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METABOLISM AND KINETICS OF SPIROLACTONES

AND ALDOSTERONE

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

PHARMACEUTICAL CHEMISTRY

in the

GRADUATE DIVISION

(San Francisco)

of the

UNIVERSITY OF CALIFORNIA

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ABSTRACT

The spirolactones are competitive antagonists of mineralocorticoids. However, it is not known what actions contribute to therapeutic results when spirolactones are used in a number of disease states with largely different etiologies. Further, the molecular mechanisms of extrarenal and toxic effects are poorly understood.

The goal of this dissertation was to investigate the metabolism of the spirolactones as well as their effects on the kinetics of endogenous aldosterone, since both might contribute to the effects of spirolactones. Studies in this dissertation include, the tissue distribution, and metabolism of the spirolactone, canrenoate-K, affinity of canrenoate-K metabolites to the cytoplasmic receptors for aldosterone and the effects of spironolactone and canrenoate-K on production and elimination of aldosterone.

The metabolism of spironolactone is extensive in man. The dethioacetylated canrenone, appears to be a major metabolite. It has an intact gamma-lactone ring, and is present in enzymatic equilibrium with the gamma-hydroxy carboxylic acid canrenoate. Metabolites may account for the clinically observed toxicity of spironolactone. Therapeutic use of canrenone and canrenoate would eliminate the chemically labile and potentially toxic sulfurous metabolites of spironolactone. However, little information is available about the further metabolism and tissue distribution of canrenone and canrenoate.

Tissue distribution studies on the distribution of

labelled canrenoate-K as well as specific fluorescence assays for canrenone and canrenoate were performed in rabbits. Canrenone was concentrated about 10-fold in tissues when compared to plasma while no such preferential uptake was found with canrenoate. Differences between measurements of radioactivity and fluorescence of canrenone and canrenoate indicated extensive formation of metablites and thus retention in the rabbit. Therefore the further metabolism of canrenoate-K was studied.

Two new major metabolites of spironolactone and canrenoate-K were isolated from rabbit liver and plasma. The structure of one metabolite was determined to be 20-hydroxy-canrenone. The second metabolied contained two oxygen atoms of unknown location and nature. Since these metabolites were identified as major metabolites in the rabbit, their contribution to the antimineralocorticoid effect of canrenoate was examined. An _in vitro_ test measuring affinities of these metabolites to the aldosterone cytosol "receptor" was used to assess their potential pharmacological activity. The metabolite 20-hydroxy-canrenone possessed moderate affinity in this _in vitro_ test system.

The spirolactones can affect the kinetics of endogenous aldosterone by stimulating or inhibiting production and elimination of aldosterone, as well as effecting cytosol receptor binding of the hormone. Conflicting results previously reported on the kinetics of aldosterone in the presence of spirolactones, may be due in part to non-specific analytical assay techniques used to measure aldosterone.

Concentrations of aldosterone in plasma were measured by a radioimmunoassay method in rabbits, dogs, and in man following single doses of spironolactone and canrenoate-K. Erratically high values of aldosterone were obtained after canrenoate-K was administered to adrenalectomized dogs. The assay for aldosterone was interferred with by 20-hydroxy-canrenone. A procedure, specific for aldosterone in the presence of this metabolite was developed. Following single doses of spironolactone and canrenoate-K, the concentrations of aldosterone in plasma were unchanged in humans and in dogs, and decreased in rabbits.

Elimination kinetics of aldosterone during treatment with spirolactones were measured. Preliminary results of this pilot study indicate no changes in clearance values of aldosterone.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

LISTING OF FIGURES i

LISTING OF TABLES iv

ABSTRACT v

Statement of OBJECTIVES 1

SECTION I: INTRODUCTION 2

ALDOSTERONE 2

PHARMACOKINETICS OF ALDOSTERONE 9

Metabolism of Aldosterone 11

Distribution of Aldosterone 14

THE PHARMACOLOGY AND PHYSIOLOGY OF ALDOSTERONE 15

The Molecular Mechanism of Action of Aldosterone 17

Extrarenal Effects of Aldosterone 19

THE SPIROLACTONES 22

Pharmacology of Spirolactones 24

Extrarenal Effects of the Spirolactones 26

Biochemical Pharmacology of Spirolactones 28

Clinical Uses 29

Clinical Problems Associated with Spirolactones 30

SPIROLACTONE PHARMACOKINETICS AND BIOPHARMACEUTICS 31

Bioavailability 31

Spirolactone Potency 35

Methods for Measurement 36

Therapeutic Plasma Levels of Canrenone 37

Tissue Distribution 38

Spirolactone Metabolism 40

Spirolactone Elimination 44

BIOCHEMICAL INTERACTIONS OF SPIROLACTONES WITH ALDOSTERONE 46

SECTION II. TISSUE DISTRIBUTION OF TRITIATED CANRENOATE-K IN RABBITS

Abstract 49

Introduction 49

Experimental 51

1. Materials 51

2. Animal Protocols 52

3. Analytical Procedures 53

4. Calculations 54

Results and Discussion 56

SECTION III. CHEMICAL STRUCTURE AND ALDOSTERONE RECEPTOR AFFINITY OF CANRENOATE-K METABOLITES IN RABBITS

Abstract 66

Introduction 66

Experimental 68

1. Chemicals and Radiochemicals 68

2. Apparatus 68

3. Analytical Procedures 69

4. Aldosterone Cytosol Receptor Binding Assay 70

Results 72

Characterization of Metabolites 72

Results of Renal Cytosol Binding Studies 81

Discussion 83

SECTION III. THE EFFECTS OF SPIROLACTONES ON CONCENTRATIONS AND PHARMACOKINETICS OF ALDOSTERONE

Abstract 86

Introduction 86

Experimental 90

1. Aldosterone Plasma Radioimmunoassay 90

2. Modified Aldosterone Plasma Radioimmunoassay 90

in the presence of Spirolactone Metabolites

3. Administration of Spironolactone and Canrenoate-K 96

4. Administration of ^3H-Canrenoate-K to Rabbits and Isolation of Metabolites 97

5. Cross-Reactivity of Spirolactones to Aldosterone Antibody 98

6. Fluorescence Measurements of Canrenone and Canrenoate 98

7. Apparatus 98

8. Materials 98

Results 99

Spirolactone Cross Reactivity to Aldosterone Antibody 103

Modified, Specific Aldosterone Radioimmunoassay 105

Cerebral Spinal Fluid Analysis 113

Discussion 114

SECTION V. Pilot Study: ELIMINATION KINETICS OF ALDOSTERONE DURING LONG TERM AND SHORT TERM SPIROLACTONE ADMINISTRATION

Abstract 117

Introduction 117

Experimental 119

1. Materials 119

2. Animal Procedures 119

3. Preparation of Doses 120

4. Analytical Procedures 121

Results and Discussion 124

SECTION V. CONCLUDING STATEMENT 129

SECTION VI. REFERENCES 131

ACKNOWLEDGEMENTS

These studies were supported in part by a research grant from G.D. Searle Co., Chicago, by a grant from Deutsche Forchungsgemeinschaft, Sonderforschungsbereich 51: Medizinische Molekularbiologie und Biochemie, and in part by grants GM 16496 and GM 0001 from the National Institute of General Medical Sciences, National Institutes of Health.

I thank Drs. Thomas N. Tozer, and Kenneth L. Melmon for their interest in my studies and their continued support.

I sincerely thank my research advisor, Dr. Wolfgang Sadée, for his patience, guidance and friendship over the years.

Finally, there are not words enough for me to thank my husband, Claude Finn, for his continued encouragement and assistance. I dedicate this dissertation to him.

***To Claude!!!!!

Listing of Figures

Figure 1: Structures of Aldosterone 3

Figure 2: Biosynthetic Pathway of Aldosterone 5

Figure 3: Structures of Some Experimentally Used Spirolactones 23

Figure 4: Structures of Known Spirolactone Metabolites 43

Figure 5: Standard Curve for the Fluorescence Procedure Used to Measure Spirolactone Levels 55

Figure 6: Plasma Elimination Time Curves for Canrenone and Canrenoate 57

Figure 7: Levels of total ^{3}H-radioactivity measured by scintillation counting for all tissues analyzed and for plasma 59

Figure 8: Concentrations of total ^{3}H-radioactivity as measured by liquid scintillation counting, and concentrations of canrenone (II) and canrenoate (III) measured by a specific fluorescence assay in rabbit liver 60

Figure 9: Concentrations of total ^{3}H-radioactivity as measured by liquid scintillation counting, and concentrations of canrenone (II), and canrenoate (III) measured by a specific fluorescence assay in rabbit plasma 61

Figure 10: Plasma and organ concentrations of Canrenone (II). 62

Figure 11: Plasma and organ concentrations of Canrenoate (III) 64

Figure 12: Ultra Violet Spectra of Metabolite M_B, Canrenone, and Metabolite M_A (Absorbance vs nm.) 75

Figure 13: Tritium Exchange Scheme 76

Figure 14: Fragmentation during Electron Impact-Mass Spectrometry showing loss of the C-17 side chain 77

Figure 15: Thermolytic dehydration of M_B to give Compound IV 78

Figure 16: 360 MHz Nuclear Magnetic Resonance Spectra of Canrenone, Compound IV, and Metabolite M_B 80

