approach normal and physiologic, unexpected results were attained from the perfused rabbit liver. *In situ* perfused rabbit livers demonstrated no apparent saturation in the extraction of either PO or POH or in the reduction of PO to POH. The hepatic extraction of prednisone approached unity, and hepatic clearance approached organ blood flow. The elimination and interconversion intrinsic clearances of PO greatly exceeded those of POH. These perfusion studies suggested that the liver of the rabbit did not contribute to the nonlinear disposition of PO and POH observed in the rabbit *in vivo*.

Prednisone was not measurable after hepatic perfusion over a wide range of POH concentrations. In all mammals studied to date, a significant fraction of the administered dose of prednisolone was measurable as prednisone *in vivo*. Hence, some extrahepatic organ of the rabbit is responsible for producing these measurable quantities of the oxidized drug.

Prednisolone was approximately half extracted across the leporine liver and no PO escaped. Roughly half of a prednisone dose was recoverable as prednisolone upon exit and very little PO passed unchanged. In other words, both drugs produced a similar

hepatic perfusion profile. Together, these results verified the clinical observation that oral administration of either PO or POH yields indistinguishable effects (Haynes and Murad, 1985).

The disposition of prednisone across the leporine liver was modeled using the well stirred and the parallel tube models. The well stirred model appeared to better predict the rabbit liver extraction of PO than did the parallel tube model over a wide variation in flow rates. This superior prediction can be logically perceived, as well, since reversible metabolic processes inherently possess some disorganized, random nature.

Intrahepatic events cannot be measured at this time, unlike kidney experiments using micropuncture techniques. Hence, the liver is a black box which we have attempted model. In the *in situ* perfused liver, in the absence of corticosteroid binding globulin, all metabolic and distribution processes were apparently linear. This implies that hepatic metabolic events are not the cause of the observed nonlinearities in the disposition of prednisolone *in vivo* in rabbits.

Chapter 6

Isolated Human Liver Perfusions

Introduction

Little is known about the metabolic capacity of human tissues; one exception is red blood cells, due to their availability. The human placenta has occasionally been examined for its metabolic function as well. Small portions of surgically excised tissues have been available from biopsy samples or tumor removal. There is even less experience with the perfusion of whole human organs. Fortunately, recent surgical advancements have increased the availability of human organs for transplantation. An extension of this work is the availability of human tissues for experimental research uses. A protocol for experimentation with livers which were not successfully matched for transplantation was approved by the Northern California Transplant Network, making possible the acquisition of human livers.

Methods

As might be expected, no information is available on the perfusion of human livers. Agius (1987) has reviewed the use of human liver for *in vitro* experimentation, primarily as isolated hepatocytes. Inter-species hepatic anatomical differences are generally small, with the exception of the presence or absence of a gallbladder. Therefore methodology for

human liver perfusion was adapted from the techniques of liver perfusion of the rabbit (Chapter 5).

Liver donors

The surgical removal of a human liver was performed by liver and kidney transplant surgeons at the University of California San Francisco Hospital or other hospitals within Northern California. The livers originated from cadavers whose organs were donated for transplantation or for research purposes. Often, the kidneys were matched for transplantation, and the liver was not matched, and subsequently was made available for biomedical research. It is also possible that the organ was removed with the intention of transplanting it, but a better match was later found for the recipient. A third source is a liver that expressed some mild disease and was rejected for transplantation; this situation was not clinically apparent in the donor but was detected in liver biopsy after removal. The two livers perfused here were not affected by disease that would alter steroid metabolism. Donors were only accepted if they were in excellent health prior to brain death. Kidney and liver function tests were performed on all potential donor organs and were strictly interpreted by the transplant surgeons before organ removal. All potential donors were tested for hepatitis and acquired immune deficiency syndrome (AIDS).

The donor was taken to surgery within a few hours of the declaration of brain death. Circulatory status may have deteriorated during this waiting period and vasopressors may have been used to ensure normal organ blood flow for a few hours before surgery. Additionally, the patient may have received antibiotics or other medications on admission. The medications which would render this organ unacceptable for our experimental purposes are compounds which alter the metabolic capacity of the liver (P_{450} and steroid dehydrogenases) or change specific binding protein (CBG) concentrations: phenobarbital, phenytoin, carbamazepine, erythromycin, rifampin, cimetidine, glucocorticoids and estrogens. A copy of the hospital record abstracted by transplant

technicians was obtained for each donor. These contained medical and social histories as well as medication administration records.

Surgery

The abdomen of the patient was opened and the relevant organs were isolated. The surgery was performed without anesthesia; pancuronium bromide (0.05 mg/kg), heparin (20,000 to 30,000 units) and phentolamine (15 mg) were administered intravenously and in some cases, mannitol was administered (50 g). Full circulatory function was maintained until the time of organ removal. The organs to be transplanted were removed first and the donor was sacrificed. Afterward, the research organs were isolated and flushed with several liters of ice-cold UW (University of Wisconsin, see Table 6.1) solution then removed. The liver received full blood flow until just prior to removal.

Table 6.1 Composition of University of Wisconsin preservation solution.

Component	**Concentration (mmol/l)**
Potassium lactobionate	100
NaH_2PO_4	20
KH_2PO_4	5
Adenosine	5
$MgSO_4$	5
glutathione	3
raffinose	30
allopurinol	1
insulin	100 units
sulfisoxazole/trimethoprim	40 mg/2.5 mg
dialyzed Hexethyl starch	50 g

pH 7.4
320-330 mosm/l

The success of transplantation is largely determined by the preparation of the organ during the period of ischemia (Humphries, 1967). Therefore, it was of particular importance to keep the liver cold during the ischemic period before perfusion. Hence, the liver was stored surrounded by ice in a cooler until the experimental details were complete.

Perfusate, apparatus and experimental details

The perfusion apparatus was similar to that for the rabbit liver, but simpler in design due to the limited availability of equipment at this stage in the project. The vasculature was composed of Tygon tubing (R-3603, Fisher, Pittsburg, PA) 5/16" I.D. x 7/16" O.D.; the polypropylene joints were of the same diameter (Cole Parmer, Chicago, IL). A 2-liter glass beaker was heated and stirred continually on a stir-heat plate (Corning, NY); temperature was monitored continuously until it reached 37 °C, then every 5 minutes with a common laboratory thermometer. The pH was adjusted initially then measured every five minutes (Chemcadet, Cole Parmer, Chocago, IL) and maintained between 7.35 and 7.45. Oxygenation was accomplished with a Variable Prime Cobe Membrane Lung (VPCML, Cobe, Lakewood, CO); the heat exchanger and filtration systems were disconnected from the oxygenator due to their large priming volume and the limited availibility of red blood cells at the times of the perfusions. Carbogen (95% 0_2/5% CO_2) was used to oxygenate through the blood-gas exchange device, acheiving a pO_2 of about 50. Perfusate flow was the rate which achieved 20 mmHg pressure; flow was adjusted to maintain the pressure at this level. A mercury manometer (Baum, New York, NY) was attached to the system for continuous pressure measurements. No filtration mechanism was used because the priming volume exceeded one liter. Depending on the surgical preparation of the organ, one or two cannula(e) were sutured in place in the portal vein(s). The multiple hepatic artery branches were ligated with silk suture and clamped. Flow freely exited the liver through the vena cava. The liver rested on a flat support device within a funnel. Perfusate was returned to the stirred reservoir.

The human liver perfusate was similar to that used in rabbit liver perfusions, as described in Chapter 5. Fifteen-hundred ml Krebs-Heinseleit bicarbonate buffer, pH 7.4, was used. Bovine serum albumin was dissolved to a concentration of 4.5% (the cost of the required quantity of human albumin is prohibitive). Glucose was the energy supply, included at 100 mg%. Twenty mM ornithine and 5 mM ammonium chloride were included. Expired human red blood cells were included to attain a hematocrit of 0.25 (500 ml). The blood type was the same as that of the organ donor, or 0-negative if the same type was not available. Due to the speed required in the preparation of the buffer after a liver was obtained, the cells were not washed in the laboratory before use. These cells were obtained as either washed red blood cells or packed red blood cells. Instead, they were filtered through about 20 layers of gauze before incorporation into the buffer. This perfusate was prepared immediately before use.

The viability tests used were the urea synthesis rate, constancy of pressure-flow relationship and constancy of extraction ratio.

The human liver was kept on ice in a bag with UW solution until the time of cannulation, at which time the liver was removed from the bag and the vessels were isolated. The arteries were ligated and clamped and the portal vein was cannulated; the bile duct was ligated. Warm perfusate flow was initiated slowly to wash out the UW solution (~50 ml/min), then gradually increased to about 200 ml/min. When the red cells appeared to flow from the vena cava in great volume, perfusate was collected for recirculation. Flow was slowly increased to yield 20 mm Hg pressure over the next 30 minutes. This gradually brought the liver to ambient temperature; 37 ^{o}C was achieved within the next hour of perfusion.

Steady state studies were not performed, because the extraction of PO and POH in the human liver were not known. Instead, a new concentration was achieved by bolus

administration of drug to reservoir, a few minutes were allowed for mixing, then two to six samples were taken quickly (over 5 to 20 minutes) and averaged.

Viability

The urea synthesis rate was the most significant viability test. Figure 6.1 depicts urea synthesis of the perfused human livers. It can be seen that the rates were not constant; the values for one preparation plateaued and the other demonstrated a slow initial rate followed by a faster rate. Ammonium chloride and ornithine were supplied in concentrations which were scaled up from rabbit liver perfusions, with little knowledge of utilization capacity; it is possible that the first preparation reached a plateau with respect to urea synthesis not because of hepatic dysfunction, but because of a lack of substrate.

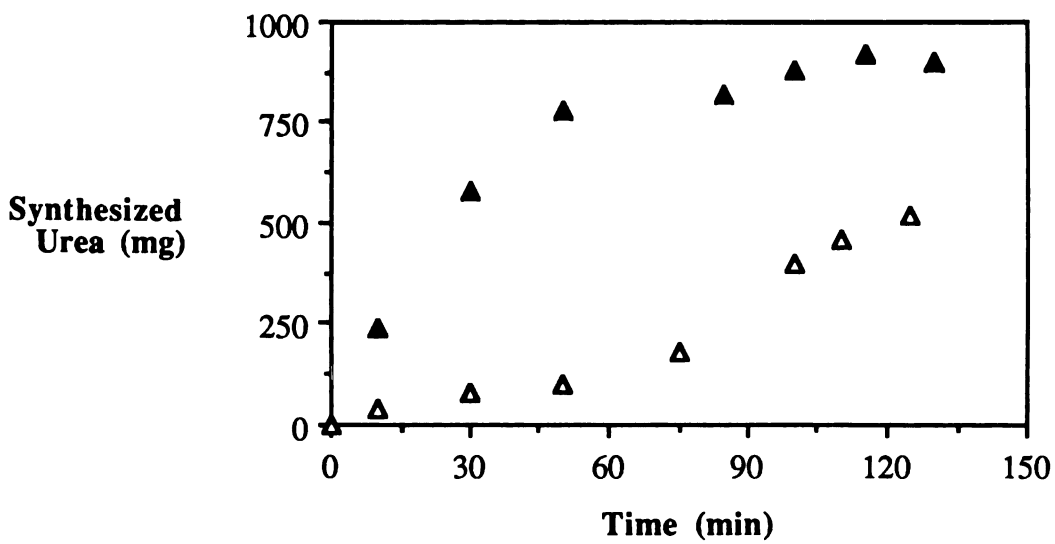

Figure 6.1. Rate of urea synthesis in human liver perfusions; preparation H1, solid triangles; preparation H2, open triangles.

Liver H1 continued to extract drug linearly and to maintain a constant pressure-flow relationship throughout this period and showed no other signs of deterioration. Eight mmol of ammonium chloride and 32 mmol of ornithine were supplied; approximately 17 mmol of urea were produced at the plateau, or utilization of half of the supplied precursor. In preparation H2, the perfusion flow rate increased continuously and began to stabilize by two hours; this may be reflected in the increasing rate of urea synthesis with time in this preparation. The low urea measurements may be expected for a liver which is poorly perfused and not functioning optimally.

Physiologic flow rates were not achieved in either preparation; this may have been a result of the cold storage period or some other unknown factor. The decision was made to perfuse the human and rabbit livers by pressure. The human livers probably should have been perfused by predicted flow rates after the initial equilibration period. The highest perfusion rates achieved in the human livers were about 1100 and 900 ml/min for preparations H1 and H2, respectively. The pressure-flow ratio remained relatively constant once equilibration was achieved in preparation H1, but the second liver appeared to stabilize only toward the end of the experiment, increasing from 560 to 900 ml/min over 2 hours.

Results

Tables 6.2 and 6.3 presents the results of the perfused human liver experiments. Two were performed: Liver H1 was perfused with POH alone; liver H2 received POH, followed by PO. Perfusion of the human liver with prednisolone in experiment H1 yielded constant extraction over the concentration range of 620 to 1870 ng/ml. The extraction ratio of prednisolone was observed to be about 0.21, with little variation. Liver H2 extracted POH to a lesser degree; for concentrations 4532 and 5320 ng/ml, extraction was 0.09 and 0.05, respectively.

Table 6.2. Human liver perfusions: prednisolone experiments

Perfusion	Prednisolone Concentration (ng/ml)	Extraction	Prednisone Concentration (ng/ml)	Prednisone: Prednisolone Ratio	Perfusate Flow (ml/min)	Apparent Hepatic Clearance (ml/min)	Viability
H1	620	0.22	39	0.06	1050	231	good
	1512	0.22	179	0.19	1050	231	good
	1870	0.19	78	0.04	1050	200	acceptable
	944	0.21	38	0.04	1100	221	acceptable
H2	4532	0.09	70	0.02	560	50	poor
	5320	0.05	68	0.01	700	35	poor

Table 6.3 Human Liver Perfusions: Prednisone experiments

Perfusion	Prednisone Concentration (ng/ml)	Extraction	Prednisolone Concentration (ng/ml)	Prednisone: Prednisolone Ratio	Perfusate Flow (ml/min)	Apparent Hepatic Clearance (ml/min)	Viability
H2	2995	0.41	?	?	760	312	good
	531	0.5	?	?	880	440	good
	104	0.43	?	?	900	387	good

Prednisone perfusion yielded an approximately constant extraction, as well, over the concentration range of 104 to 2995 ng/ml, at about 0.45 (preparation H2). It was not possible to measure POH concentrations produced, as POH had been perfused prior to initiation of PO perfusion in the preparation and was still present in high concentrations. Prednisolone concentrations remained relatively constant, indicating conversion of PO to POH was of about the same magnitude as the extraction of POH (data not shown).

Prednisone was measurable consistently in both POH perfusions H1 and H2; the concentrations were low and ranged from about 40 to 160 ng/ml. Figure 6.2 shows the concentration of PO resulting from the two POH perfusions. It can be seen that with one exception, PO concentrations were relatively constant; this is a conclusion based on preliminary results, as only two livers were perfused.

Table 6.2 shows the PO/POH ratio resulting from experiments H1 and H2; within the first experiment, the ratio is quite variable, ranging from 0.04 to 0.19; the values for the second liver are 0.02 and 0.01. Table 6.3 does not display the prednisolone concentrations resulting from perfusion with PO, nor the ratio of the concentrations. This omission was necessary because perfusion with prednisone followed perfusion with prednisolone, and as prednisolone was still present in the perfusate in high concentrations, it was not possible to accurately estimate POH concentrations resulting from conversion of prednisone.

The apparent hepatic clearance of POH in liver H1 ranged from 200 to 231 ml/min; the values for liver H2 were 35 and 50 ml/min. The flow rates and extraction were both reduced in preparation H2 relative to H1 and combined to produce this difference between preparations. The apparent hepatic clearance of PO in preparation H2 ranged from 312 to 440 ml/min, as a function of both flow and extraction changes.

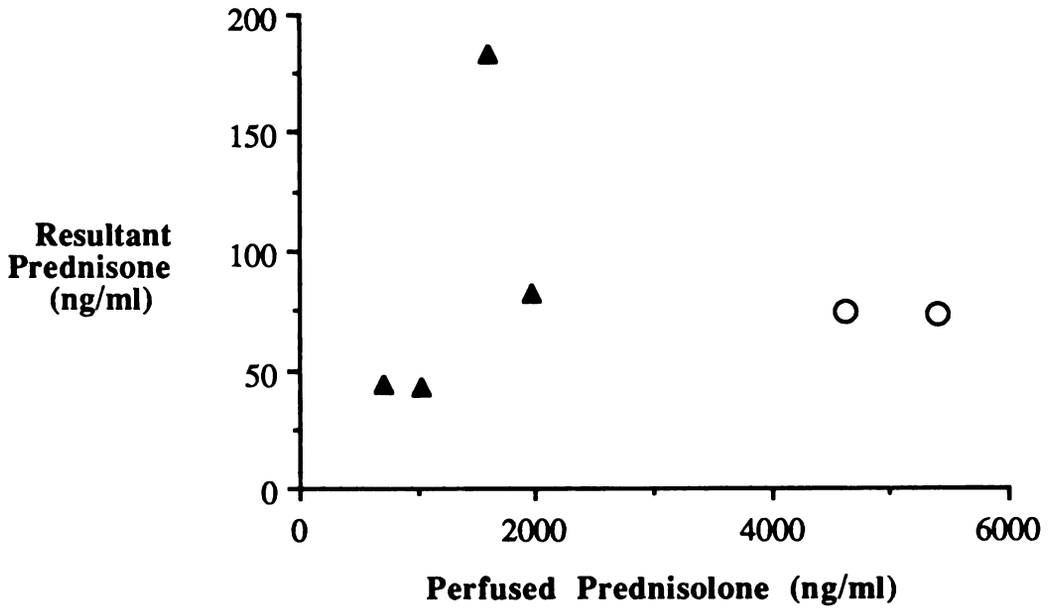

Figure 6.2 Steady state concentration of prednisone produced by the human liver perfused with prednisolone; preparation H1, solid triangles; preparation H2, open circles.

Discussion

The objective of perfusion of the human liver with PO and POH was to determine whether the extraction of these compounds was similarly to that in the rabbit liver. Seven human livers were obtained over a several month period. Two of these were perfused; the remainder were used in *in vitro* studies or frozen for future use. The human livers were obtained with little or no notice, yielding minimal preparation time. Additionally, red cells could not be obtained on all occasions. Nevertheless, important results were derived from two perfused human liver preparations.

It is not appropriate to compare these preparations as they were treated differently prior to perfusion, that is, preparation H2 was stored on ice for 14 hours longer than preparation H1 (twenty versus six hours). In the transplantation of livers in humans, preservation technology has advanced to the point that livers may be stored *ex vivo* for up to 24 hours without deleterious effects; the use of UW solution has contributed significantly to this result.

The urea synthesis viability measure was the major determinant of hepatic function and, as can be seen in Tables 6.2 and 6.3, not all data was acceptable for use; the last column of the table indicates whether data met acceptance criteria. All of the acquired data were included in the table because so little information is available on the capacity of perfused human organs to extract glucocorticoids.

The extraction of prednisone and prednisolone was apparently constant *within each preparation.* The extraction of POH was about 0.21 over a 3-fold concentration range, 600 to 1900 ng/ml in H1. In the second preparation, POH concentrations were higher, about 4500 to 5300, and extraction was reduced, at about 0.07. The extraction of prednisone was examined over a wider range, 100 to 3000 ng/ml, and extraction was about 0.45, with little variability.

It appeared that PO and POH were interconverted reversibly to a small extent. This was observed directly with POH perfusions, and the formation of PO. Concentrations of PO varied from 38 to 179 ng/ml, with no apparent relationship to POH concentration. This might suggest some potential saturable formation of PO from POH, but it is too early to to conclude from these experiments alone (see Fig. 6.2). Indirectly, POH concentrations increased after PO perfusion, but determination of their exact concentration was difficult because POH was present in high concentration prior to PO perfusion.

The apparent hepatic clearance of POH in liver H1 averaged about 220 ml/min with little variability; stable flow and constant extraction combined to yield this observation across the concentration range of 600 to 1900 ng/ml POH. Values for liver H2 were about

43 ml/min, a function of both reduced flow rates and lower relative extraction ratios. The apparent hepatic clearance of PO in preparation H2 averaged about 375 ml/min. Note that all clearance values were low, unlike in the rabbit where the extraction of these glucocorticoids approached or exceeded hepatic blood flow.

Conclusions

The two perfused human livers offered preliminary information on glucocorticoid extraction in humans. These were not ideal preparations, in that they were stored on ice prior to perfusion, steady state studies were not performed, and physiologic flow rates were not achieved. Nevertheless, valuable information was obtained. The extraction of both compounds was linear within each preparation, over a range of concentrations, and with time; the second preparation was less stable with regard to extraction but showed no discernible pattern of saturation.

Prednisone and prednisolone were interconverted. The reaction was primarily oriented in the reductive direction. The potential for saturable formation in either direction cannot be surmised at this time. The results from perfused human liver studies show great similarities and small differences when compared to other species.

Significance

The extraction and interconversion of PO and POH was examined in perfused rabbit, rat and human livers. Traditionally, species differences in steroid metabolism have been perceived to be great, but the rabbit, rat and human demonstrated significant similarities in hepatic glucocorticoid disposition. Some of the differences may be attributable to methodological variation. A comparison of the results of liver perfusion experiments with prednisone and prednisolone in three species is presented in Table 6.4. It can be seen that the human liver extracted PO and POH at about half the capacity of the rabbit and rat; this may have been a species difference, or it may have been associated with

different treatment prior to perfusion, as discussed above. The extraction of both compounds was not limited by concentration any of these species at the concentrations examined.

Table 6.4. Species comparisons of perfused liver parameters.

Parameter	Species Rabbit	Rat (b)	Human (a)
Extraction of PO	0.96	0.95	0.45
Reduction to POH?	yes	yes	yes
Resultant POH/PO ratio	0.5	0.5	?
App Hep CL PO * (ml/min/kg)	15.2	114	5.40
Extraction of POH	0.49	0.63	0.22
Oxidation to PO?	not detectable	not detectable	yes
Resultant PO/POH ratio	0	0	0.08
App Hep CL POH * (ml/min/kg)	8.2	76 (b)	3.3

* apparent hepatic clearance of PO or POH
? not possible to calculate value due to experimental factors
a organ stored on ice for several hours prior to perfusion
b perfusions were performed at flow rates which greatly exceeded physiologic rates due to the absence of an oxygen carrier in the perfusate.

Interconversion showed slight differences between species in the oxidative direction only; PO was reduced to POH by the livers of all three species. The rabbit and rat did not appear to oxidize POH to PO; the human liver produced a small amount of PO, with no clear POH concentration association.

It is difficult to include rat liver perfusion data in the comparison of apparent hepatic blood clearances as high flow rates were used with the cell-free media, but the numbers were calculated nevertheless. The pattern of organ clearance of PO and POH shows an decrease with body size from the rat to the rabbit to the human. This observation is compatible with knowledge of species differences in drug metabolism in general.

Implications for *in vivo* experiment interpretation

The liver is responsible for a substantial portion of the systemic elimination and a large part of the interconversion of glucocorticoids. An accurate characterization of the disposition of prednisone and prednisolone in the liver is essential for success of the model assembled from perfused organs to predict the *in vivo* situation. The rabbit, rat and human liver perfusion results show little species differences in hepatic metabolism of the glucocorticoids. The similarity between the rabbit and human is encouraging for future modeling attempts.

The findings of linear extraction of both compounds in the liver of three species is of great significance, particularly since nonlinearities observed in humans have been attributed to saturation of hepatic metabolic pathways. The liver's preferred directionality of interconversion toward reduction is also a significant finding in that an extra-hepatic organ must be responsible for the oxidation of POH peripherally.

Chapter 7

In Situ Leporine Kidney Perfusions

Background

The kidney has been implicated as the source of nonlinearities in the disposition of prednisone and prednisolone (Rose et al., 1981). Apparent urinary clearance measurements have demonstrated increases with dose and concentration (Rose et al., 1981). Renal glucocorticoid transport processes and excretory patterns have been investigated both *in vivo* and *in vitro*. Results from urinary excretion studies performed *in vivo* are complicated by several factors. Renal processes cannot be examined *in vivo* because of the dynamic autoregulation of pressure, flow and oxygenation. Control of regional blood flow by the kidney appears to be essential for normal excretory function (Ross, 1972a), as this mechanism enables the kidney to control its energy and oxygen supplies. The kidney is unusual in that it possesses two sequential capillary networks, the glomeruli and the capillary plexus which surrounds the loops of Henle. Renal arterial pressure must be sufficient so as to perfuse both regions to ensure normal biochemical and physiologic function. Finally, the kidney of the rat can metabolize the glucocorticoids (Rocci et al., 1981), resulting in intra-renal concentrations which differ from those measured in blood.

The perfused kidney is an ideal system with which to simultaneously examine renal metabolic and excretion processes because arterial concentrations of drug are measured, venous effluent and urine are collected, and pressure, flow, and oxygenation are regulated. In this study, the rabbit kidney was perfused with prednisone and prednisolone to characterize the organ-specific interconversion, excretion and irreversible metabolism of prednisone and prednisolone.

Methodology

The rat has been the species of choice for kidney perfusion studies since the rat's right kidney can be cannulated without blood loss or cessation of blood flow. The rat's mesenteric and right renal arteries branch perpendicularly from the aorta at the same level. When the mesenteric artery is cannulated first, the cannula can subsequently be slid across the aorta into the renal artery with minimal disturbance of the vessel and without cessation of flow. The rabbit vascular anatomy differs substantially, such that direct cannulation of the renal artery is required. Complications arise when this sensitive vessel is directly cannulated, as will be discussed. In the rabbit, either kidney may be studied but the left kidney was chosen for these experiments because the left renal artery and vein provide an additional centimeter of length for cannulation.

Perfusate, apparatus and experimental conditions

The kidney perfusion method of Bowman and Maack (1972) is frequently referenced. These workers accessed the right renal artery of the rat via the mesenteric artery, cannulated the ureter, removed the kidney, and allowed the venous perfusate (recirculated homologous blood) to drip freely into the reservoir of the perfusion apparatus. Weiss and coworkers were the first to perfuse the rat kidney with red cell-free perfusate (Weiss et al., 1959); Bahlmann (1967) allowed the perfused rat kidney to remain *in situ*.

The method of rabbit kidney perfusion used here is modified from that of Bowman and Maack (1972). The perfusate is modeled after that of Rocci et al. (1981) because their results with prednisolone will be compared with the results of these studies. The perfusate is composed of Krebs-Heinseleit bicarbonate buffer (Krebs et al., 1932), with added dextrose (100 mg%), bovine serum albumin fraction V (70 mM, 4.5%), and creatinine (initial concentration = 1 mg/ml). (All chemicals were obtained from Sigma, St. Louis, MO.) The solution was then filtered (Whatman, No.1, Maidstone, England), and the pH was brought to 7.4 by the addition of sodium bicarbonate. The buffer-protein solution was prepared one to three days prior to use. A total volume of 300 ml was used in the perfusion experiments.

Urine replacement solution (atrificial urine) was prepared by dissolving 2.52 g NaCl and 0.92 g KCl in 1 liter distilled water; the solution was filtered and stored refrigerated. This hypotonic solution was developed to replace what was lost in urine from rat kidney perfusion experiments.

The kidney perfusion apparatus consisted of the following: a 400 ml glass reservoir containing the perfusate which was continuously stirred magnetically (mini model 200, VWR, San Francisco, CA), a pump (Masterflex, model N-07553-20, Cole Parmer, Chicago, IL) that delivered non-pulsatile flow and a blood transfusion filter (SQ40S, Pall, Fajardo, PR) was incorporated to trap clumps of red cells which might have exited from the kidney. An oxygenation chamber was created from a glass jar, continuously perfused with carbogen, 95% 0_2 / 5% CO_2. Perfusate flowed through five meters of coiled thin walled medical grade silicon tubing, 0.058" ID x 0.077" OD (Scientific Products, McGaw Park, IL); the pO_2 was maintained at or above 100. The path of flow then traversed an in-line temperature probe, an in-line pH electrode (Cole Parmer, Chicago, IL), a manometer (W.A.Baum, New York, NY), a flow meter (Gilmont, Great Neck, NY), and a bubble trap. The artificial vasculature was composed of Tygon tubing, 1/8" I.D. x 3/16" O.D. (Fisher, Pittsburg, PA). Sampling ports (Argyle, St. Louis, MO) were placed in-line prior

to entrance into and upon exit from the kidney. Perfusate exiting the animal was returned to the reservoir. The ureter cannula emptied into a pre-weighed collection vial (microcentrifuge tube).

The components were housed inside a two-tiered plexiglass chamber (Air Control Inc., Huntingdon Valley, PA). The animal and sampling ports remained on the benchtop, adjacent to the chamber. One milliliter of perfusate was taken from the in and out sampling ports at specified times and frozen until analyzed (usually within one week). The perfusate temperature was maintained at 38 °C; in this manner, a drop of one degree was anticipated as the perfusate travelled outside the perfusion chamber and into the animal. The pH of the perfusate was continuously monitored and manually adjusted to remain between 7.35 and 7.45 by the addition of 0.5 N HCl or saturated $NaHCO_3$ (both prepared with 0.9% NaCl) as needed. The flow, about 50 ml/min, was adjusted to maintain perfusate pressure at about 110 mmHg.

Urine water and electrolytes were replaced by the addition of the artificial urine at a rate equivalent to urine production every 20 minutes. This solution replaced water, sodium and potassium.

At the start of an experiment, a bolus dose of prednisone or prednisolone dissolved in a minimal volume of methanol was added to the reservoir to achieve a specific concentration. To maintain this concentration at steady state, it was necessary to infuse drug into the system. Knowing the extraction of the drug from preliminary experiments and the flow rate of the perfusate, it was possible to predict the infusion rate of drug required to maintain steady state conditions in such organ perfusion experiments. This quantity was continuously infused (Harvard infusion pump, Model 975, Mills, MA), in a volume which was calculated to replace the perfusate removed in sampling. Concentrations were not randomized but were increased within each preparation, as decreasing concentrations required lengthy equilibration periods for concentrations to drop due to the

low extraction of both drugs in the kidney. Eleven rabbit kidneys were perfused, five with prednisone, five with prednisolone, and one was perfused without drug (control).

Prednisone and prednisolone were quantified in plasma and urine using a normal phase high performance liquid chromatographic (HPLC) assay. Urine was diluted about 10-fold for analysis. Equilibrium dialysis was used to estimate the unbound fractions of prednisone and prednisolone present in the perfusate samples. A colorimetric, enzymatic method was used to quantitate creatinine present in perfusate and urine samples. (See Chapter 3 for details of all of these methods.)

Urine sodium was measured by flame photometry (Chemistry Laboratory, University of California Hospital). Urinary protein and glucose were estimated with Ames Reagent Strips (Multistix 8SG, Ames, Miles Inc., Elkhart, IN).

Surgery

New Zealand White rabbits, of both sexes, weighing 1.8 to 2.6 kg were used (Nitabell, Hayward, CA). The animal was anesthetized by intramuscular injection of 50 mg/kg ketamine hydrochloride (Vetalar, Parke-Davis, Morris Plains, NJ) and 10 mg/kg xylazine hydrochloride (Rugby, Rockville Center, NY); fractional doses of the anesthetic agent were administered subsequently as needed. The rabbit's abdomen was shaved. Diazepam (Elkins Sinn, Cherry Hill, NJ), 0.5 mg/kg, was injected into the ear vein immediately prior to initiation of surgery to prevent hyper-reflexia. A midline abdominal incision was made from the bladder to the sternum and the left abdominal wall was cut to expose the surgical field. The intestines were moved to the right of the rabbit, revealing the vena cava and left kidney. A solution of mannitol (25% solution, Abbott, Chicago, IL; 2 g/kg) and normal saline (10 ml/kg) was administered through the vena cava over one to two minutes. This combination promoted urine flow and the added volume helped to overcome some of the local vasoconstriction.

Adipose and connective tissues were removed from the left renal artery, vein and hylum; the kidney was anchored by leaving some connective tissue on the renal capsule. The vessels were manipulated as gently as possible while two sutures were placed around the renal artery and vein, respectively. A few drops of a solution of nitroglycerin in saline (2 mg/ml) was applied topically to the renal artery and hylum; this reversed some of the vasoconstriction, and limited the pre-perfusion ischemic period. The left renal artery was clamped at the aorta and quickly cannulated (Angiocath, 18 ga, 2 in, Deseret, Sandy, UT); the cannula was advanced to the level of the hylum and secured. A solution of warm (37 °C) Krebs buffer and heparin (20 ml, 50 Units/ml) was immediately flushed through the kidney which prevented clot formation and removed rabbit blood from the renal vasculature. Additional nitroglycerin was dripped onto the hylum as needed over the next few minutes to reduce resistance. Perfusate flow was then initiated at a pressure of 80 mm Hg. The renal vein was cannulated (Angiocath, 14 ga, 2 in) and collection of the perfusate began. The pressure was subsequently increased to 110 mm Hg over the next one to two minutes. The kidney was without flow for less than one minute prior to cannulation. The kidneys were allowed to equilibrate for 10 minutes before drug was added to the reservoir.

The ureter was isolated, a small incision was made about three cm below the kidney and cannulation was performed (Angiocath, 18 ga, 2 in). The catheter was inserted to within a few mm of the kidney and secured with silk sutures.

The inferior vena cava was cut and the animal died of circulatory failure under full anesthesia. Complete isolation of the renal circulation was achieved and the kidney remained *in situ*.

Modeling intra-renal disposition

The pharmacokinetic model presented in Figure 7.1 describes the disposition of prednisone and prednisolone. Knowledge of renal steroid metabolism and excretion was applied to develop this model. The formation of the metabolites is irreversible and

interconversion requires enzyme catalysis, so there is no interconversion in the urine or perfusate. The kidney perfusions were performed at steady state where net distribution does not occur and is therefore omitted from the model. Interconversion and irreversible elimination (metabolic and excretory) of PO and POH occur. The perfusion rate limitation was assumed and unbound drug concentrations were used in the calculations, yielding intrinsic clearance estimates.

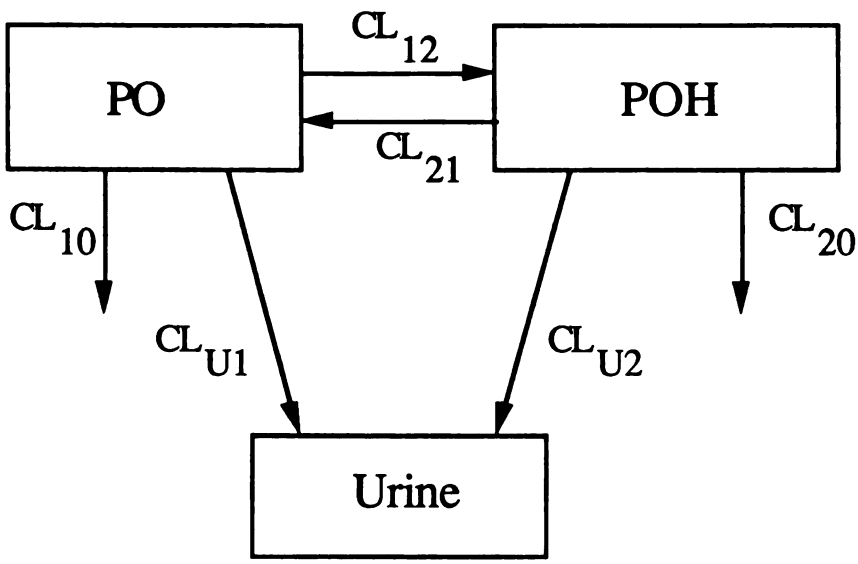

Figure 7.1. Model of the interconversion and elimination of prednisone and prednisolone in the perfused rabbit kidney.

CL_{10} = irreversible metabolic elimination of prednisone
CL_{12} = reductive conversion of prednisone to prednisolone
CL_{U1} = excretion of prednisone into urine
CL_{20} = irreversible metabolic elimination of prednisolone
CL_{21} = reductive conversion of prednisolone to prednisone
CL_{U2} = excretion of prednisolone into urine

Mass balance for perfusion with each drug results in two differential equations:

$$V_R \frac{dC^{PO}}{dt} = Q_R \left(C_{IN}^{PO} - C_{OUT}^{PO} \right) - C_R^{PO} CL_{12} + C_R^{POH} CL_{21} - C_R^{PO} CL_{10} - C_R^{PO} CL_{U1} \quad (1)$$

$$V_R \frac{dC^{POH}}{dt} = Q_R \left(C_{IN}^{POH} - C_{OUT}^{POH} \right) - C_R^{POH} CL_{21} + C_R^{PO} CL_{12} - C_R^{POH} CL_{20} - C_R^{POH} CL_{U2} \quad (2)$$

where C^{PO} and C^{POH} are unbound perfusate concentrations of prednisone and prednisolone, respectively; Q_R is the renal perfusate flow rate; entering and exiting unbound concentrations of POH and PO are designated C_{in} and C_{out}, respectively, while V_R and C_R are the volume of the kidney and the renal tissue concentrations of unbound drug. The clearance terms were defined in Figure 7.1. Two sets of equations 1 and 2 are required, one set following perfusion of POH and the other set following PO perfusion. The well stirred and parallel tube models of organ disposition were used to predict C_R according to these definitions:

$$C_{R,ws} = C_{out} \quad (3)$$

while for the parallel tube model (pt), the concentration is:

$$C_{R,pt} = [(C_{in} - C_{out}) / \ln (C_{in} / C_{out})] \quad (4)$$

The values for the concentration term were substituted into the corresponding physiologic model differential equations, allowing the solution of intrinsic clearance values. Because studies were performed at steady state, the left side of the equations were set equal to zero.

As demonstrated below, the renal conversion of POH to PO in the rabbit was apparently nonlinear; Michaelis kinetics were assumed, and equations 1 and 2 are modified to reflect this observation:

$$V_R \frac{dC^{PO}}{dt} = Q_R\left(C_{IN}^{PO} - C_{OUT}^{PO}\right) - C_R^{PO}CL_{12} + \frac{\upsilon_{21} \cdot C_R^{POH}}{K_{M21} + C_R^{POH}} - C_R^{PO}CL_{10} - C_R^{PO}CL_{U1} \quad (5)$$

$$V_R \frac{dC^{POH}}{dt} = Q_R\left(C_{IN}^{POH} - C_{OUT}^{POH}\right) - \frac{\upsilon_{21} \cdot C_R^{POH}}{K_{M21} + C_R^{POH}} + C_R^{PO}CL_{12} - C_R^{POH}CL_{20} - C_R^{POH}CL_{U2} \quad (6)$$

The υ_{21} is the equivalent of V_{max}, or the maximal rate at which the reaction proceeds; its units are ng/min. The K_{M21} is analagous to the Michaelis constant, the concentration of drug at which the reaction reaches half-maximal rate; its units are ng/ml.

The urinary clearances of PO and POH, CL_{U1} and CL_{U2}, respectively, can be solved directly from urine data. However, the intrinsic clearances of compounds which are renally interconverted are actually *apparent* terms because intra-renal concentrations cannot be determined. The apparent urinary clearances for PO and POH are defined as:

$$CL_{U1} = \frac{C_{UR}^{PO} \cdot Q_{UR}}{C_R^{PO}} \quad (7)$$

$$CL_{U2} = \frac{C_{UR}^{POH} \cdot Q_{UR}}{C_R^{POH}} \quad (8)$$

where C_{UR} is the urine drug concentration and Q_{UR} is the urine flow rate. Rearranging equations 7 and 8, the rate of excretion of drug into urine is the product of C_{UR} and Q_{UR}, which can be substituted into equations 5 and 6:

$$V_R \frac{dC^{PO}}{dt} = Q_R\left(C_{IN}^{PO} - C_{OUT}^{PO}\right) - C_R^{PO}CL_{12} + \frac{\upsilon_{21} \cdot C_R^{POH}}{K_{M21} + C_R^{POH}} - C_R^{PO}CL_{10} - C_R^{PO}Q_{U1} \tag{9}$$

$$V_R \frac{dC^{POH}}{dt} = Q_R\left(C_{IN}^{POH} - C_{OUT}^{POH}\right) - \frac{\upsilon_{21} \cdot C_R^{POH}}{K_{M21} + C_R^{POH}} + C_R^{PO}CL_{12} - C_R^{POH}CL_{20} - C_{UR}^{POH}Q_{UR} \tag{10}$$

The result is five intrinsic clearance parameters within four equations: CL_{12}, υ_{21}, K_{M21}, CL_{10} and CL_{20}. Equations 9 and 10 can be modeled with the measured variables Q_R, C_{in}, C_{out}, C_{UR} and Q_{UR}; C_R is predicted for the well stirred and parallel tube models.

Viability and renal function

Renal function tests of the perfused rabbit kidney are presented in Table 7.1, along with literature values for comparison. In the evaluation of renal function tests, it is important to consider the composition of the perfusate because glomerular filtration rate (GFR), urine flow rate, and sodium and glucose reabsorption are directly affected by perfusate components and their concentrations [Epstein et al.(1982); Segal and Guttman (1983); De Mello and Maack (1976); Pacini and Bocci (1983)]. Perfusate composition and/or experimental conditions differed within each experiment presented in Table 7.1. Some kidneys were perfused with blood and others with cell-free perfusate modified to contain various additional components, so functional tests are expected to vary.

Table 7.1. Functional behavior of the perfused rabbit kidney.

Parameter	Rabbit	Author
GFR ml/min/g	0.15	Segal
	0.16	Fonteles
	0.5	Pacini
	0.03-0.30	present study
Urine Flow ml/min/g	0.1	Epstein
	0.5	Pacini
	0.15-0.5	Pieroni
	0.13	Segal
	0.002 -0.290	present study
Sodium Reabsorption (%)	38	Segal
	65	Pacini
	30-67	present study
Glucose Reabsorption (%)	79	Segal
	90	Pacini
	88-99	present study
Proteinuria (mg/ml)	10	Pacini
	0 - 0.4	present study

Autologous blood is the ideal perfusion medium but it contains endogenous substances which adversely affect the designed experiments, namely endogenous glucocorticoids and corticosteroid binding globulin (CBG). Homologous blood is a reasonable alternative in that endogenous glucocorticoids can be dialyzed away over the several days before use, but CBG would remain. Because studies were designed to incorporate linear protein binding, blood could not be used. The presence of an efficient oxygen carrier such as red blood cells is desirable so that physiologic flow rates can then be used. Rabbit red blood cells could have been added to cell-free perfusate, but an effort was made to reduce the number of animals used for these experiments, and thus human red

blood cells were incorporated in two perfusion experiments. Extreme vasoconstriction was observed within a few minutes. Pacini and Bocci (1983) determined that platelets and leukocytes are primarily responsible for the extreme vasoconstriction observed with kidney perfusion; perhaps some platelets and white blood cells remained in the washed human red blood cell preparation. Immune-mediated reactions may have also been involved. Because of these difficulties, cell-free medium was chosen for use in these experiments. The great majority of published kidney perfusion experiments have employed synthetic cell-free medium and much is known about organ viability within this experimental system.

Cannulation of the rabbit renal artery results in immediate vasoconstriction such that the cannula cannot be advanced. It is therefore necessary to clamp the vessel while the artery is entered and the cannula is positioned. This harsh manipulation of the artery necessitates the application of pharmacologic vasodilators which will moderate the auto-regulatory vasoconstriction. Some investigators have included vasodilators in the perfusion medium but since their effect on metabolism and/or excretion of the glucocorticoids is unknown, this approach was not used. Alternatively, isoprenaline and phenoxybenzamine have been applied topically to lessen arterial spasm (Ross, 1978); Segal and Guttman (1983) applied papaverine (30 mg/ml) and lidocaine (1% solution) in equal volumes topically with good results. Topical nitroglycerin solution (5 mg/ml in normal saline) was used in these experiments as it appeared to be the most effective relaxant.

Several tests of renal function have been applied to the perfused kidney: creatinine clearance, sodium reabsorption, potassium excretion and urine flow are most commonly encountered. These measures are generally derived from urinary analyses and viability is not known until after completion of the experiment. An indicator of general function of the preparation which is immediately apparent during an experiment is the flow rate achievable under conditions of constant pressure. Vasoconstriction results in inadequate flow rates, and therefore insufficient oxygen delivery, rendering an inferior preparation. If constant flow can be maintained at an acceptable and constant rate for 1 to 2 hours, Nishiitsutsuji-

Uwo et al. (1967) claim that the experiment is probably a viable one (1967). Experimental periods which required in excess of a 10% reduction in flow rate to maintain constant pressure were excluded from this analysis.

As might be expected for any perfused organ, the perfused kidney is deficient in some mediators of cellular metabolism for several minutes after surgery. A reduction in ATP was observed in the rat kidney by the end of the surgical period, but within 30 minutes normal values were obtained (Nishiitsutsuji-Uwo et al., 1967). This was thought to be associated with the brief period of pre-perfusion ischemia. For this reason, an equilibration period of 10 minutes was integrated into these experiments before drug was added to the perfusate in the first experiment.

Normal renal blood flow in the rabbit is 1 ml/min per g kidney (5 to 8 ml/min) (Pacini and Bocci, 1983). Here rabbit kidneys received 32 to 61 ml/min perfusate flow when pressure was maintained at 110 mmHg, equivalent to 6.1 to 7.8 ml/min per g kidney. This is consistent with flows of 27 to 54 ml/min achieved at constant pressure of 80 mmHg in rabbits by Pacini and Bocci (1983). Weiss et al. (1959) and Ross (1978) perfused at rates of 8 and 10 ml/min per g rat kidney, respectively.

The glomerular filtration rate is an indicator of renal function and is most commonly estimated by creatinine or inulin clearances. Creatinine clearance estimates of GFR ranged from 0.03 to 0.30 ml/min per g rabbit kidney. Literature values were generally higher, at 0.15 to 0.5 ml/min per g kidney. These values were obtained upon perfusion of blood or red cell-supplemented perfusate, however. The perfusate employed here was buffer containing albumin and glucose. It is known that higher concentrations of albumin generally increase the GFR, as do red cells (Epstein et al., 1982; Pacini and Bocci, 1983).

Urine flow rate is a nonspecific indicator of renal function. Anuric and oliguric kidneys are dysfunctional, but estimation of the normal urine flow rate is difficult as it is a function of multiple factors including hydration status, diet, stress, position, and environmental conditions. *In vitro*, urine flow is also intimately associated with perfusate

composition and has been shown to decrease with increasing albumin concentrations (Bekersky, 1972), can be reduced by the addition of amino acids to the perfusate (Epstein et al., 1982), and the rate changes with hematocrit (Pacini and Bocci, 1983). Physiologic leporine urine flow rates are approximately 0.07 ml/min (Pacini and Bocci, 1983) but Pieroni (1988) measured urine flow as high as 0.5 ml/min per g perfused kidney. Urine flow rates in these experiments were quite variable and ranged from 0.005 to 1.820 ml/min, averaging 0.217 ± 0.326. This translates to 0.002 to 0.290 ml/min per g kidney. The urine flow rates achieved here encompassed and exceed the physiologic range as was expected because renal flow rates were higher than normal physiologic rates.

A consequence of elevated perfusate flow rates is a reduced passage time along the proximal convoluted tubule, allowing less time for sodium reabsorption which is often reduced in the perfused kidney preparation. The rabbit kidney perfused in other laboratories reabsorbed from 38 to 65% of its tubular load of sodium (Table 7.1), while 30 to 67% reabsorption was observed here. Pacini and Bocci (1983) have demonstrated that sodium reabsorption is dependent on the energy source, lactate being superior to butyrate or glucose. Glucose was the sole energy source provided in these experiments.

Glucose reabsorption is also an energy-requiring process. It is higher compared to sodium in the perfused kidney of the rabbit, with 88 to 99% reabsorbed in our studies. This exceeds the values obtained by Pacini and Bocci (1983) and Segal and Guttman (1983).

Proteinuria and glucosuria have been observed in the healthy rat and rabbit *in vivo* and may be normal for these species (Spector, 1956) but gross protein spillage is indicative of renal dysfunction. Pacini and Bocci (1983) observed 10 mg/ml protein in the urine of the perfused rabbit kidney; up to 2 mg/ml was measured in our urine samples and more than half of the samples had no measurable protein (detection limit = 0.03 mg/ml albumin).

Overall, the viability of the perfused rabbit kidney is adequate, although not optimal. It is apparent that the surgical cannulation procedure has a significant effect on

renal hemodynamics which is not overcome with time. This observation is unavoidable in the rabbit because of the requirement of direct arterial cannulation. In the rat, where indirect cannulation can be achieved, viability measurements are improved. Vasodilators are beneficial but the normal dynamic processes have been altered significantly and functional tests will reflect these manipulations.

Results and discussion

Renal extraction

Prednisone and prednisolone were individually perfused in the rabbit kidney; sixteen PO and fifteen POH steady state concentrations were examined, ranging from 138 to 13392 ng/ml. Tables 7.2 and 7.3 present the results of these experiments.

The binding of PO and POH in these samples was found by equilibrium dialysis to be linear, as expected, since albumin was the sole binding protein present in the perfusate. The average fractions unbound were 0.54 for PO and 0.47 for POH (see Figure 7.2), with little variability.

The extraction of both prednisone and prednisolone was apparently linear in the perfused rabbit liver over the entire concentration range where extraction (E_R) is defined as:

$$E_R = (C_{in} - C_{out}) / C_{in}$$

C_{in} is the arterial concentration of drug and C_{out} is the venous concentration. Figure 7.3 shows the extraction of PO over the concentration range of 138 to 7192 ng/ml total drug concentration; extraction ratio ranged between 0.01 and 0.10, and averaged 0.04 ± 0.03.

Table 7.2
Steady state drug concentrations and calculated apparent renal clearance for prednisone perfusions of the rabbit kidney

Rabbit No	Total Steady State Concentration Prednisone (ng/ml)	Unbound Steady State Concentration Prednisone (ng/ml)	Total Steady State Concentration Prednisolone (ng/ml)	Unbound Steady State Concentration Prednisolone (ng/ml)	Extraction Ratio	Apparent Renal Clearance (ml/min)	Apparent Renal Clearance (ml/min/kg)
3	1481	622	15	7	0.04	1.4	0.86
	1734	728	43	20	0.01	0.4	0.25
	7192	3021	38	18	0.03	1.1	0.67
5	1420	809	0	0	0.01	0.4	0.15
	3221	1836	0	0	0.04	1.7	0.64
	5566	3173	0	0	0.06	2.5	0.94
8	616	320	12	6	0.07	2.5	1.15
	1459	759	14	7	0.03	1.1	0.50
	2348	1094	59	28	0.01	1.8	0.83
	3431	1613	66	31	0.05	2.2	1.02
9	485	272	11	5	0.03	1.7	0.61
	904	506	12	6	0.01	0.6	0.22
	3170	1775	11	5	0.02	1.0	0.36
	4121	2308	12	6	0.03	1.4	0.50
10	138	70	0	0	0.10	6.1	2.48
	4940	2519	0	0	0.02	1.2	0.49
				Mean	**0.04**	**1.7**	**0.73**
				S.D.	**0.03**	**1.3**	**0.55**

Table 7.3
Steady state drug concentrations and calculated apparent renal clearance for prednisolone perfusions of the rabbit kidney

Rabbit No.	Total Steady State Concentration Prednisolone (ng/ml)	Unbound Steady State Concentration Prednisolone (ng/ml)	Total Steady State Concentration Prednisone (ng/ml)	Unbound Steady State Concentration Prednisone (ng/ml)	Extraction Ratio	Apparent Renal Clearance (ml/min)	Apparent Renal Clearance (ml/min/kg)
1	1328	624	110	63	0.11	3.3	1.38
2	6513	3061	99	53	0.09	2.8	1.33
4	2087	981	209	113	0.09	2.9	1.17
	4252	1998	440	238	0.07	2.2	0.89
	8176	3843	679	367	0.07	2.2	0.89
	13392	6294	746	403	0.12	3.8	1.54
6	474	218	233	126	0.10	4.7	1.52
	997	459	385	208	0.13	6.1	1.97
	2655	1221	649	350	0.08	3.8	1.23
	7916	3641	1243	671	0.12	5.3	1.72
7	169	79	81	44	0.15	7.8	3.17
	660	310	228	123	0.12	6.2	2.52
	1291	607	495	267	0.09	4.4	1.79
	2088	981	618	334	0.06	2.9	1.18
	3673	1726	838	453	0.06	2.9	1.18
				Mean	**0.10**	**4.1**	**1.57**
				S.D.	**0.03**	**1.6**	**0.62**

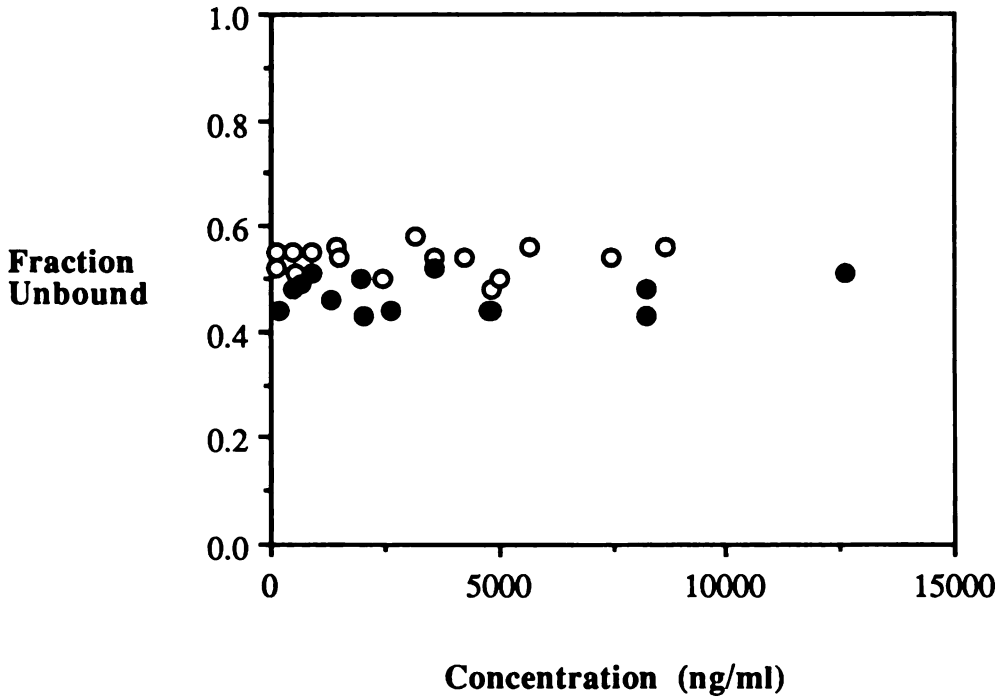

Figure 7.2. Unbound fractions of prednisone (open circles) and prednisolone (solid circles) in perfusate of rabbit kidney perfusion experiments.

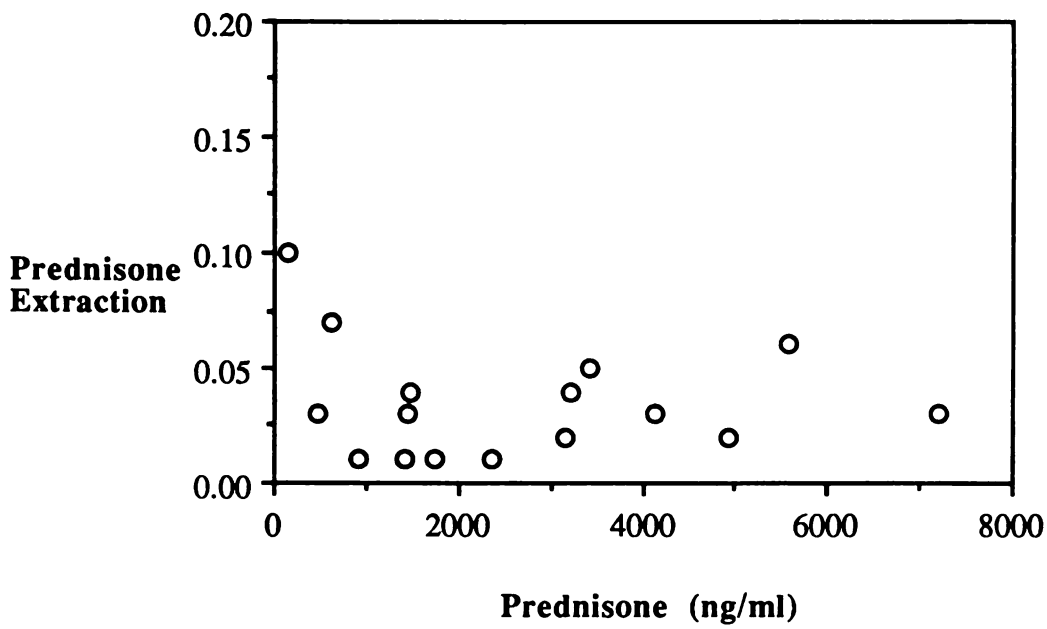

Figure 7.3. Extraction ratio of prednisone in the perfused rabbit kidney as a function of total perfused drug concentration.

The extraction of POH is depicted in Figure 7.4 where the concentration range of 169 to 13392 ng/ml POH was examined. Prednisolone extraction in the rabbit kidney was double that of PO, at 0.10 ± 0.03 and values ranged from 0.06 to 0.15. The mean value favorably compares to that of Rocci and coworkers (1981) who measured the renal extraction of POH in the perfused rat kidney to be 0.044 ± 0.014.

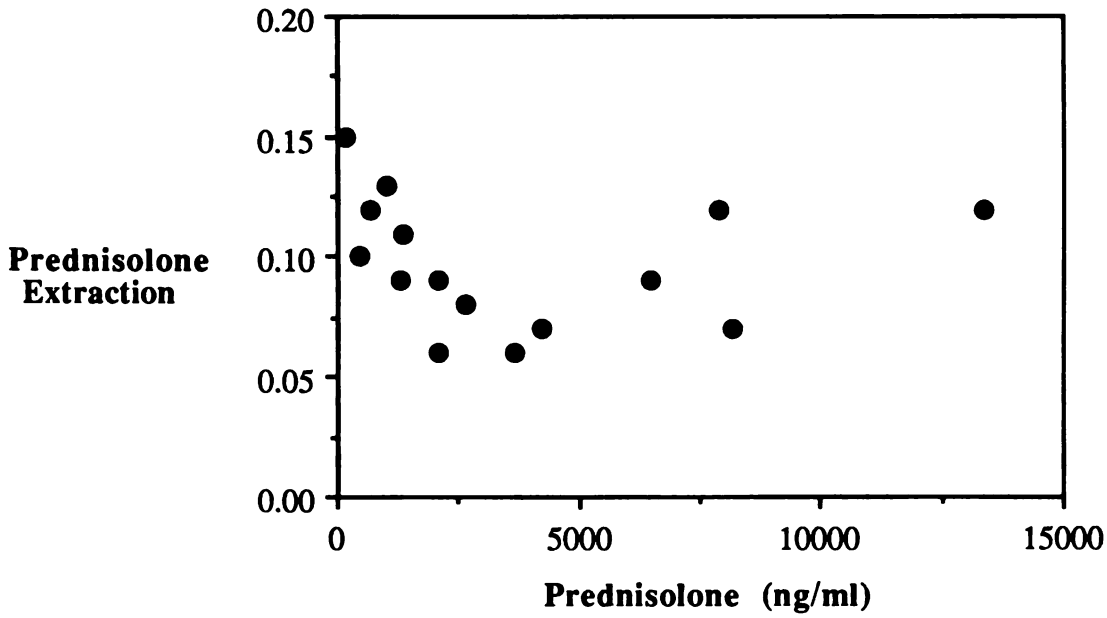

Figure 7.4. Extraction ratio of prednisolone in the perfused rabbit kidney as a function of total perfused drug concentration.

Variability in extraction ratio was quite high for both PO and POH (C.V.% = 40 and 33, respectively). The extraction ratio ranged 0.09 units for both compounds. This range is similar to that observed in the liver perfusion experiments implying that some experimental factor, rather than biologic, may be involved in the development of this variability.

Apparent renal clearance

Because interconversion of prednisone and prednisolone occurs in the kidney, all calculated clearances are apparent and do not necessarily reflect true metabolic events. The apparent renal clearances, $CL_{R,app}$, of PO and POH by the rabbit were linear (data not plotted; see Tables 7.2 and 7.3). This parameter is defined as:

$$CL_{R,app} = Q_R \cdot E_R$$

where Q_R is the renal perfusate flow rate and E_R is the extraction ratio of drug. Values averaged 1.7 ml/min for PO and 4.1 ml/min for POH; this is equivalent to 0.73 and 1.57 ml/min per kg, respectively. The perfused rat kidney experiments of Rocci et al. (1981) produced a POH clearance value of 6.51 ml/min per kg. The rat value is approximately 4-fold higher than the value we found in rabbits. These differences could potentially relate to species-specific elimination pathways in the rat and rabbit.

If PO and POH were eliminated by filtration alone, apparent renal clearances would be approximately equal, as the the unbound fractions of PO and POH averaged 0.54 and 0.47, respectively. The apparent renal clearance of POH was 2.5 times as large as that of PO, implying that prednisolone undergoes further renal handling. Within a smaller concentration range, Rocci and coworkers also observed linearity in the extraction and renal clearance of POH in the perfused rat kidney (Rocci et al., 1981). They performed recirculating non-steady state experiments using initial concentrations of 100 to 1000 ng/ml POH. This group did not measure the extraction and/or the apparent renal clearance of prednisone.

Metabolic activity of the kidney

The three metabolic routes to be addressed are reductive and oxidative interconversion and irreversible elimination. The perfused rabbit kidney demonstrated the

ability to interconvert prednisone and prednisolone; conversion to the partner did not account for all of the extracted drug, hence irreversible elimination also occurred. Perfusion of the whole kidney was performed to ascertain apparent renal metabolic capacities in terms of relative magnitudes.

Reductive interconversion

The capacity for reductive conversion of PO to POH was low (Figure 7.5). The highest POH concentration achieved was 66 ng/ml at 3431 ng/ml PO; increasing the perfused PO concentration further failed to increase POH concentrations. The capacity to produce POH appeared to be related to an individual preparation, rather than the concentration of perfused drug, as two of the five kidneys did not produce any measurable prednisolone in venous perfusate.

The ratio of formed POH to perfused PO is plotted versus perfused PO concentration in Figure 7.6. A line connects the data collected from each of the five experiments, represented by different symbols. The ratio demonstrated high variability, so it was assumed that linearity was obeyed. The highest value of POH/PO was 0.025 at 1734 ng/ml PO. This is a small number and reflects the dominance of the oxidation reaction in the kidney.

Five of the sixteen samples examined contained no measurable POH in the venous perfusate. Prednisolone may have been formed in the kidney, but sequentially converted to PO, irreversibly metabolized, or excreted before exiting in the perfusate. In fact, of these five perfusion periods, POH was recovered in the urine on four occasions, proving that these kidneys did form POH. No kidneys were without the ability to reduce PO to POH as all produced measurable urinary POH during at least one experimental period.

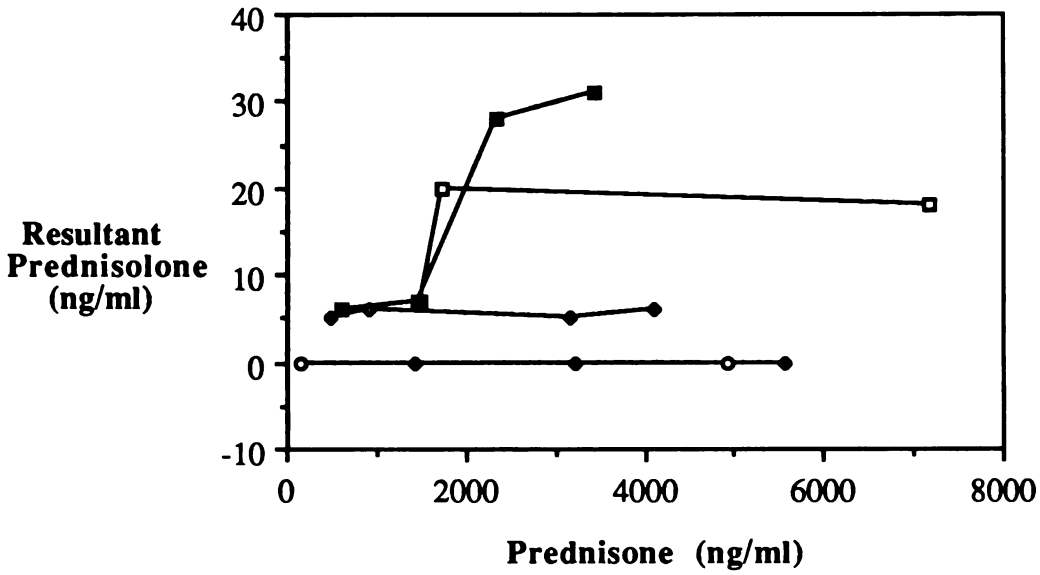

Figure 7.5. Prednisolone concentrations resulting from prednisone perfusion of the rabbit kidney; each experiment is represented by a different symbol and each determination within an experiment is connected by a line.

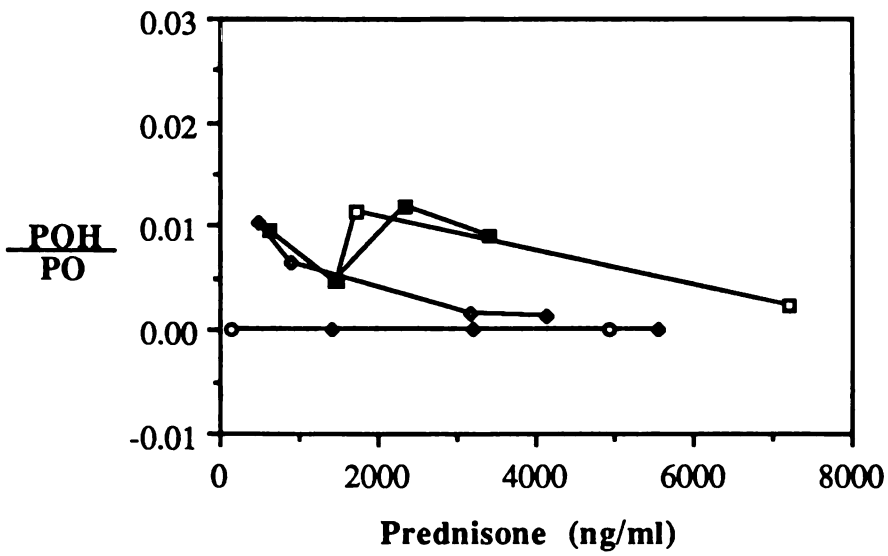

Figure 7.6. Ratio of prednisolone and prednisone steady state concentrations formed in prednisone perfusion of the rabbit kidney; each experiment is represented by a different symbol and each determination within an experiment is connected by a line.

The perfused rat kidney has also demonstrated reductive conversion capacity toward the glucocorticoids (Rocci et al., 1981; Reach et al., 1977). Both of these experiments were recirculating, nonsteady-state experiments and few details were presented as to the amounts or ratios of reduced compounds produced.

Oxidative interconversion

The prednisone concentrations formed from POH perfusions are depicted in Figure 7.7. Five kidneys were perfused and prednisone was measurable in all fifteen experiments. Within each preparation, prednisone concentrations increased consistently; concentrations ranged from 81 to about 1243 ng/ml as POH concentrations increased from 169 to 13392 ng/ml total drug. These concentrations were 10 to 15-fold greater than the opposing reaction, reduction of PO to POH (Figure 7.5), implying that the kidney is preferentially oxidatively oriented toward the glucocorticoids.

The resultant ratio of PO to POH is plotted in Figure 7.8. The ratio very clearly decreased in the three kidneys which were perfused with multiple concentrations. Two were very similar, the ratio decreasing from about 0.5 to 0.15; the third decreased from 0.1 to 0.07 with increasing POH perfusion concentrations. Perhaps this last experiment examined concentrations which were too high to express nonlinearities, as the lowest concentration was 2099 ng/ml POH. Regardless, this observation suggests that the oxidative conversion pathway in the kidney is saturable. For modeling purposes, it will be assumed that this enzymatic system obeys Michaelis-Menten kinetics. No other group has studied this process or reported this nonlinearity.

Unlike the case for prednisolone, prednisone was measurable in every venous perfusate and urine sample. This result is compatible with the results of the rabbit kidney incubation studies of Chapter 4; in those experiments, the kidney was strongly oriented toward oxidative interconversion and demonstrated little or no reductive interconversion capacity.

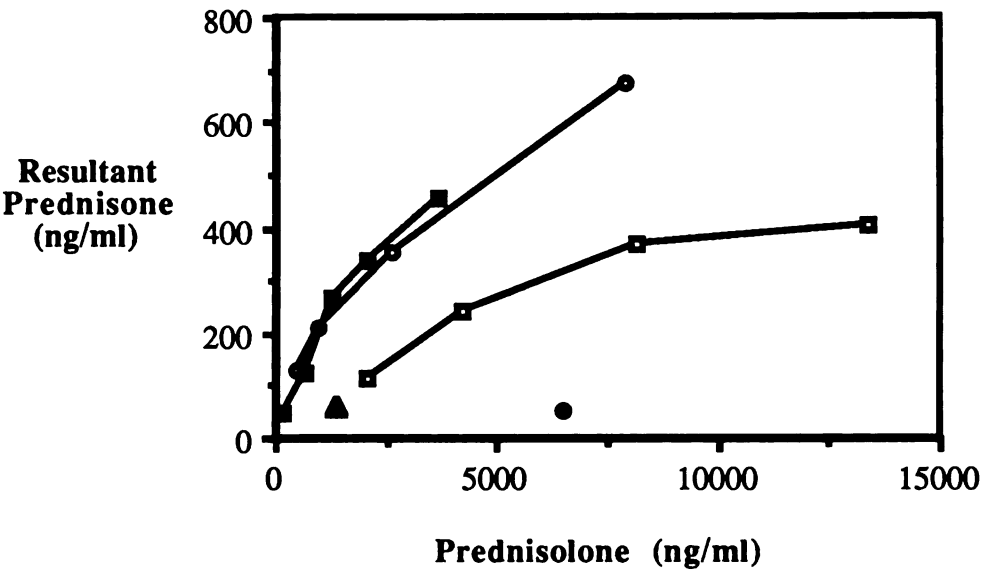

Figure 7.7. Steady state concentration of prednisone resulting from perfusion of the rabbit kidney with prednisolone; each experiment is represented by a different symbol and each determination within an experiment is connected by a line.

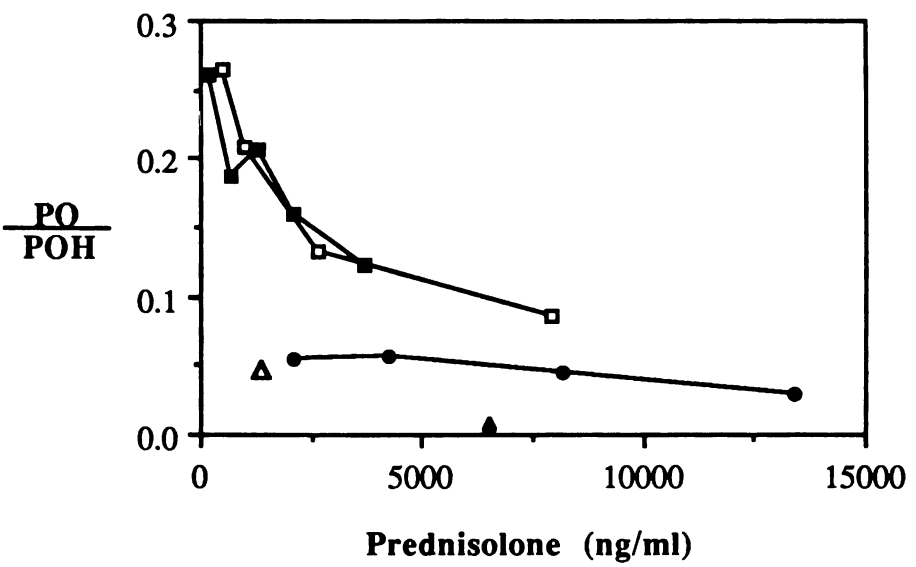

Figure 7.8. Ratio of prednisone and prednisolone steady state concentrations formed in prednisolone perfusion of the rabbit kidney. Legend is same as Figure 7.5.