Figure 17: Metabolite M_B 81

Figure 18: Structures of Spironolactone, Canrenone and Canrenoate-Potassium 87

Figure 19: Column Retention (LH-20) Sephadex of Spirolactones and Aldosterone 92

Figure 20: Standard Curve For the Aldosterone Radioimmunoassay 94

Figure 21: Antibody Dilution Curve for the 21-hemisuccinyl-BSA antibody for Aldosterone 95

Figure 22: Aldosterone Plasma Levels in Rabbits Dosed with spironolactone (orally) and canrenoate-K (IV) 101

Figure 23: LH-20 Sephadex Column Retention for spirolactones, aldosterone and the new spirolactone metabolites 102

Figure 24: Cross Reactivity Curves for the Spirolactones, Spironolactone (I), Canrenone (II), Canrenoate (III), and the new metabolites 104

Figure 25: Aldosterone Plasma Levels in a rabbit dosed with 50 mg canrenoate-K (III) IV as measured by the Ito et al., method and by the modified radioimmunoassay procedure 107

Figure 26: Aldosterone Plasma Levels in a dog dosed with 50 mg/kg canrenoate-K IV as measured by the Ito et al., method and by the modified radioimmunoassay procedure 108

Figure 27: Aldosterone Plasma Levels in man (400mg spironolactone, orally) as measured by the Ito et al., method and by the modified radioimmunoassay procedure 109

Figure 28: Canrenone (II) and Canrenoate (III) levels in rabbit 110

Figure 29: Canrenone (II) and Canrenoate (III) levels in a dog 111

Figure 30: Canrenone (II) and Canrenoate (III) levels in man 112

Figure 31: Standard Curve for the $^{3}H/^{14}C$ Double-Isotope Assay with 300 dpm (25λ) ^{14}C added 122

Figure 32: Standard curve for the $^{3}H/^{14}C$ Double-Isotope Assay with 600 dpm (50λ) ^{14}C added 123

Figure 33: Steady State Curves observed Following infusions of ^{3}H-aldosterone in control experiments in rats 125

Figure 34: Prorenoate 129

Listing of Tables

Table 1:	Factors Affecting Aldosterone Biosynthesis (short-term and perfusion experiments).	7
Table 2:	Factors Affecting Aldosterone Biosynthesis (Long-term and disease states)	8
Table 3:	Comparison of Some Parameters of the Spirolactones, Spironolactone, Canrenone and Canrenoate	33b
Table 4:	Counts per minute in Kidney Homogenate	56
Table 5:	Levels of Canrenone and Canrenoate in plasma and liver at 2 hours following administration of Canrenoate-K to rabbits.	63
Table 6:	Outline of the Kidney Slice Experiments.	71
Table 7:	Electron Impact Mass Spectra of Canrenone, Compound IV, M_A and M_B.	74
Table 8:	Molecular ion cluster in the Electron Impact Mass Spectra of Canrenone, Compound IV, and M_B prior to and after deuterium exchange.	78
Table 9:	Clearance Values of Aldosterone in Rats	128

OBJECTIVES

This investigation was designed to examine the physiological disposition of the 17-gamma spirolactones and to study the effect of spirolactones on aldosterone kinetics. This was accomplished by dividing the work into four specific objectives:

1. To determine the tissue distribution of tritiated canrenoate-potassium in rabbits (1)

2. To elucidate the chemical structure of canrenoate-potassium metabolites isolated from rabbit liver and plasma and to determine the affinities of metabolites to the aldosterone rat kidney cytosol receptor *in vitro* (2)

3. To determine the effects of spirolactones on endogenous aldosterone levels and kinetics (3)

4. To determine the effect of spirolactones on the elimination kinetics of aldosterone.

INTRODUCTION

ALDOSTERONE

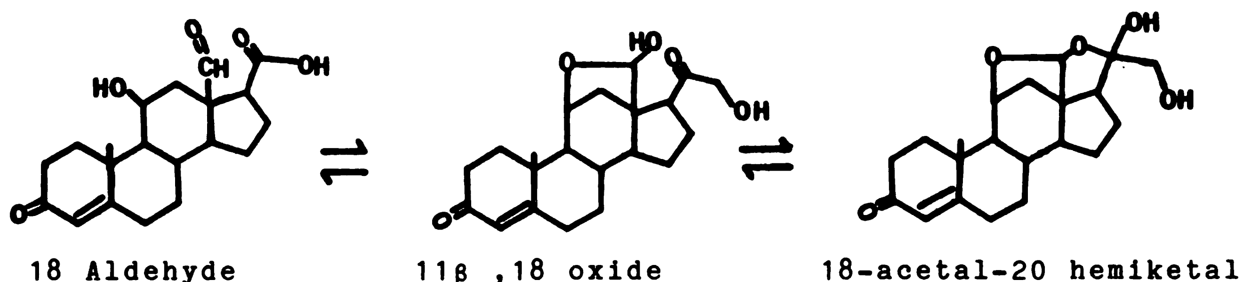
The vital importance of the adrenal gland has been recognized since 1855 but not until some seventy years later was the function of this organ in the regulation of mineral metabolism observed (4,5,6,7,8). Evidence pointed to an adrenal hormone, but it was not until 1953 that discovery of this substance was finally made by Simpson and Tait (9,10,11,12), Mattox et al., (13) and one year later by Leutscher (14). This substance possessed sodium retaining and kaliuretic activity (9) and was called "electrocortin." Chemical structure of this compound was elucidated in 1954 and it was found to contain an aldehyde group in the 18 position of the steroid unlike the methyl group usually seen with most steroid compounds (12,10). The name aldosterone was adopted for this substance in lieu of "electrocortin" (10). The d-isomer is the naturally occuring hormone (15).

A mineralocorticoid is capable of stimulating active sodium transport across the epithelium at physiological concentrations (5). Aldosterone, 11β ,21-dihydroxy-30,20-dioxopregn-4-ene-18-al lactal (16), a $C_{21}H_{28}O_5$ steroid, possesses about 500 times the sodium retaining activity of cortisol and between 50-100 times that of 11-deoxycorticosterone (15,12,17,18,11,13). Aldosterone has been isolated from many species (5,19,12,13) and has also been found in the peripheral blood of lower vertebrates (12).

Racemic aldosterone was synthesized in 1955 (20) and the L-isomer has been shown to have less activity than the naturally occuring D-form (12). Synthesis of pure D-aldosterone was accomplished by Vischer et al., in 1956 (21). Daux and Hauptman found by x-ray analysis that aldosterone in a monohydrated crystalline form existed as the 18-acetal-20-hemiketal isomer (22). Aldosterone equilibrates in three different forms; the 18 aldehyde, the 11β, 18 oxide, and the 18-acetal-20 hemiketal form (23,24,12,25,10,22) as seen in Figure 1.

FIGURE 1

Three Forms of Aldosterone



18 Aldehyde 11β ,18 oxide 18-acetal-20 hemiketal

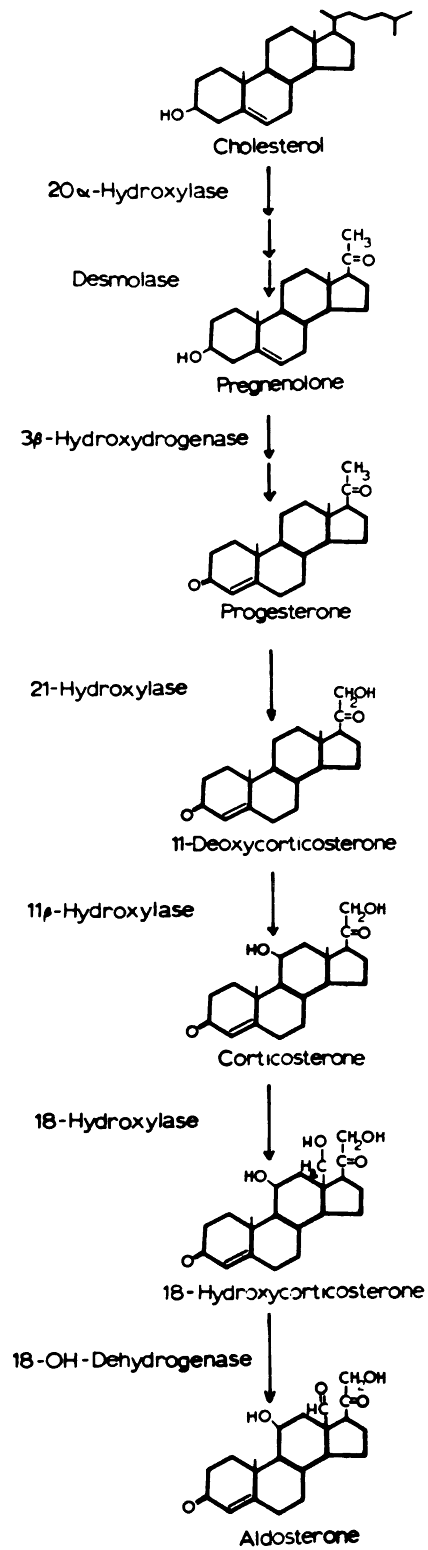
The biosynthesis of aldosterone in man as well as in most other species is accomplished by the zona glomerulosa, the outermost region of the adrenal cortex (12,26). Under some circumstances and in some species aldosterone can be synthesized in other zones of the adrenal cortex, and by other organs of the body (12,26). Biosynthesis of aldosterone utilizes a variety of substrates, for example, progesterone, deoxycorticosterone, cholesterol, 11β -hydroxycorticosterone etc. depending upon the species (19,12).