Irreversible elimination

Four disposition routes are available for glucocorticoids in the kidney: interconversion, excretion into the urine, irreversible metabolism and distribution into tissues. At steady state, distribution into tissues equals that leaving tissues, the result being no net distribution. Therefore mass balance is complete with the metabolic and excretory factors:

$$\text{Extraction} = \text{Irreversible metabolism} + \text{Conversion} + \text{Excretion}$$

The extraction and excretion rates are easily calculated:

$$\text{Extraction rate} = (C_{in} - C_{out}) \cdot Q_R \qquad (11)$$

$$\text{Excretion rate} = C_{urine}\,(PO + POH) \cdot Q_{UR} \qquad (12)$$

where C_{urine} (PO + POH) is the sum of the concentrations of both drugs in the urine and Q_{UR} is the urine flow rate. The conversion and irreversible elimination rates are not easily calculated because the drug can be back-converted to the parent compound and extracted. If it is assumed that sequential metabolism does not occur, the converted partner is not back converted nor is it eliminated. Then the relative contribution of these two pathways may be approximated. The steady state concentration of the formed (converted) species may be compared to the concentration of perfused drug which was extracted at steady state:

$$\frac{C_{ss}^{\text{metabolite}}}{\left(C_{in}^{\text{parent}} - C_{out}^{\text{parent}}\right)}$$

In prednisolone perfusions, the relative magnitude of the oxidative conversion pathway was 0.4, implying that almost half of the extracted POH was recovered as PO in the venous

perfusate. In the opposite direction, reduction produced no measurable PO, which suggested that nearly all of the extracted PO was irreversibly eliminated and very little was converted to POH. These results are approximations but give insight into the relative importance of the interconversion and elimination pathways. This result is supported by data from *in vitro* incubation of rabbit kidney tissue wherein little or no PO was reduced to POH but substantial amounts were oxidized to PO. Rocci et al. (1981) determined that an average of 56% of the extracted prednisolone was converted to products other than prednisone during a 90 minute recirculating rat kidney perfusion. Similarly, Reach and coworkers observed that 95% of cortisol recirculated through the perfused rat kidney was converted to 20β-hydroxylated derivatives after 120 minutes (Reach et al., 1977).

Urinary excretion

The percent of dose excreted of PO and POH are presented in Table 7.4 and is calculated as:

$$\% \text{ excreted} = (C_{urine} \cdot Q_{UR}) / (Css \cdot Q_R)$$

The mean value for the excretion of PO was 0.43 ± 0.67% and that of POH was 0.24 ± 0.35%. (Percent excretion was calculated for the drug only for the experiments when it was perfused.) There was wide variability in the excretion of the glucocorticoids: prednisone excretion ranged from 0 to 2.2% and POH excretion, from 0 to 1.05%. In the perfused rat kidney, Rocci et al. (1981) measured higher excretion of POH, between 1.9 and 6.4 % . This observed value could be associated with the higher urine flow rates achieved in the rat (see discussion of diuresis-dependent excretion below). Species differences would not be expected to significantly affect this process.

Table 7.4
Urinary Excretion and Apparent Urinary Clearance*
Rabbit Kidney Perfusions

Total Steady State Concentration Prednisone (ng/ml)	% Excreted	Prednisone Apparent Urinary Clearance (ml/min)	Total Steady State Concentration Prednisolone (ng/ml)	% Excreted	Prednisolone Apparent Urinary Clearance (ml/min)
1481	0.05	0.00	1328	0.01	0.00
1734	0.10	0.08	6513	0.02	0.01
7192	0.05	0.03	2087	0.90	0.38
1420	0.35	0.46	4252	1.05	0.38
3221	0.40	0.48	8176	0.65	0.44
5566	0.05	0.36	13392	0.50	0.40
616	0.25	2.10	474	0.07	0.01
1459	1.20	1.40	997	0.05	0.01
1248	1.70	2.30	2655	0.05	0.01
3431	2.20	1.80	7916	0.05	0.03
485	0.00	0.00	169	0.00	0.00
904	0.00	0.00	660	0.00	0.00
3170	0.05	0.01	1291	0.12	0.01
4121	0.00	0.00	2088	0.08	0.02
138	0.05	0.00	3673	0.10	0.07
4940	0.50	0.00			
mean	**0.43**	**0.56**	**mean**	**0.24**	**0.12**
S.D.	**0.67**	**0.83**	**S.D.**	**0.35**	**0.18**

* calculated based on unbound concentration of PO and POH in perfusate

It is generally believed that the glucocorticoids are passively reabsorbed with no apparent transport maximum within the range of normal doses or the physiologic range (Beisel et al., 1964; Lindholm, 1973; Franke et al., 1982). Supporting these theories, no apparent concentration-dependency of urinary excretion of either PO or POH was observed in the rabbit kidney (data not plotted). Saturation of an active secretion or reabsorption process(es) would have demonstrated a relationship to perfused drug concentration, although renal interconversion could mask this relationship. There was a difference in excretion between preparations but within preparations, excretion (percentage of dose) was nearly constant.

Apparent urinary clearance

It is believed that prednisone, prednisolone and most glucocorticoids undergo glomerular filtration and passive reabsorption. Therefore, the unbound drug concentration (Cu) is used in the calculation of $CL_{urinary}$ as it is assumed that only the fraction not bound to albumin is filtered. $CL_{urinary}$ is a component of the total renal clearance, and reflects excretion of unchanged drug in the urine. It is actually an apparent term, as concentrations of the glucocorticoids at the nephron cannot be known with certainty due to renal interconversion of the two species.

The apparent urinary clearance is defined as:

$$CL_{urinary} = \text{rate of excretion} / C_{U,ss}$$
$$= (C_{UR} \cdot Q_{UR}) / C_U$$

The clearance values are presented in Table 7.4. The mean values for PO and POH $CL_{urinary}$ were 0.56 and 0.12 ml/min, respectively. The values demonstrated great variability, C.V.% = 150 for both sets of data. These values may be compared to the total kidney clearances: the apparent renal clearances for PO and POH were 1.7 and 4.1 ml/min.

The fraction of the prednisone apparent renal clearance accounted for by excretion was 10-fold that of POH, 0.30 versus 0.03. This implies that a significantly greater portion of PO is excreted unchanged in the urine than is POH. Being so similar structurally, this is probably not true, and therefore, the significance of renal interconversion must be considered. Before strict conclusions are drawn, however, it should be noted that if the four very high excretion values (>1.4) are omitted from the average PO excretion calculation, the mean is equivalent to that of POH, 0.12. It is probable that this latter value is more comparable to the total results as urine flows were then similar between preparations.

The glucocorticoids are small, lipophilic, nonpolar moieties and are believed to undergo flow-dependent excretion. Franke et al. (1982) observed with micropuncture studies that this was true for cortisol reabsorption in the perfused rat kidney. Reach et al. (1977) and Rocci et al. (1981) have also demonstrated flow-dependent reabsorption of cortisol and prednisolone, respectively, in perfusion experiments. *In vivo*, Lloyd (1952) observed that corticosteroid excretion increased in humans when the urine flow rate was high. A relationship between urine flow and urinary clearance would be indicative of diuresis-dependent excretion. This was the case in our studies, as a high correlation was observed for both drugs (Figure 7.9): POH exhibited a correlation coefficient of 0.923 and PO, 0.875.

Figure 7.10 compares the urinary clearance to the steady state concentration of perfused drug in the rabbit kidney; no apparent relationship exists between urinary clearance and concentration (or dose). The clearance of POH in the perfused rat kidney was also found to be independent of concentration (Rocci et al., 1981). The results of the urine flow and perfusate concentration analyses imply that filtration and passive reabsorption mechanisms are sufficient to characterize the renal excretion of PO and POH. These lipophilic compounds would not be expected to undergo active reabsorption as these processes are generally reserved for polar or hydrophilic compounds which otherwise

would be lost into the urine. However, the existence of secretion and reabsorption processes cannot be ruled out on the basis of these experiments alone.

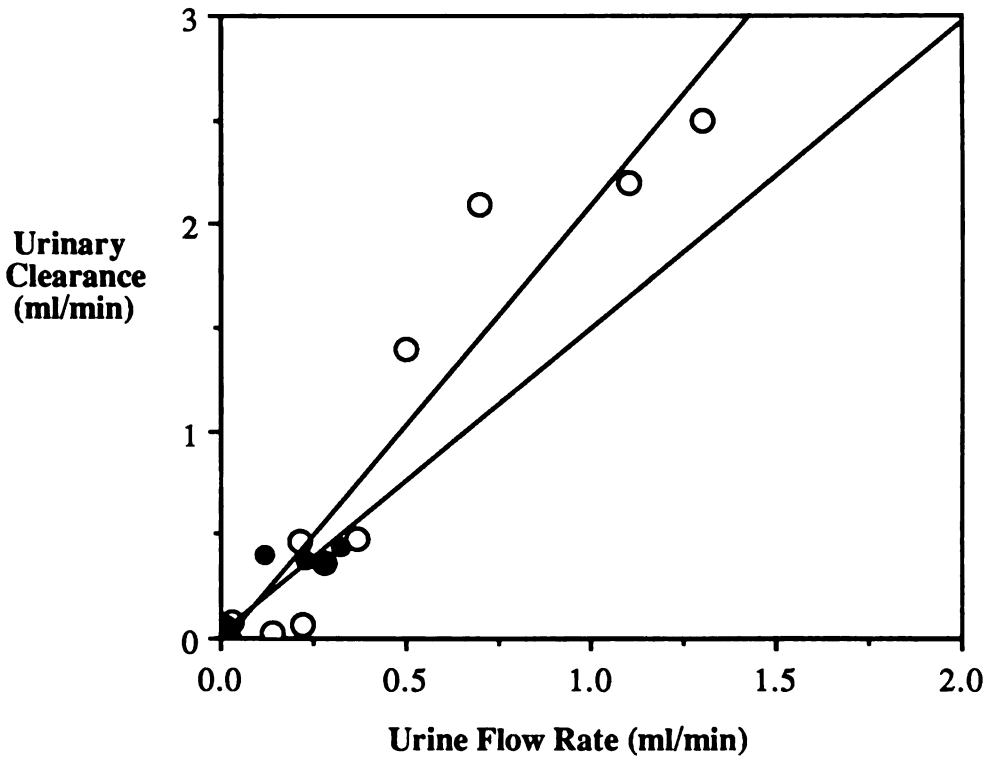

Figure 7.9. Apparent urinary clearance of prednisone (solid circles) and prednisolone (open circles) in the perfused rabbit kidney as a function of urinary flow rate. Linear regression lines are shown.

A composite model of the results of these analyses is presented in Figure 7.11. Oxidative interconversion and irreversible elimination are of apparently relatively greater magnitude than the other processes. Oxidative conversion is apparently saturable.

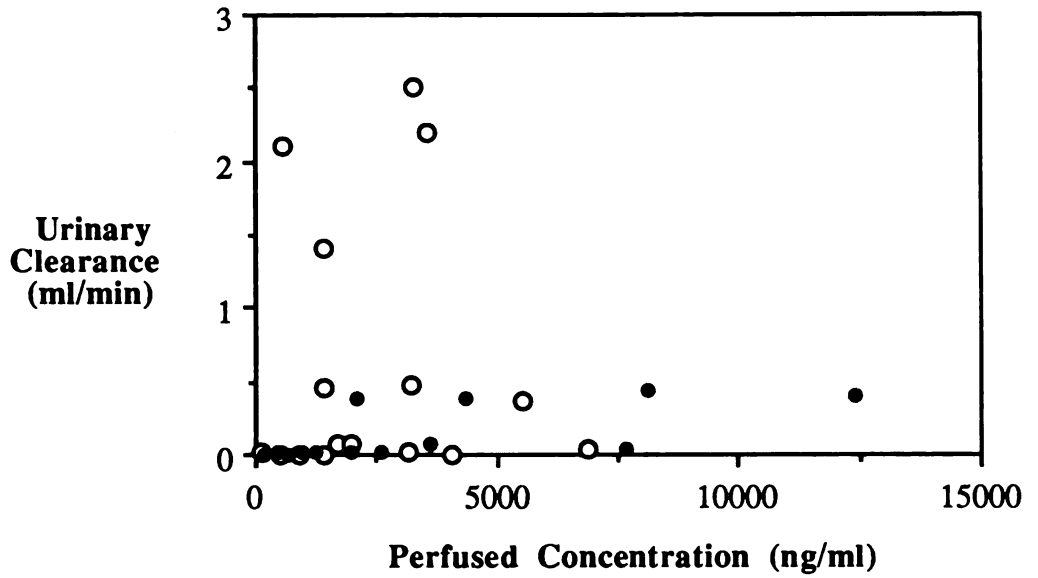

Figure 7.10. Apparent urinary clearance of prednisone (solid circles) and prednisolone (open circles) in the perfused rabbit kidney as a function of perfused drug concentration.

Significance

Strong autoregulation was expressed by the perfused kidney. It was therefore difficult to maintain rabbit kidney perfusion conditions which resembled those achieved *in vivo*. Fortunately, topically-applied vasodilators were effective in reversing the constriction for a short time and multiple tests of renal function assured viability for short experimental periods.

Prednisone and prednisolone demonstrated apparently linear renal extraction ratios in the leporine kidney. The values were less than 0.10 and independent of concentration over the range of 130 to 12,000 ng/ml total drug. Apparent renal and urinary clearances were calculated for both compounds.

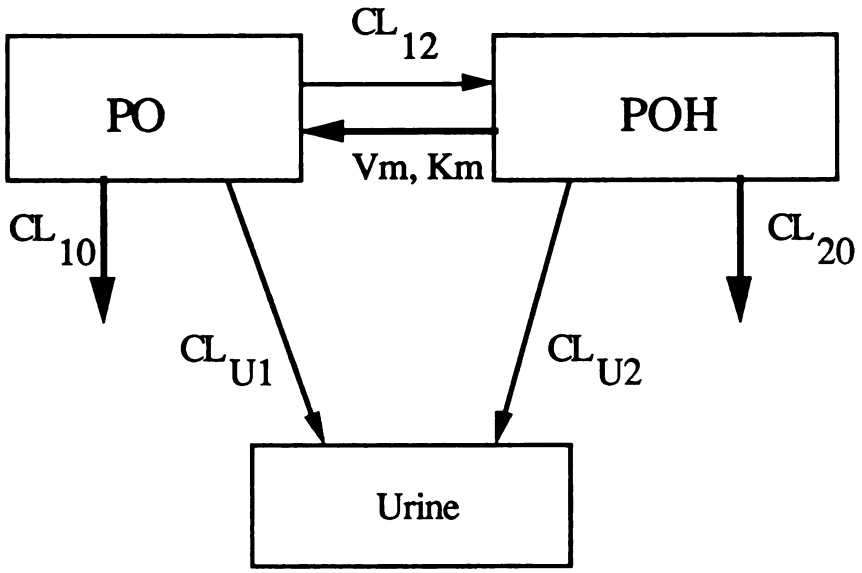

Figure 7.11. Model of the interconversion of prednisone and prednisolone in the perfused rabbit kidney. Terms are as defined in Figure 6.1. Boldness of the lines indicates relative magnitude of the clearance parameters. Nonlinearity is represented by V_m, K_m.

The urinary excretion of PO and POH was observed to be independent of dose and accounted for a very small percentage of the total dose (< 1%). The excretion of PO and POH appeared to be diuresis-dependent, as both demonstrated highly significant increases in excretion with urine flow rate. Consequently, flow-dependent excretion is an additional complication in the analysis of the *in vivo* disposition of PO and POH.

The kidney was primarily oxidatively oriented in the interconversion of PO and POH, exceeding reduction 10-fold. The observed reductive capacity varied greatly between preparations such that linearity was assumed to exist. Although oxidation was greater, it demonstrated nonlinearity with increasing POH concentrations.

Irreversible elimination appeared to be linear and accounted for the largest fraction of eliminated drug. Exact rates could not be calculated as metabolites were not measured in

these experiments. With some assumptions, approximations as to the relative magnitude of these pathways were made. These suggested that the extent of irreversible metabolism greatly exceeded net reductive conversion in the perfused rabbit kidney, but the extent of oxidative conversion was comparable to irreversible elimination.

A model for glucocorticoid disposition in the rabbit kidney was developed which reflected the results of these perfusion experiments. One nonlinearity was included, the oxidative interconversion pathway. The results of modeling these experiments will provide exact values for the six intrinsic clearances associated with pathways of elimination and interconversion.

The nonlinear disposition of prednisone and prednisolone may be partially explained by the kidney. However, due to the relatively low metabolic activity of the rabbit kidney, an additional organ must also contribute significantly, the subject of the next chapter.

Chapter 8

In Situ Leporine Lung Perfusions

Background

The lung performs several critical nonrespiratory functions such as filtration and water balance but it is not often appreciated for its significant metabolic capacities: the endogenous compounds histamine, "slow reacting substance", heparin, and prostaglandins are synthesized in the lung (Heinemann and Fishman, 1969). Additionally, the pulmonary endothelium metabolizes serotonin, bradykinin, epinephrine, angiotensin I, and prostaglandins (Thurlbeck & Abell, 1978). The metabolic activity of the lung was first recognized in 1925 by Starling and Verrey who observed that the rapid vasoconstriction of the renal circulatory bed in kidney perfusions could be eliminated if the blood perfusate was first passed through a "heart-lung" preparation. In 1953, perfused cat lungs were shown to inactivate the renal arterial vasoconstrictor, serotonin (Gaddum et al., 1953).

The pulmonary disposition of the glucocorticoids has been characterized with studies designed primarily to ascertain the role of glucocorticoids in lung development (Murphy, 1978; Torday et al., 1976; Nicholas and Kim, 1975). It is believed that glucocorticoids promote lung maturation and secretion of surfactant which allow a newborn to breathe and oxygenate its own blood. Studies have been performed in fetuses of various gestational ages, infants (or young animals) as well as adults. Most work has examined the disposition of cortisol and cortisone, as these are the major endogenous human

glucocorticoids. Results from these studies can be extrapolated to prednisolone and prednisone (refer to Chapter 1).

Rabbit lungs were perfused with prednisone and prednisolone to characterize the pulmonary disposition of these compounds in the whole organ. While it was anticipated that metabolic capacity would be reduced as compared to the liver and kidney, relative perfusion rates could impart greater significance to the lung than any other organ.

Methodology

Techniques of lung perfusion are still in the developmental stage and the lung has been perfused much less readily than the liver or the kidney. There are few tests of pulmonary metabolic function and it is therefore difficult to assess the viability of the perfused lung. Not unexpectedly, methodologies for investigation of pulmonary metabolism vary substantially. The lung may be perfused *in situ* or as an isolated organ. The *in situ* technique minimizes the anoxia period and for this reason, was adapted for the rabbit.

Perfusate, apparatus and experimental conditions

The perfusate was prepared as follows: to Krebs-Heinseleit bicarbonate buffer (1932), 100 mg% dextrose and bovine serum albumin fraction V, 70 mM (5.5%) (both from Sigma, St. Louis, MO) were added. The pH was brought to 7.4 with sodium bicarbonate. The solution was then gravity filtered (Whatman, No.1, Maidstone, England). The perfusate was prepared one to three days prior to use. The total volume used in the perfusion experiments was 400 ml.

The lung perfusion apparatus was similarly designed to those of the rabbit liver and kidney (described in Chapters 5 and 7). The major difference is the substitution of mechanical ventilation for the perfusate membrane oxygenator; as with the kidney, the animal remains outside of the thermostatically-controlled chamber. The apparatus and

experimental conditions consisted of the following. Within a 400 ml glass beaker, the perfusate was continuously stirred magnetically (mini model 200, VWR, San Francisco, CA). The pump (Masterflex, model N-07553-20, Cole Parmer, Chicago, IL) delivered non-pulsatile flow. A blood transfusion filter (SQ40S, Pall, Fajardo, PR) trapped clumps of red cells, but permitted single cells to pass. An in-line temperature probe, pH electrode (Cole Parmer, Chicago, IL), manometer (W.A.Baum, New York, NY), flow meter (Gilmont, Great Neck, NY), and a bubble trap were placed in order. The artificial vasculature was composed of Tygon tubing, 1/8" ID x 3/16" OD (Fisher, Pittsburg, PA); the tubing to and from the ventilator was latex (Primeline Industries, Cuyahoga Falls, OH). Sampling ports (Argyle, St. Louis, MO) were placed prior to entrance into and upon exit from the lung. Perfusate exiting the animal was returned to the reservoir for recirculation. The mechanical respirator (Harvard Rodent Ventilator, model 683, Harvard Apparatus, South Natick, MA), sampling ports and the rabbit remained on the table surface; other components of the apparatus were housed in a temperature-controlled plexiglass chamber (Air Control Inc., Huntingdon Valley, PA). One and one-half ml of entering and exiting perfusate was sampled at specified times then frozen until analyzed (usually within one week).

The perfusate temperature was maintained at 38 °C to allow some cooling of the perfusate as it exited the chamber and perfused the lung. The surface of the lung was moistened with warm saline and the chest cavity was covered with aluminum foil. A lamp was then placed over the chest to help maintain the temperature. The flow was adjusted to maintain pressure at or below 17 mm Hg. The pH of the perfusate was continuously monitored and maintained between 7.35 and 7.45 by adjustment of the respiratory rate. Respiration was performed with room air; the use of carbogen (95% O_2 / 5% CO_2) lead to an increasing pH with time, which was believed to be due to excessive removal of carbon dioxide. The respiratory rate, 40 to 50 breaths/min and the tidal volume was estimated

from the literature accompanying the Harvard Apparatus ventilator, and set at 9 ml/min for rabbits of about two kg. These rates were compatible to those of the literature.

At the start of an experiment, a bolus dose of PO or POH (Sigma, St. Louis, MO) dissolved in a minimal volume of methanol was added to the reservoir to achieve a specific concentration. To maintain this concentration at steady state, it was necessary to infuse drug into the system. Knowing the extraction of the drug from preliminary experiments and the flow rate of the perfusate, it was possible to predict the infusion rate of drug needed to maintain steady state conditions in these organ perfusion experiments. This quantity was continuously infused (Harvard infusion pump, Model 975, Mills, MA), at a rate and concentration which maintained the steady state concentration of drug and replaced the volume of perfusate removed in sampling. Equilibration of lung tissue with perfusate was very rapidly achieved, as might be expected for this low-density organ.

A normal phase high performance liquid chromatographic (HPLC) assay was used for the detection and quantitation of prednisone and prednisolone in perfusate samples. Equilibrium dialysis was used to measure the free concentrations of prednisone and prednisolone present in the perfusate samples. Both methods are described in detail in Chapter 3.

Surgery

Several surgical procedures exist for perfusion of the rat, rabbit and guinea pig lung (Nicholas and Kim, 1975; Smith and Bend, 1981; Orton et al., 1973; Niemeier, 1984). The method employed here minimizes as much as possible the time the organ is without flow and is adapted primarily from the *in situ* rat lung perfusion of Leary (1969).

The animal was anesthetized with 50 mg/kg ketamine and 10 mg/kg xylazine as intramuscular injections. The abdomen, chest and under side of the neck were shaved. One mg diazepam was slowly infused intravenously into the auricular artery of one ear. The front neck was opened and the trachea was exposed, carefully avoiding the vessels

which lie beside the trachea. Two silk sutures were placed around the trachea, below the thyroid cartilage and a razor blade was used to slice through half of the trachea, between discs. The trachea cannula was inserted (Pharmaseal, tube connectors, American Scientific, Sunnyvale, CA) and secured. (Ventilation was not initiated until both perfusion cannulae were secured and perfusate flow was started.) Heparin was administered (1000 U/kg) through the auricular artery of the ear opposite to the one which received diazepam and three to four minutes elapsed to allow complete circulation of the anticoagulant. The abdomen was opened and the vena cava was cut, resulting in death due to circulatory failure. The chest was then accessed through the diaphragm and opened along the sternum. The pericardium was removed, the right ventricle was cut on the anterior wall, and the pulmonary artery was cannulated (Angiocath, 14 ga, Deseret, Sandy, UT). The tip of the cannula was positioned about five mm into the artery and secured to the right ventricular wall with two silk sutures (Harvard Apparatus, South Natick, MA). The inferior and superior vena cavae were clamped to prevent loss of perfusate due to back flow into the right atrium. After a slow (20 ml/min) perfusion rate was initiated then the vena cava was clamped above the diaphragm. The aorta was clamped above the pulmonary artery branch to avert perfusate loss from the left ventricle. A small incision was made in the anterior wall of the left ventricle and the cannula was passed to the level of the mitral valve. This cannula was composed of rigid plastic tubing, 6 cm long, 4 cm O.D. x 3 cm I.D.; the tip was filed to smoothness to prevent an atrial tear. A purse-string suture in the left ventricle secured this cannula. Artificial respiration was initiated and perfusate flow was slowly increased over the next five minutes until a pressure of 17 mmHg was achieved.

Flow rates in this preparation were dictated by blood pressure, which was very sensitive to the cannula position. It was important to manipulate the cannulae so as to yield true pressure readings. The pulmonary artery cannula is very sensitive to the distance it is advanced, as the artery curves approximately 180° between the heart and lungs. The effluent cannula must not be advanced into the left atrium, but remains at the level of the

mitral valve. Otherwise, pulmonary venous drainage is impeded and pulmonary vascular pressure quickly rises, resulting in the formation of edema. The positioning of both cannulae was continually monitored throughout the experiment.

Modeling intra-pulmonary disposition

The pharmacokinetic model presented in Figure 8.1 describes the disposition of prednisone and prednisolone in the perfused rabbit lung, including nterconversion and irreversible elimination. The perfusion rate limitation is assumed and unbound drug concentrations are used in the calculations, yielding estimates of intrinsic clearance. (Chapter 5 describes in greater detail the model's assumptions.) Studies were performed at steady state to eliminate the potential complication of concentration gradients and distribution phenomena.

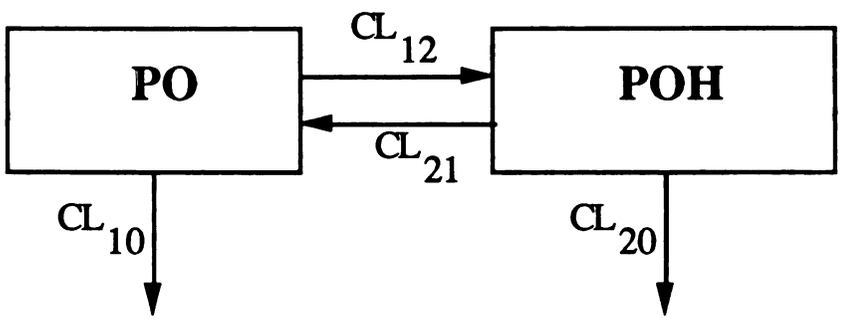

Figure 8.1. Model of the interconversion and elimination of prednisone and prednisolone in the perfused rabbit lung. (Clearance terms represent intrinsic clearance.)

CL_{10} = irreversible elimination of prednisone
CL_{12} = reductive conversion of prednisone to prednisolone
CL_{20} = irreversible elimination of prednisolone
CL_{21} = oxidative conversion of prednisolone to prednisone

Mass balance for perfusion with each drug results in two differential equations:

$$V_P \frac{dC^{PO}}{dt} = Q_P\left(C_{IN}^{PO} - C_{OUT}^{PO}\right) - C_P^{PO}CL_{12} + C_P^{POH}CL_{21} - C_P^{PO}CL_{10} \quad (1)$$

$$V_P \frac{dC^{POH}}{dt} = Q_P\left(C_{IN}^{POH} - C_{OUT}^{POH}\right) - C_P^{POH}CL_{21} + C_P^{PO}CL_{12} - C_P^{POH}CL_{20} \quad (2)$$

C^{PO} and C^{POH} are unbound perfusate concentrations of prednisone and prednisolone, respectively; Q_P is the pulmonary perfusate flow rate; entering and exiting unbound concentrations of POH and PO are designated C_{in} and C_{out}, respectively, while V_P and C_P are the volume of the lung and the pulmonary tissue concentrations of unbound drug, respectively. The well stirred and parallel tube models of organ disposition are used to predict C_P. The four clearance parameters are defined in Figure 8.1 and represent intrinsic clearance.

For perfusion of two drugs, the result is four differential equations with four parameters to solve. Experimental results dictated which clearance terms were represented by nonlinear parameters. As discussed below, it was apparent that leporine pulmonary conversion of POH to PO was nonlinear. This process was assumed to obey Michaelis-Menten kinetics, such that equations 1 and 2 are modified in this manner:

$$V_P \frac{dC^{PO}}{dt} = Q_P\left(C_{IN}^{PO} - C_{OUT}^{PO}\right) - C_P^{PO}CL_{12} + \frac{\upsilon_{21} \cdot C_P^{POH}}{K_{M21} + C_P^{POH}} - C_P^{PO}CL_{10} \quad (3)$$

$$V_P \frac{dC^{POH}}{dt} = Q_P\left(C_{IN}^{POH} - C_{OUT}^{POH}\right) - \frac{\upsilon_{21} \cdot C_R^{POH}}{K_{M21} + C_R^{POH}} + C_P^{PO}CL_{12} - C_P^{POH}CL_{20} \quad (4)$$

where υ_{21} (analogous to V_{max}) is the maximal velocity of the oxidative conversion reaction and K_{M21} is the Michaelis constant or the concentration at which half-maximal rate is achieved. The result is five metabolic parameters within four differential equations. The experiments were performed at steady state such that the right hand side of the equations were equal to zero. Q_P, C_{in} and C_{out} were measured. C_P was estimated with the well stirred or parallel tube model approximations of tissue concentrations according to equations 5 and 6:

$$C_{P,ws} = C_{out} \quad (5)$$

$$C_{P,pt} = [\ (C_{in} - C_{out}) / \ln (C_{in} / C_{out})\ ] \quad (6)$$

Only the metabolic parameters remain to be solved simultaneously: CL_{12}, CL_{10}, CL_{20}, υ_{21} and K_{M21}.

Results and discussion

The results of rabbit lung perfusions are presented in Tables 8.1 and 8.2. Five prednisolone perfusions were performed (Table 8.2), examining twelve steady state concentrations of POH, ranging from 164 to 112,497 ng/ml total drug. Concentrations for which there are no PO values listed (dashes) are those in which lower steady state concentrations of POH were run following high ones, yielding artificially elevated PO concentrations.

Table 8.1 contains data from prednisone perfusions. Five experiments yielded 15 steady state concentrations of PO which ranged from 296 to 10,844 ng/ml (total drug). A dash is indicative of artificially elevated values of POH (described above), as opposed to a zero, which is a measured concentration of POH.

Table 8.1
Steady state drug concentrations and calculated apparent pulmonary clearance for prednisone perfusions of the rabbit lung

Rabbit No	Total Steady State Concentration Prednisone (ng/ml)	Unbound Steady State Concentration Prednisone (ng/ml)	Total Steady State Concentration Prednisolone (ng/ml)	Unbound Steady State Concentration Prednisolone (ng/ml)	Extraction Ratio	Prednisone Apparent Pulmonary Clearance (ml/min)	Prednisone Apparent Pulmonary Clearance (ml/min/kg)
1	17652	6708	--	-	0.04	2.4	1.2
3	522	193	0	0	0.06	5.1	2.2
6	2358	196	196	80	0.07	4.6	2.0
	4498	341	341	140	0.06	3.9	1.7
	10844	722	722	296	0.1	5.5	2.4
7	577	219	1	0	0.06	3.3	1.1
	713	271	6	3	0.03	1.7	0.6
	1041	396	10	5	0.04	2.2	0.7
	3383	1286	21	10	0.03	1.6	0.5
	5023	1909	49	24	0.03	1.6	0.5
9	296	110	36	17	0.05	4.0	1.2
	875	324	57	27	0.04	3.2	1.0
	2005	742	79	37	0.08	6.4	2.0
	6755	2499	109	51	0.07	5.6	1.7
	5893	2180	247	116	0.05	4.0	1.2
				Mean	**0.05**	**3.7**	**1.3**
				S.D.	**0.02**	**1.6**	**0.6**

Table 8.2
Steady state drug concentrations and calculated apparent pulmonary clearance for prednisolone perfusions in rabbit lungs

Rabbit No.	Total Steady State Concentration Prednisolone (ng/ml)	Unbound Steady State Concentration Prednisolone (ng/ml)	Total Steady State Concentration Prednisone (ng/ml)	Unbound Steady State Concentration Prednisone (ng/ml)	Extraction Ratio	Prednisolone Apparent Pulmonary Clearance (ml/min)	Prednisolone Apparent Pulmonary Clearance (ml/min/kg)
1	3906	1641	36	14	0.12	5.4	2.6
	21002	8821	123	47	0.06	2.7	1.3
2	472	198	37	14	0.05	3.0	1.3
	3809	1600	322	122	0.08	4.4	2.0
4	210	95	14	6	0.05	3.9	1.7
	164	74	-	-	0.06	5.1	2.2
5	309	127	32	12	0.06	5.1	2.3
	1294	531	88	34	0.04	3.1	1.4
	2004	822	90	35	0.05	3.8	1.7
	1530	627	-	-	0.04	3.0	1.4
8	10020	3908	64	22	0.06	2.6	0.9
	35706	13925	950	333	0.09	3.6	1.2
	70230	27390	1175	411	0.08	3.1	1.1
	112497	43874	2548	892	0.07	2.9	1.0
				Mean	**0.07**	**3.7**	**1.6**
				S.D.	**0.02**	**1.0**	**0.5**

The binding of prednisone and prednisolone was linear due to the absence of corticosteroid binding globulin in perfusate (Figures 8.2 and 8.3). The fractions unbound of PO and POH averaged 0.39 and 0.44, respectively. Small variability was observed between determinations.

Viability

The rabbit lung was perfused *in situ* with prednisone and prednisolone. A good test of endogenous pulmonary metabolic function would assess synthetic or metabolic capacity, but such a procedure has not been developed at this time. Nonspecific indices such as the constancy of perfusate flow rate under constant pressure, pH stability and weight gain are the most commonly monitored. Formation of edema and decreasing perfusate flow are the primary problems associated with this experimental system. As these lungs were perfused *in situ*, it was not possible to monitor weight gain. Instead, viability of the perfused rabbit lung was assessed by three criteria: flow rate and pH stability, as well as physical appearance.

Most investigators accept visual observation of the perfused lung as an indicator of its viability. A healthy lung perfused with cell-free media is uniformly very white. A poor preparation is immediately obvious, as edematous areas appear translucent and are poorly ventilated. Areas which are not perfused (due to an embolism, for example) retain the pink color imparted by red blood cells which have been retained in the vasculature. Heparin minimizes the risk of this occurrence. Collapsed areas develop if the lung is punctured or physically damaged in some other way and these areas demonstrate no expansion or contraction upon ventilation. Therefore, the entire tissue should expand and contract in unison.

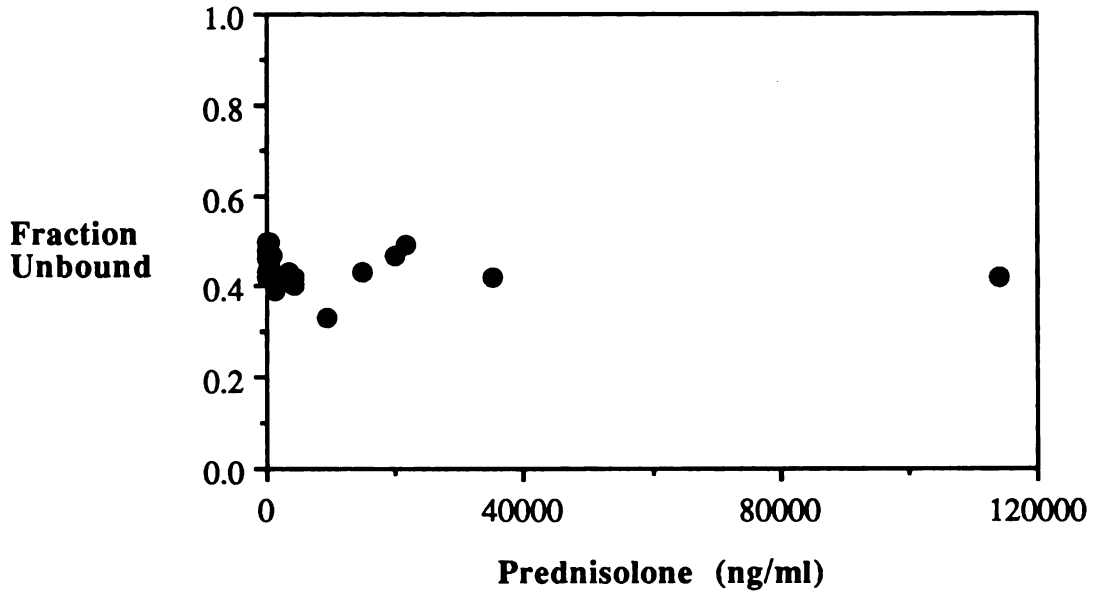

Figure 8.2. Unbound fraction of prednisone in perfusate of rabbit lung perfusion experiments.

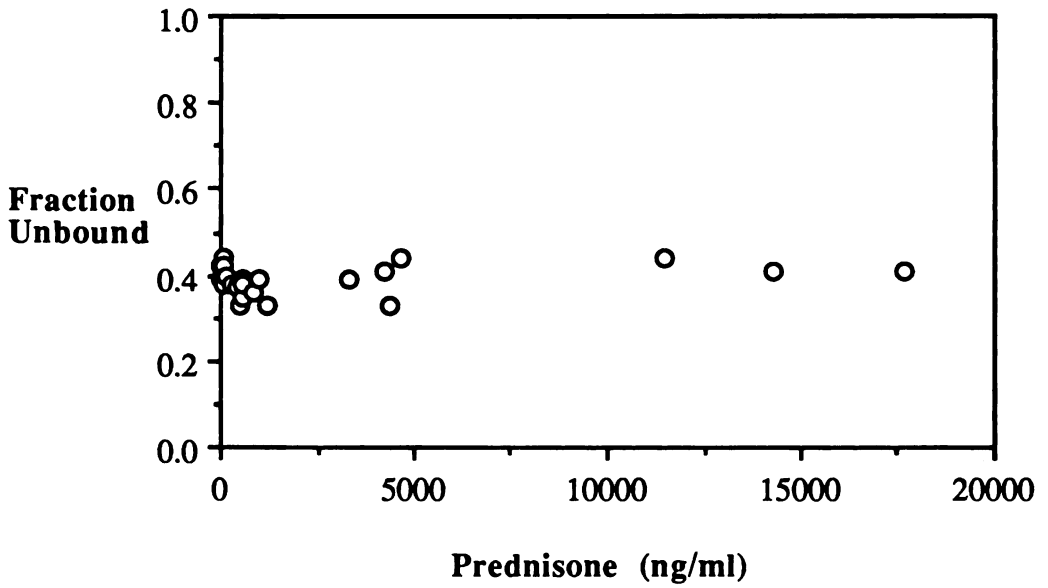

Figure 8.3. Unbound fraction of prednisolone in perfusate of rabbit lung perfusion experiments.

Optimization of ventilation gas, tidal volume and respiratory rate is usually sufficient to yield stable pH measurements. In the perfused leporine lung, pH stability was the first indication of failure. When the pH began to drop or rise, the preparation was discontinued.

Perfusate flow rate (pressure) plays a large role in edema development. Neimeier (1984) and Ross (1972) state that flow rates above physiologic (those which produce a pressure greater than 23 cm H_2O or 17 mm Hg) result in the rapid onset of edema. Constant-pressure perfusion is therefore preferable to constant-flow perfusion of the lung. Short experimental periods generally minimize the risk of edema development. The perfusate flow rates required to maintain pressure at 17 mm Hg were significantly lower than estimated leporine cardiac output, approximately 250 ml/min for 2 kg rabbits (Spector, 1956b). Flows were 45 to 85 ml/min and attempts to increase the flow rates resulted in the development of edema and the cessation of the experiment within a few minutes.

Pulmonary extraction

Figures 8.4 and 8.5 depict the extraction ratios of PO and POH, respectively, as a function of total perfused drug concentration. The pulmonary extraction ratio (E_P) was calculated as:

$$E_P = (C_{in} - C_{out}) / C_{in}$$

The perfused rabbit lung extracted less than 10% each of prednisone and prednisolone in an apparently linear manner. Concentrations of total PO extended to about 20,000 ng/ml. The extraction ratio of prednisone was 0.05 and ranged from 0.03 to 0.10. The extraction of POH ranged from 0.04 to 0.12 with a mean 0.07 over the 500-fold concentration range of 200 to 100,000 ng/ml.

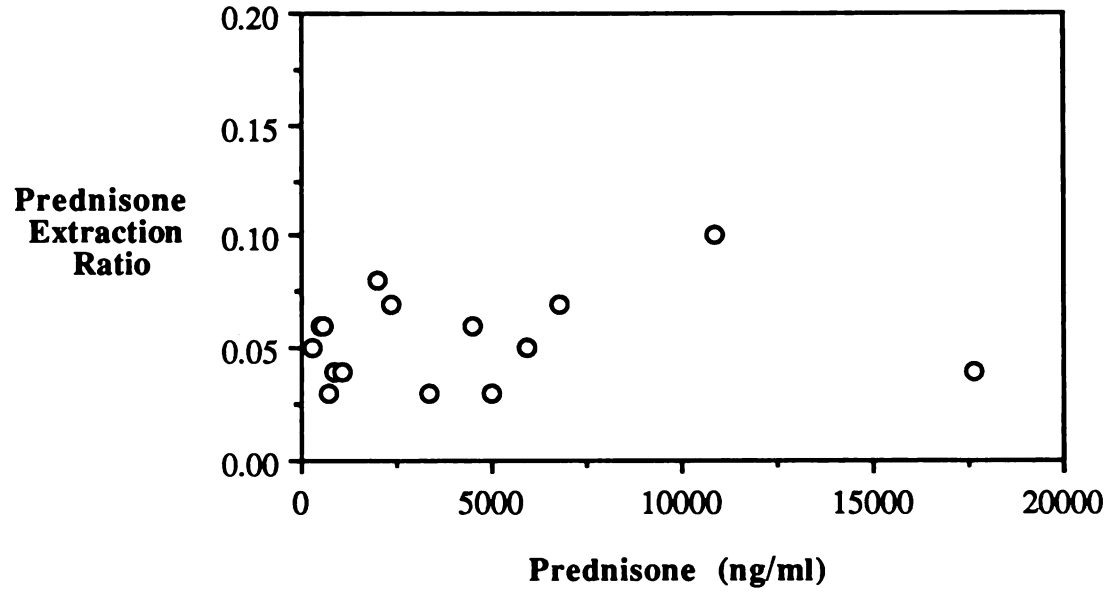

Figure 8.4. Extraction of prednisone in rabbit lung perfusions as a function of total perfusate drug concentration.

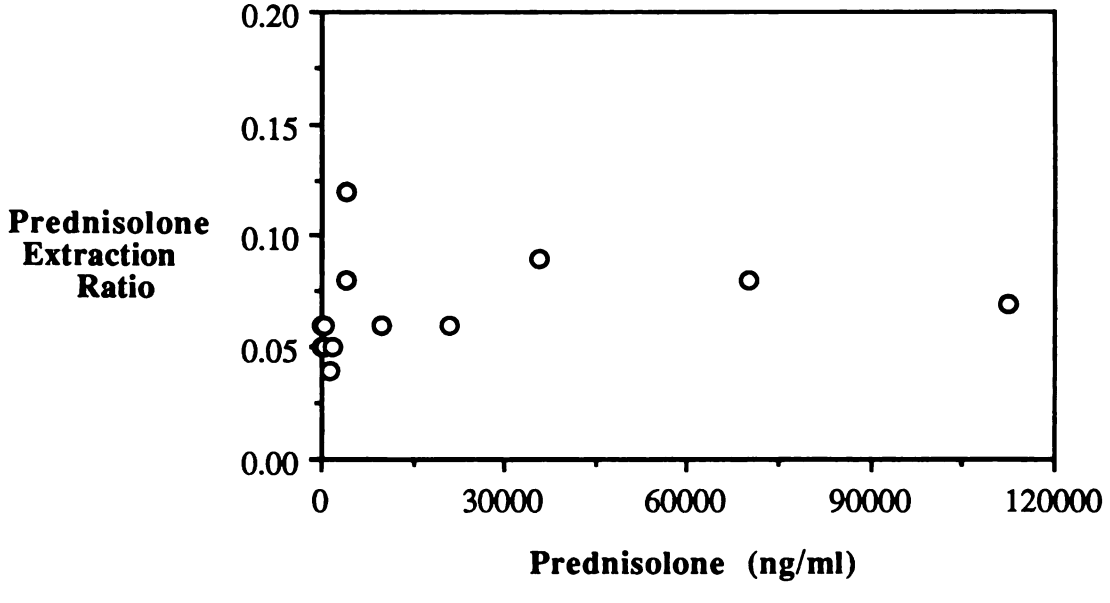

Figure 8.5. Extraction of prednisolone in rabbit lung perfusions as a function of total perfusate drug concentration.

As the endogenous glucocorticoids cortisol and cortisone are believed to be metabolized by the same enzymes, it would be of interest to compare the results of experiments with such compounds to these results. However, no perfusion studies with these compounds have calculated the pulmonary extraction.

Apparent pulmonary clearance

Apparent organ clearance, $CL_{P,app}$ is presented in Tables 8.1 and 8.2. It is calculated as:

$$CL_{P,app} = Q_R \cdot E_P$$

Pulmonary clearances were small, as a function of the both the low extraction of the glucocorticoids and the sub-physiologic flow rates. Clearance was, however, independent of concentration (data not plotted). Despite the numerous perfusion references examining the pulmonary disposition of the glucocorticoids, values for the pulmonary blood clearance of these compounds could not be found for comparison.

Interconversion

Prednisone and prednisolone were reversibly interconverted by the perfused rabbit lung. Formed prednisone was measurable in all samples and ranged from 14 to 2548 ng/ml. Prednisolone was present in perfusate at concentrations up to 722 ng/ml and was not detectable in one case (detection limit, 10 ng/ml).

The ratio of product to substrate ratio for prednisolone perfusions (PO/POH) was concentration-dependent (Figure 8.6), and decreased from 0.10 to 0.006 over the concentration range of 164 to 112,497 ng/ml. Figure 8.7 expands the data for concentrations below 12,000 ng/ml. The greatest decrease in the ratio occurred between

164 and 4000 ng/ml. This nonlinearity occurs throughout the usual therapeutic concentration range, defined for this purpose to be between 100 and 1000 ng/ml.

The opposing reaction, reductive interconversion, was apparently linear through a smaller concentration range (Figure 8.8). Prednisone was perfused at concentrations from 296 to 10,844 and POH was measurable in all but one period. The ratio of formed to perfused drug, POH/PO, was constant at about 0.07 or 0.08 in all but that one period over the 35-fold concentration range. This observation is consistent with the results of Nicholas and Kim (1975) who observed nonsaturable reduction of cortisone to cortisol in the perfused rat lung over the concentration range of 0.4 to 360 ng/ml cortisone.

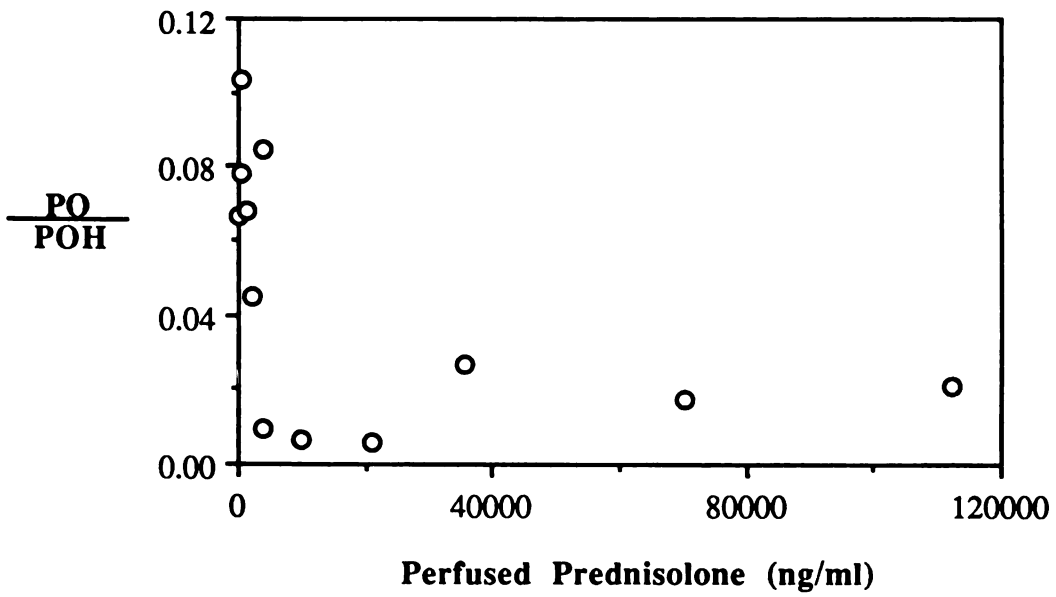

Figure 8.6. Ratio of formed prednisone to perfused prednisolone produced by the perfused rabbit lung, all experiments.

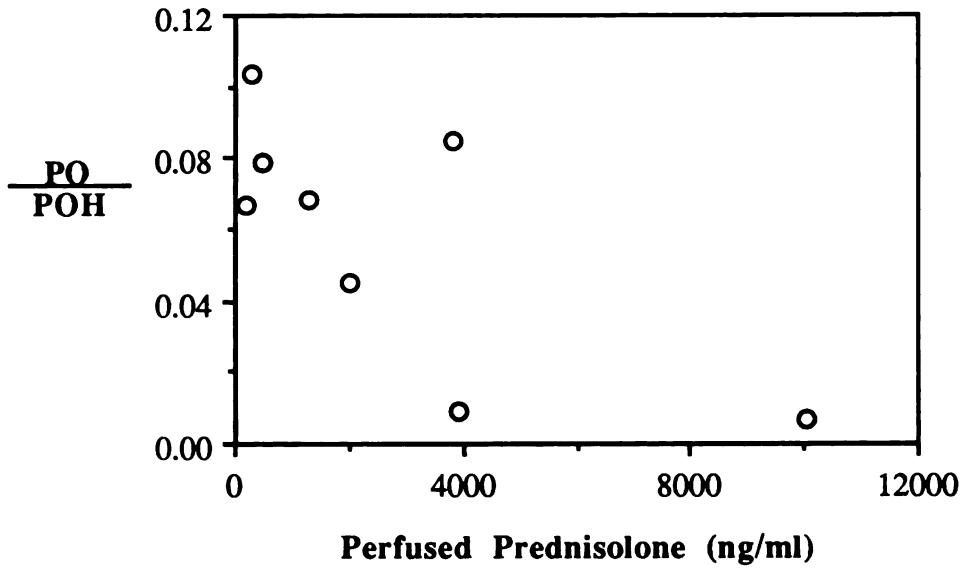

Figure 8.7. Ratio of formed prednisone to perfused prednisolone produced by the perfused rabbit lung, concentrations < 12,000 ng/ml.

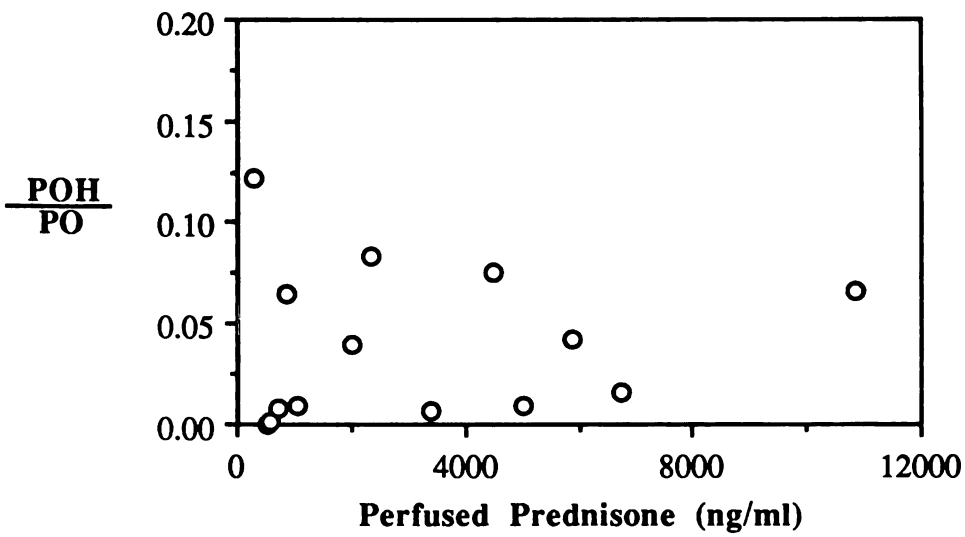

Figure 8.8. Ratio of formed prednisolone to perfused prednisone produced by the perfused rabbit lung.

These results suggest that the pulmonary conversion of POH to PO is saturable in the rabbit. The oxidation of POH to PO is believed to be saturable in humans and dogs *in vivo*, as demonstrated by a concentration-dependent decrease in the PO/POH ratio in plasma (Rose et al., 1981; Frey et al., 1980; Legler et al., 1982). Saturation of the pulmonary metabolic conversion pathway of these species could explain this observation. This nonlinearity has earlier been attributed to hepatic metabolism, but no investigation of the capacity for pulmonary metabolism of PO and POH has been performed until this time. The drug concentrations measured in antecubital venous blood are probably not significantly affected by metabolic processes in the arm. Therefore, it can be assumed that the dose-dependent changes in apparent systemic clearance are a function of metabolic events in the lung and organs proximal to the lung, such as the liver and kidney.

Species differences in the pulmonary interconversion of cortisol/cortisone or prednisolone/ prednisone have been observed. Brooks et al. (1977) demonstrated a strong oxidative directionality with the glucocorticoids in the perfused rabbit lung. The ratio of cortisone to cortisol was 2.6 after two hours of cortisol perfusion over the concentration range of 4 to 3600 ng/ml. Nicholas and Kim (1975) perfused the guinea pig lung with cortisone, observing that only 7% of cortisone was converted to cortisol. But the rat lung reversibly interconverted the endogenous glucocorticoids (Nicholas and Kim, 1975) with a reductive preference: the cortisone to cortisol ratio was dependent on the compound perfused, being higher after cortisol than cortisone perfusion, 3.5 versus 1.9.

Irreversible elimination

In *in vitro* studies, the lung possesses a spectrum of xenobiotic metabolizing enzymes nearly as large as the liver (Bend and Hook, 1976). Pulmonary mixed function oxidases, glutathione-dependent transferases and sulfotransferases are involved in steroid metabolism (Heinemann and Fishman, 1969). The endothelium is believed to be responsible for the majority of metabolic activity (Harris and Heath, 1978). As the

glucocorticoids prednisone and prednisolone are synthetic derivatives of the endogenous glucocorticoids, metabolic pathways evolved to biotransform cortisol and cortisone could also affect the disposition of PO and POH. The perfused lung of the rat, rabbit and guinea pig has been shown to significantly eliminate cortisol and cortisone (Nicholas and Kim, 1975; Torday et al., 1976). It was not possible to quantitate irreversible elimination pathways of PO and POH in the current study, as other metabolites were not measured.

It is possible that low elimination activity was present in the lung. Comparison of the extraction ratio and the fraction of drug recovered as the interconverted pair lends insight into the relative magnitude of irreversible loss and conversion pathways. No conclusions can be drawn from this comparison, however, as these studies do not compare masses, but fractions. For PO perfusion, extraction was 0.05 and the POH/PO ratio averaged 0.07. Extraction of POH was 0.07 and the PO/POH ratio decreased from 0.10 to 0.01. If elimination processes exist, they are apparently small relative to conversion processes, as the fractions are of similar magnitude.

The irreversible elimination of cortisol and cortisone demonstrates small species differences. In the perfused rabbit lung, Brooks et al. (1977) recovered 5 to 10% of perfused cortisol as compounds of increased polarity, such as hydroxylated or conjugated metabolites. Nicholas and Kim (1975) documented similar elimination by the perfused rat and guinea pig lung; about 10% of both cortisol and cortisone were eliminated as nonglucuronidated polar metabolites.

Significance

For some compounds, the lung determines the peripheral concentrations. It regulates circulating concentrations of many hormones and vasoactive substances. If any of the substance is to reach the target organ, low pulmonary metabolic activity is necessary, because if the lung extracted high fractions of the compound, arterial concentrations would be very low. For other drugs, hepatic and/or renal enzymatic capacity commonly exceeds

that of the lung. However, because organ blood (perfusate) flow rates determine the rate of substrate delivery, the lung may be a more significant moderator of total body disposition than other organs. This observation is true because despite having a lower capacity than other organs, it receives total cardiac output.

Pulmonary extraction of the glucocorticoids prednisone and prednisolone was low but significant. Extraction below 0.10 was expected, as the liver and kidney have been recognized to be the most important contributors to glucocorticoid disposition. Linearity in extraction was observed over a very large concentration range which greatly exceeded those seen therapeutically.

Prednisone and prednisolone were reversibly converted by the rabbit lung, as well. The pulmonary oxidation of POH to PO was apparently saturated at low concentrations, whereas reductive conversion was linear. This observation may explain, in part, the apparent reductive preference and nonlinearity observed *in vivo*.

Chapter 9

The Pharmacokinetics of Prednisone and Prednisolone in Humans After Intravenous and Oral Administration

Introduction

Studies in the previous chapters were designed to characterize the organ-specific disposition of prednisone and prednisolone in a species which demonstrates pharmacokinetics of the glucocorticoids similar to the human. The profile is characterized by dose-dependent increases in the apparent systemic clearances of prednisone and prednisolone and apparently saturable formation of prednisone (Frey et al., 1980; Rose et al., 1981; Legler et al., 1982). It is hypothesized that the nonlinear kinetics of these compounds can be attributed to organ-specific metabolic processes. The objective of the study described here is to characterize the dependency of the pharmacokinetics of the glucocorticoids *in vivo* in humans on the route of administration, with particular emphasis on hepatic clearance processes.

Several well-designed kinetic studies of prednisone and prednisolone have been performed, but none of these has considered all of the factors which are important for organ-specific analysis of glucocorticoid disposition. For instance, some investigators did not perform protein binding measurements, urine may not have been collected, or only one of the two drugs may have been administered by a particular route. This study involves the administration of the same dose of both prednisone and prednisolone orally and intravenously with plasma and urine collection and determination of unbound

concentrations of both compounds. Conclusions regarding hepatic metabolic processes may be made from the results of pharmacokinetic analyses.

Equipment and supplies

Twenty-five mg doses of each drug were administered; this dose is intermediate in the range of those prescribed to most patients in the outpatient setting. Prednisone (PO) and prednisolone (POH) tablets were provided by The Upjohn Co. (Kalamazoo, MI). The intravenous solutions of prednisone, prednisolone and prednisolone phosphate were prepared by the University of California Sterile Products Laboratory. Prednisone U.S.P. and prednisolone U.S.P. (Pharma-Tek, Inc., Huntington, NY) were dissolved in a cosolvent system of ethanol U.S.P., 5%, and propylene glycol, 1%, in 0.45% sodium chloride (Travenol, Chicago, IL). The concentration of each solution was 0.2 mg/ml, so that 125 ml delivered 25 mg steroid. The solutions were then autoclaved to ensure sterility. Prednisolone disodium phosphate U.S.P. (supplied by Merck, Sharp & Dohme Research Laboratories, Rahway, NJ) was dissolved in 0.45% sodium chloride and was sterilized by filtration due to its thermal instability. The concentration of the phosphate ester was 0.28 mg/ml, so that 125 ml delivered 25 mg-equivalents of prednisolone. All three solutions were tested for microbial contamination and pyrogen content according to regulatory standards.

The solutions were infused at a rate of 12.5 ml/min (AVI Inc., 3M Health Care Group, St. Paul, MN). Catheters (18 gauge, 2 inches, Becton Dickinson, Rutherford, NJ) were placed in the antecubital veins of subjects on infusion days. Blood samples were drawn from the catheters using a syringe (10 ml, Becton Dickinson) and immediately transferred to a heparinized glass vacuum tube (Vacutainer, product 6480, Becton Dickinson, Rutherford, NJ). The samples were mixed by gentle inversion and centrifuged within a few minutes (Beckman Instruments, model TJ-6, Palo Alto, CA). The prednisolone phosphate sample was divided between two tubes, one of which contained

the esterase inhibitor sodium arsenate (Sigma, St. Louis, MO) in water (final concentration, 0.1 M), following the procedure of Unadkat and Rowland (1985).

Study design

The participants were ten healthy male volunteers, ranging in age from 20 to 35 and weighing 67 to 80 kg (Table 9.1). Each subject underwent a physical exam before the study and no subject was receiving chronic medication or had a history of chronic disease. None were smokers and all were low to moderate consumers of alcohol. No alcohol was permitted two days prior to drug administration. The clinical research protocol was approved by the University Committee on Human Research and each subject gave informed consent to participate.

Table 9.1. Study participants

Subject	Age (yr)	Weight (kg)
1	26	70.8
2	24	80.1
3	27	68.3
4	29	75.5
5	28	75.1
6	29	76.2
7	32	77.4
8	20	67
9	31	83
10	35	79.5

Subjects were given five treatments at one-week intervals. These treatments were: (A) 25 mg intravenous prednisone; (B) 25 mg intravenous prednisolone; (C) 25 mg-equivalents intravenous prednisolone phosphate; (D) five 5 mg prednisone tablets (Deltasone®, Upjohn); (E) five 5 mg prednisolone tablets (Delta-Cortef®, Upjohn). The dosing schedule followed a randomized, cross-over design. The oral treatments were administered with 120 ml water. The parenteral forms were administered as short infusions over ten minutes via the antecubital vein of one arm and samples were collected from the other arm. Drug was administered between 7 and 8 a.m. after an overnight fast and subjects were not permitted to eat for two hours after dosing. After this time, a carbohydrate breakfast was provided (breads, juice, decaffeinated coffee); a typical hospital lunch and dinner followed later in the day (vegetable, meat, starch, dessert, beverage). Subjects were encouraged to drink water throughout the day, particularly if they had difficulty voiding at the scheduled urine collection times.

Plasma sample collection differed between treatments. After the oral treatments D and E, seven ml blood samples were collected at 15, 30, and 45 minutes, and at 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10, and 12 hours. For intravenous administration, treatments A, B and C, sampling was performed 5 minutes into the infusion period, and after the infusion at 2, 5, 10, 20, 30 and 45 minutes, and at 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 hours. The separated plasma samples were stored frozen until analyzed. Urine was collected at the same time for all treatments: 1, 2, 3, 4, 6, 8, 10, 12, and 24 hours. The volume was recorded and an aliquot was frozen until analyzed. Both plasma and urine were stored at -20 °C and analyzed for drug content within one month.

Analysis

The plasma samples were simultaneously analyzed for prednisone and prednisolone using the normal phase high performance chromatographic procedure described in Chapter 3. Urine samples were analyzed with a similar procedure, modified to include an additional

extraction and drying step. Urine samples were usually diluted 2 to 10-fold, to yield concentrations below 3000 ng/ml. Prednisolone phosphate was measured in the esterase-inactivated plasma samples, using the reverse-phase chromatographic system described in Chapter 3. Direct measurement of prednisolone phosphate in plasma was not part of the original study design but was begun two weeks into the study; therefore, esterase inactivation was performed in only six of the ten subjects. Two pharmacokinetic parameter calculations were performed for treatment C; the first used the prednisolone concentrations measured with the normal phase assay of non-stabilized samples. The second method (corrected method) subtracted the measured prednisolone phosphate (reverse phase method; corrected for molecular weight differences) from prednisolone (normal phase assay), assuming that all of the ester was hydrolyzed in the nonstabilized samples. The detection limit for both prednisolone and prednisone was 10 ng; prednisolone phosphate could be detected down to 100 ng.

Equilibrium dialysis was performed on approximately one fourth of the plasma samples to estimate prednisone and prednisolone binding. The tritiated compounds ([2,4,6,7-^{3}H]prednisolone and [1,2(n)-^{3}H]prednisone) were custom synthesized by Amersham (Arlington Heights, IL). The method, as described in Chapter 3, used plexiglass dialysis cells manufactured by the Research and Development Laboratory of the University of California, with Spectra/Por*2 membrane tubing (American Scientific Products, McGaw Park, IL) as the semipermeable barrier. The volume shifts and dilution of drug were corrected by the method of Tozer et al. (1983). Binding parameters were estimated by MINIM (R.D. Purves, University of Otago, Denedin, New Zealand) with least squares regression and weighting of $(C_{obs})^{-2}$. Prednisone binding was fitted to one linear term; that of prednisolone was fitted to one linear and one Langmuir-type function as described by Tozer et al. (1983).

Pharmacokinetic parameters were calculated as follows: the average maximum concentration, C_{max}, and time to reach this concentration, t_{max}, were obtained directly from

the data. Half-life, $t_{1/2}$, was calculated from the rate constant of elimination, k, resulting after logarithmic linear regression of the terminal phase of the concentration time profile, using at least three points. The absorption rate constant, k_a, was calculated by curve-peeling for two exponents using SIPHAR (SIMED, Centre d'Etudes et de Recherches en Statistiques et Informatique Medicale, Creteil Cedex, France).

The area under the concentration time curve, AUC, was calculated using the linear trapezoidal rule for the oral treatments and with the log-linear trapezoidal rule for the intravenous treatments. The AUMC (area under the first moment curve) was calculated using the linear method for all treatments. The apparent urinary clearance was calculated as: (amount of drug excreted into urine)/AUC for each drug.

The bioavailability of the oral tablets, F, and the phosphate injection were calculated with various treatments as reference, comparing either PO or POH concentrations in the AUC ratios. A calculation was also made based on the comparison of the sum of PO and POH areas.

Following noncompartmental analysis methods, the apparent clearance, CL_{app}, was calculated as Dose/AUC for treatments A and B; for treatments C, D and E, the parameter is actually CL_{app}/F. The mean residence time, MRT, was calculated as AUMC/AUC of the dosed compound. For the oral doses, the mean absorption time, k_a^{-1}, was subtracted from the MRT. The steady state volume of distribution, V_{ss}, was calculated as $Dose \cdot AUMC/AUC^2$ for intravenous dosing data.

For interconverted species, pharmacokinetic parameters should be calculated to incorporate the contribution of conversion. Methods exist to calculate kinetic parameters of drugs which undergo linear interconversion. The fundamental clearance values were calculated by the method of Hwang et al. (1981), as described in Table 2.1 (Chapter 2) for intravenous administration of PO and POH, treatments A and B, respectively. The true clearance, CL_{true}, of a reversibly metabolized compound must consider the interconversion

clearances as well as the elimination clearances. CL_{true} is actually the sum of clearance parameters exiting a drug compartment, as depicted in Figure 9.1 such that:

$$CL_{true,1} = CL_{10} + CL_{12}$$
$$CL_{true,2} = CL_{20} + CL_{21}$$

where $CL_{true,1}$ is the true clearance of prednisone, $CL_{true,2}$ is the true clearance of prednisolone and the fundamental clearances are defined in Figure 9.1. The MRT and the V_{ss} were calculated according to the method of Ebling and Jusko (1986), with the assumption that linear kinetics were observed. Data were calculated by this method as well as the traditional one (noncompartmental method described above) for comparison purposes.

The apparent urinary clearance, CL_{UR}, was calculated as the amount of drug excreted/AUC of both PO and POH for each treatment. The term urinary clearance is used instead of renal clearance because renal excretory events but not renal metabolic events are included in this calculation. This distinction is made to avoid confusion when kidney perfusion results are discussed where both renal and urinary clearances are determined.

Results

A typical plasma concentration time profile after intravenous (IV) administration of 25 mg prednisolone (subject 5, treatment B) is presented in Figure 9.2. The maximum measured concentration of prednisolone (two minutes after infusion) was 536 ng/ml total and 115 ng/ml unbound drug. Total prednisone concentrations peaked at 40 ng/ml after 1.5 hours. The terminal phases of the two drugs' concentration-time profiles declined with different rate constants and the elimination of POH exceeded that of PO.

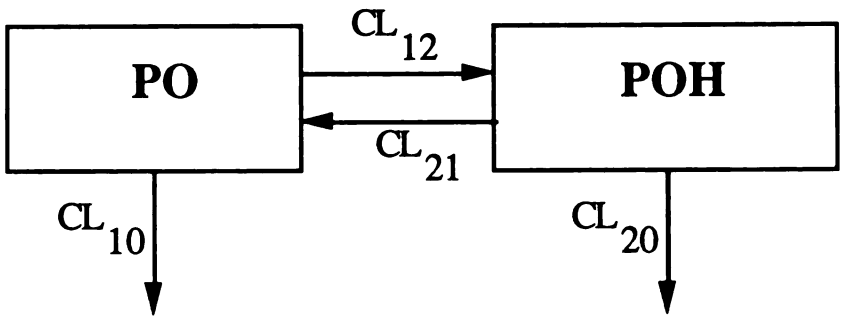

Figure 9.1. Model of the interconversion and elimination of prednisone and prednisolone in human subjects. (Clearance terms represent intrinsic clearance.)

CL_{10} = irreversible elimination of prednisone
CL_{12} = reductive conversion of prednisone tp prednisolone
CL_{20} = irreversible elimination of prednisolone
CL_{21} = oxidative conversion of prednisolone to prednisone

Plasma concentrations resulting after the administration of 25 mg-equivalents of prednisolone phosphate to subject 4 are presented in Figure 9.3. Prednisolone phosphate concentrations decreased rapidly, while those of prednisolone peaked in the first minutes after intravenous administration. Five minutes after completion of the infusion, POH concentrations reached 264 ng/ml (POH phosphate concentrations subtracted from the measured POH values). The phosphate ester concentrations decreased linearly from 671 to 99 by ten minutes and the ester was undetectable after 20 minutes. (This was true in all six subjects.) Prednisone concentrations reached their maximum, 46 ng/ml, at 3.2 hours.

Figure 9.4 is a concentration-time profile for volunteer 8 following oral administration of 25 mg prednisone (treatment D). Prednisone concentrations reached their maximum, 51 ng/ml, at 2 hours. The prednisolone concentration peaked earlier, at 45 minutes and 297 ng/ml total drug.