Substrate to product conversion in different species was used in an attempt to work out the biosynthetic pathway for aldosterone (12). Reviews of this literature are found in the following references (12,26).

Cytochrome P-450 is involved in aldosterone synthesis by adrenal cortical mitochondria (26,27,28), as shown by the involvement of a carbon monoxide combining substance absorbing light with a maximum of 450 mu in the 18-hydroxylation reaction of deoxycorticosterone. The route by which aldosterone is formed from corticosterone may involve the C-18 methyl group of corticosterone going to the C-18 aldehyde group of aldosterone by way of the C-18 alcohol group of 18-hydroxycorticosterone (27,28), Figure 2. Cytochrome P-450 is required for other reactions in aldosterone biosynthesis, such as 11β-hydroxylation (28,29). It should be recognized that cytochrome P-450 reactions are a vital part in aldosterone biosynthesis, and spirolactones represent potential inhibitors of this enzyme.

FIGURE 2

Biosynthetic Pathway of Aldosterone



A number of factors influence aldosterone biosynthesis and secretion (15,19,26,30,12,5). Lists of some of these factors are provided in Tables 1 and 2. Reviews in this area can be found in the following references (15,19,26,12,5). Four of these many factors have been widely investigated, namely the renin-angiotensin-aldosterone system, plasma potassium, and plasma sodium concentration and adrenocorticotropic hormone (30,15,19,26,5). The spirolactones also influence aldosterone biosynthesis and secretion (31). Some of the substances listed in Table 1 are capable of both stimulation and inhibition of aldosterone production and secretion depending upon the species used for the experiments and the conditions under which the experiments are performed. The mechanism by which even classical stimulating substances such as ACTH act is yet to be fully understood.

TABLE 1

Factors Affecting Aldosterone Biosynthesis and Secretion
(as determined by short-term incubation or perfusion experiments)

A. Stimulating Factors

Angiotensin I
Angiotensin II
Renin Extract
Monovalent Cations
Bivalent Cations (Ca^{++}, Mg^{++})
ACTH
Serotonin
Prostaglandin A_1, A_2, E_2 (32,33,5)

B. Inhibitors of Aldosterone Biosynthesis

1. Inhibitors of Enzymes Involved in Aldosterone Biosynthesis

Metopyrone
SU 8000
SU 9055
SU 10603
SU 4885
Cyanoketone
Triparanol (Mer-29)
Aminoglutethimide

2. Inhibitors of Protein Synthesis-Which also inhibit Aldosterone Production

Actinomycin D
Cycloheximide
Puromycin

3. Steroid Hormones which inhibit Aldosterone Biosynthesis

18-Hydroxycorticosterone
cortisol
cortisone
19 nor-testosterone
19 hydroxytestosterone
testosterone

4. Other Substances which inhibit Aldosterone Production

ouabain
Spirolactones

TABLE 2

Factors which affect aldosterone biosynthesis and secretion (as determined by long-term experiments and disease states)

Alterations in sodium balance
Alterations in potassium balance
Exogenous angiotensin and renin
Renal hypertension
Malignant Hypertension
Pheochromocytoma
Blood Loss
Bilateral nephrectomy
Hypophysectomy and hypopituitarism
Leisons in the central nervous system
Mineralocorticoids
Glucocorticoids and ACTH
Estrogens and Progestogens
Heparin and Heparinoids
o,p' DDD-dichlorodiphenyldichloroethane
Spirolactones

PHARMACOKINETICS OF ALDOSTERONE

A pharmacokinetic model for aldosterone was proposed by Tait et al., (34) which consisted of an "inner pool" (extracellular space and part of the intracellular space) and an "outer pool" (the rest of the intracellular space). This model was derived from disappearance curves of radiolabelled aldosterone given intravenously to normal subjects (34,12). With this model the calculated V_1 for aldosterone was found to be 20-30 liters, and the $V_1 + V_2$ about 40 liters (34). The alpha half-life of aldosterone in man is about 14 minutes while the beta half-life is about 33 minutes (34,15,35). Other, more complex models for aldosterone have been proposed (12,36) for the distribution and transport in the body. More complex models may be needed (34) to include additional compartments into which aldosterone might diffuse more slowly such as the cerebrospinal fluid.

Between 50-68% of aldosterone is bound to albumin and a small amount is reversibly bound to transcortin or corticosteroid binding globulin (15,12,37,38). Aldosterone has a low affinity to corticosteroid binding globulin and that which is bound is easily displaced by other steroids such as cortisol, and corticosterone (12,37), which are bound to about 90%.

As the concentration of biologically active hormone in plasma is dependent upon its secretion rate, the volume of distribution, the hepatic extraction rate, the degree of protein binding, and renal clearance of unbound steroid (15,39),

the plasma concentration of aldosterone should be expected to be altered by liver disease (15,35,5,39), renal failure (15,5,40), pregnancy (41) and other situations where alterations in the secretion rate are affected by protein binding and other stimuli. ACTH has been shown to cause an increase in the metabolic clearance rate of aldosterone (42). This occurred along with a sharp decrease in the protein bound fraction of aldosterone (42).

The relatively low degree of aldosterone protein binding may account for the very high percentage of hepatic extraction (15,43,42,12,44,41,35,5). The hepatic extraction during a single passage of aldosterone has been reported to be almost 100% (41,34,44). Extrasplanchnic clearance of aldosterone was reported by Tait et al., (44,45) to be a significant but minor component of total metabolic clearance in subjects with minimal and marked cardiac dysfunction. The lowered metabolic clearance rate seen in these subjects was attributed to decreased hepatic extraction or hepatic plasma flow or both (44,5). Hepatic extraction in patients with advanced congestive heart failure fell to between 50-75% and plasma clearance rates of aldosterone were decreased (46).

Tait et al., (116) calculated the plasma clearance of aldosterone to be about 1620 liters/day. The mean adrenal formation rate, (34,41,35,39,47,48,49,50) was found to be 128 ug/day, with a range of 50-200 ug in normal subjects (34,51,12).

Aldosterone secretion follows circadian rhythm (19,12,52,53,34,54,55), with plasma aldosterone values being

lowest between 4 pm and midnight and the highest values at 4 am or 8 am in normals (52,12). Alterations in posture appear to bring about changes in aldosterone metabolite excretion (12,53,54). Lying down appears to lower the amount of aldosterone metabolite(s) excreted into the urine.

Brodie et al., (51), using double-isotope dilution techniques reported a value of about 7 ng/100ml for subjects on normal sodium intake. Slightly higher aldosterone plasma values have been reported by radioimmunoassay techniques. Mayes et al., (56) reported values of about 14ng/100ml plasma, while Bayard (57) reported values of similar magnitude in subjects who were upright. Kowarski et al., (49) measured aldosterone plasma concentrations in adults, children and infants and found by radioimmunoassay that adults had much lower plasma levels than children and that infants had the highest levels of all.

Metabolism of Aldosterone

Eighty five to 92% of total administered radioactive aldosterone undergoes liver metabolism with about 5-10% being metabolized in the kidneys and a small but significant fraction of aldosterone being metabolized in other organs (12,5,44,36,58,59). The renal clearance of unmetabolized aldosterone is 5-15% of glomerular filtration rate (60). Tetrahydro-aldosterone glucuronide, the principal metabolite of hepatic venous blood (36,61,5), has had no appreciable extra-hepatic formation reported (36,61,59). This metabolite

is used for determination of aldosterone secretion rate if urine is collected over a 48 hour interval (5). THA is excreted mainly as a conjugate freed mainly by beta-glucuronidase (62,12,35). It is biologically inactive (35,19). Renal tissue is capable of producing acid-hydrolyzable conjugates of aldosterone (36,61). In renal venous blood from 13 patients the average renal production of acid-labile conjugate was about 58% of a labelled dose (36). Bledsoe et al., (36,61) showed that about 33-50% of this conjugate is formed at extra-hepatic sites, most likely in the kidneys.

The 18-glucuronide metabolite of aldosterone, which has previously been referred to as the 3-oxo-conjugate, as the pH 1 conjugate or as the acid labile conjugate (63), and the tetrahydroglucuronide metabolite of aldosterone are the major metabolites of aldosterone (64). A number of other metabolites of aldosterone have been isolated, however. Some of them have not been fully characterized (5,19,64,63,12). Human urine contains tetra-hydrogenated derivatives of aldosterone (62). Aldosterone and its reduced metabolites have been shown to exist in small amounts in the sulfate form in human plasma and urine (64).

Sex differences in the metabolism of aldosterone were demonstrated by Morris et al., in both intact and adrenal-ectomized rats (65,66,67). In intact rats the rates of clearance of aldosterone and its metabolites from the plasma via the bile is also sex-dependent (65). This is significant as in rats, biliary excretion of aldosterone is the major

pathway of excretion (65). Female rats excreted 82% of an injected dose via the bile within one hour, while male rats excreted only 49% (65).

In adrenalectomized rats the percentage of dichloromethane extractable plasma radioactivity is greater in female than in male rats from 5 minutes to 90 minutes after an IV injection of labelled aldosterone (66). Non-extractablepolar metabolites (NEPD) of aldosterone were higher in the plasma of male rats and in female rats these metabolites were rapidly cleared from the blood (66).

Morris et al., (65,66) concluded that in both adrenalectomized and intact male and female rats, sex hormones may influence the metabolism of aldosterone, the plasma levels of unmetabolized aldosterone and its metabolites (65,66).

Steroid reductases have been located in both the soluble fraction and the microsomal fraction of rat liver (68). The Δ^4-5 -reductase is found in liver microsomes, while the Δ^4-5 reductase is found in the soluble fraction (68,69). Differences in the effective concentration of Δ^4-reductases in female rat livers when compared to that of male rat livers have been found to exist for aldosterone (19,70), and a number of other hormones, for example corticosterone, deoxycorticosterone, testosterone, progesterone and so on (19). Three to ten fold increases in reductase enzyme concentrations have been seen in female rat livers compared to male rat livers (19,70,69). In young rats estrogens apparently increase theΔ^4-reductase content of liver while androgens produce the opposite effect (19). After 75 days the

estrogens loose their influence however, the androgens appear to be an important factor in older male rats (19).

Read et al., (68) postulated that the increase in aldosterone metabolism seen in sodium restricted rats could be due to enzyme induction. Also, Samuels and Tompkins classified aldosterone itself, as an optimal inducer as judged by its ability to induce the enzyme tyrosine aminotransferase (71).

Distribution of Aldosterone

Aldosterone distributes to various organs (12,72,73,40, 6,74). Autoradiographic studies in mice showed high concentrations of aldosterone and its metabolites in liver, bile, kidney, ductus deferens, caput epididymidis, and adrenal cortex (72). Sulya et al., (74), found that in the rat higher uptake of labelled aldosterone was seen in kidney, heart, lungs than in blood. Fifteen minutes after injection kidney, heart, muscle, spleen, aorta, lung, liver and duodenum had a higher concentration than in blood (74). Only brain and adipose tissues did not show levels of radioactivity higher than those seen in blood (74).

Subcellular fractionation of tissues to locate the intracellular sites of aldosterone led some investigators to report presence of labelled compound mainly in the cytoplasm (12,40). In the early work however, it was not possible to distinguish labelled aldosterone from its metabolites (12). Edelman et al., (268, 134,316,263,265) found aldosterone to

be selectively localized in the nucleus of toad bladder epithelial cells in *in vitro* studies. By autoradiography, aldosterone has been shown to be localized in the tubular epithelial cells of the kidney, along muscle fibers in skeletal and cardiac muscle, in the alveolar walls of the lungs and in the parenchymal cells of the liver (74). Recently, ^{3}H-aldosterone has been found *in vitro* in the cytosol fraction of adrenalectomized rat brain (45). The subcellular localization of aldosterone becomes important in the search for "receptors" which might play a role in the physiological mechanism of action of aldosterone.

THE PHARMACOLOGY AND PHYSIOLOGY OF ALDOSTERONE

A primary function of aldosterone is the regulation of active sodium and potassium secretion and transport in a number of tissues in the body including the kidney, sweat glands, intestinal mucosa and salivary glands (12,15,5,77, 4,78,79,80,81,82,83,84). In mammals, aldosterone's role in sodium retention is met by its ability to cause a reduction of sodium losses (77). Aldosterone can cause sodium concentration to be decreased in the lumen of the distal parts of the nephron and intestine and in the lumen of the excretory ducts of sweat glands (77,81,84). Aldosterone also promotes the excretion of potassium, however, the antinaturetic and kaliuretic effects of this hormone appear to depend on experimental conditions (4,67). Barger (4,78,85) in the adrenalectomized dog and Fimognari et al., (4,86) in the

adrenalectomized rat on low potassium diets, showed that aldosterone produced coordinate antinatiuretic and kaliuretic responses. On normal diets in the adrenalectomized rat, the only effect observed was on the excretion of sodium (4,86). In the adrenalectomized dog and in the salt-loaded intact dog, aldosterone showed no effect on sodium excretion, but caused potassium excretion (4,15,78,86). Morris et al., (67,82) demonstrated that at physiological levels aldosterone promoted the antinatiuretic and kaliuretic responses of this hormone in adrenalectomized rats, but only a kaliuretic response in intact rats. These two effects may be separable (82). Both of these responses to aldosterone in adrenalectomized and intact rats were greater in males than in females (82,66,65).

In the kidney the major site of action of aldosterone, is in the distal renal tubule (5,15,51,87,88). This was demonstrated with the use of stop-flow techniques in the dog (5,15,51,87,88). Aldosterone may stimulate the reabsorption of sodium and chloride in the ascending limb of the loop of Henle (5,89), but it probably has no effect on the proximal tubular reabsorption of sodium (5,15,88) as shown by stop-flow techniques.

Short term administration of aldosterone to human subjects produces different effects than long term administration on the renal handling of sodium and potassium (15,5, 51,90). Subjects given mineralocorticoids retain sodium with associated expansion of the extracellular fluid and concomitant weight increase. This is not seen if dietary sodium

is restricted (5). Continued administration of aldosterone results in the establishment of a sodium steady state with excretion of sodium into the urine equalling daily salt intake. This has been called the "escape phenomenon" (15,5, 51,90). After the "escape", stable sodium balance occurs in normal subjects but the potassium continues to be lost (5,15). In the major edematous states, characterized by chronic depletion of intravascular volume, such as congestive heart failure, nephrotic syndrome, cirrhosis with hypoalbuminemia, the "escape phenomenon" fails to occur (5,15, 51).

The Molecular Mechanism of Action of Aldosterone

Studies on the mechanism of aldosterone action have been numerous (75,86,6,76,77,5,91). Edelman et al., (75,76) presented evidence that aldosterone was located in the nucleus of epithelial cells and acts by promoting DNA-dependent RNA synthesis. This in turn yields an increased rate of protein synthesis (75,91). The current hypothesis is that aldosterone stimulates transepithelial transport of sodium by inititiating a sequence of biochemical events (6,75,86,6, 76,91).

It appears that aldosterone enters a specific target cell, binds to a specific cytoplasmic "receptor",temperature-sensitive activation of the complex takes place, the active complex binds to the chromatin, RNA induction leading to protein synthesis takes place, and finally the induced protein

acts to produce the physiological expression of the hormone (92,4).

Marver et al., (93) proposed a three-step sequence for the transfer of cytoplasmic aldosterone-"receptor" complexes to the nucleus. Using a rat kidney slice technique, Marver et al., (93) were able to follow the time course of intracellular aldosterone binding. Three ^{3}H-aldosterone receptor complexes were found in rat kidneys by these investigators; cytosol, tris-soluble nuclear and chromatin bound. Funder et al., (94) using kidney slices and parotid slices of adrenalectomized rats also found a three step time sequence of specific intracellular binding. Thus, it would seem, if this 3 step mechanism is valid, that interactions of receptor complexes with the chromatin should initiate the induction phase of the physiological response to aldosterone (93). The precise relationship if any, between the binding process and the induction of transcription for mRNA and rRNA remains to be seen (93,5). The conclusive demonstration of increased RNA synthesis has yet to be shown to support this hypothesis (5).

There have been several hypotheses reported on the mode of action of the aldosterone-induced protein (AIP) (5,4,95). The "sodium-pump" hypothesis suggests that the AIP activates the sodium pump via the enzyme Na^{+}-K^{+} activated adenosine triphosphatase (5,4). The "permease hypothesis" suggests alterations of cell permeability to facilitate sodium entry from the luminal surface (5,4). The effects of the AIP on intermediary cellular metabolism to cause an increase in

rate-limiting supply of energy to the sodium pump has also been proposed (96,4,5). Edelman et al., (75,4) showed that in the substrate depleted state, isolated toad bladder has almost no response to aldosterone. Snart, suggested, that a dual mechanism, a "two-stage mechanism" might be operating in which both the ion pump and mucosal barrier are affected (97).

Extra-Renal Effects of Aldosterone

Besides its renal effects aldosterone has a variety of extra-renal effects (98,5,15,99,100). In sheep parotid saliva, aldosterone lowered the normal Na^+/K^+ ratio (83). The 3-step time sequence of specific intracellular binding takes place in parotid from adrenalectomized rats (94). In the sweat glands aldosterone appears to decrease sodium concentration (15,5). This effect is slower in onset than renal effects (5,101). Aldosterone may have influence on the sodium and potassium balance and transport across the intestinal wall (81). Aldosterone given by continuous infusion enhanced net K^+ secretion by the intestine showing an increase in the rate at which K^+ entered intestinal lumen (84), while sodium and water movements were not affected. Aldosterone appears to stimulate sodium transport across the intestinal mucosa in both directions leaving the net flux unchanged (15).

Aldosterone elevates K^+ and Na^+ ratios in both brain and muscle (80). Muscular potassium decreases and muscle sodium

increases when aldosterone is administered to intact animals (5), although this effect is not seen during sodium restriction. Much of the *in vitro* work shows the reverse effect on muscle electrolytes; a rise in potassium, and a fall in sodium concentration (5).