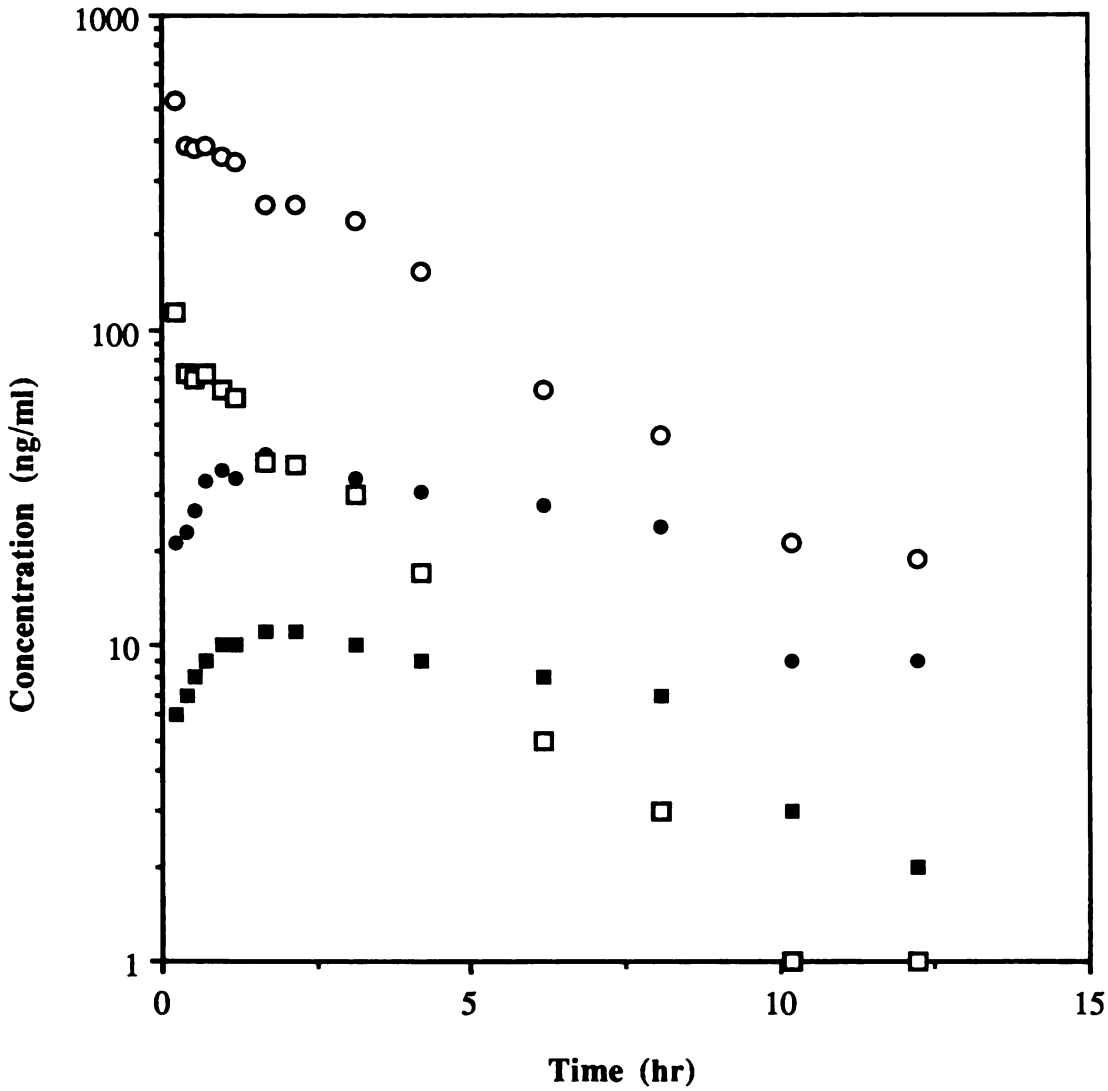

Figure 9.2. Plasma concentration-time profile of prednisone and prednisolone after intravenous bolus administration of 25 mg prednisolone to subject 5. Open symbols, prednisolone; solid symbols, prednisone; circles, total drug; squares, unbound drug.

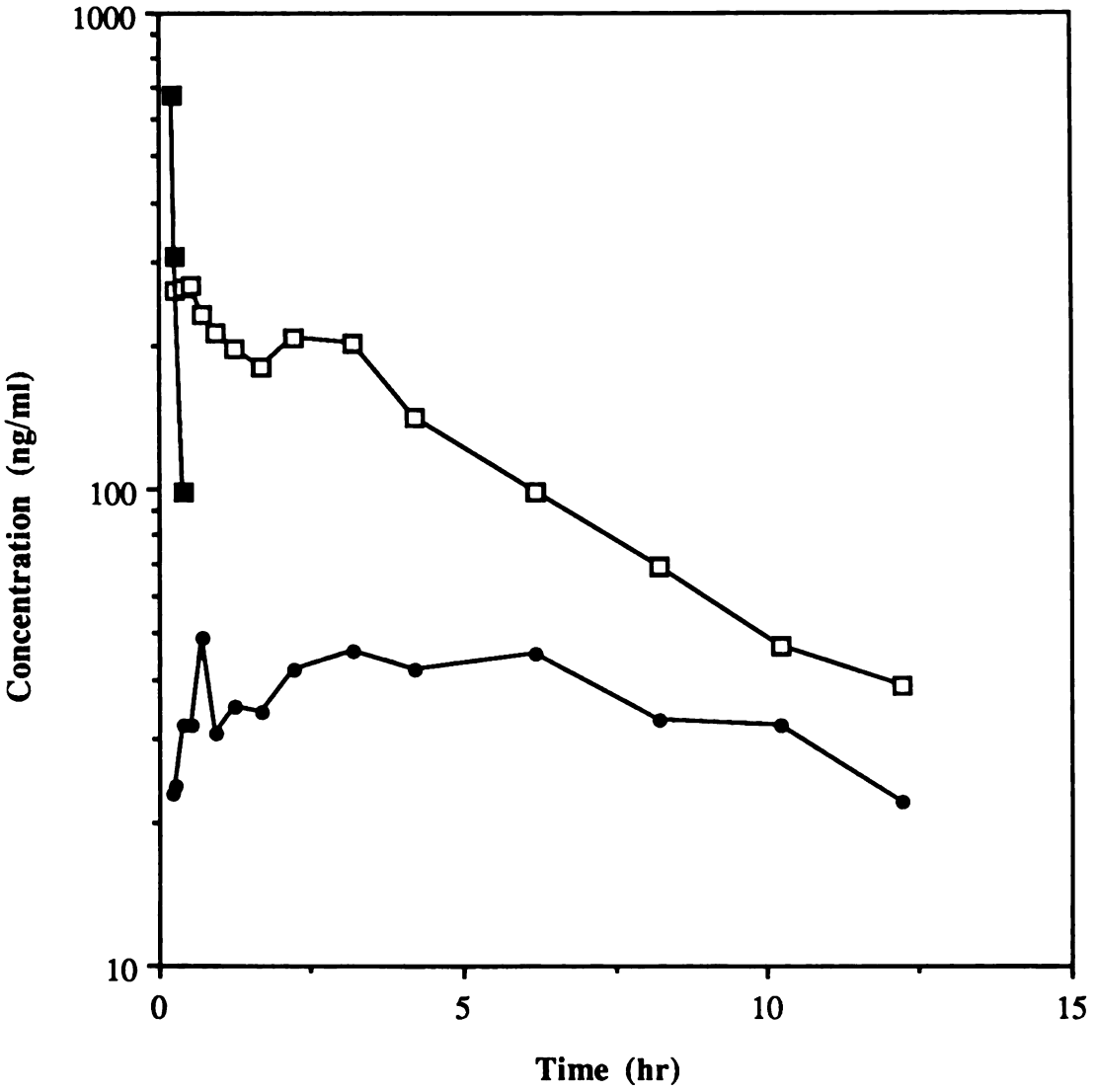

Figure 9.3. Plasma concentration-time profile after administration of 25 mg-equivalents of prednisolone phosphate by intravenous bolus to subject 4. Solid squares, prednisolone phosphate; open squares, prednisolone; solid circles, prednisone.

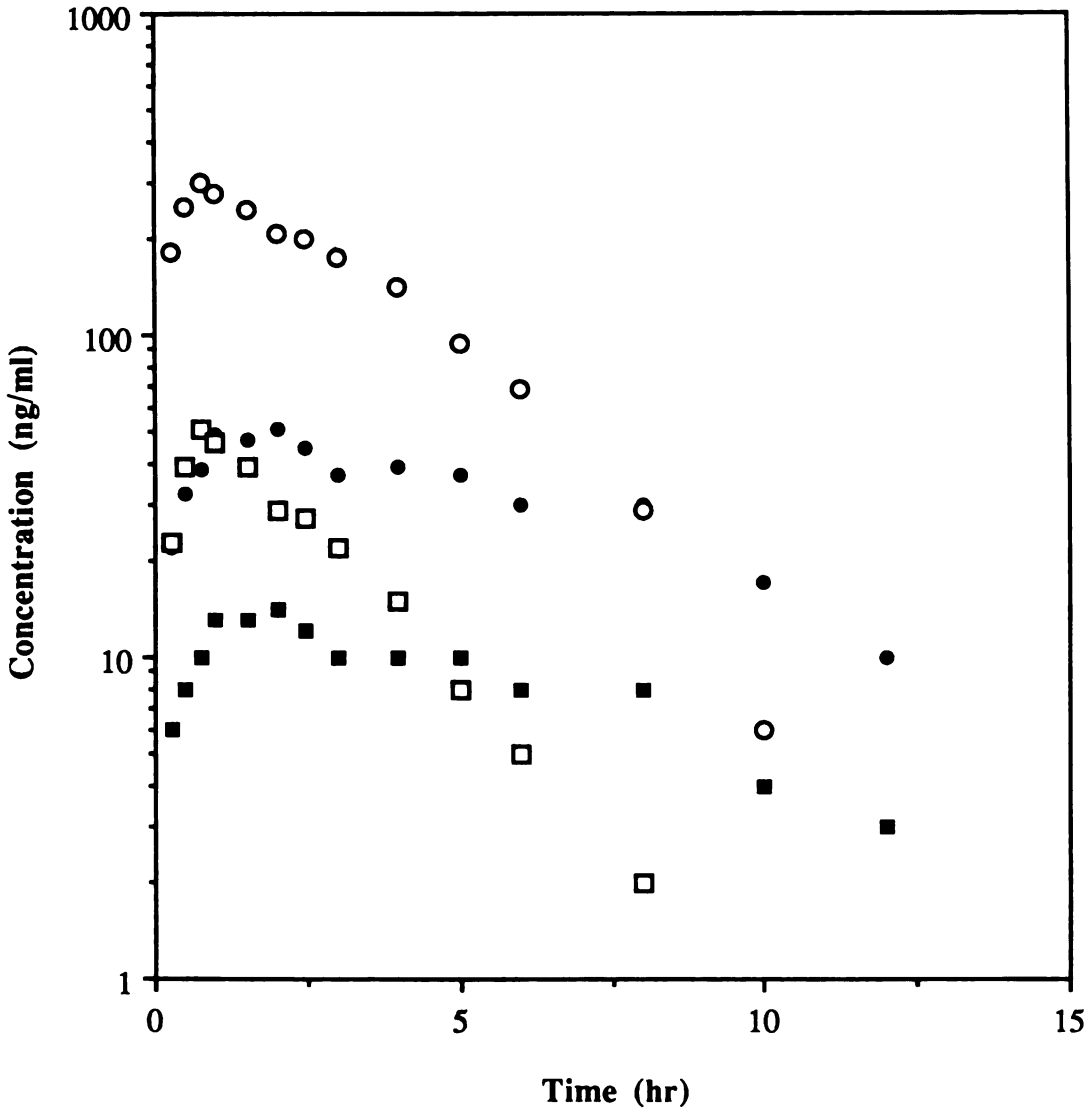

Figure 9.4. Plasma concentration-time profile of prednisone and prednisolone after administration of 25 mg prednisone orally to subject 5. Open symbols, prednisolone; solid symbols, prednisone; circles, total drug; squares, unbound drug.

Plasma protein binding

The plasma protein binding parameters for PO and POH are presented in Table 9.2. Prednisolone was modeled assuming saturable binding to corticosteroid binding globulin (CBG) and linear binding to albumin according to the following relationship (discussed in Chapter 3):

$$C_B = \frac{CAP \cdot C_U}{\frac{1}{K_d} + C_U} + S_{POH} \cdot C_U$$

where C_B is the bound concentration and C_U is the unbound concentration of prednisolone. The capacity of CBG for POH (CAP) was observed to range between 3.44 and 7.47 x 10^{-7} M with a mean value of 4.81 x 10^{-7} M; the dissociation constant (K_d) for the CBG binding averaged 3.01 x 10^{-8} M. The binding constant for POH from albumin (S_{POH}) averaged 2.37.

Prednisone was assumed to bind in a nonsaturable manner to one protein, namely albumin (Chapter 3):

$$C_B = S_{PO} \cdot C_U$$

The prednisone binding constant (S_{PO}) averaged 3.31.

The relationship of the unbound fraction of both prednisone and prednisolone as a function of total drug concentrations for subject 3 (all treatments) is presented in Figure 9.5. Prednisone concentrations were lower than prednisolone concentrations for all cases except treatment A, resulting in a smaller range of values. While the unbound fraction of prednisone was linear at about 0.19, the unbound fraction for prednisolone increased from 0.05 to 0.16 through the concentration range of 0 to 995 ng/ml.

Table 9.2. Binding parameters for prednisone and prednisolone in human subjects.

Subject	CAP x 10^{-7} M	K_d x 10^{-8} M	S_{POH}	S_{PO}
1	6.14	4.42	0.57	3.63
2	6.08	3.97	1.92	4.02
3	5.44	3.44	3.11	3.6
4	3.72	3.25	3.26	2.53
5	4.64	3.50	2.34	2.48
6	3.75	2.67	2.59	2.89
7	7.47	3.25	1.43	3.13
8	3.47	2.03	2.71	2.77
9	3.94	1.86	2.56	4.08
10	3.44	1.72	3.23	3.94
Mean	**4.81**	**3.01**	**2.37**	**3.31**
S.D.	**1.40**	**0.91**	**0.86**	**0.62**

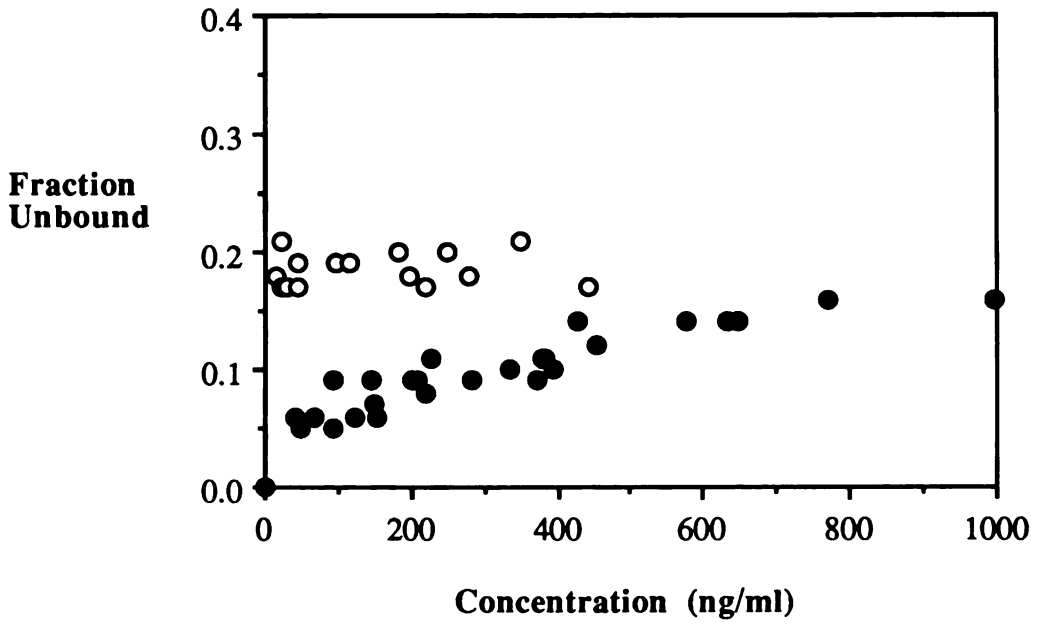

Figure 9.5. Unbound fraction of prednisone (open circles) and prednisolone (solid circles) as a function of total drug concentration; data from subject 3 for all five treatments.

Pharmacokinetic parameters

The C_{max} and t_{max} for the five treatments are compared in Table 9.3. Prednisone C_{max} values were about 40 ng/ml for multiple treatments, including the three POH and the oral PO treatments. Prednisone maximum concentrations were 15-fold greater after IV administration than after oral administration, 568 vs. 38 ng/ml. The maximal POH concentration achieved after IV administration of POH was 585 ng/ml, a value similar to that for prednisone after IV PO administration.

Table 9.3
Summary of maximal values and rate constants

		Treatment				
		A	**B**	**C**	**D**	**E**
parameter	**drug**	**IV PO**	**IV POH**	**IV POH phos.**	**oral PO**	**oral POH**
C max (ng/ml)	PO	568 ± 156	42 ± 12	42 ± 9	38 ± 10	36 ± 11
	POH	314 ± 73	585 ± 151	1055 ± 412	345 ± 75	349 ± 88
t max (hr)	PO	-	2.4 ± 1.1	3.1 ± 1.4	3.5 ± 1.3	1.8 ± 0.7
	POH	0.44 ± 0.26	-	0.09 ± 0.06	1.2 ± 1.2	1.3 ± 0.5
t 1/2 (hr)	PO	3.78 ± 1.65	3.14 ± 1.21	4.01 ± 1.59	3.04 ± 1.81	4.07 ± 1.51
	POH	2.57 ± 0.87	2.19 ± 0.33	2.85 ± 1.36	2.45 ± 0.83	2.77 ± 0.73
ka (hr-1)	PO	-	-	-	1.16	
	POH	-	-	-		1.49

Oral administration of each drug produced similar C_{max} and t_{max} values. After oral PO and oral POH, respectively, PO concentrations averaged 38 and 36 ng/ml, while mean POH concentrations reached 345 and 349 ng/ml. The t_{max} values varied between 1.2 and 3.5 hours for both drugs for both oral treatments.

The elimination half-life of prednisone ranged between 3.04 and 4.07; the half-life of prednisolone was smaller, ranging from 2.19 to 2.85 hours. The elimination half-life of prednisolone phosphate was 2.38 ± 2.15 minutes and ranged from 0.98 to 6.51 minutes, as determined by log-linear regression of all of the available data points. The concentration of prednisolone phosphate with time is displayed in Figure 9.6. The phosphate ester was measurable only in samples up to 10 minutes.

The absorption rate of prednisone was 1.16 ± 0.48 hr^{-1} compared to 1.49 ± 0.71 hr^{-1} for prednisolone, which translates to absorption half-lives of 0.69 and 0.55 hr for PO and POH, respectively. These differences were not statistically different.

The AUC of prednisone ranged nearly 2-fold, between 371 and 695 ng·hr/ml (Table 9.4); the high value was for intravenous prednisone (treatment A) and the low for oral PO (treatment E) ($p<0.01$). The AUC of prednisolone varied from 1383 ng·hr/ml after intravenous prednisone to 2136 ng·hr/ml after intravenous prednisolone phosphate. The corrected prednisolone AUC, for the six subjects whose blood was treated with the esterase inhibitor, was reduced from 2136 to 1886 ng·hr/ml.

The AUC ratio of PO to POH varied between 0.20 and 0.25 for four of the five treatments, B, C, D and E (Table 9.4). Only for intravenous prednisone was the ratio significantly different, about 2-fold higher, or 0.54 ($p<0.01$). This difference was a result of both an increased AUC of PO and a reduced AUC of POH.

The AUC sum of prednisone and prednisolone ranged between 2061 and 2524 ng·hr/ml, with the lowest values observed for the oral and intravenous prednisone treatments and the high values associated with the intravenous prednisolone treatments.

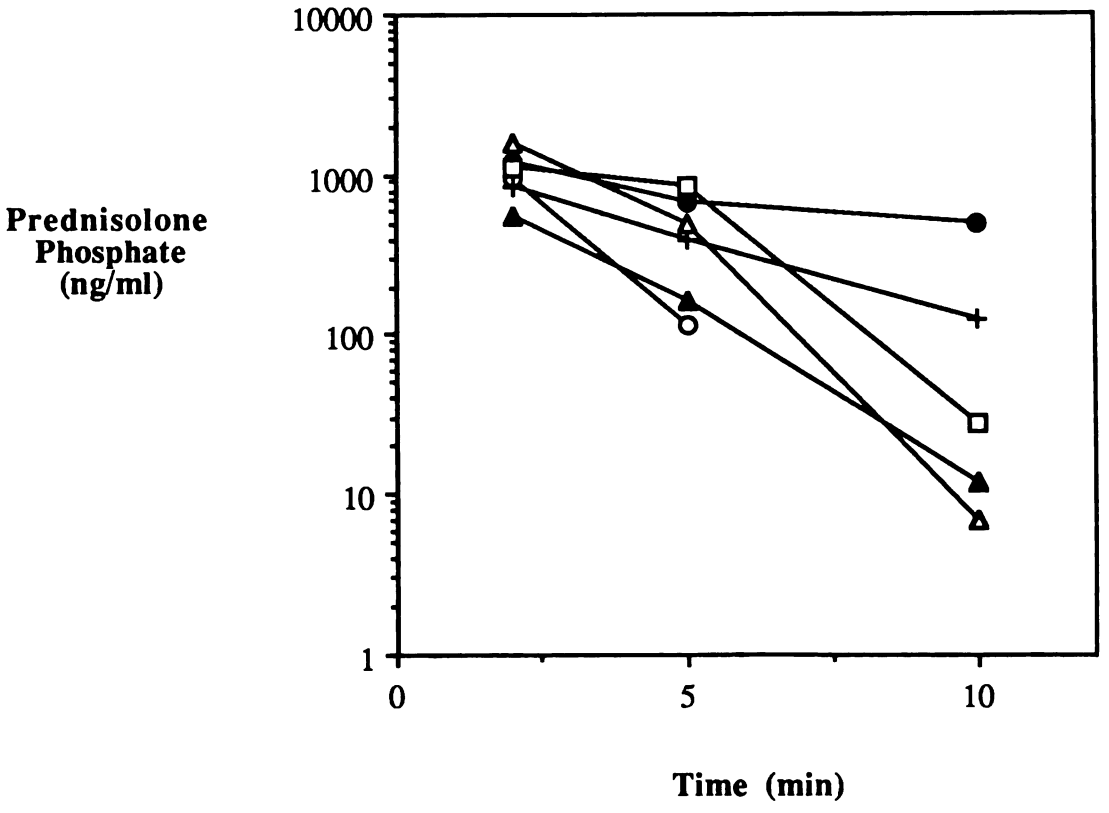

Figure 9.6. Concentration time profile of prednisolone phosphate in the six subjects examined.

The apparent systemic clearance calculated by noncompartmental methods as Dose/AUC is presented in Table 9.4. As described above, the value for the two oral doses is actually CL_{app}/F. For the three POH treatments, the value was similar and means ranged from 12.2 to 14.4 L/hr, suggesting that F approaches 1 for oral prednisolone. The CL_{app} after intravenous prednisolone with corrected prednisolone concentrations was 12.9 L/hr, compared to 12.2 L/hr when concentrations were not corrected.

Table 9.4
Summary of noncompartmental pharmacokinetic parameters

		Treatment					
		A	B	C	D	E	corrected C
parameter	drug	IV PO	IV POH	IV POH phos	oral PO	oral POH	IV POH phos
AUC	PO	695 ± 235	412 ± 131	482 ± 177	383 ± 185	371 ± 116	482 ± 177
(ng*hr/ml)	POH	1383 ± 382	2112 ± 382	2136 ± 466	1678 ± 500	1810 ± 405	1886 ± 366
AUC PO/POH		0.54 ± 0.23	0.20 ± 0.06	0.24 ± 0.11	0.25 ± 0.11	0.21 ± 0.06	0.25 ± 0.12
AUC sum *		2078 ± 417	2524 ± 413	2618 ± 402	2061 ± 402	2180 ± 448	2341 ± 336
Dose/AUC	PO	39.7 ± 13.3	-	-	82.2 ± 41.3	-	-
(L/hr)	POH	-	12.2 ± 2.1	12.2 ± 2.4	-	14.4 ± 2.9	12.9 ± 2.7
Dose/unbound AUC	PO	172.1 ± 76.9	-	-	364.6 ± 216.9	-	-
(L/hr)	POH	-	27.1 ± 9.1	22.5 ± 10.3	-	24.2 ± 8.8	22.7 ± 10.3
MRT	PO	14.7 ± 10.6	16.9 ± 11.9	27.1 ± 11.4	16.5 ± 7.5	17.6 ± 9.3	
(hr)	POH	8.2 ± 6.7	6.9 ± 4.0	8.9 ± 6.5	7.9 ± 3.5		8.7 ± 6.3
Vss	PO	542 ± 437	-	-	-	-	-
(L)	POH	-	75 ± 30	105 ± 68	-	-	128 ± 68

dash indicates no calculated value
* not normalized, as molecular weights differ by one unit.
** prednisolone phosphate concentrations subtracted from prednisolone concentrations (see text)

The apparent clearance of PO was substantially greater and route-dependent ($p<0.01$): after IV prednisone administration, the CL_{app} of PO was 39.7 L/hr and after oral dosing, Dose/AUC was 82.8 L/hr, so F was approximately 0.5 for oral prednisone.

The unbound CL_{app} demonstrated an even greater difference between the two drugs. The CL_{app} of POH ranged from 22.5 to 27.1; that of PO was 172.1 L/hr for treatment A and 364.6 L/hr for treatment D.

The MRT values based on noncompartmental methods were 14.7 ± 10.6 hr for PO and 6.9 ± 4.0 hr for POH (Table 9.4). The Vss based on noncompartmental methods was 542 ± 437 L for PO and much lower for POH, at 75 ± 30 L.

The fundamental clearances were calculated for treatments A and B, intravenous PO and POH (Table 9.5; defined in Figure 9.1). These values exhibited high variability: CL_{10} was 397 ± 257, 125 ± 78 ml/min for CL_{20}; CL_{12} was 678 ± 378 and CL_{21} was 196 ± 82 ml/min.

The true clearance was calculated for treatments A and B, as well, with consideration of the contribution of interconversion processes (Table 9.5). The value for the true clearance of PO, $CL_{true,1}$, was 1075 ± 506 ml/min; that of POH was substantially reduced: $CL_{true,2}$, was 321 ± 65 ml/min.

The MRT calculated with the contribution of interconversion was 14.7 ± 10.6 hr for PO and 7.0 ± 4.0 hr for POH. The apparent steady state volume calculated to include the contribution of conversion was 453 ± 498 L for PO and that of POH was 28 ± 57 L.

The two methods of calculating clearance, volume and mean residence time are compared in Table 9.6. The methods differed for both CL and V_{ss}, but not MRT calculations. The true clearance of both PO and POH was underestimated by about half when interconversion was ignored. The volume was overestimated, and displayed a C.V.% of >100%.

Table 9.5
Fundamental clearances (ml/min) *

Subject	CL10	CL20	CL12	CL21	CL true, 1	CL true, 2
1	145	195	331	67	476	262
2	557	71	4256	1287	4813	1358
3	231	132	897	196	1128	328
4	288	134	673	240	961	374
5	394	171	438	160	832	331
6	679	36	517	245	1195	280
7	837	-36	505	301	1342	265
8	154	206	405	168	559	374
9	638	142	1558	296	2196	438
10	206	144	782	95	988	239
Mean	**413**	**119**	**1036**	**306**	**1449**	**425**
S.D.	**248**	**75**	**1186**	**354**	**1275**	**334**

* calculated according to Hwang et al. (1981)

Table 9.6. Comparison of some pharmacokinetic parameters calculated using noncompartmental and interconversion methods.

Parameter	units	**Method 1***	**Method 2***
CL PO	L/hr	39.7 ± 13.3	64.5 ± 30.4
CL POH	L/hr	12.2 ± 2.1	25.5 ± 20.0
V_{ss} PO	L	542 ± 437	453 ± 498
V_{ss} POH	L	75 ± 30	28 ± 57
MRT PO	hr	14.7 ± 10.6	14.7 ± 10.6
MRT POH	hr	6.9 ± 4.0	7.0 ± 4.0

* Method 1 = traditional, noncompartmental method
* Method 2 = consideration of interconversion in calculations

Urinary excretion

Urinary excretion data and apparent urinary clearance values are presented in Table 9.7. Prednisone excretion varied between 1.2 and 1.5 mg, equivalent to about 5% of the 25 mg dose. Prednisolone excretion was dependent upon which drug was administered, as excretion ranged between 2.1 and 4.3 mg. Excretion was lowest for the prednisone treatments A and D; the percentage of the dose excreted as POH was between 8 and 17%. Route dependencies were not demonstrated for the excretion of either drug.

The apparent urinary clearance, CL_{UR}, of prednisone ranged from 36.9 to 62.3 ml/min. In all but the intravenous prednisone treatment, the mean values were between 52 and 62 ml/min. Prednisolone apparent urinary clearance was variable and average values ranged from 27.1 to 41.7 ml/min. The low value was observed after oral prednisone and the high value after oral POH dosing.

Table 9.7
Summary of urinary excretion data and urinary clearance values

		Treatment				
		A	B	C	D	E
parameter	drug	IV PO	IV POH	IV POH phos.	oral PO	oral POH
amt excreted	PO	1.4 ±1.5	1.2 ± 1.3	1.5 ± 0.9	1.2 ± 0.6	1.3 ± 0.4
(mg)	POH	2.1 ± 0.8	4.2 ±1.0	3.5 ± 0.9	2.5 ± 0.8	4.3 ± 1.4
excreted	PO	5.8 ± 2.1	4.6 ± 1.2	5.9 ± 3.4	4.7 ± 2.4	5.0 ± 1.6
(%)	POH	8.3 ± 3.1	16.6 ± 7.9	14.1 ± 3.6	10.2 ± 3.1	17.3 ± 5.7
CLur	PO	36.9 ± 14.5	52.1 ± 26.4	58.1 ± 34.5	61.3 ± 37.1	62.3 ± 28.4
ml/min	POH	27.8 ± 16.6	33.4 ± 17.2	28.8 ± 8.9	27.1 ± 10.1	41.7 ± 16.2
CLur	PO	0.49 ± 0.18	0.69 ± 0.34	0.75 ± 0.42	0.81 ± 0.50	0.84 ± 0.40
ml/min/kg	POH	0.37 ± 0.22	0.46 ± 0.27	0.38 ± 0.12	0.37 ± 0.16	0.57 ± 0.25
CLur	PO	34.3	48.3	52.5	56.7	58.8
ml/min/70 kg	POH	25.9	32.2	26.6	25.9	39.9

Bioavailability

The bioavailability, F, of the tablets was calculated in several ways: Determinations were made using prednisone AUC's (Table 9.8) and also with prednisolone AUC's (Table 9.9). These PO or POH AUC's could come from dosing IV prednisone or IV prednisolone solutions. The comparisons were performed using both reference solutions while measuring both drug concentrations. Bioavailability was also calculated based upon the sum of PO and POH areas (Table 9.10), again using both intravenous solutions as standards.

The two tablets yielded similar F values, regardless of the reference solutions or compared concentrations. In each case, PO tablets were slightly less bioavailable than the POH tablets, but the variability was such that they may be presumed bioequivalent.

The estimates differed substantially between the reference solutions and reference concentrations, however. For prednisone AUC ratios (Table 9.8), the mean bioavailabilities of the tablets were 0.56 and 0.61 for infused PO as the reference and 1.01 and 1.06 when intravenous POH was the reference.

When POH AUC's were compared, PO administration as reference yielded mean values of 1.28 and 1.40; the mean values were 0.81 and 0.88, respectively, for intravenous POH.

The bioavailability calculation based upon the sum of the areas under the curve for both drug concentrations estimates bioequivalence for PO and POH tablets as well (Table 9.9) because of the reversible metabolism. Compared to IV prednisone, PO and POH mean tablet bioavailabilities were 1.04 and 1.12; IV prednisolone comparison yielded lower values, or 0.83 and 0.89 for PO and POH tablets, respectively.

Table 9.8. Bioavailability of prednisone and prednisolone tablets based on prednisone area measurements.

Subject Reference	Prednisone Tablets Intravenous Prednisone	Prednisolone Tablets Intravenous Prednisone	Prednisone Tablets Intravenous Prednisolone	Prednisolone Tablets Intravenous Prednisolone
1	0.66	0.31	2.58	1.20
2	0.95	0.63	1.00	0.66
3	0.27	0.26	0.45	0.44
4	0.43	0.51	0.67	0.80
5	0.86	0.65	1.78	1.35
6	0.40	0.69	0.46	0.79
7	0.87	0.38	0.77	0.33
8	0.39	0.36	0.87	0.79
9	0.46	1.59	0.68	2.34
10	0.35	0.74	0.89	1.86
Mean	**0.56**	**0.61**	**1.02**	**1.06**
S.D.	**0.25**	**0.38**	**0.67**	**0.64**

Table 9.9. Bioavailability of prednisone and prednisolone tablets based on prednisolone area measurements.

Subject Reference	Prednisone Tablets Intravenous Prednisone	Prednisolone Tablets	Prednisone Tablets Intravenous Prednisolone	Prednisolone Tablets
1	1.04	0.96	0.72	0.67
2	1.13	1.23	1.00	1.09
3	0.74	0.85	0.59	0.67
4	1.48	1.33	1.04	0.93
5	1.59	1.82	0.84	0.96
6	0.87	2.00	0.38	0.86
7	2.45	1.71	0.92	0.64
8	0.97	1.30	0.70	0.95
9	1.60	2.10	1.14	1.49
10	0.94	0.74	0.74	0.58
Mean	**1.28**	**1.40**	**0.81**	**0.88**
S.D.	**0.51**	**0.48**	**0.23**	**0.27**

The absolute bioavailability of prednisolone phosphate solution, treatment C, was determined versus intravenous prednisolone (treatment B). Table 9.11 demonstrates that the estimate of the bioavailability of prednisolone phosphate was dependent upon which drug areas were compared. When PO AUC's were used, the mean F of prednisolone phosphate solution was 1.23; the value was 1.03 when based on POH. These mean values exhibited great variability.

The average error in the AUC of prednisolone by not subtracting the measured phosphate was 6 ± 3%. The individual values were 2, 4, 5, 6, 8 and 10% in the six subjects tested.

Table 9.10. Bioavailability of prednisone and prednisolone tablets based on the sum of prednisone and prednisolone area measurements.

Subject Reference	Prednisone Tablets Intravenous Prednisone	Prednisolone Tablets	Prednisone Tablets Intravenous Prednisolone	Prednisolone Tablets
1	0.87	0.67	0.95	0.73
2	1.09	1.08	1.00	1.00
3	0.61	0.69	0.56	0.64
4	1.11	1.04	0.96	0.91
5	1.28	1.32	0.99	1.02
6	0.70	1.54	0.39	0.85
7	1.91	1.25	0.89	0.59
8	0.69	0.85	0.74	0.91
9	1.35	1.99	1.08	1.59
10	0.80	0.74	0.76	0.70
Mean	**1.04**	**1.12**	**0.83**	**0.89**
S.D.	**0.40**	**0.42**	**0.22**	**0.29**

Table 9.11. Absolute bioavailability of prednisolone phosphate solution.

Subject	Reference Concentrations	
	Prednisone	Prednisolone
1	2.19	0.86
2	1.11	0.99
3	1.04	1.24
4	1.27	0.88
5	1.64	1.21
6	0.49	0.68
7	0.83	0.84
8	1.53	1.13
9	0.87	1.39
10	1.34	1.04
Mean	**1.23**	**1.03**
S.D.	**0.48**	**0.22**

*reference solution = intravenous prednisolone

Discussion

The concentration time curves and binding profiles resulting from these studies are similar to those obtained by other investigators and in our laboratory (Rose et al., 1981; Gustavson and Benet, 1985b).

Absorption

Prednisone and prednisolone demonstrated similar apparent absorption rates: that of PO was 1.16 hr^{-1} compared to 1.49 hr^{-1} for prednisolone. These rates were approximations, as there was interconversion throughout the absorption period, and both drugs were always measurable.

Metabolic interconversion

Metabolic interconversion of the glucocorticoids was examined by comparison of the areas under the plasma concentration-time curves for each treatment. The resultant AUC's of PO and POH after oral administration of each drug were not statistically different: the ratio of PO/POH AUC's was 0.25 for PO tablets and 0.21 for POH tablets. This is in contrast to IV administration of both drugs for which the PO/POH AUC ratio was 0.54 after IV PO but only 0.20 after IV POH. In fact, for four of the five treatments, the AUC ratio was between 0.20 and 0.25.

The implications of these results are that the liver and/or gut are reductively oriented. This is demonstrated by the similar AUC ratios between oral administration of either drug and intravenous prednisolone. Clinically, it has been observed that either drug can be administered orally to achieve the same effect (Gustavson and Benet, 1985). Furthermore, the liver and/or gut are primary sites of reduction of PO to POH in the body; when the first pass effect is bypassed, the body slowly reduces the circulating PO, as was the case in treatment A. Additionally, neither the gut nor liver produce much PO. If they did, there would have been an increase in the AUC ratio of PO to POH after oral administration of POH versus intravenously administered POH, but the values were equal: 0.20 and 0.21, respectively. Rose and coworkers (1981) observed similar ratios after dosing 20 mg oral PO and intravenous POH phosphate; the ratios were 0.16 and 0.14, respectively.