Recently Anderson and Fanestil (45) reported the existence of high and low affinity mineralocorticoid binding "receptors" in *in vitro* cytosol preparations from adrenalectomized rat brains. These authors suggest that the presence of these "receptors" might offer a possible extra-renal mechanism of action of aldosterone in or mediated through the central nervous system. In neurosurgical patients with brain tumors being treated for edema with aldosterone, a positive response to therapy was seen (102). Adrenalectomized rats and mice show water and sodium accumulation similar to the findings in some forms of brain edema (103). The fluid is probably distributed in the extracellular space (103). Aldosterone apparently antagonizes the effects seen in the CNS of adrenalectomized animals. Schmiedek et al., (104) recently re-evaluated short term steroid therapy for perifocal brain edema. These studies confirm earlier findings for the use of mineralocorticoids in the treatment of brain edema (104). That aldosterone stimulates active sodium transport across epithelial cells in toad bladder, frog skin or renal tubular cells does not imply necessarily that the same effect will be seen in non-epithelial cells such as nervous tissue of brain (102). Woodbury and Koch (102,80) suggest that aldosterone stimulates the active pumping of

sodium out of brain cells. Baethmann et al., (102) confirmed this result.

Baethmann et al., (105) studied another extra-renal property of aldosterone, that of immunosuppressive ability. Aldosterone was able to prolong survival time of allogeneic skin grafts from 10-14 days (105). These authors suggest that this ability is independent of the glucocorticoid component of aldosterone (105).

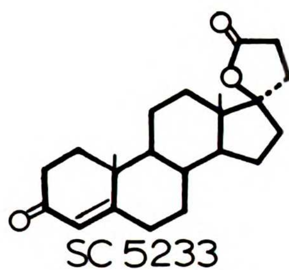
Aldosterone possesses two circulatory effects, a peripheral effect on blood pressure and a cardiac effect (12). Aldosterone can potentiate the pressor effect of sympathomimetic amines such as norepinephrine (15,12). Concentrations of aldosterone added to isolated rat heart preparations have been shown to cause the heart to work longer and more efficiently than preparations without added aldosterone (106).

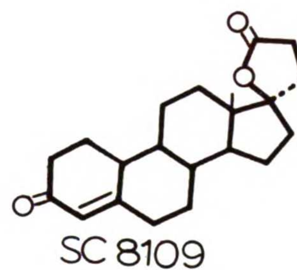
THE SPIROLACTONES

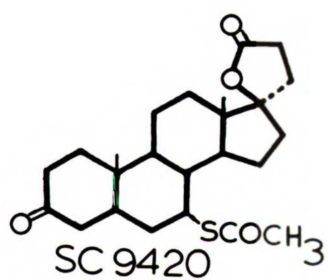
The synthesis of the first steroidal spirolactones was reported in the late 1950's by Cella and Kagawa (107). Development of this new class of drugs was spurred on by the fact that progesterone was capable of reversing the effect of deoxycorticosterone in a bioassay which measured the reversal of the effect of deoxycorticosterone on urinary Na^+/K^+ ratios of adrenalectomized rats (107). Also, the search for agents capable of counteracting the effects of mineralocorticoids offered a promise of new approaches to the control of edema and other effects caused by these hormones (108). The first two steroidal spirolactones synthesized were found to be better mineralocorticoid antagonists than progesterone and were able to reverse the electrolyte effects of aldosterone (107) in rats (109,110,111,112,113), dogs (112,114,88,115), and in man (116,109,117,114) under various experimental conditions. The name "spirolactone" is derived from the spiro configuration of the gamma-lactone ring in position 17 of the steroid moiety (24). Subsequently one of these compounds (SC-5233) was shown to possess antihypertensive activity in rats (109), Figure 3. In an attempt to find derivatives of steroidal spirolactones which possessed the antimineralocorticoid effect when given orally, spironolactone, 3-(3-oxo-7 α-acetylthio-17 -hydroxy-4-androsten-17α-yl) propanoic acid lactone (SC-9420, Figure 3) was synthesized (118).

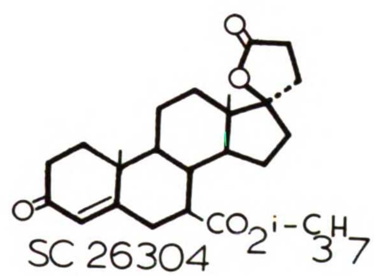
FIGURE 3

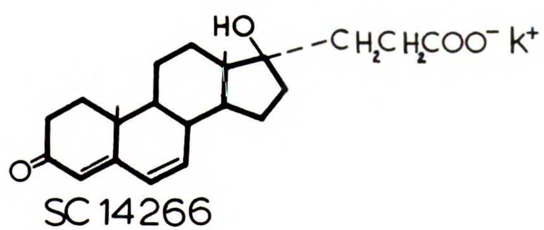
Structures of Some Experimentally Used Spirolactones











Spironolactone was found to be the most potent of a series of compounds tested when administered orally (118). Spironolactone possessed activity orally and parenterally and reversed the urinary electrolyte effects of aldosterone (119). The search for aldosterone antagonists led to the discovery of many compounds (118,120,121,122,112,123,124,125, 73) including SC-14266 (Figure 3), a water soluble open lactone salt congener of spironolactone (112). SC-14266 or potassium 3-(3-oxo-17α-hydroxy-4,6-androstadien-17β-yl) propanoate, is soluble in water to about 20% (112). Spironolactone is given orally and is available in 25 mg tablets in the United States as Aldactone[R] (Searle and Co.). It can also be found in the United States in fixed combination of 25 mg with 25 mg of hydrochlorothiazide, marketed as Aldactazide[R] (Searle and Co.). Canrenoate-potassium, is not available in the United States for clinical use, but in other countries it is given orally. It can also be given by the IV route due to its water solubility.

Pharmacology of Spironolactones

Much evidence has been accumulated to show that the spirolactones produce their effects by antagonism of the mineralocorticoids. The bioassay developed by Kagawa et al., (110) uses adrenalectomized rats injected with aldosterone and measurement of urinary Na^+/K^+ ratios. To test for mineralocorticoid antagonism this ratio is measured for the compound suspected of having blocking ability. On the basis

of results from this bioassay Gantt (126) classified diuretic agents into three categories; physiologic antagonists, non-competitive antagonists, and competitive antagonists. Most diuretics are considered to be physiologic antagonists, having no true antimineralocorticoid activity (126). Included in this class are thiazide diuretics, furosemide and ethacrynic acid. Non-competitive antagonists are compounds such as triamterene. Competitive antagonists increase the urinary sodium excretion with no change or a decrease in urinary potassium excretion. Progesterone, testosterone, and the 17-γ-spirolactones exhibit competitive antagonism (126).

A number of studies are available supporting the idea that spirolactones are effective as natriuretic agents only in the presence of sodium retaining steroids (110,114, 111,112,113). Ross, et al., (117) developed a human bioassay to test the antimineralocorticoid properties of steroidal spirolactones. This bioassay measures the ability of these compounds to reverse the sodium retaining, potassium excreting ability of 9-α-fluorohydrocortisone given orally or aldosterone given by injection. Approximately 300mg of Aldactone^{R} can produce complete antagonism. Gantt and Dyneiwicz expanded on Ross' studies noting a reduction in the urinary Na^{+}/K^{+} ratio of subjects given 9αfluorocortisol (127). This lowered ratio returned towards control values when subjects were given Aldactone^{R} or Aldactone-A^{R} (127).

The spirolactones produce different effects in adrenalectomized vs. nonadrenalectomized animals and man (15,110, 111,112,113,114,87,128,115,116,60). These compounds block

mineralocorticoid renal effects in adrenalectomized animals and are inactive as sodium-loosing agents when given alone. It can thus be concluded that these compounds act by antagonizing the effect of mineralocorticoids on the kidney (113).

Extra-renal Pharmacology of the Spirolactones

Extra-renal effects of spirolactones have been shown on the heart and central nervous system. Spirolactones also possess anti-inflammatory activity and catatoxic properties.

The inotropic effects of spirolactones were investigated in patients with coronary or pulmonary heart disease (129,130,128) and in animals (131,132). Positive inotropic effects of spirolactones were demonstrated (130). It is thought that canrenone and canrenoate are cardioactive metabolites of spironolactone (130). Effects of canrenoate-sodium were investigated in rat atria and ventricular strips and on isolated perfused hearts (131). A dose-dependent reversible negative inotropic effect was seen on isolated atria and ventricular strips, however a small positive inotropic effect could be seen on perfused hearts possibly related to a decrease in coronary resistance produced by the drug (131).

Spirolactones can correct cardiac arrhythmias (133). Spironolactone is reportedly the drug of choice in treating serious cardiac insufficiency when complicated by myocardial hyperexcitability (129).

Baethmann et al., (105) demonstrated immunosuppressive properties of spirolactones. Spirolactones prolonged survival time of allogeneic skin grafts, an ability thought related to stimulation of endogenous aldosterone production (105).

Spirolactones have various effects on the central nervous system. Canrenone was found in the CSF and in brain (134). IP injections of spirolactones into rats produced convulsions (135), but oral administration via gastric tube did not. Simon et al., (136) found that IV injections of canrenoate-K in dogs showed dose-dependent reproducible and reversible EEG changes (137,136). In adrenalectomized dogs the same changes were seen indicating that the mechanism for these changes is independent of the effect of aldosterone and is probably a direct result of the action of spirolactones on the CNS (136,137). Also, in neuro-surgical patients pre-treated with spirolactones peri-focal brain edema was reduced (138).

The spirolactones interact with aldosterone on the renal and on the extra-renal level. On the extra-renal level both compounds have positive inotropic effects on the heart, reduce peri-focal brain edema, and have immunosuppressive properties.

Spirolactones oppose mineralocorticoid effects on the salivary glands (79), sweat glands (101), the gastro-intestinal tract (139) and muscle (100).

The spirolactones, primarily spironolactone, have been shown to be catatoxic in animals against a wide variety of

chemicals including digitoxin (197,252.254,248,255,256, 257,259,260,261,262), digoxin (140,132,141,142), indomethacin (140,143,144,145,146), mercury (140,147,148), pentobarbital (145), aminopyrine (145), bishydroxycoumarin (145), as well as many more toxicants differing in their chemical structure and pharmacological actions (147). Spironolactone decreased the anesthetic effect of steroids and barbiturates as well as the convulsions caused by diphenylhydantoin (143,149,144, 145,6,150,151).

Biochemical Pharmacology of Spirolactones

Several theories have been suggested as to the biochemical mechanism of action of spirolactones (113,38). Aldosterone binds to a specific cytoplasmic "receptor" (152). Spirolactones have been shown to compete for these cytoplasmic binding sites (153). The cytoplasmic receptor is thought to equilibrate between an active and an inactive form (152,154,92). Inactive complexes when formed, are assumed to be incapable of entering the nucleus (92,154). The complexes are incapable of initiating the transcriptional event even if they do attach to chromatin binding sites (154). Alberti and Sharp (155) identified spirolactones as inhibitory compounds because they bind to mineralocorticoid receptors, do not stimulate sodium transport, inhibit active steroids and can displace active steroids. SC-14266 (Figure 3) reduces renal cytoplasmic binding of aldosterone (153,154). Fanestil showed that SC-14266 competitively inhibits aldosterone

interaction with renal mineralocorticoid receptors (156). After injection of aldosterone *in vivo* to adrenalectomized rats uptake of aldosterone by the nuclear fraction was reduced to 35% of control (156). Also, uptake in the supernatant fraction was decreased to 60% of control with simultaneous administration of 10^4 fold molar excess of SC-14266. SC-26304 (Figure 3) also inhibited binding of ^{3}H-aldosterone to renal cytoplasmic and nuclear receptors (154).

Clinical Uses of Spirolactones

The spirolactones are useful in treating congestive heart failure, hepatic ascites, primary aldosteronism and essential hypertension (157,158,15). Spirolactones are indicated in primary aldosteronism as an aid to diagnosis and in treatment (12,159,160,161,128,162). Spirolactones correct only the metabolic abnormalities of secondary aldosteronism and not the hypertension (159,163).

Patients refractory to other diuretic preparations may respond to spirolactones for the control of edema and ascites (164,12). When spirolactones alone fail to reduce these conditions, combination with thiazides or ethacrynic acid has proven useful (12,165).

Spirolactones have been used to treat hypokalemia (12,15), cardiac edema (164,12), nephrotic syndrome edema (15,12,164), and brain edema (166).

Spironolactone has been used to treat essential hypertension (167,12,168,163,158,15,162,169). It is especially

effective in treating low renin hypertension (170,171, 169,167,158,163) where such patients rarely show excess mineralocorticoid levels. It may be that extracellular fluid volume reduction is important in alleviating low renin essential hypertension (170).

Other areas where the spirolactones have been used clinically are in the treatment of idiopathic edema (15,12), pregnancy (108,163), familial periodic paralysis (23,15), acute mountain sickness (172), as diuretic therapy in children (173), in treating licorice induced hypertension (163,174), as an anti-androgen in men with prostatic cancer (175,176), and as a "provocative test" for gonadotrophin secretion in children and adolescents (175).

Clinical Problems Associated with the use of Spirolactones

The spirolactones are known to cause a number of side effects and adverse reactions (115,177,159,178,163). The endocrine abnormalities seen with spirolactone therapy are estrogenic like side effects such as gynecomastia (179,180,181,182,183), impotence, androgenic like side effects including hirsuitism, irregular menses and deepening voice (175,184). Breast cancer has been associated with spirolactone therapy (185), although evidence to the contrary is also available (186). The mechanisms by which these reactions are thought to occur involve changes in the normal gonadal-hypothalamic-pituitary inter-relationships (175). The spirolactones affect testosterone biosynthesis. They

destroy cytochrome P-450 in microsomes of a number of laboratory animals (187). 17-Hydroxylase and other cytochrome P-450 enzymes may convert spironolactone to a metabolite capable of destroying the heme portion of the cytochrome molecule and thereby decrease 17 hydroxylation (175). Spirolactones also affect adrenal microsomal cytochrome P-450 (188).

Canrenoate-K causes a decrease in plasma testosterone and a decrease in urinary 17-ketosteroids in patients (189). This decrease was the result of effects on testicular secretion rate. Canrenone was shown to significantly decrease plasma testosterone concentration in normal males (190).

Spironolactone can competitively inhibit interaction of dihydrotestosterone and its cytoplasmic receptor protein in rat and human tissues (175), indicating that a peripheral androgen antagonism is part of the mechanism by which spirolactones affect testosterone biosynthesis (51,191,175,192).

PHARMACOKINETICS AND BIOPHARMACEUTICS OF SPIROLACTONES

Bioavailability

Spironolactone was first marketed by Searle and Co., as 100 mg tablets. It has a low water solubility (2.8 mg/100mlat 25°C) and bioavailability problems were noted soon after its introduction (193,194,158,195,196). Micronization

was used to increase the absorption further and the current product on the market exhibits this characteristic in its manufacturing process.

Gantt (126) reported rapid conversion to the lactone form following administration of canrenoate-K IV and orally. Gantt was unable to find consistent ring opening in normals following administration of Aldactone[R] (126). The small gut mucosa of rats hydrolytically cleaves the lactone ring of canrenone in *in vitro* studies (197). Canrenone is stable in acid and hydrolyzes in alkali to its corresponding canrenoic acid salt (198). At 25°C canrenone is soluble in water to about 32 ug/ml (9.4×10^{-5}M). The half-life of hydrolysis or lactonization at pH 7.4 is 24 days (198).

Canrenoate-K was quantitatively absorbed within 20 minutes and bypassed the liver practically unchanged in one study (199). Canrenoate-K IV in man produced levels of the hydroxy acid and canrenone that were similar (200). Thus enzymatic processes are inferred and explain the rapid *in vivo* equilibrium seen between canrenone and canrenoate.

Canrenone levels presented in the literature are used for bioavailability measurements (201). Sadée et al., (199) reported that the oral bioavailability of Aldactone[R] tablets (Boehinger-Mannheim micronized) was 96% in the same patient relative to administration of spironolactone in PEG solution, but only 60% relative to the oral administration of canrenoate-K, when measured by the area under the plasma concentration time curves of canrenone. A preparation of 100 mg spironolactone tablets was compared with the equivalent dose

of marketed 25 mg tablets (202). The mean peak plasma metabolite level, and the peak times were similar (202). Karim et al., (193) studied the bioavailability of commercial 25 mg tablets relative to drug administered in solution (PEG 400) and found that the absorption rate of drug from solution was more rapid than from the tablets. Completeness of absorption is important for drugs like spironolactone which is usually administered over long periods of time (193). Karim et al., (193) reported relative bioavailability measurements of tablets to solution similar to those seen by Sadee et al. (199). Karim et al., (193) also showed statistical bioequivalency between eight 25 mg tablets and two experimental 100 mg spironolactone tablets in agreement with the results of Hofmann et al., (202). *In vitro* and *in vivo* bioavailability testing of spironolactone from oral dosage forms was performed by Chao et al., (203). Spironolactone was combined with hydrochlorothiazide, and no effects were seen on the availability of spironolactone (203).

TABLE 3

Comparison of Pharmacokinetic Parameters of Spirolactones

	Spironolactone	Canrenone	Canrenoate
1. Plasma Elimination Half lives			
a) in man	below assay detection	α phase= 4-5 hours (177,206) β phase= 17-22 hours (199,206)	β phase= 17-22 hours (199)
b) in rats	4-5 min (207)		2.5 hrs (207)
c) in dogs	10 minutes	5 hours	5 hours
2. Multiple Dosing Accumulation Time		1-7 days	
3. Volume of Distribution	not determined*	not determined*	not determined*
4. Bioavailability (oral)	** 96% (60%) (199)**		100%***
5. Solubility in Water at 25°C	2.8 mg/100ml	3.2mg/100ml	>1g/100ml

* difficulties in assessing V_d arise from the rapid enzymatic equilibrium between canrenone and canrenoate.

** Spironolactone bioavailability measured as available canrenone. 96% in tablets compared to spironolactone in PEG soln, but only 60% relative to p.o. administration of canrenoate-K.