The human liver perfusion results proved that the human liver easily reduces PO to POH (Chapter 6). The reverse reaction, oxidation, occurred only to a small extent: the ratio of PO to POH averaged 0.08 over five determinations.

Apparent systemic clearance

The apparent systemic clearance of POH varied slightly for the three POH treatments, namely 12.2, 12.2 for treatments B and C, and CL_{app}/F was 14.4 L/hr for

treatment E. As the value for the oral treatment was higher than the intravenous values, it can be assumed that first pass clearance is responsible for metabolism of about 20% of the POH. This result agrees well with perfused human liver experiments which showed the extraction of POH to be 0.22 and 0.09 in two different experiments (Chapter 6).

The apparent clearance of PO was route-dependent, as the value was 39.7 L/hr for intravenous PO and 82.2 L/hr for oral PO. As above, this difference was probably a function of bioavailability, as the Dose/AUC value calculated for treatment D is actually CL_{app}/F. The bioavailability of orally administered PO (based on PO concentrations) is about 0.5. This corresponds well with observations in the perfused human liver (Chapter 6), as well. In those experiments, the extraction of PO averaged 0.45 through concentrations of 2000 ng/ml.

As PO and POH tablets were bioequivalent and about 20% of POH was metabolized, the discrepancy between the two routes may be explained by significant hepatic conversion of prednisone to prednisolone, rather than irreversible elimination of half of the prednisone dose. The individual and summed AUC values (Table 9.4) are the same for prednisone and prednisolone tablets. Furthermore, it may be concluded that the apparent systemic clearance of PO is greater than that of POH because the body preferentially forms POH from PO.

Modeling apparent clearance

The model of interconversion and elimination of prednisone and prednisolone used in these calculations is depicted in Figure 9.1, together with definitions of the fundamental clearance parameters. These clearance parameters were calculated according to Hwang and coworkers (1981). One problem with this method is that these methods require linear kinetics. As PO and POH do not express linear kinetics like methylprednisolone (Ebling and Jusko, 1986), the values obtained from these calculations may be expected to reflect estimates of the actual processes. Taken one step further, these fundamental clearance

parameters do not reflect actual metabolic events, because it has been shown that interconversion and elimination do not occur in one organ, but in at least three organs of the rabbit (Chapters 5, 7 and 8). When this modeling approach is applied to one organ in isolation, then the calculated individual fundamental parameters may begin to approximate clearances through specific pathways. The individual fundamental clearance values were calculated so that these clearance values might be compared to those calculated noncompartmentally, so as to determine the magnitude of the contribution of interconversion to clearances.

Fundamental clearances

Table 9.5 presents the fundamental clearance values for interconversion and elimination of prednisone and prednisolone *in vivo* in humans. These were calculated using the data obtained following intravenous administration of PO and POH (treatments A and B). The values for the irreversible elimination of PO and POH were 413 ± 248 and 119 ± 75 ml/min, respectively. The variability was large, but reasonable, considering the assumptions made in these calculations. For interconversion, however, the C.V. (%) exceeded 100; CL_{12} was 1036 ± 1186 and CL_{21} was 306 ± 354 ml/min. Thus, reductive conversion is the most active pathway, followed by irreversible elimination of prednisone; elimination of POH was the smallest clearance. As suspected, reduction exceeded oxidation by more than 3-fold; irreversible elimination of PO exceeded that of POH by about the same factor.

Ebling and Jusko (1986) calculated these parameters for prednisolone administration in humans based on the data of Beitins et al. (1972) and observed very different values. The CL_{10} and CL_{20} values were 60 and 258 ml/min, respectively, and CL_{12} and CL_{21} were 70 and 575 ml/min, respectively.

The interconversion clearance values for subject 2 were skewed to high values due to very high estimates of CL_{12} and CL_{21}; the values in this subject were more than 10-fold

higher than the lowest values obtained and exceeded the highest values by more than 3-fold. With these values omitted, CL_{12} averaged 678 ± 378 and CL_{21} averaged 196 ± 82 ml/min. The ranking of values with subject 2 omitted produces the same rank order of clearance values, but in this case, CL_{12} is only 1.6-fold greater than CL_{10}, instead of 2.5-fold greater.

The true clearance values of prednisone and prednisolone are the sum of clearances the drug is subjected to *in vivo*. The clearance of prednisone greatly exceeded that of prednisolone (Table 9.5), 1449 vs. 425 ml/min. This is probably because a large fraction of PO is converted to POH *in vivo*, although clearance values show that elimination of PO was also greater than that of POH.

Comparison of calculation methods

The results of the two methods of calculating CL, V_{ss} and MRT are compared in Table 9.6. Assuming that model 9.1 is sufficient to describe the disposition of PO and POH, noncompartmental analysis underestimated the apparent clearance of both PO and POH by about a factor of two. CL_1 was 64.5 versus 39.7 L/hr; CL_2 was 25.5 vs. 12.2 L/hr. This is due to the omission of the clearance due to interconversion. These results imply that the clearanvces associated with interconversion are approximately as large as those of elimination.

The mean residence time was the same for the two methods, 14.7 and 7.0 hours for PO and POH, respectively. The mean residence time is a measure of the whole body disposition of the drug and considers reversible processes whether they are specifically calculated or not. Hence, it can be expected that methods would produce equivalent results.

Urinary excretion

Prednisolone excretion was observed to be a function of the drug administered (Table 9.7), as the percentage excreted was 8.3 and 10.2 for prednisone administration but 16.6, 14.1 and 17.3 when prednisolone was administered. Rose et al. (1981) observed increased excretion, 11.7, 18.8, 24.4%, respectively, as the IV POH phosphate dose progressed from 5 to 20 to 40 mg. The same phenomena was observed by Hsueh et al. (1979). This common observation could be a result of the nonlinear binding of prednisolone resulting in an increasing free fraction with concentration. The free fraction of POH increased from 0.05 to 0.16 for plasma concentrations resulting from the 25 mg doses administered in this study. Prednisolone excretion may also increase with dose as a result of the low elimination of this compound and its poor conversion to PO (Chapter 7). In addition, this observation may be due to renal metabolic processes which obscure actual renal blood drug concentrations.

In contrast to that of prednisolone, the renal excretion of prednisone was relatively constant (Table 9.7). For all five treatments, the excretion ranged from 1.2 to 1.5 mg. This translated to 4.6 to 5.9 % of the administered dose of PO or POH. Rose and coworkers observed that 2 to 5% of orally administered PO or intravenously administered POH was recovered in the urine as prednisone (Rose et al., 1981). The subjects in the present study were administered 25 mg of both drugs orally and intravenously compared to 5, 20 and 40 or 50 mg in the study of Rose et al. In both studies, dose and route dependency were not observed for the excretion of PO.

Apparent urinary clearance

Traditionally, excretion of drug measured as clearance *in vivo* has been termed renal clearance. Renal clearance is the sum of excretory and metabolic clearances and for most drugs, only the former is significant. This assumes that there is no renal metabolism of drug. Renal metabolism of PO and POH has been documented in the perfused rat kidney

(Rocci et al., 1981) and in the perfused rabbit kidney (Chapter 7). Therefore, the correct term for this measure would be *urinary* clearance. To allow reference to kidney perfusion experiments and to avoid confusion with regard to renal versus urinary clearance, this clearance parameter will be termed urinary clearance.

Few recent prednisone-prednisolone pharmacokinetic studies have sampled urine to quantify urinary excretion. This parameter is a significant fraction of systemic clearance, however, and it could contribute to the observed nonlinearity in CL_{app}.

The apparent urinary clearance of PO exceeded that of POH in all five treatments (Table 9.7), although variability was high. The mean apparent urinary clearance of PO was lower after IV prednisone than for any other treatment, 36.9 ml/min vs. 52.1 to 62.3 ml/min, but the difference was statistically insignificant.

The urinary clearance of prednisolone was unaffected by the route of administration or the drug administered (Table 9.7). The mean values ranged from 27.1 to 41.7 ml/min and displayed high variability. The highest mean value was observed after oral POH and the lowest after oral PO. These urinary clearance values compare favorably to those of Rose et al. (1981): for 20 mg IV POH phosphate, POH urinary clearance was 34.6 ml/min per 1.73 m^2 and after 20 mg oral PO, the value was 31.7 ml/min per 1.73 m^2.

For intravenous POH phosphate administration of 20 mg (Rose et al., 1981) or 25 mg (present study), the CL_{ur} values were similar but PO urinary clearance in this study was greater. Rose observed the urinary clearance of PO to be 39.4 and for POH, 34.6 ml/min per 1.73 m^2; the results in the present study were 52.5 and 26.6 ml/min per 70 kg for PO and POH, respectively. The differences between the two studies are probably not significant.

Urine flow dependence analysis

The glucocorticoids are small, neutral and lipophilic, possessing no known active secretion or reabsorption processes. It is therefore possible that urinary clearance of these

compounds is a function of the urine flow rate. Lloyd (1959) was the first to document the diuresis-dependent excretion of corticosteroids in humans. Rocci et al. (1981) demonstrated this occurrence in the perfused rat kidney and the same was observed in the perfused rabbit kidney (Chapter 7). Analyses of the data from this study with humans shows that the urinary excretion of both PO and POH was not related to the urine flow rate (data not shown).

Phosphate ester hydrolysis

Traditional bioavailability studies of both PO and POH have compared orally administered tablets to IV POH phosphate; a recent exception is Ferry et al.(1988), who used an alcoholic cosolvent system composed of ethanol:water (1:1). Bioavailability determinations which compared a particular dosage form to the intravenous ester actually estimated the *relative* bioavailability of the steroid; the *absolute* bioavailability measurement would result from coadministration of a drug in a dosage form and the same drug (non-esterified) intravenously.

Many bioavailability studies have assumed that the *in vivo* hydrolysis of prednisolone phosphate and hemisuccinate is rapid and complete. Few of these studies have tested this assumption by measuring plasma and urine concentrations of the ester. Plasma esterases differ greatly in specificity and concentration amongst species; whereas hydrolysis is rapid in a number of species, it is not correct to assume that rapid hydrolysis also occurs in the human without testing this assumption.

The measured half-life of prednisolone phosphate in these subjects was 2.38 minutes and exhibited a six-fold range of values. No prednisolone phosphate was measurable in the plasma of any subject at 20 minutes in the six subjects in which this drug was quantitated. This is a short time relative to the half-lives of PO and POH, which are approximately three hours. It was assumed that all of the phosphate ester was hydrolyzed while sitting in the heparinized tube at room temperature and during centrifugation. No

phosphate was measurable in the non-stabilized samples after one month of frozen storage. The assumption of instantaneous and complete hydrolysis of prednisolone phosphate resulted in an over-estimation of the AUC of prednisolone of 6 ± 3%.

Estimates of the *in vivo* hydrolysis rates of glucocorticoid esters have been performed in two ways. Direct measurement entails quantitation of the ester in plasma; indirect measurement requires the addition of an esterase inhibitor to a portion of the sample while allowing the hydrolysis to go to completion in the other portion. Then, the difference in the concentrations of drugs present in the two samples is equivalent to the amount of non-ester present in the original sample.

An indirect estimate of the rate of hydrolysis of prednisone succinate in rabbits has been performed by Unadkat and Rowland (1985). They observed that the hydrolysis of prednisone succinate in the rabbit was not instantaneous and was incomplete. They recognized that if the ester was metabolized by routes other than hydrolysis or was excreted unchanged, calculation errors would result. A second group, Ebling and coworkers (1985), indirectly measured the hydrolysis rate of methylprednisolone hemisuccinate in rabbits; they observed a hydrolysis half-life of 10 minutes.

Methylprednisolone sodium succinate was measured by Antal et al. (1983) who claimed to observe no methylprednisolone sodium succinate after administration of 40 mg via various routes to humans. An error was made, however, in that these investigators did not add an esterase inhibitor to plasma samples to stabilize the ester during storage prior to analysis. Unless the sample was frozen immediately, the ambient temperature could have been sufficient to allow complete hydrolysis of the succinate ester, yielding their conclusion. Furthermore, any remaining ester would probably be hydrolyzed upon thawing.

Clearly, of the studies of glucocorticoid ester hydrolysis, either lack of stabilization of samples or use of an indirect method of ester measurement may have resulted in calculation errors. Overestimates of the amount of PO or POH or its time course in the

body would result in lower relative bioavailability than would be obtained from absolute availability measurements. Clearance and volume measurements would be affected, as well. As this effect on the concentration-time profile is only significant at early time periods, the AUMC is not affected; the AUMC's in this study were decreased 0.1% by the correction.

Bioavailability

Gambertoglio and coworkers (1980) reviewed studies of the bioavailability of PO and POH in healthy volunteers and patients. The reported values agree with those obtained here when conditions are matched for reference drug and measured concentrations. Meikle et al. (1975) administered 10 mg PO orally and 12 mg prednisolone phosphate intravenously; the availability of the tablet based on POH quantitation was 0.69 ± 0.12. Rose et al. (1980) gave 10 mg oral PO and 40 mg IV prednisolone phosphate to volunteers. Their estimate of the tablet F based upon POH plasma concentrations was 0.80 ± 0.11. Tanner et al. (1979) tested the bioavailability of oral prednisolone tablets versus IV prednisolone phosphate. For 20 and 100 mg doses by both routes, the availability averaged 98.5 ± 16%. Uribe et al. (1978) were the first to compare IV and oral routes of prednisone administration. The bioavailability of 10 mg doses by both routes averaged 1.01 ± 0.42.

There was great variability in the bioavailability results between and within the subjects in this study. There was no consistent trend in the bioavailability comparison within an individual between the two tablet formulations. The mean values for the PO tablets (treatment D) were lower than those of treatment E, POH tablets, in all cases but there were no consistencies within subjects. The standard deviations associated with the mean values were consistently high.

Table 9.8 presents the bioavailability of the tablets based on PO areas; POH area calculation results are presented in Table 9.9. Consider first the bioavailability calculations

when comparisons are made using prednisone AUC's. Here, it is first obvious that the tablets are bioequivalent. This is because the liver is apparently extremely reductive toward the glucocorticoids and forms mostly PO upon first pass. However, the results differ with the intravenous reference solution. The PO and POH tablet formulations, treatments D and E, gave bioavailabilities of 0.56 and 0.61 with IV PO as the standard; the liver converts much of the PO to POH, resulting in low F values based on PO concentrations compared to IV PO. Comparable results were obtained by Ferry et al. (1988) for 10 mg tablets of PO and POH who calculated F to be 0.64 and 0.76, respectively.

The respective bioavailabilities increased to 1.02 and 1.06 when POH was the reference solution. Intravenous prednisone yielded PO concentrations which were much higher than any other route of administration, hence the low POH availability values. Intravenous prednisolone yielded PO values which did not differ substantially from those after oral administration, and produced availabilities near unity. Ferry et al. (1988) calculated similar F values to thses for 10 mg doses; in parallel comparisons, the F values were 1.04 and 1.27, for PO and POH tablets, respectively.

Table 9.9 provides the bioavailabilities based on prednisolone area measurements. As for PO area comparisons, the two tablet formulations produce approximately equal values. When IV POH was the standard solution, availability exceeded unity, 1.28 and 1.40 for treatments D and E, respectively. This is because prednisolone is probably formed primarily in the liver; therefore, intravenous PO requires increased circulation time for equivalent reduction to POH.

In the case of the IV POH reference, the values in this study were 0.81 and 0.88 for PO and POH tablets, respectively. This implies that about 20% of the dose of PO or POH is eliminated during first pass.

Similarly, Ferry et al. (1988) observed bioequivalence for 10 mg tablets of PO and POH. With IV POH as the reference, F was 0.92; it increased to 1.04 when IV PO was used as the reference solution.

The relative and absolute bioavailabilities of 10 mg doses of PO and POH administered orally and intravenously were determined by Ferry et al. (1988). They also observed bioequivalence between the two tablets. Unlike the results presented here, prednisolone area measurements demonstrated equivalent bioavailability.

The bioavailability of PO from PO tablets using intravenous PO as the reference and measuring PO AUC's yielded an F of 0.56 ± 0.25. This is due to first pass elimination and reductive conversion of PO. Traditionally investigators measure POH availability from PO tablets using POH as the reference. This value, for comparison, was 1.02 ± 0.67. The bioavailability of POH from POH tablets using IV POH as reference and measuring POH AUC's was 0.88 ± 0.27. This value agrees with other estimates using this method.

It may be important to consider the sum of areas of both drugs as a measure of bioavailability. This has been done for procainamide and N-acetylprocainamide (Strong et al., 1975). The comparison of the sum of AUC's of PO and POH for all treatments showed differences between the routes of administration for the 25 mg dose of PO and POH (Table 9.4). Treatments B and C were the highest, at 2524 and 2618 (2314, corrected for phosphate) ng·hr/ml. Lower sums were observed for IV PO and the two oral treatments: 2078, 2061 and 2180 ng·hr/ml. From this data it can be estimated that 20% of PO and POH are extracted upon first pass.

The calculated bioavailability of the tablets based on the sum of PO and POH concentrations is presented in Table 9.10. The availability using IV PO resulted in larger values than in the use of IV POH as the reference solution when areas of both PO and POH were summed. The values based on treatment A, IV PO were 1.04 and 1.12 for oral PO and oral POH, respectively. Treatment B, IV POH, gave values of 0.83 and 0.89 for the two tablet formulations, respectively.

The bioavailability of prednisolone phosphate is presented in Table 9.11 with IV prednisolone as the standard, with calculations based on both PO and POH areas. The F of prednisolone phosphate based on PO was 1.23 ± 0.48; with POH concentrations, the value

was 1.03 ± 0.22. One large value contributes to this observed difference. When it is omitted, the average based on PO areas is reduced to 1.12 ± 0.36. Obviously, the F of the phosphate solution is complete, using either drug concentration measurements.

A thorough presentation of the problem of calculating the bioavailability of PO and POH (and reversibly metabolized drugs, in general) was published recently by Ferry and coworkers (1988). The authors determined the absolute and relative bioavailability of the glucocorticoids prednisone and prednisolone with 10 mg doses of both orally administered PO and POH using intravenously administered PO and POH phosphate as the reference compounds. The appropriate method of calculating the bioavailability of compounds which are interconverted, metabolically or chemically *in vivo* is a difficult concept. As each of the compounds is a prodrug of the other, a unique situation arises. In this case, only prednisolone is active. The present method of determining the bioavailability of tablet formulations of these drugs is to measure the prednisolone concentrations (areas) and compare them to prednisolone measures obtained after intravenous administration of prednisolone phosphate. This is true for the bioavailability of prednisone as well. This situation might be different if prednisone also possessed pharmacologic activity. If this were the case, what would be the correct calculation? The sum of areas of both drugs might be considered when the activities are comparable, or perhaps two or more estimates based on the two drug concentrations could be used.

It would seem logical to adopt a systemic availability that only used active drug as reference. However, individual organ clearances may differ in their ability to convert the inactive drug to its active form.

One other important consideration in the case of determining the bioavailability of PO and POH, but not necessarily other interconverted species is the nonlinear apparent clearance of the drugs. Rose et al. (1980) first stated the importance of comparing doses which gave equivalent plasma concentration-time profiles, not necessarily equivalent oral and intravenous doses. This is because the clearances of PO and POH are concentration-

dependent. It was assumed in this study that at 25 mg doses, the concentration-time profiles would be close enough to accept an equivalent dose of IV drug for comparison.

Conclusions

This study has demonstrated the utility of traditional bioavailability studies in the determination of the metabolic function of the liver in complex metabolic systems. With the administration of both drugs which are reversibly interconverted by two routes, orally as well as intravenously, it is possible to make preliminary conclusions as to the role of the liver and/or gut in metabolism of the compounds. This is fortunate in that otherwise, the characterization of organ-specific function in humans cannot be elucidated.

The role of the liver (it will be assumed that all activity resides in the liver, rather than elements of the gastrointestinal tract) in the interconversion of PO and POH was determined to be primarily reductive, based on AUC ratios after administration of the four drug-route combinations.

The bioavailabilities of PO and POH tablets were determined relatively and absolutely; these estimates varied as a function of the drug administered as well as the concentrations which were compared.

Although modeling fundamental clearance parameters based on *in vivo* data yields no correlation with specific pathways, some general conclusions may be made. It appears that the metabolic pathway with the greatest capacity is CL_{12}, or the reduction of PO to POH. The smallest clearance was observed to be the irreversible elimination of POH, CL_{20}. The true clearance of PO exceeded that of POH by more than 3-fold, primarily due to the contribution of reductive conversion, CL_{12}.

Chapter 10

Dose-Dependent Pharmacokinetics of Prednisone and Prednisolone Examined at Steady State in Humans

Introduction

The discovery of the dose-dependent pharmacokinetics of prednisone and prednisolone followed soon after the development of sensitive and specific analytical methodologies. Meikle et al. (1975), Pickup et al. (1977) and Tanner et al. (1979) were first to document the nonlinear pharmacokinetics of prednisolone after intravenous bolus doses of the phosphate ester. Rose and coworkers (1981) showed that the apparent systemic clearance of prednisone increased with escalation of oral doses of PO. Rose also documented the nonlinear urinary clearance of both drugs.

This study was performed to characterize the concentration-dependent pharmacokinetics of the glucocorticoids prednisone (PO) and prednisolone (POH) under steady state conditions after intravenous infusion of both compounds on separate occasions. Nonlinear pharmacokinetics of prednisolone have been demonstrated under steady state conditions, with two infused doses (Legler et al., 1982). The study described here characterizes the disposition of both PO and POH at three infusion rates each, encompassing a 10-fold dosage range.

The pharmacokinetics of prednisone have been studied less frequently than that of prednisolone, primarily due to the lack of an intravenous prednisone dosage form. The apparent systemic clearance of prednisone would be expected to show nonlinearities, as PO

interconverts to a drug whose kinetics are nonlinear, namely prednisolone. Dose-dependent kinetics have only been documented following oral administration of prednisone (Rose et al., 1981).

The concentration-dependent changes in the steady state apparent urinary clearance were also examined. This parameter has been shown to be nonlinear for both PO and POH (Rose et al., 1981) and is a contributor to the overall changes of the apparent systemic clearance with dose.

The interconversion of prednisolone and prednisolone is believed to be complicated by nonlinear processes. Several groups have reported that the conversion of POH to PO is apparently saturable (Rose et al., 1981; Frey et al., 1980; Legler et al., 1982). This nonlinearity is manifested by a decreasing ratio of PO to POH concentrations with increasing prednisolone dose. The reverse process, reduction of PO to POH has not been studied prior to this work.

Methods

Equipment and Supplies

Each drug was infused at three dosing rates, encompassing the range normally associated with observed nonlinearity in humans. An investigational new drug application (IND 28020) was obtained to administer prednisone parenterally and to administer prednisolone as non-esterified drug. Intravenous solutions of prednisone and prednisolone were manufactured by the University of California Sterile Products Laboratory. Prednisone U.S.P. and prednisolone U.S.P. (Pharma-Tek, Inc., Huntington, NY) were dissolved in a cosolvent system of ethanol U.S.P., 5%, and propylene glycol, 1%, in 0.45% sodium chloride (Travenol, Chicago, IL). The concentrations of the intravenous solutions were approximately 16, 85 and 170 μg/ml. The solutions were autoclaved to ensure sterility, tested for microbial contamination and were subjected to pyrogen testing

according to U.S.P. standards. The solutions were stored at room temperature to prevent precipitation of the prednisone from solution. The concentrations of the solutions were 16, 85, and 170 μg/ml for the low, medium and high doses, respectively.

The intravenous solutions were administered with AVI infusion pumps (AVI Inc., 3M Health Care Group, St. Paul, MN). Catheters (18 gauge, 2 inches, Becton Dickinson, Rutherford, NJ) were placed in the antecubital veins of each arm on study days. Blood samples were drawn from the catheters using a syringe (10 ml, Becton Dickinson) and immediately transferred to a heparinized glass vacuum tube (Vacutainer, Becton Dickinson). The samples were mixed by gentle inversion and centrifuged within a few minutes (Beckman Instruments, model TJ-6, Palo Alto, CA).

Study design

The participants were six healthy volunteers, ranging in age from 26 to 40 and weighing 57 to 81 kg, and consisting of three males and three females (Table 10.1). Each subject underwent a physical exam with a detailed medical history and was free of medication for two weeks before beginning the study. None was a smoker and all drank alcohol conservatively on occasion, although no alcohol was consumed 48 hours prior to a treatment. The University Committee on Human Research approved the clinical research protocol and each subject gave voluntary and informed consent to participate.

Subjects were administered one of six infusion treatments at one-week intervals. Three treatments were with prednisone: (A) 4 mg PO; (B) 20 mg PO; (C) 40 mg PO. Three treatments were with prednisolone: (D) 4 mg POH; (E) 20 mg POH; (F) 40 mg POH. A randomized, cross-over design for dosing was used. Drug administration was performed in two stages to shorten the time required to achieve steady state conditions. A rapid infusion of 125 ml/hour was administered for one hour, followed by a slow five-hour infusion of 25 ml/hour. The infusion was initiated between 7 and 8 a.m. after an overnight fast and subjects were not permitted to eat until 11a.m. when they were served lunch;

dinner followed later in the day. Subjects were encouraged to drink water throughout the day, particularly if they had difficulty voiding for the scheduled urine collection periods. The subjects remained supine for the first hour and then remained sitting for the next five hours of infusion.

Seven milliliters of blood was collected at 15 and 30 minutes, and at 1, 2, 3, 4, 5, 6 hours during the infusion and at 6.25, 6.5, 7, 8, 9, 10, and 11 hours. Urine was collected at 1, 2, 3, 4, 6, 8, 10, 12, and 24 hours. The urine and separated plasma samples were stored at -20 °C and were analyzed for drug content within one month.

Table 10.1. Characteristics of study participants.

Subject	Age (yr)	Sex	Weight (kg)
1	33	Female	56.7
2	42	Female	61
3	39	Female	60.4
4	29	Male	80.8
5	31	Male	73.8
6	26	Male	63.1

Analysis

The plasma samples were simultaneously assayed for prednisone and prednisolone using the normal phase high performance chromatographic procedure described in Chapter 3. A solid phase extraction step using Chem-Elut tubes (# E 1003, Analytichem, Harbor City, CA) was performed and separation was achieved on a silica column. Diluted urine

samples were analyzed with a similar procedure, modified to include an additional extraction and drying step.

Equilibrium dialysis was performed on approximately one fourth of the plasma samples to estimate prednisone and prednisolone binding using the tritiated compounds [2,4,6,7-^{3}H]prednisolone and [1,2(n)-^{3}H]prednisone (Amersham, Arlington Heights, IL), as described in Chapter 9.

The prednisone infusion rates averaged 90, 469 and 936 ng/min per kg for treatments A, B and C, respectively; for the three prednisolone treatments, the rates were 102, 532 and 1067 ng/min per kg. The steady state concentration of each drug, C_{ss}, was calculated for the total and unbound concentrations as the average of concentrations from the 3, 4, 5 and 6 hour samples.

Pharmacokinetic parameters were calculated in the following manner: the area under the concentration time curve, AUC, and AUMC (area under the first moment curve) were calculated using the linear trapezoidal rule. The apparent systemic clearance, CL_{app}, was calculated as the rate of infusion, R_{inf}, divided by C_{ss} for the infused drug. The apparent urinary clearance, CL_{ur}, was calculated as the (rate of drug excretion between 2 and 6 hours)/C_{ss} for each drug. The fractional urinary clearance was calculated as: CL_{ur}/CL_{app} for each drug.

Certain pharmacokinetic parameters required calculation methods which incorporated the contribution of reversible metabolism. The four fundamental clearance parameters (Figure 10.1) were calculated as described in Table 2.1 (Chapter 2), substituting the drug infusion rate and steady state plasma concentrations for dose and area measurements, respectively. The steady state volume of distribution, V_{ss}, and the mean residence time, MRT, were calculated according to Ebling and Jusko (1986). Statistical analyses of dose-dependencies were performed with repeated measures analysis of variance.

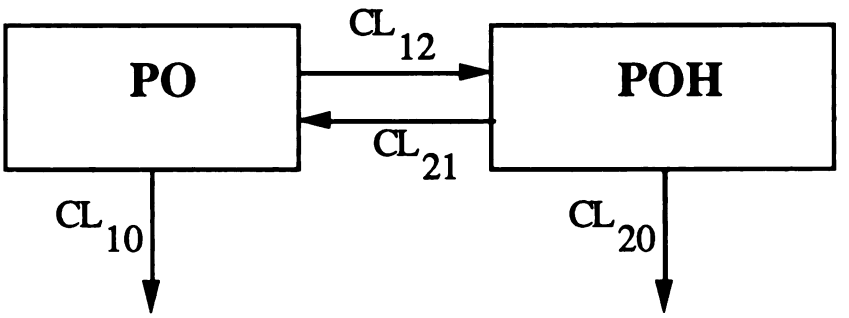

Figure 10.1. Model of the interconversion and elimination of prednisone and prednisolone at steady state in human subjects.

CL_{10} = irreversible elimination of prednisone
CL_{12} = reductive conversion of prednisone to prednisolone
CL_{20} = irreversible elimination of prednisolone
CL_{21} = oxidative conversion of prednisolone to prednisone

Results

The plasma concentration-time profiles after treatments A, B and C (PO infusions) in subject 2 are presented in Figure 10.2. The PO concentrations dropped between 1 and 2 hours, reflective of the reduced infusion rate. For the 4, 20 and 40 mg doses of PO, the corresponding steady state plasma concentrations were 52, 93 and 195 ng/ml. Prednisolone concentrations decreased after one hour for the low dose; in the other two cases, POH concentrations continued to increase until about 2 hours. The steady state concentrations of prednisolone were 112, 272 and 560 ng/ml. The steady state concentrations of both PO and POH increased with increasing infusion rate. The PO and POH concentrations declined in parallel from 6 to 11 hours.

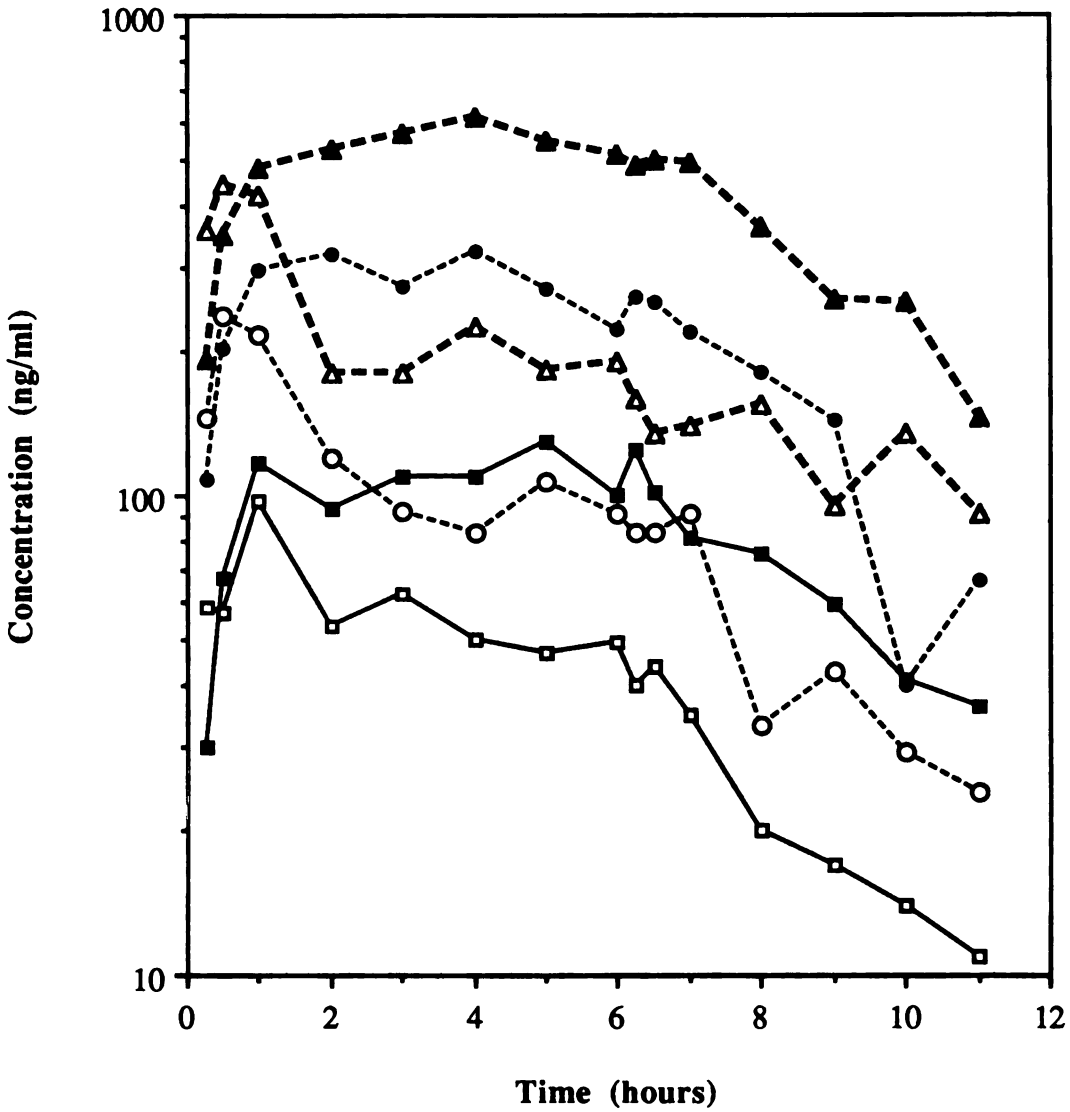

Figure 10.2. Plasma concentration-time profiles in subject 2 after three prednisone infusions: squares, 4 mg PO, low dose, treatment A; circles, 20 mg PO, medium dose, treatment B; triangles, 40 mg PO, high dose, treatment C; open symbols, prednisone concentrations; solid symbols, prednisolone concentrations.

Plasma drug concentrations for the three prednisolone infusions in subject 1 are presented in Figure 10.3. For the three infusions, 4, 20 and 40 mg of prednisolone, the steady state concentrations achieved were 90, 301 and 417 ng/ml, respectively. Prednisone accumulated slowly, as plasma concentrations continued to increase for at least two hours. They were nearly constant between the three treatments: over the 10-fold dose range, these were 60, 36 and 48 ng/ml, for the low, intermediate and high doses, respectively. Prednisolone and PO concentrations declined in an apparently parallel manner at the completion of the infusion.

The average steady state concentrations of prednisone were dependent upon the drug infused. In prednisone infusions, A, B and C, the respective mean PO concentrations were 62, 116 and 145 ng/ml total drug (Table 10.2). The corresponding values following infusion of prednisolone, treatments D, E and F, were 38, 62 and 68 ng/ml, respectively. Prednisone concentrations did not increase proportionately to prednisolone infusion rates, nor did they equal those achieved upon infusion of prednisone.

Unlike those observed for prednisone, the prednisolone steady state concentrations were similar after both PO and POH infusions. With administration of POH, the mean values were 88, 373 and 577 ng/ml; for PO administration, the mean values were 133, 328 and 422 ng/ml. The prednisolone concentrations were lower after PO infusion than after POH, but with the inherent variability, there were no significant differences in the POH concentrations achieved with the two drugs.