*** in aqueous solution (199).

The onset of action of the spirolactones is rather slow (108,196,204,205,126). There appears to be an increasing response over the first three days when a maximal effect is seen (108,196,126). The accumulation of canrenone may continue for as long as six days (196). Sadée et al., (204) reported the cumulation in patients recovering from myocardial infarction without congestive heart failure or cirrhosis to be 1-4 days (204), and in one patient with congestive heart failure cumulation of canrenone and canrenoate occured for 7 days. The biological half-life of canrenone from plasma concentration-time curves has been reported in man to be 4-5 hours (177), and 8 hours (200) in another study. Sadée et al., (199) found that the half-lives of canrenone and canrenoate following doses of spironolactone and canrenoate-K ranged from 17-22 hours in their terminal log-linear phase of elimination. In another study canrenone levels declined in two phases with half-lives of about 4 hours and 17 hours after single oral doses of spironolactone (206). Spironolactone has a half-life of 4-5 minutes in rats (207), less than 10 minutes in dogs (197), and in man levels of spironolactone following oral doses were below the detection limit of available analytical procedures (199,208,197).

The first pass phenomenon does not appear to play a major role in the bioavailability of spironolactone (197). The high oral activity of spironolactone may be explained in part by the dethioacetylation to canrenone, which is metabolized with a slower rate than spironolactone and escapes

effective hepatic clearance (197). Canrenoate-K given orally was rapidly and quantitatively absorbed from the gastrointestinal tract and bypassed the liver unchanged (199).

Spirolactone Potency

Spironolactone and canrenoate-K are reportedly equally potent in rats (209,112) and in man (126,204,199). The question of equipotency was recently reexamined by Ramsay et al., (157,210,211). Comparisons of plasma levels of canrenone in normals with the antimineralocorticoid properties of spironolactone and canrenoate by a modified Ross (117) procedure were performed. Spironolactone and canrenoate-K were given in tablet form orally, but the bioavailability of the canrenoate-K tablet was incomplete so equipotency was not demonstrated (157). However, with the modified bioassay, it was suggested that canrenone is not the only active metabolite of spironolactone and canrenoate. Metabolites other than canrenone appear to account for the potency of spironolactone, as the potency of spironolactone was greater than measured levels of canrenone would predict. Kagawa et al., (209) found that while spironolactone and canrenoate given orally to rats and dogs produced equal plasma levels of canrenone, the mineralocorticoid effects were unequal. Thus, antimineralocorticoid activity of spironolactone is only partly accounted for by levels of canrenone. In reviewing the Ramsay study (157), Sadée (212) pointed out

that single doses of drugs might not reflect the situation clinically where the drugs are given over long periods of time. Canrenone accumulates in plasma with time before reaching steady state (204). In replying to this comment Ramsay (213) reviewed the data obtained from testing the renal antimineralocorticoid potency of spironolactone and canrenoate. The data showed that with single doses a relative potency of .31:1 (canenoate-K:spironolactone) is observable. Unpublished observations of this ratio after dosing till steady state showed it to be .68:1 suggesting that even at steady state canrenoate-K is less potent than spironolactone (213). No comment was made about the bioavailability of canrenoate however. Ramsay estimated that at steady state, 70% of spironolactone's antimineralocorticoid activity could be due to canrenone.

Methods for Measurement of Spirolactones

A fluorimetric assay for spirolactone measurement was introduced by Gochman and Gantt (214) based on the method of Silber et al., (215), utilizing the chemical properties of steroids to fluoresce in sulfuric acid. It is possible by this method to measure .005 ug or more of canrenone in plasma. Sadée et al., (216) published the mechanism by which steroids fluoresce in sulfuric acid through the formation of trienones. Fluorescence can be correlated with the 4-ene-3-one system present in spirolactone compounds. The spirolactones, spironolactone and canrenone fluoresce in a 60%

concentration of sulfuric acid (216). This is important as other endogenous steroids require higher sulfuric acid concentrations to form fluorescent products and this reduces interference. Sadée et al (217) published a fluorimetric microassay for spironolactone and its metabolites in biological fluids such as bile, plasma, gastric fluid and urine. This method allows measurements of spironolactone canrenone and canrenoate with high sensitivity (about 10 ng/ml in plasma) and specificity. Chamberlain described a gas-chromatographic method to measure levels of canrenone in human plasma and urine (218). This method was reported sensitive and specific through the use of GC-electron capture. However, the specificity is questionable as spironolactone is thermolytically dethioacetylated to canrenone (217). Williamson reported a high-speed liquid chromatographic (HPLC) method to measure canrenone in pharmaceutical dosage forms (219). Such a procedure was necessary because the trienone formed in the fluorescence reaction could not be used to test for stability in dosage forms. GC methods were also found unsatisfactory for this purpose.

"Therapeutic" Concentrations of Canrenone in Plasma

"Plasma levels" of spirolactones have been reported however, data relating plasma levels with effects is minimal. Very few studies have measured plasma levels of spirolactones after chronic dosing (196,204,50). Sadée et al., (204) measured multiple dose kinetics of spironolactone and

canrenoate-K in cardiac and hepatic failure patients. Results of these studies showed canrenone plasma concentrations of about .2ug/ml at 50 mg/day for at least 3 weeks, and at 400 mg/day for 3 weeks, levels were about 2.7 ug/ml. Differences in canrenone levels between the cirrhotic patients and the congestive heart failure patients were not statistically significant. Steady state levels of canrenone following treatment with spironolactone 100 mg twice daily ranged from 340-950 ng/ml in 6 patients with myocardial infarction (204). Differences in the rate of spirolactone metabolism is suggested as a reason for the observed interindividual differences in steady state plasma levels of canrenone following spironolactone treatment (204). Half lives in the 6 patients with MI following cessation of therapy ranged from 13-24 hours. Karim et al., (50) measured mean plasma concentrations of canrenone at 4 hours and 24 hours following 200 mg spironolactone every day and compared these levels to those obtained when healthy males were given 50 mg four times a day (50). From his data it can be concluded that a twice daily regimen or once daily dosing regimen is a reasonable alternative to the currently used dosing intervals.

Tissue Distribution

The tissue distribution of the lipophilic gamma-lactones, spironolactone and canrenone should be different from that seen with canrenoate, the more polar

compound. Autoradiography in mice showed that labelled spironolactone was "enriched" in liver, bile ducts, and in the content of the small intestine (220). ^{3}H-canrenoate-K crosses the blood brain barrier in dogs (134). The tissue distribution of canrenoate-K is discussed elsewhere in this report (Section II).

The apparent volumes of distribution cannot be accurately estimated from plasma level data. Sadée et al (208,197) reported that plasma levels following IV administration of canrenone and canrenoate in dogs indicate that canrenone possessed a larger volume of distribution than canrenoate.

Metabolism of Spirolactones

The metabolism of spironolactone is extensive in man, with the dethioacetylated canrenone being present in enzymatic equilibrium in blood with canrenoic acid. In 1962, Gochman and Gantt (214) reported that what appeared to be extractable from plasma, after oral administration of spironolactone in man, was the dethioacetylatedΔ^6-derivative, the 4,6 diene. 79% of a dose of spironolactone was metabolized to canrenone as judged by measuring areas under the curve of canrenone after oral doses of spironolactone and canrenone (199), however, canrenone accounts for only about 4% of an administered dose of spironolactone in a 0-24 hour urine collection (221). Sadée et al., (207) showed in rats that the conversion of spironolactone to canrenone proceeded in 2 steps, a fast step of hydrolysis to the 7α-SH and a rate limiting step involving the elimination of H_2S to form canrenone. It has been suggested that spironolactone may be hydrolyzed to its open chain congener, spironoate, however, Sadée et al., (207) were unable to detect this compound in rat plasma due to the rapid hydrolysis of the 7-alpha thioacetyl moiety. Karim and Brown (221) isolated and identified from urine canrenone and a new sulfur retaining metabolite identified as 3-(3-oxo-7α-methyl-sulfinyl-6 β,17β -dihydroxy-4-androsten-17 α-yl) propionic acid γ-lactone (221). Three other minor metabolites were also found in this urine work up, but were labile and readily converted to canrenone. These minor metabolites were found to be 7α -sulfoxide

spirolactone (2 epimers) and 7 α sulfone-spirolactone (222,221). Karim et al., (222) reported identification of additional metabolites of spironolactone in blood and urine of 3 healthy men. Of an oral dose of labelled spironolactone (20-^{3}H-spironolactone), canrenone, represented 2.9% of the dose and the previously identified 6β hydroxy sulfoxide, represented 1.8% of the dose. Two new metabolites were identified as 15 -hydroxycanrenone (0.8% of the dose) and 6 hydroxy thiomethyl derivative (0.5% of the dose). Canrenoate-ester glucuronide represented 4.5% of the dose and was the principal water soluble urinary metabolite found. Karim et al., (206) found no unchanged spironolactone in the urine of 5 healthy men given oral doses of spironolactone in solution. The major urinary metabolites found were canrenone (5% of the dose), 6β -OH-sulfoxide (5%) and the canrenoate ester glucuronide (6.2% of the dose). Abshagen and Rennekamp (223) recently isolated a metabolite of spironolactone from human urine. This metabolite is reported to be 3-(3-oxo-7α -methyl sulfonyl-6 β ,17 β -dihydroxy-4-androsten-17 α yl) propionic acid gamma lactone (223).