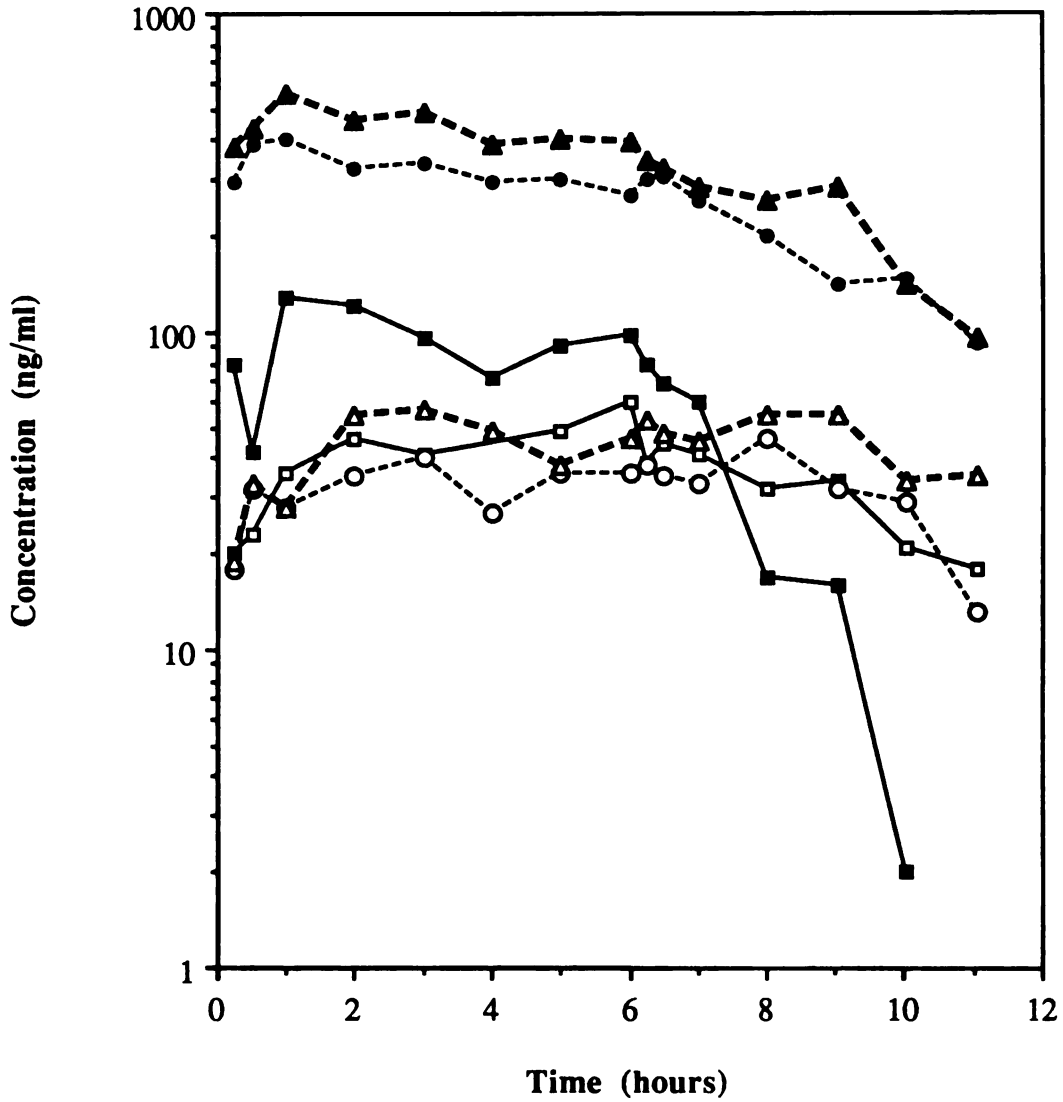

Figure 10.3. Plasma concentration-time profiles in subject 1 after three prednisolone infusions: squares, 4 mg POH, low dose, treatment D; circles, 20 mg POH, medium dose, treatment E; triangles, 40 mg POH, high dose, treatment F; open symbols, prednisone concentrations; solid symbols, prednisolone concentrations.

Table 10.2. Resultant steady state concentrations of prednisone and prednisolone for the six treatments averaged for the six subjects (± s.d.).

Prednisone Infusions

PO	4 mg (A)	20 mg (B)	40 mg (C)
CL_{app} (L/hr)	7.7 ± 4.4	16.8 ± 4.5	29.4 ± 13.6
CL_{app} (L/hr per kg)	0.12 ± 0.07	0.26 ± 0.09	0.46 ± 0.24
CL_U (L/hr)	28.0 ± 15.0	62.9 ± 16.8	110.4 ± 0.24
CL_U (L/hr per kg)	0.43 ± 0.24	0.98 ± 0.32	1.71 ± 0.85

Prednisolone infusions

POH	4 mg (D)	20 mg (E)	40 mg (F)
CL_{app} (L/hr)	4.95 ± 1.59	6.06 ± 1.76	7.88 ± 2.40
CL_{app} (L/hr per kg)	0.074 ± 0.017	0.093 ± 0.031	0.119 ± 0.031
CL_U (L/hr)	46.0 ± 15.1	35.1 ± 18.6	33.6 ± 16.9
CL_U (L/hr per kg)	0.69 ± 0.16	0.55 ± 0.32	0.51 ± 0.26

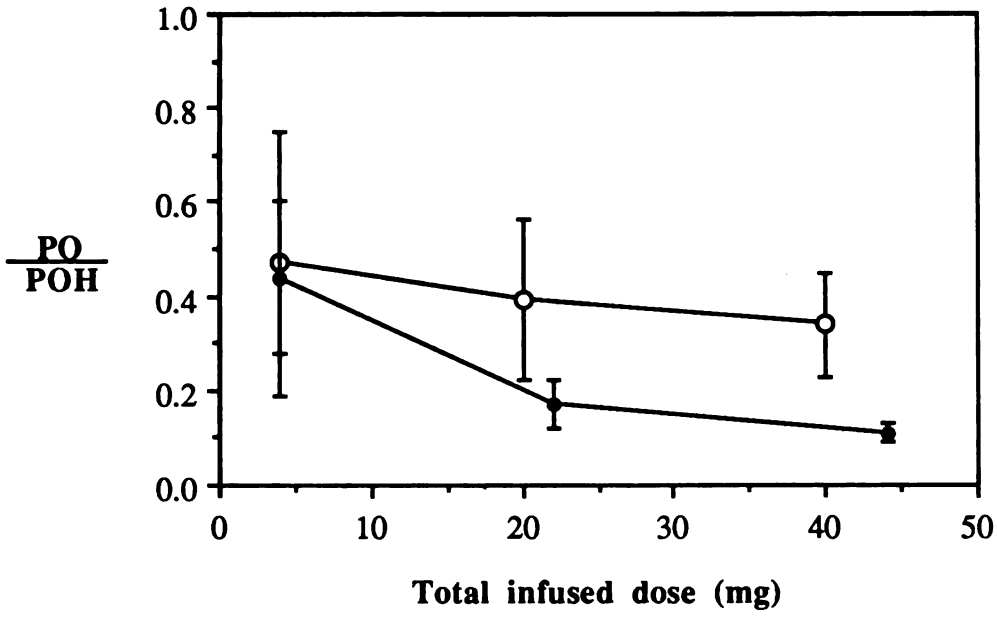

Figure 10.4. Average ratio of steady state concentrations of prednisone and prednisolone in six treatments. open symbols, PO infusions (A,B, C); solid symbols, POH infusions (D, E, F).

The ratio of steady state concentrations of PO and POH are presented in Table 10.2 and are plotted in Figure 10.4. The ratio of PO to POH decreased slightly with infusion of increasing doses of PO (treatments A, B and C): 0.47, 0.39 and 0.34. The variability between and within subjects was high, such that the decreases were statistically insignificant.

In contrast, prednisolone infusions, treatments D, E and F, produced a PO to POH concentration ratio which was significantly decreased for two of the three comparisons. The most significant decrease was between the low and intermediate doses, 0.44 to 0.17; the ratio at the high dose was 0.11. Statistically significant decreases were observed between treatments D and E, $p<0.01$, as well as D and F, $p<0.01$, but not for treatment E versus F.

The results of plasma protein binding determinations measured by equilibrium dialysis agree with those of other laboratories. The binding of prednisone was linear over the entire concentration range (up to 319 ng/ml) and the fraction unbound averaged 0.25 ± 0.04. The binding of POH was nonlinear over the range of 24 to 598 ng/ml, where the unbound fraction varied from 0.07 to 0.21.

The apparent systemic clearances of prednisone and prednisolone are presented in Table 10.3, for both total and unbound drug; the values are plotted in Figures 10.5 A and 10.5 B. The apparent systemic clearance of prednisone demonstrated statistically significant increases between the three treatments. With increasing infusion doses, the clearance increased from 0.12 to 0.26 to 0.46 L/hr per kg ($p<0.01$ and $p<0.01$). As the binding of prednisone was linear, unbound clearances increased in parallel.

The apparent systemic clearance of prednisolone increased slightly between treatments. For the three doses of prednisolone, 4, 20 and 40 mg, the clearance values averaged 0.074, 0.093 and 0.119 L/hr per kg. Only the increase between treatments D and F (10-fold difference in infusion rate) was significant ($p<0.01$). Due to the nonlinear binding of prednisolone and the small increase in CL_{app} of total drug, the apparent unbound clearance of POH decreased between treatments: 0.69, 0.55 and 0.51 L/hr/kg. This decrease was not significant, however.

Comparing the apparent systemic clearances of PO and POH, the clearance of prednisone was 2, 3 and 4-fold greater than that of prednisolone for the 4, 20 and 40 mg dose infusions, respectively.

The apparent urinary clearances of prednisone and prednisolone at steady state are presented in Table 10.4. The urinary clearance of prednisone increased with dose for both prednisone and prednisolone infusions. Due to great variability, only the changes observed for prednisone infusion studies were statistically significant. For treatments A, B and C, the mean apparent CL_{ur} for PO were 24.5, 38.4 and 44.0 ml/min ($p<0.01$ for treatments A

versus C), respectively; the mean CL_{ur} PO values for treatments D, E and F were 20.5, 56.8 and 88.2 ml/min ($p<0.01$ for treatments D versus F).

Table 10.3. Average apparent systemic steady state clearances of prednisone and prednisolone resulting from infusions of both PO and POH.

Prednisone Infusions

	4 mg (A)	20 mg (B)	40 mg (C)
C_{ss} PO	62 ± 42	116 ± 32	145 ± 59
C_{ss} POH	133 ± 50	328 ± 96	422 ± 77
PO/POH	0.47 ± 0.28	0.39 ± 0.17	0.34 ± 0.11

Prednisolone infusions

	4 mg (D)	20 mg (E)	40 mg (F)
C_{ss} PO	38 ± 17	62 ± 23	68 ± 33
C_{ss} POH	88 ± 30	373 ± 134	577 ± 193
PO/POH	0.44 ± 0.16	0.17 ± 0.05	0.11 ± 0.02

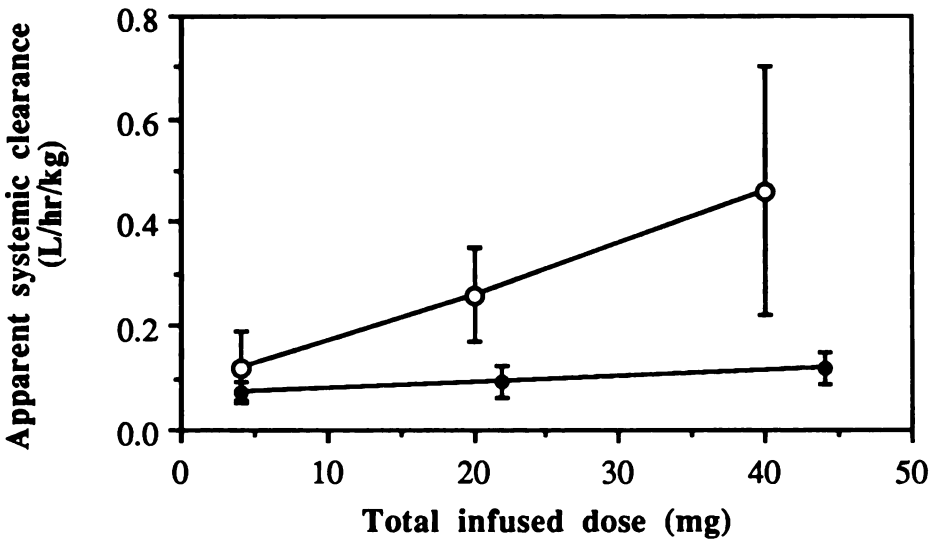

Figure 10.5A. Relationship between apparent steady state clearance of total drug and dose infused: open symbols, prednisone; solid symbols, prednisolone

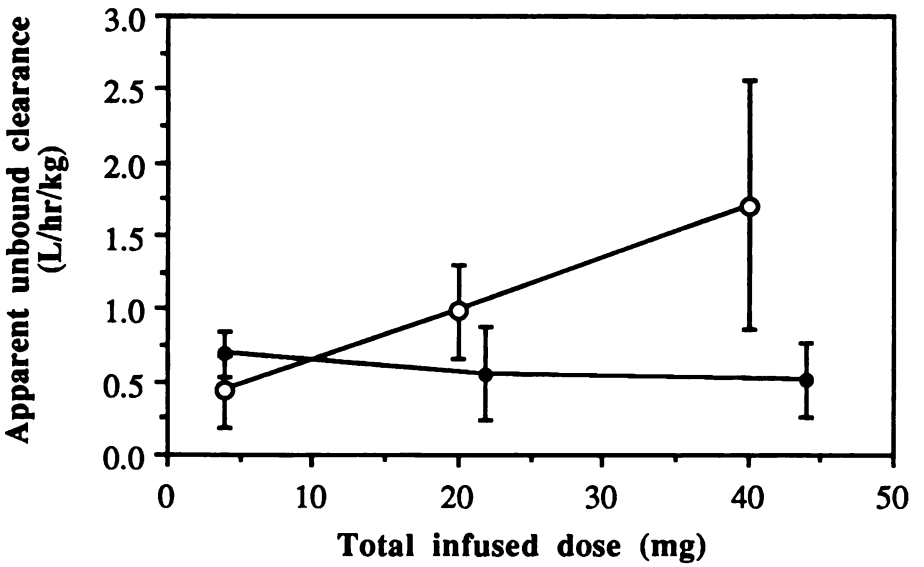

Figure 10.5B. Relationship between apparent steady state clearance of unbound drug and dose infused; open symbols, prednisone; solid symbols, prednisolone.

The apparent steady state urinary clearance of prednisolone increased for both PO and POH treatments. For prednisone infusions A, B, and C, the mean values were 6.2, 19.4 and 38.8 ml/min, respectively; statistically significant increases were observed in each of the comparisons ($p<0.01$ for all comparisons). The prednisolone excretion increased 3-fold when the dose was increased 5-fold dose, and excretion of PO increased 6-fold over the 10-fold dose increase. For prednisolone treatments D, E and F, the mean prednisolone CL_{ur} values also increased: 10.7, 35 and 52.8 ml/min ($p<0.01$ for treatment D versus E and F).

The fractional urinary clearance of prednisone decreased with increasing infused PO dose: 0.18, 0.14 and 0.09 (Table 10.4). The change was significant only for the low versus high dose, $p<0.01$. In contrast, the fractional urinary clearance of prednisolone for the three POH infusions increased from 0.13 to 0.35 to 0.40 with dose ($p<0.01$ for the low versus the intermediate and high doses).

A model of the interconversion of prednisone and prednisolone is presented in Figure 10.1. The values calculated for the fundamental clearances are presented in Table 10.5, where the individual and mean values are shown. Negative clearances were calculated for all three doses. Ten negative values in the 4 mg dose calculation and three negative values each in the 20 and 40 mg doses were encountered; two negative averaged clearance values were obtained, as well. Variability was greatest in the 4 mg treatment, as the C.V.% was between 109 and 1032 for the four clearance values. Consequently, none of the parameters showed a consistent pattern between treatments; nor did any of the subjects demonstrate consistencies in the relative magnitude of the parameters.

10.4
Steady state urinary parameters

	Prednisone			Prednisolone		
	4 mg	**20 mg**	**40 mg**	**4 mg**	**20 mg**	**40 mg**
CLur PO (ml/min)	24.5 ± 17.2	38.4 ± 14.6	44.0 ± 19.3	20.5 ± 12.2	56.8 ± 40.7	88.2 ± 73.9
CLur PO (ml/min/kg)	0.37 ± 0.26	0.58 ± 0.20	0.68 ± 0.33	0.30 ± .013	0.91 ± 0.70	1.25 ± 0.87
CLur POH (ml/min)	6.2 ± 4.3	19.4 ± 5.9	38.8 ± 7.4	10.7 ± 3.7	35.0 ± 11.7	52.8 ± 22.6
CLur POH (ml/min/kg)	0.09 ± 0.06	0.31 ± 0.12	0.60 ± 0.17	0.16 ± 0.05	0.54 ± 0.21	0.80 ± 0.32
Fractional CLur (PO)	0.18 ± 0.07	0.14 ± 0.07	0.09 ± 0.02	-	-	-
Fractional CLur (POH)	-	-	-	0.13 ± 0.03	0.35 ± 0.07	0.40 ± 0.08

Table 10.5. Fundamental clearances (ml/min).

4 mg dose

Subject	CL_{10}	CL_{20}	CL_{12}	CL_{21}
1	96	10	-528	-288
2	21	41	481	182
3	-398	168	1455	211
4	551	-97	-900	-137
5	295	-67	-391	-56
6	-35	75	106	31
Mean	**88**	**22**	**37**	**-9**
S.D.	**320**	**97**	**848**	**191**

20 mg dose

Subject	CL_{10}	CL_{20}	CL_{12}	CL_{21}
1	-80	122	411	38
2	-159	167	737	85
3	312	18	673	120
4	-2875	657	6177	628
5	46	89	241	54
6	149	51	253	95
Mean	**-435**	**184**	**1415**	**170**
S.D.	**1207**	**238**	**2342**	**226**

40 mg dose

Subject	CL_{10}	CL_{20}	CL_{12}	CL_{21}
1	84	145	669	87
2	-63	128	539	60
3	890	2	968	184
4	-153	197	768	63
5	-83	166	914	79
6	161	59	215	57
Mean	**139**	**116**	**679**	**88**
S.D.	**386**	**73**	**276**	**48**

Discussion

The infusion of both prednisone and prednisolone was designed to study the dose-dependency of two processes: interconversion and the apparent systemic clearance of PO and POH. The interconversion of prednisone and prednisolone was examined under steady state conditions to assure that distributional factors did not complicate the analysis of this process. In this manner, the steady state concentrations and the concentration ratios provided information regarding this complicated metabolic system.

Interconversion

The formation of prednisone from prednisolone under steady state conditions has been shown to be a nonlinear process *in vivo*: Legler et al. (1982) observed that prednisone concentrations did not exceed 60 ng/ml upon infusion of prednisolone to humans. A similar observation has been made in dogs (Frey et al., 1980). In the present study, the prednisone steady state concentrations achieved with prednisolone infusions (D, E, F) averaged 38, 62 and 68 ng/ml, respectively. The values for all six volunteers are presented in Figure 10.6, where it can be seen that prednisone concentrations demonstrated no clear pattern with increasing infusion dose. In five of the six subjects, however, an apparent maximum concentration of PO was reached between 40 and 70 ng/ml. This result is similar to that of Legler et al. (1982).

Additionally, the plot in Figure 10.4 shows the ratios of PO to POH concentrations. Statistically significant decreases were observed with POH infusion, from 0.44 to 0.17 to 0.11. This is in agreement with the work of other investigators. In intravenous bolus and oral administration studies, Rose et al. (1981) demonstrated that the ratio of the areas under the curve of PO to POH decreased with dose. After administration of 5, 20 and 50 mg PO oral tablets, the ratio decreased from 0.21 to 0.16 to 0.09, respectively; after intravenous administration of 5, 20 and 40 mg prednisolone phosphate, the ratio decreased from 0.20 to 0.14 to 0.10.

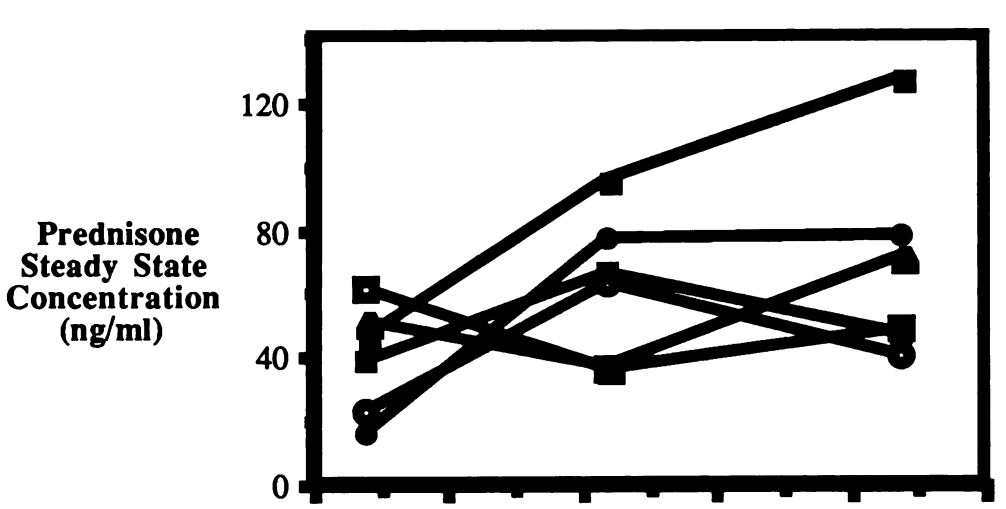

Figure 10.6. Steady state concentrations of prednisone as a function of infused prednisolone dose (treatments D, E and F); each symbol represents a different subject.

It can be concluded from the maximal concentration and ratio analyses that the oxidation of POH to PO is an *apparently* saturable process. If conversion were known to occur in one organ, it could be concluded that this process in this organ is saturable. But in the rabbit, at least two organs, the kidney and lung (Chapters 7 and 8), perform this reaction. Furthermore, it has been demonstrated that the rabbit and rat livers do not form measurable prednisone concentrations during prednisolone perfusions (Chapter 5), although the reverse reaction does occur, i.e. measurable POH during PO perfusions. Because of this observation, the reaction is not uniform in all organs, and it is difficult to ascertain the source of the nonlinearity from *in vivo* data. An additional complication which is superimposed onto these results is a consequence of reversible metabolism: reduction obscures apparent oxidative conversion activity and it is therefore impossible to determine oxidative capacities directly.

The liver has been proposed as the site of the apparently saturable formation of PO from POH (Frey et al., 1980). The perfused rabbit and rat liver studies (Chapter 5) challenge this hypothesis, as no measurable PO was present in hepatic venous blood upon prednisolone perfusion. It is likely that more than one organ contributes to the observation of apparent saturation of the oxidative formation of PO from POH.

It is interesting to note that the perfused human liver was able to oxidize POH to PO to a small extent (<10%), as described in Chapter 6; as these studies were preliminary, conclusions regarding human hepatic activity cannot be made at this time. The results in the human liver differed from those of the perfused rabbit and rat livers; the difference may be partially attributable to pre-perfusion conditions. The human livers were stored in ice for several hours, whereas the animal livers were cannulated *in vivo* and perfused *in situ* with only a few seconds of anoxia. The extended period of ischemia in the human organ could have resulted in altered metabolic profiles. A reduction in the activity of the reductive conversion pathway is a possibility.

An interesting analysis is the dependency of the PO/POH concentration ratio on the drug infused (Figure 10.4). At the low dose, 4 mg, the ratio did not differ with the drug administered, and was between 0.4 and 0.5. With increasing dose, the relationship was a function of the drug infused. Insignificant decreases in the PO/POH ratio were measured after PO infusion; highly significant decreases were measured after POH administration.

The reduction of PO to POH was apparently linear across this dosage range. The Css of POH increased from 133 to 328 to 422 ng/ml with infusion of PO. This increase was proportional to the dose increase: a change of 5-fold and 2-fold between the 4 and 20 and the 20 and 40 mg doses produced steady state ratios of 2.5 and 1.3, respectively. A similar increase was observed with infusion of prednisolone itself: POH concentrations increased from 88 to 373 to 577 ng/ml. The ratios differed 4-fold and 1.6 fold for equivalent comparisons with prednisolone infusions. Hence, these two treatments were not distinguishable.

From these results, it can be concluded that the formation of prednisolone from prednisone was apparently linear over the dosage range examined.

Apparent steady state systemic clearance

Prednisone apparent clearance

The apparent systemic clearance of prednisone was measured across a 10-fold infusion dose range, with infusion rates between 100 and 1000 ng/min/kg. The total administered doses were approximately 4, 20 and 40 mg prednisone. The apparent clearance of PO increased with dose for the three treatments. An average value of 0.12 L/hr per kg was calculated for treatment A and 0.26 L/hr per kg for treatment B. Hence, over the 5-fold dose-range, the apparent clearance of prednisone increased 2-fold. This same observation was made for the unbound apparent clearance of prednisone, which increased from 0.43 to 0.98 L/hr per kg. This was to be expected as the plasma protein binding of PO was linear.

Between treatments B and C, an increase was also observed; over the two-fold dosage range, CL_{app} of PO increased from 0.26 to 0.46, or 1.8-fold. The observation of increased apparent clearance of prednisone has not been rigorously documented. Rose et al. (1981) observed that the Dose/AUC of prednisone increased after three oral doses of prednisone, but this finding could not be distinguished from bioavailability (first-pass) effects. In this study, a measured increase in the apparent clearance of prednisone, total and unbound, was documented after intravenous infusion of non-esterified prednisone to steady state.

The apparent clearance of prednisone increased 4-fold over the 10-fold range of infusions and the apparent clearance of unbound prednisone increased in parallel. This large dose-dependency of unbound clearance strongly suggests the involvement of metabolic factors. Interconversion has been shown to be apparently saturable in the

formation of PO, but not in the reverse direction. The elimination of PO has not been studied adequately. At this point, such a large increase in unbound clearance would have to be regarded as an apparent increase, and not necessarily related to specific metabolic events.

Prednisolone apparent clearance

The apparent systemic clearance of prednisolone increased with dose over the 10-fold dosage range. For 4, 20 and 40 mg of intravenously administered prednisolone, the mean apparent clearance of total drug increased from 0.074 to 0.093 to 0.119 L/hr per kg. The variability in these numbers was so great, however, that significance was only associated between the low and high doses, treatments D and F, where the clearance increased 1.6-fold.

The unbound apparent clearance of prednisolone is an important parameter in that nonlinearities in the plasma protein binding of POH result in an increasing unbound free fraction of drug with increasing concentrations. Hence, dose-dependencies observed for pharmacokinetic parameters based on total prednisone may reflect changes associated with binding versus metabolic factors. The mean unbound apparent clearance of prednisolone decreased over the 10-fold dose range from 0.69 to 0.55 to 0.51 L/hr per kg. The changes were not statistically significant, however, such that the apparent clearance of unbound prednisolone was constant. The unbound fraction of prednisolone increased from 0.07 to 0.21 over the entire concentration range and the 1.6-fold change in apparent clearance of total prednisolone can easily be accounted for by the changes in binding alone.

These results differ substantially from those of Legler et al. (1982) who infused prednisolone phosphate to steady state at two rates: 5.5 and 64 μg/hr per kg. The apparent clearance of POH increased from 36.5 to 178 ml/min. Binding changes alone could not explain these results, as even the CL_{app} of unbound prednisolone increased an average of 29% with an increase noted in 9 of the 10 subjects. The source of the large differences in

results from the same laboratory cannot be explained, but may relate to interindividual differences.

Modeling Clearances

The fundamental clearances of PO and POH were determined by the method of Hwang et al. (1981), with the substitution of infusion rate and steady state plasma concentration for dose and area, respectively. This theory assumes that the pharmacokinetics of these compounds is linear. As has been demonstrated above, nonlinearities in the apparent clearance and interconversion of PO and POH exist within this dosage range.

Table 10.4 presents the results of the fundamental clearance calculations. None of the four clearance values demonstrated a relationship to dose. The irreversible elimination of prednisone, CL_{10}, varied from 88 to -435 to 139 ml/min and the elimination of POH, CL_{20}, from 22 to 184 to 166 ml/min for the 4, 20 and 40 mg doses, respectively. CL_{12} and CL_{21} were distributed equally widely. From PO/POH concentration ratio analyses, it appeared that the interconversion approached linearity at the 4 mg dose, because the ratios of drug concentrations were nearly equivalent for infusion of both drugs. Yet, the highest number of negative values was obtained for this calculation. Negative values were included in the determination of the average value, as they modeled the data as well as any other values, although they are obviously not valid clearance estimates. There were no trends in the relative magnitude of the fundamental clearance values, which would be expected for a system which undergoes nonlinear kinetics.

Urinary Analyses

What is traditionally termed the renal clearance as associated with *in vivo* pharmacokinetic studies is actually a measure of the urinary clearance of a drug; this is because the kidney may exhibit metabolic activity toward a drug and it is not possible to quantitate renal metabolic events with *in vivo* studies. In the kidney perfusion experiments

of Chapter 7, both renal and urinary clearances were calculated. To avoid confusion, urinary clearance is used with the human studies to refer to the parameter which measures urinary excretion of unchanged drug relative to plasma drug concentrations.

Another terminology issue is the appendage *apparent*. The apparent urinary clearance (CL_{ur}) is named this because of the metabolism of PO and POH in renal tissue, particularly the interconversion.

Apparent urinary clearance

The apparent mean urinary clearance of prednisone for the three doses was not constant, but increased from 24.5 to 38.4 to 44.0 ml/min for treatments A, B and C, respectively. For the three POH infusions, the mean values also increased, but were highly variable: 20.5, 56.8 and 88.2. The values for the 4 mg doses were statistically different from those of the other two doses. It would be expected that urinary clearance would not be a function of the drug administered, but only of the plasma drug concentrations. Ignoring the variability, this was the case. Rose et al. (1981) also observed changes in the CL_{ur} of PO after oral administration and after intravenous prednisolone administration.

The apparent urinary clearance of prednisolone was dose-dependent, as well. The PO treatments yielded mean values of 6.2, 19.4 and 38.8 ml/min; prednisolone infusions produced mean POH urinary clearances of 10.7, 35.0 and 52.8 ml/min. Significant differences were observed upon comparison of the low and high dose treatments. These values compare favorably to those of Rose and coworkers (1981) who measured the mean apparent urinary clearance of POH, 23.2, 59.9 and 87.4 ml/min for three intravenous bolus doses of POH phosphate of about the same magnitude.

The increase in the apparent urinary clearance of both PO and POH is difficult to explain. The lung and the kidney of the rabbit eliminate and interconvert PO and POH (Chapters 8 and 7, respectively). If human lung and kidney behave in a like manner to that seen in rabbits, it follows that the CL_{ur} calculations are incorrect. However, the analysis of

this process is difficult; simulation of these events is required, as not only does interconversion occur, but saturation of interconversion may occur as well.

It is possible that renal metabolic processes are saturable and at high concentrations more of prednisone and prednisolone are excreted unchanged, rather than metabolized.

Fractional urinary clearance

The fraction of the systemic clearance of PO contributed by urinary clearance decreased with dose. That is, metabolic elimination of PO plays a larger role in drug disposition with increasing PO concentrations or doses. The values of the fractional contribution of urinary clearance to systemic clearance for treatments A, B and C were 0.18, 0.14 and 0.09; the low and high dose values were statistically different. The CL_{ur} of PO increased with increasing dose, but did not keep pace with the increase in apparent clearance of PO, such that fractional urinary clearance decreased.

In contrast, the fractional urinary clearance of prednisolone increased significantly with increasing doses of POH: 0.13 to 0.35 to 0.40. Statistical significance was observed for the low versus the 5- and 10-fold infusion dose changes. This implies that prednisolone was increasingly excreted unchanged in the urine as concentrations of the drug in plasma increased. A 3-fold increase in fractional clearance was observed over the 10-fold dosage range.

The fact that the fractional urinary clearance of one drug increased and the other behaved oppositely is a clue to understanding these renal excretion processes. Because the apparent urinary clearance and apparent systemic clearance are inversely proportional to the steady state plasma drug concentration, the fractional urinary clearance may be simplified:

$$\text{Fractional } CL_{ur} = \frac{\text{excretion rate}}{\text{infusion rate}}$$

Hence, the increased excretion of POH and decreased excretion of PO may simply reflect changes in plasma drug concentrations, wherein the oxidative formation of PO is reduced due to saturation and POH concentrations consequently increase with dose. This would explain the increase in the fractional urinary clearance of prednisolone where this pattern in concentration was observed. But for PO infusions, the PO/POH ratio was constant and the fractional urinary clearance decreased; a decrease in renal drug metabolism is one possible explanation.

Urine-flow dependence analysis

The dependence of the excretion of prednisone and prednisolone on the urine flow rate at steady state was determined. Within one treatment, excretion rates and urine flow rates between 2 to 4 hours and 4 to 6 hours were plotted. The results of linear regression were poor; the best data was with prednisone excretion in treatment D. Here, $r^2 = 0.465$ and the worst fit was with prednisolone excretion in treatment E, $r^2 = 0.001$. A much stonger relationship was observed for PO data than POH data, however. The mean correlation coefficients across all six treatments were $r^2 = 0.233$ for PO and $r^2 = 0.053$ for POH.

It is clear from these results that the excretion of PO and POH at steady state was not urine-flow dependent. Rose et al. (1981) also observed no relationship between urinary excretion rate and urine flow in humans. These results differ from results in the perfused rabbit (Chapter 6) and rat kidneys (Rocci et al., 1981), where high correlations between excretion rate and urine flow rate were observed.

Conclusions

In conclusion, steady state investigation of the interconversion of prednisone and prednisolone demonstrated that the formation of PO from POH was saturable. The reverse reaction, reductive formation of POH, was apparently linear over the dosage range

examined. The administration of both drugs, on separate occasions, by intravenous infusion was essential for this conclusion.

The apparent systemic clearance of prednisone at steady state was proven to increase with dose; that of prednisolone increased slightly over the 10-fold range of infusion rates. The unbound apparent clearance of prednisone increased to the same degree as total prednisone due to this drug's linear protein binding; the implications of this observation are unknown, but it is important to emphasize that this is an apparent clearance. The unbound apparent clearance of prednisolone was unchanged with dose.

The apparent urinary clearance of prednisone and prednisolone increased with dose. This observation could not be attributed to urine flow-dependence and the significance of the term *apparent* is demonstrated in this result. Pulmonary and renal metabolism of these compounds has been documented in animals and may be assumed to occur in humans, as well. The calculated values are therefore confounded by alterations due to irreversible metabolism as well as interconversion; consequently, arterial drug concentrations probably differ from venous concentrations. The fractional urinary clearance of PO decreased but that of POH increased. This result is in agreement with the pattern of plasma drug concentrations, which exhibited reduced PO and increased POH concentrations with increasing dose.

Chapter 11

Conclusions:
Human and Leporine Studies with Prednisone and Prednisolone

Combination of results of human studies

Many studies have been performed in humans *in vivo* to characterize the dose-dependent pharmacokinetics of prednisone and prednisolone, as reviewed in Chapter 2. Two additional studies in humans, both of which involve the intravenous administration of prednisone are presented in Chapters 9 and 10. The analysis of these studies reveals several aspects of the disposition of prednisone and prednisolone which are difficult to explain through *in vivo* experimentation alone. These findings are briefly outlined below.

Interconversion

Rose and coworkers (1981) very elegantly documented the nonlinear conversion of POH to PO in humans after administration of intravenous prednisolone phosphate and oral prednisone tablets. They showed that the AUC ratio of PO to POH decreased to half its original value over a 10-fold range of oral prednisone and over the 8-fold dosage range of intravenously administered prednisolone. Under steady state conditions, Legler et al. (1982) documented the same limited oxidation after infusion of prednisolone phosphate, where a maximum of about 60 ng/ml PO was measured. Three infusion doses of prednisolone were administered to steady state conditions, as described in Chapter 10,

where a maximum PO concentration of less than 70 ng/ml was observed in five of the six subjects. Together, these three studies document the apparently saturable formation of PO from POH in humans.

It is equally important to study the opposite process through the administration of intravenous prednisone as interconversion probably plays a major role in the nonlinear disposition of PO and POH. Such studies were performed and are described in Chapter 10. For the three PO doses administered, encompassing a 10-fold range, the respective steady state concentrations achieved differed approximately 8-fold. In other words, the formation of prednisolone from prednisone was not apparently saturable within this dosage range.

It is interesting to note that at low doses, the PO/POH ratio is independent of which drug given; at higher doses, the PO to POH ratio became a function of whether PO or POH was dosed.

Route-dependence

The pharmacokinetics of prednisone and prednisolone are a function of the route of administration. It is accepted that equivalent clinical results are obtained after the oral administration of either PO or POH (Gustavson and Benet, 1985). Ferry and coworkers (1988) have demonstrated that prednisone and prednisolone are bioequivalent when administered orally as 10 mg doses; similar results were found with 25 mg doses as described in Chapter 9. This implies that the liver metabolizes prednisone and prednisolone such that approximately equivalent amounts of each drug exit the liver after administration of either drug. That is, the concentration-time profiles of both drugs are similar.

Intravenous and oral administration of prednisolone produce nearly the same result; however, this equivalence was not observed when prednisone is administered by the two routes. Prednisone concentrations were much greater after parenteral PO administration than after oral administration. Hence, the apparent clearance of PO was significantly

increased following oral administration, suggesting that PO possesses a high first pass clearance.

Prednisone vs. prednisolone administration

Whereas after oral administration of either PO or POH the concentration-time profiles of both drugs are similar, the same is not true after administration by the intravenous route. It was not until both prednisone and prednisolone were administered intravenously that this discovery was made. The profiles for the two drugs after intravenous administration differed with respect to prednisone concentrations. The ratio of steady state concentrations were a function not only of the dose but also of the drug administered (Figure 10.4).

Apparent systemic clearance

The apparent systemic clearance of prednisolone increases with the dose administered. Numerous groups have documented this, the first being Meikle et al. in 1975. Legler et al. (1982) studied this phenomenon under steady state conditions, where constant values were obtained rather than time-averaged estimates. In general, most investigators agree that a change in POH clearance is observed over the two fold increase in dose. A different result, however, was observed in Chapter 10, were a 10-fold change in dose was required to document a statistically significant increase in the Cl_{app} of POH.

Rose et al. (1981) first demonstrated the dose-dependent clearance of prednisone, where the clearance increased 4-fold over a 10-fold increase in the oral dose. Under steady state conditions, this was documented after intravenous PO infusion to healthy volunteers as reported in Chapter 10. Over the 10-fold dosage range, the clearance of PO increased 4-fold.

The apparent clearance of PO and POH is actually a complicated term, as defined in Chapter 2, and is a function of the model presented in Figure 2.1:

$$CL_{app,1} = CL_{10} + CL_{12}\left(\frac{CL_{20}}{CL_{20}+CL_{21}}\right) \quad (1)$$

$$CL_{app,2} = CL_{20} + CL_{21}\left(\frac{CL_{10}}{CL_{10}+CL_{12}}\right) \quad (2)$$

The apparent clearance can not be characterized by a single metabolic site or process, but is rather an average of the clearance processes occurring throughout the body. Due to the existence of organ-specific metabolism of these compounds, the four fundamental clearance values presented above have no explicit correlate *in vivo*, but express net metabolic processes. Only within an isolated organ can these values be estimated. One or more of these intercompartmental clearance values may be concentration-dependent and contribute to the observed whole body nonlinearities.

Apparent renal clearance

The apparent renal clearances of prednisone and prednisolone were shown to be nonlinear by Rose and coworkers (1981) who designed the first comprehensive urine analysis of drug excretion over a range of prednisone and prednisolone doses. It was difficult to conclude unambiguously that dose-dependency occurred with these results, as variability was very high. As early as 1959, studies in the endocrinology literature demonstrated that the excretion of these compounds was urine-flow dependent (Lloyd, 1959). This factor has been analyzed by a few laboratories, but neither urine flow rate nor urinary dose-dependency explain all of the variability. Therefore, another factor must be involved.

Restatement of hypothesis

It is impossible to predict plasma concentrations of prednisone and prednisolone in humans after intravenous or oral administration of either drug over a range of doses; it can

be expected that the compounds will demonstrate nonlinear pharmacokinetics, however. At present, these drugs are administered primarily based on the avoidance of side effects (Haynes et al., 1985). It is the goal of this project to develop a rational approach for administration of these compounds so that site-specific targeting may be achieved. As the target is commonly a particular organ or tissue, knowledge of organ-specific disposition is necessary. When pharmacodynamic measures of glucocorticoid activity are developed, it will then be possible to target a particular concentration of prednisolone to achieve a desired effect. This could minimize the severe side effects and toxicities of these compounds, in other words, optimize therapeutics by the minimization of dose administered and/or administration with drug delivery approaches.

This chapter combines all of the experimental and background material associated with this project. The underlying hypothesis is that the nonlinear disposition of PO and POH is a function of the organ-specific disposition of the glucocorticoids. Furthermore, *in vivo* analyses cannot help to elucidate the sources of these complexities since it is probable that multiple, reverse-acting processes are occurring simultaneously in various organs.

Animal studies were designed to better define the glucocorticoids' metabolic processes and characterize the organ-specific pathways. Using perfusion techniques and pharmacokinetic principles, the objectives were to determine the organ-specific extraction of PO and POH, and to characterize the organ-specific interconversion of the two compounds in the perfused rabbit liver, kidney and lung *in situ*. This information would allow the development of a model to explain the nonlinear disposition of prednisone and prednisolone *in vivo* in rabbits. This work might later be scaled up to humans through the use of physiologically-based pharmacokinetic modeling techniques.

The results from animal work would provide an understanding as to how interconversion depends on the drug and dose administered. The dose-dependent increases in the apparent clearance may then be correlated with specific metabolic events in a particular organ. The apparent renal clearance can be examined when renal arterial

concentrations are measured explicitly. Thus, an explanation and characterization of the multiple nonlinear processes could follow.

Review of results of animal studies

The first well-designed studies of the pharmacokinetics of the glucocorticoids were performed in humans. When complex dose-dependencies resulted, an animal model was sought which would demonstrate similar kinetic profiles as humans, particularly the dose-dependent increases in apparent clearance.

Results of tissue incubation studies suggested that different species expressed different and unpredictable metabolic characteristics (Monder and Shackleton, 1984). Furthermore, in *in vivo* studies, the rat (Boudinot and Jusko, 1986) and dog (Frey et al., 1980) demonstrated dose-dependent decreases in the apparent clearance, opposite to what was observed in humans (Table 11.1). The rabbit demonstrated an increase in the apparent clearance of both PO and POH with increasing concentrations (Unadkat and Rowland, 1985; Rocci and Jusko, 1981) and was selected for further study.

Effect of tissue disruption

The disposition of PO and POH in rabbit organs could have been examined in a number of tissue preparations. As was demonstrated in Chapter 4, the interconversion of prednisone and prednisolone is highly dependent upon the degree of integrity of the tissue studied. In general, oxidation was increased and reduction was reduced as tissue structure progressed from minces to homogenates to microsomes for the rabbit liver (Table 11.2). Based on these observations, the decision was made to retain as much normal tissue architecture as possible, resulting in the selection of organ perfusions for experimentation.

Table 11.1

Species comaprison of apparent systemic clearance

(ml/min per kg)

Species	Prednisone	↑, ↓ *	Prednisolone	↑, ↓ *	Author
Human	2.4 - 8.8	↑	11.0 - 13.5	↑	Hammarlund-Udenaes and Benet, 1986
	1.7 - 3.3	↑	8.8 - 12.4	↑	Rose et al., 1981
			1.1 - 2.5	↑	Legler et al., 1982
Rabbit			6.8 - 11.1	↑	Rocci et al., 1981
	30 - 72	↑			Ferry and Wagner, 1988
	49 - 80	↑	3.3 - 8.5	↑	Unadkat and Rowland, 1985
Rat			49 - 105	↓	Boudinot and Jusko, 1986
Dog			6.5 - 10.9	↓	Frey et al., 1980

Table 11.2. Effect of tissue disruption on the interconversion of prednisone and prednisolone in the rabbit liver

	% Conversion in 10 minutes		
	Mince	Homogenate	Microsomes
Oxidation of POH	18	77	81
Reduction of PO	18	22	3

Organ-specific disposition

Organ-specific disposition was suspected as the cause of the complex pharmacokinetics of prednisone and prednisolone. Incubation studies showed that there were indeed great differences between organs in the disposition of the glucocorticoids, which were subsequently confirmed with perfusion experiments. Some perfusion work had been performed with the glucocorticoids in the past, but the studies were not designed to obtain useful conclusions regarding the metabolism and interconversion of PO and POH. Even if such studies were widely available, comparison of these results would be difficult as experimental conditions varied greatly between laboratories. Here, studies were designed for three organs of the rabbit with standardized methods. Table 11.3 presents a summary of the results of the present and past perfusion experiments relevant to this discussion.

The enzymes which metabolize PO and POH are shared by cortisol, cortisone and corticosterone and can therefore can be expected to be distributed throughout the body. In fact, every organ of the body can interconvert these compounds and only the brain is unable to eliminate the glucocorticoids (Miyabo et al., 1973).

11.3

Results of relevent organ perfusion experiments
studying glucocorticoid interconversion and elimination

Species/Organ	Reference	Drug	Results
Rabbit Liver	Ch. 5	PO	E (PO) = 0.96; POH/PO = 0.5
	Ch. 5	POH	E (POH) = 0.49; no measurable PO.
Human Liver	Ch. 6	PO	E (PO) = 0.45; POH/PO = 0.08.
	Ch. 6	POH	E (POH) = 0.22; very low PO conc detectable
Rat Liver	Ch. 5	PO	E (PO) = 0.95; POH/PO = 0.36
	Ch. 5	POH	E (POH) = 0.63; no measurable PO.
Rabbit Kidney	Ch. 7	PO	E (PO) = 0.04; POH/PO = 0.01.
	Ch. 7	POH	E (POH) = 0.10; PO/POH saturable, ≈0.10
Rat Kidney	Rocci et al., 1981	POH	* E (POH) = 0.044; PO measurable
	Reach et al., 1977	cortisol	* cortisone measurable
Rabbit Lung	Ch. 8	PO	E (PO) = 0.05; POH/PO = 0.07.
	Ch. 8	POH	E (POH) = 0.07; PO/POH saturable, ≈0.005
Rabbit Lung	Brooks, 1981	cortisol	* CO/COH = 2.6
Rat Lung	Nicholas and Kim, 1975	cortisone	* CO/COH = 3.5
Guinea Pig Lung	Nicholas and Kim, 1975	cortisol	* CO/COH = 1.9
	Nicholas and Kim, 1975	cortisone	* 7% cortisol converted to cortisone

* studies performed in nonsteady state conditions;

measurements reflect values after two hours of recirculation;
CO/COH = ratio of cortisone to cortisol concentrations.

Species differences

It has been generally accepted that steroid metabolism varies substantially between species (Monder and Shackleton, 1984). The studies carried out here do not necessarily corroborate this belief. In incubation studies, the rabbit and human showed great similarity in their metabolic profiles in the liver and kidney (Chapter 4). Additionally, the liver perfusions in rat, rabbit and humans demonstrated great similarities when the clearance parameters were normalized to ml/min per $kg^{0.7}$ (Table 11.4), as described by Adolf (1949) in scaling biochemical processes between species.

Table 11.4. Comparative hepatic blood clearance from organ perfusion experiments.

Species	Prednisone ml/min/kg	ml/min/$kg^{0.7}$	Predisolone ml/min/kg	ml/min/$kg^{0.7}$
Human	6.3	19.4	3.6	11.2
Rabbit	16	19	8.6	10.3
Rat	114	75	76	49.7
Rat*	22.8	15	15.2	9.9

* clearance determined for normal flow rate instead of true perfusate flow rate.

Interconversion

The data in Figures 11.1 and 11.2 compare the interconversion ratios of PO and POH achieved in the three perfused organs of the rabbit. The reduction of perfused prednisone occurs predominantly in the liver, with about half of the perfused PO measurable as POH in the exiting perfusate. Oxidation occurs in both the kidney and the lung, in a saturable manner in both cases. The kidney is primarily oxidative. The lung possesses approximately equivalent bidirectional capacities at low doses, but the oxidative capacity is apparently saturable.

Irreversible elimination

The capacity for irreversible elimination differs between organs. This may be partially explained by the existence of numerous metabolic pathways available to the glucocorticoids (see Chapter 1). Figure 11.3 compares the fraction of extraction accounted for by irreversible loss processes in the three perfused rabbit organs. Prednisolone was eliminated but not aparently oxidized to PO in the liver; half of the extraction of PO was irreversible. In the kidney, more prednisone was eliminated than was reduced to POH; prednisolone was preferrentially converted to PO versus eliminated unchanged. The lung expressed equivalent elimination capacity toward both compounds, slightly greater than the conversion capacities.

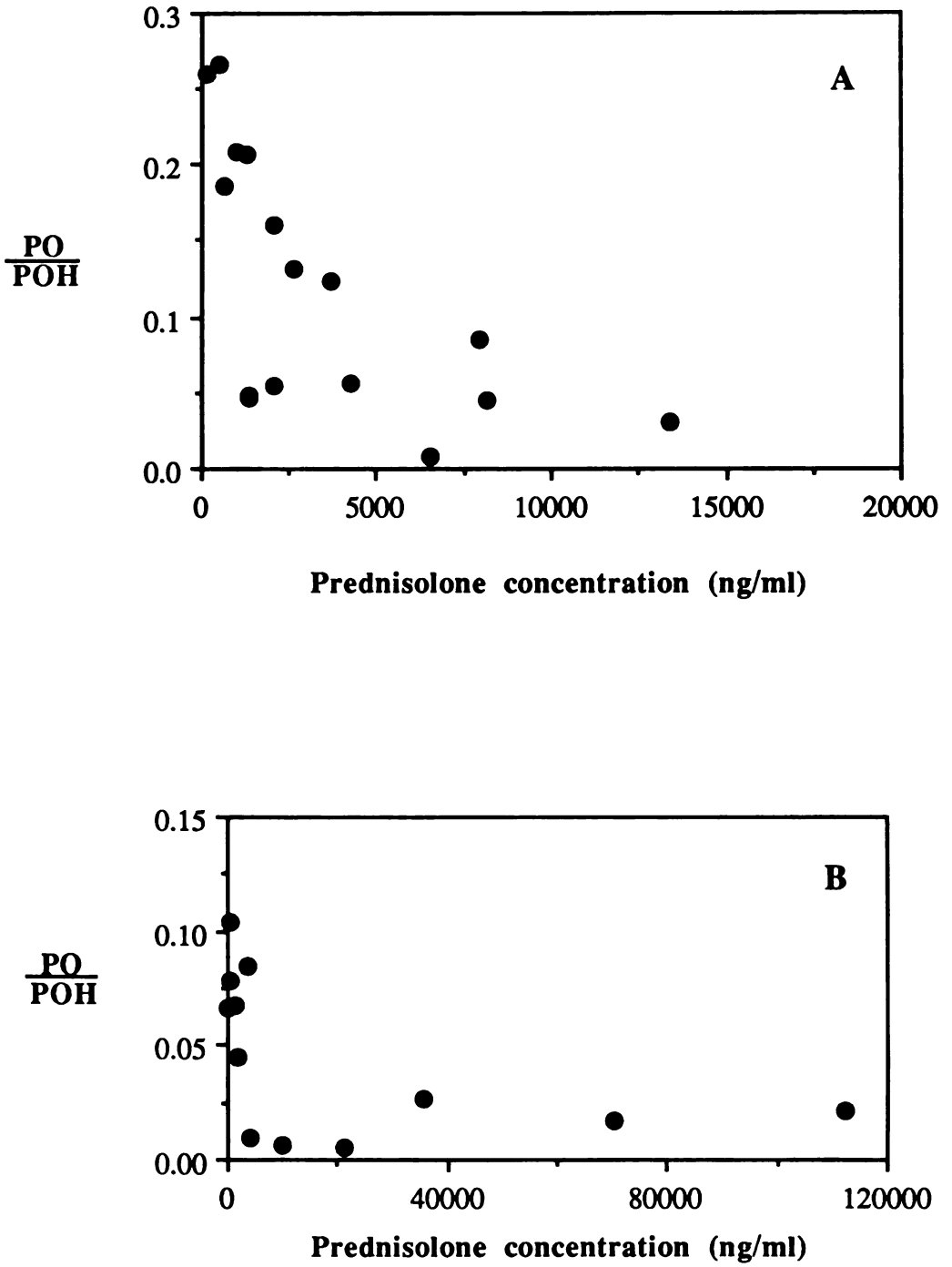

Figure 11.1. Ratio of produced prednisone to perfused prednisolone for the rabbit kidney (A) and lung (B) perfused under steady state conditions.

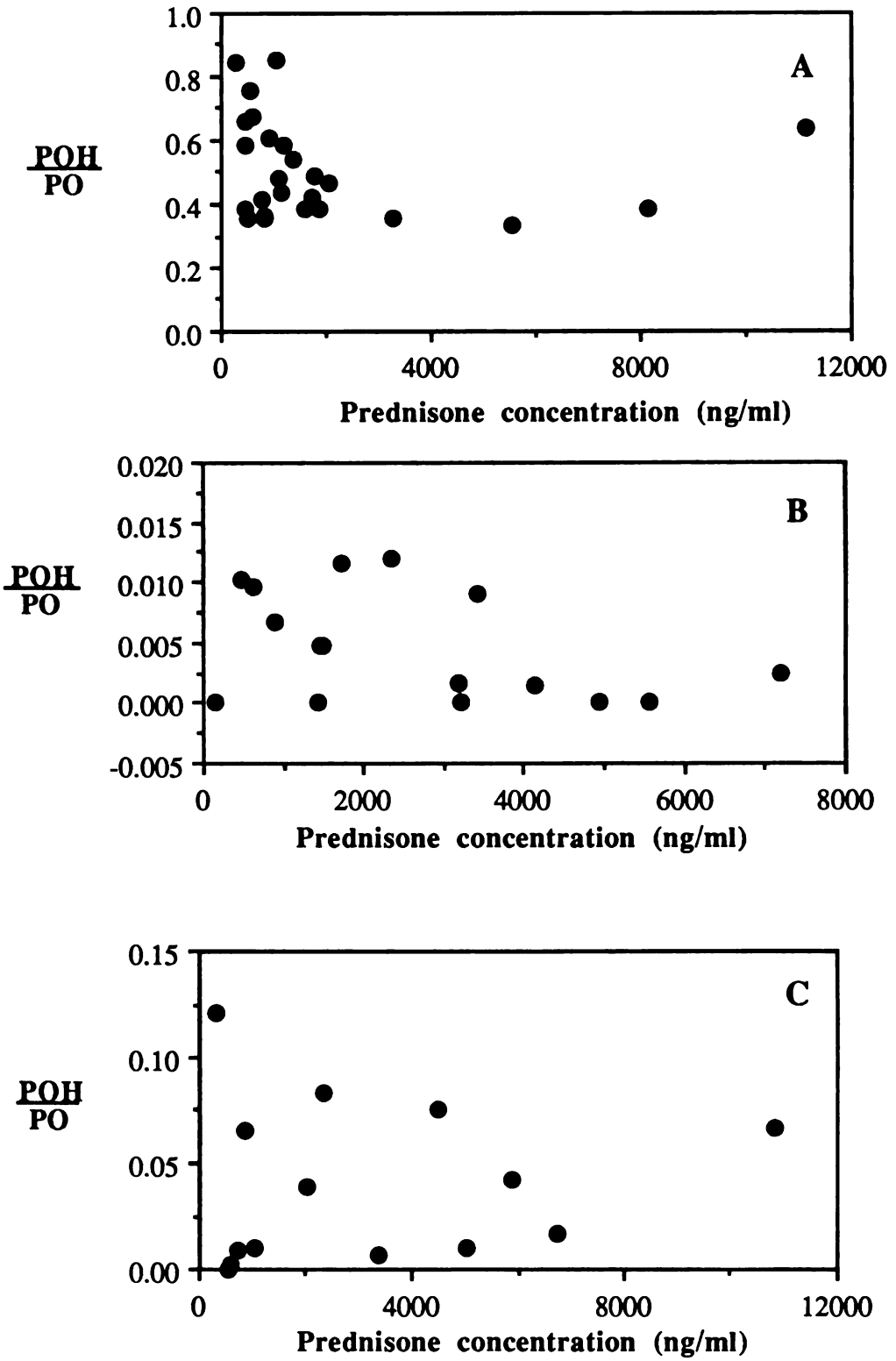

Figure 11.2. Ratio of formed prednisolone to perfused prednisone in the rabbit liver (A), kidney (B) and lung (C) perfused under steady state conditions.

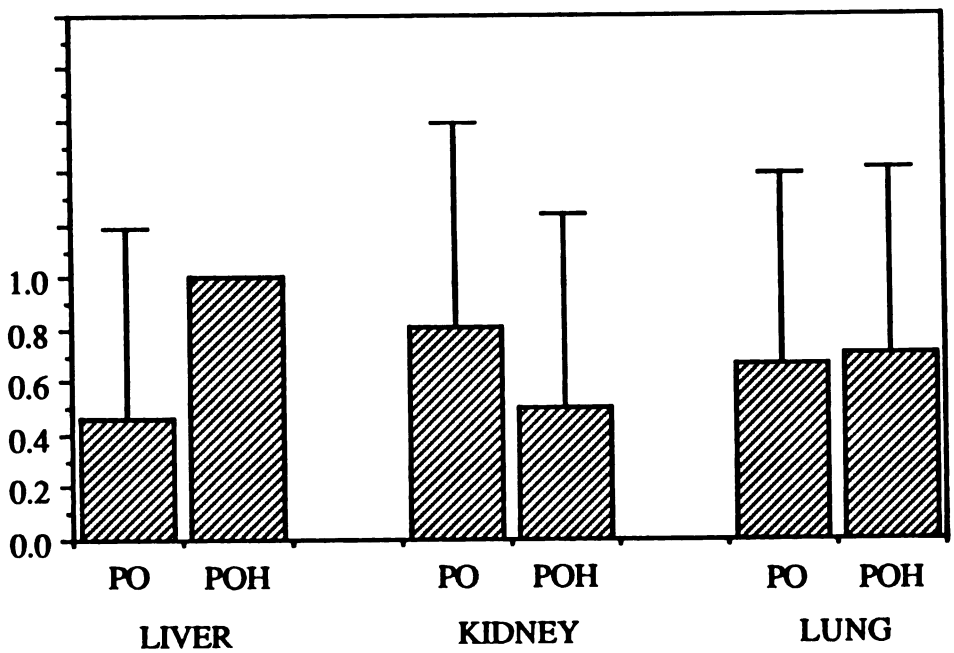

Figure 11.3. Fraction of drug eliminated which was irreversibly eliminated in the perfused rabbit organs, calculated under steady state conditions.

The relative significance of organ-specific processes may be approximated: as the extraction was greatest in the liver (Table 11.3), it would be expected that the greatest proportion of irreversible elimination processes occur hepatically. The kidney and lung were approximately equivalent in their magnitude of contribution to elimination and conversion, due to their similar extraction and fractional elimination profiles.

Hepatic disposition

Glucocorticoid disposition has been infrequently studied in the perfused liver. The apparent unidirectional conversion (reduction) was not expected, as incubated liver minces, microsomes and homogenates exhibited conversion of POH to PO (refer to Table 11.2).

Oxidation of POH to PO probably occurs in the perfused liver, but the drug formed may be immediately back converted so that reduction apparently predominates. Because oxidation occurs *in vivo*, it therefore must occur extrahepatically.

The liver has been implicated as the source of the nonlinear disposition of the glucocorticoids *in vivo* (Frey et al., 1980; Legler et al., 1982), and it is recognized as the most metabolically active organ toward these compounds. However, the liver is not the source of the apparently saturable formation of PO *in vivo*.

Apparent renal clearance

The kidney of the rabbit excretes, metabolizes and interconverts the glucocorticoids PO and POH. It is primarily oxidatively oriented in interconversion, but this capacity of the kidney is saturable. Reduction occurs to a lesser extent and is apparently linear. A distinction is made here regarding the difference between renal and urinary clearance: urinary clearance is the parameter measured *in vivo* with the collection of urine samples. It is only a portion of renal clearance which also includes metabolic contributions.

Analysis of the urinary clearance of PO and POH is complicated by renal metabolism. For this reason, this parameter must be designated as apparent urinary clearance. The excretion of both compounds is diuresis-dependent, as demonstrated in Chapter 7.

Pulmonary disposition

The pulmonary extraction of PO and POH is low but due to the high blood flow delivered to the lung, this organ significantly contributes to total body disposition. The lung was originally chosen to represent a poorly eliminating organ, but its activity rivals that of the kidney. The rabbit lung is reductively oriented toward the glucocorticoids; oxidation occurs and is apparently saturable. The lung produces a metabolic profile which is close to that of the whole body measured intravenously, with reductive predominance

and saturable oxidation. Collins and Dedrick (1982) demonstrated that relatively low pulmonary metabolic activity can produce a large reduction in arterial drug concentrations. The lung and liver are in series, which results in a synergistic increase in total body clearance.

Reinterpretation of human results

With the combined observation of species similarity in hepatic and renal metabolism and knowledge of the organ-specific disposition of these compounds, it is now possible to propose the source of the nonlinearities observed *in vivo* in humans with these compounds.

Interconversion

Two aspects of the interconversion of prednisone and prednisolone in the human must be considered: the reductive predominance and the apparently nonlinear oxidative formation of PO.

The liver is the most reductive of the three perfused organs and its activity is probably sufficient to account for most of the reduction of PO to POH observed *in vivo*. Oral administration of prednisone and prednisolone (Chapter 9) results in similar profiles, probably as a result of first pass conversion of a substantial fraction of PO to POH. One half of the perfused PO was recovered as POH in the perfused rabbit, rat and human liver perfusions.

Prednisone concentrations resulting from intravenously administered prednisone are elevated for extended periods of time relative to those observed after intravenous prednisolone dosing. By avoiding the first pass, prednisone concentrations are only slowly converted to prednisolone *in vivo*.

A maximum prednisone concentration was achieved *in vivo* upon administration of prednisolone to humans, as discussed above. It does not appear from the rabbit, rat and human liver perfusion experiments that this observation is due to saturation of hepatic

oxidative conversion processes. A high fraction of the prednisone present in the portal vein or hepatic artery is extracted and reduced to POH; little or no PO is produced from perfused prednisolone.

The liver probably has the greatest glucocorticoid elimination capacity of any organ of the body. Although some controversy exists, liver disease does not greatly effect the kinetics of PO and POH (In liver disease, the ability to convert orally administered PO to POH is well-conserved, most authors agree: Araki et al. (1966), Powell and Axelsen (1972), Schalm et al. (1977), Uribe et al. (1978) and Davis et al. (1978).). NADPH-linked steroid dehydrogenases do not appear to be affected by cirrhosis, hepatitis or other commonly encountered hepatic diseases. Cytochrome P_{450}, which is commonly affected in such conditions, plays a minor role in elimination of these compounds. Interconversion is catalyzed by the dehydrogenases and the bidirectional reaction remains relatively unaffected in these diseases.

First-pass effect

The opportunity to perfuse the human liver provided very valuable information regarding the most metabolically active organ in humans. The results were quite similar to those of the rabbit and rat liver despite different storage and perfusion conditions between the three preparations. Greater confidence is thus afforded in extrapolating the rabbit liver perfusion results to the interpretation of data in humans.

The discussion of the first pass effect is directed primarily toward the liver, although the intestinal wall possesses some significant effects on metabolism of the glucocorticoids, as well (Macdonald et al., 1983). Prednisone and prednisolone are affected differently by hepatic first-pass processes. Orally administered prednisolone produces AUC's nearly equivalent to those after intravenous administration. In contrast, prednisone's AUC is route-dependent yielding increased concentrations of POH after first pass through the liver (Chapter 9; Ferry et al., 1988).

The liver is the most important organ for reduction, based on the results of experiments in rabbits. The reductive activity in terms of ratios of POH to perfused PO concentrations is 10-fold greater than found in the lung and 100-fold greater than that of the kidney (Figure 11.1).

Prednisone and prednisolone are bioequivalent at doses of either 10 or 25 mg due to the high reductive and unidirectional capacity of the liver. The rabbit and rat livers extracted nearly all of the PO and converted approximately half to POH; half of the POH was extracted and none was measurable as PO.

Apparent systemic clearance

The source of the nonlinear pharmacokinetics of PO and POH is difficult to localize. The increases in the apparent clearance with dose reflect a nonlinearity in one or more of the intercompartmental clearances of one or more organs. From equations 1 and 2 above, it can be seen that a decrease due to saturation of a particular parameter may be responsible for the increase in apparent clearance.

Collins and Dedrick (1982) have described the effect of pulmonary clearance on total body clearance. Because the lungs are in series and not in parallel, the influence on systemic drug disposition is great. The apparent clearance with concurrent pulmonary (P), hepatic (H) and renal (R) metabolism is:

$$CL_{app} = \frac{CL_P}{(1 - E_H - E_R)} + CL_H + CL_R \qquad (3)$$

where CL_P, CL_R and CL_H are apparent organ clearances, and E_P, E_R and E_H are organ extraction ratios. Table 11.5 provides a summation of the organ clearances of the three organs perfused in the rabbit. The apparent clearance of PO in the rabbit was 49.1 ml/min per kg. This value is consistent with that calculated by two different laboratories: Ferry

and Wagner (1988) and Unadkat and Rowland (1985) measured the apparent clearance of PO in the rabbit to be between 30 and 72 and 49 and 80 ml/min per kg (Table 11.1), respectively.

The apparent clearance of POH was calculated to be 18.9 ml/min/kg. This estimate compares favorably with the experimental values presented in Table 11.1. Rocci and Jusko (1981) measured 6.8 to 11.1 ml/min per kg and Unadkat and Rowland (1985) measured 3.3 to 8.5 ml/min per kg for apparent clearance of prednisolone.

The calculated apparent clearance of PO and POH based on pulmonary, hepatic and renal contributions are very close to those of the literature. However, the apparent clearance of PO could be increased slightly, and that of POH reduced slightly to achieve better comparisons to literature values. An organ which converts PO to POH (CL_{12}) would bring both of these clearance values close to the systemic clearances measured *in vivo* in rabbits. One organ that might be important for the rabbit is muscle, which constitutes a large mass in the rabbit. Figure 11.4 is the physiologic pharmacokinetic model which can be used to model the pharmacokinetics of PO and POH in the rabbit, with eventual scale-up to the human. The "other" organ in this case would probably have a relatively large CL_{12}.

Table 11.5. Combined organ clearances (ml/min per kg)

Clearance	Prednisone	Prednisolone
CL_H	16	8.6
CL_R (2)*	1.8	3.2
CL_P	2	1.9
sum CL	19.8	13.7
CL combined*	49.1	18.9
CL_{app}	**30 - 80**	**3.3 -11.1**

* renal clearance for one kidney doubled
** clearances of organs in series combined according to equation 3 after Collins and Dedrick (1982)

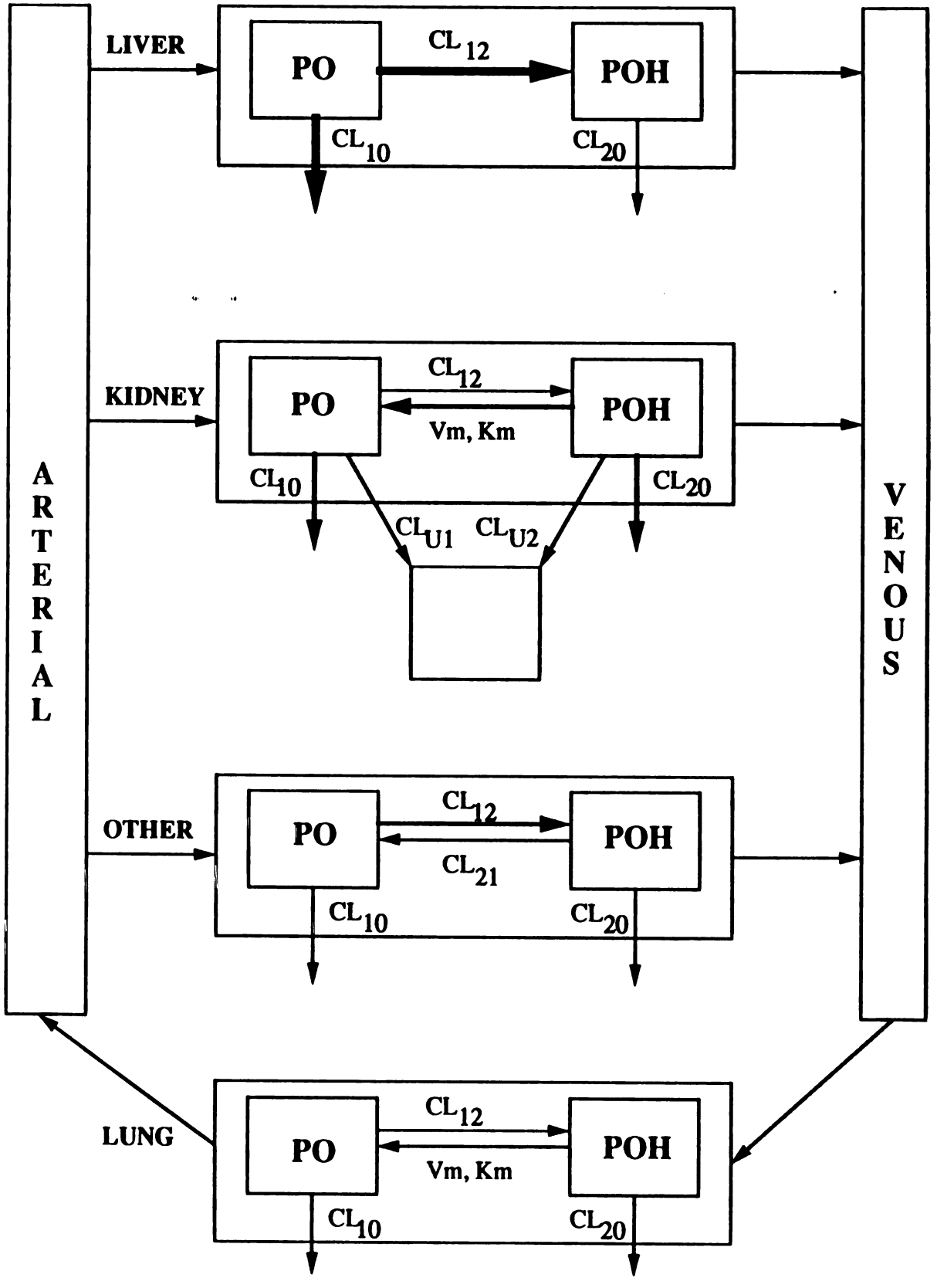

Figure 11.4. Physiologically based pharmacokinetic model of the disposition of prednisone and prednisolone in the rabbit.

Apparent renal clearance

The measured apparent renal clearance in humans is complicated by renal metabolism and is probably not reflective of actual renal excretory function. The concentrations of PO and POH perfusing the renal capillary beds are not necessarily the measured arterial (antecubital vein) concentrations because of interconversion within renal tissue. It is not possible to determine the contribution of renal metabolism without performing micropuncture experiments. Secondly, reabsorption occurs along the nephron and has been demonstrated to be diuresis-dependent in rat and rabbit kidney perfusion experiments (Rocci et al., 1981; Chapter 7). Failure to document this characteristic in the human studies may reflect the uncertainty in the C_{ss} and AUC measures of drug concentration.

General accomplishments

The objecitves of this work were to elucidate the nonlinear pharmacokinetics of the glucocorticoids prednisone and prednisolone. Through the use of organ perfusion in the rabbit, details regarding the organ-specific disposition of PO and POH have been obtained. It was our hypothesis that the dose-dependent kinetics of these compounds were a result of multiple factors, including saturable protein binding of prednisolone to corticosteroid binding globulin, metabolic interconversion and organ-specific interconversion and elimination. The factor of organ-specific metabolism had not previously examined because it was assumed that the liver was the only significant contributor to the elimination of PO and POH.

The results obtained here are significant in that prednisone and prednisolone are commonly prescribed for disease of one organ, as in organ transplantation or imflammatory disease. With knowledge of the specific hepatic, renal and pulmonary disposition of PO and POH, rational dosing of these compounds may follow. Optimization of drug therapy is the ultimate goal of this research. When combined with pharmacodynamic measures of

glucocorticoid function, it will be possible to target a specific concentration of the active moiety, prednisolone. This strategy can be further optimized by the adjunctive use of drug delivery systems which may be placed proximal to the target site.

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