Canrenoate-K is reportedly metabolized to a water soluble metabolite, the glucuronic acid ester conjugate (200), which is found in urine. Sadée et al., (199) suggested that a "fluorigenic conjugate" such as this glucuronide of canrenoate is rapidly cleared by the kidneys and does not represent a major metabolite in plasma. In dogs, this metabolite accounts for less than 1% of the dose (197). Thus, all the known metabolites account for only a

fraction of the metabolism of spironolactone, canrenone, and canrenoate. Figure 4 shows the structures of these metabolites.

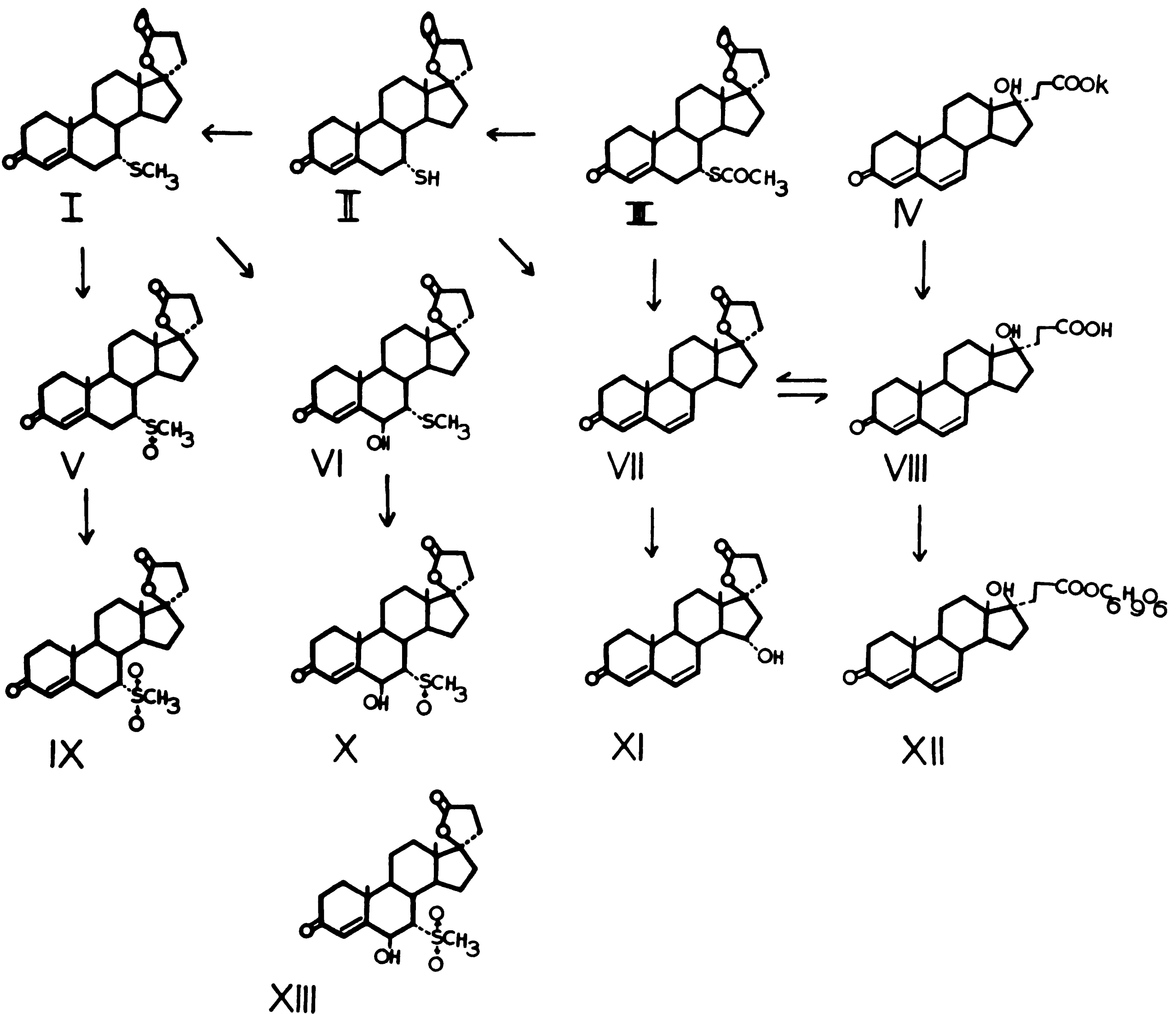


FIGURE 4: Structures of Known Spirolactone Metabolites

Elimination of Spirolactones

The urinary excretion of spironolactone and canrenoate has been investigated in the rat (207), dog (197), and in man (224,206,199,200,60). In rats with bile fistulas total urinary excretion after IV doses of ^{3}H-spironolactone and ^{3}H-canrenoate, was shown to be less than 3% (207). The kidney appears to play a minor role in the excretion of spironolactone and canrenoate in the rat (207). In bile fistula dogs total urinary excretion was low, about 1% of an oral dose of spironolactone was excreted in 6 hours (197). Urinary excretion in dogs was about 2% in an 80 hour time period (197). In man, 47% of a dose of ^{3}H-canrenoate-K was recovered in the urine within five days in one study (200), and 32% of a radioactive dose of spironolactone was recovered in the urine in 5 days in another study (206). Measuring fluorogenic metabolites, Sadée et al., (199) found that within 5 days 14% of a dose of spironolactone, 20% of a dose of canrenoate-K IV, and 24% of a dose of canrenoate-K given orally were excreted in human urine samples. Abshagen et al., (224) reported 47-57% of a given dose of spironolactone could be detected in human urine within 6 days. The t 1/2 of urinary excretion rate was 0.9 days.

Canrenoate administered to normal men showed canrenone renal clearance exceeded GFR by 70% (60). Hofmann et al.,(60) presented evidence that canrenone may be actively secreted into the proximal tubular urine. These workers also suggest net tubular reabsorption of spirolactones (60).

Biliary excretion of spirolactones has been studied in various species. Studies in mice with labelled spironolactone showed localization in the liver, bile ducts, and small intestine indicating biliary excretion (220). In the rat excretion of IV doses of labelled spironolactone and canrenoate occured primarily by biliary excretion and urinary excretion of the polar conjugated metabolites (207). This was followed by enterohepatic cycling. Sadee et al., (197) estimated total biliary excretion of about 60% of an administered dose of labelled spironolactone given IV. Abshagen et al., (224) suggested enterohepatic recirculation of lipophilic compounds to explain the short urinary excretion rate and observed increase in the polar fraction from the urine in normals given spironolactone orally.

Spirolactones have been measured in the feces in mass balance studies. After doses of labelled canrenoate IV in normals 14-36% of the dose was found in the feces within 5 days (200,224,206). Total recovered compound from urine and feces amounted to about 90% (224).

BIOCHEMICAL INTERACTIONS OF SPIROLACTONES WITH ALDOSTERONE

Spirolactones affect a number of microsomal and mitochondrial enzymes involved in aldosterone biosynthesis and secretion. *In vitro* experiments in rats showed spironolactone inhibition of aldosterone production by blockade in the conversion of corticosterone to aldosterone (225). Erbler (226) found that SC-5233 and its 11β-hydroxy analogue inhibited aldosterone production. The 11β-hydroxy compound showed a selective inhibition for the 11-hydroxylating enzyme, whereas the compound without the hydroxyl group had an unselective inhibitory effect. Canrenone, has been shown to inhibit production of aldosterone, corticosterone, 18-OH-corticosterone and 18-OH-desoxycorticosterone in a dose-dependent manner in *in vitro* experiments (227). Canrenone was shown to be hydroxylated in the 11 and 18 positions after being incubated with adrenal tissue (227). Inhibition of corticosteroid and aldosterone synthesis was found to be due to competition for mitochondrial hydroxylating enzymes (227). Sundsfjord et al., (228) provided evidence in support of Erbler's data by showing that spironolactone when administered in therapeutic doses decreases aldosterone secretion *in vivo* in man. In a case of primary aldosteronism these authors report a decrease in aldosterone secretion rate during spironolactone treatment with maintenance of a normal metabolic clearance rate of aldosterone (228).

Menard et al., (229) studied the effect of spirono-

lactone on adrenal 21-hydroxylating enzymes in male cortisol and corticosterone producing animals. Spironolactone caused a 70-80% decrease in the activity of this enzyme in guinea pigs, and dogs and thus decreased hydroxylation of progesterone (229). In male rats and rabbits (corticosterone producers) spironolactone administration caused a significant increase or no change in 21-hydroxylase activity (229). Greiner et al., (230) also found a decrease in microsomal 21-hydroxylase in *in vitro* slice experiments using adrenals of guinea pigs pretreated with spironolactone (230). However, in adrenal microsomal incubations spironolactone addition showed no effect on the rate of 21-hydroxylation (230).

Several mitochondrial enzymes are affected by spirolactones (229,227,230,29). Spironolactone, canrenone and canrenoate compete with the intermediates of corticosteroid biosynthesis for 11 and 18-hydroxylation (227,29). Spironolactone and canrenone are themselves hydroxylated possibly causing reduction in aldosterone production.

Cholesterol side chain cleavage is the rate-limiting step in corticosteroid production and is a cytochrome P-450 dependent reaction of adrenal mitochondria (230,231). Spironolactone decreases CSCC concentration or activity by about 50% in adrenal slices of pretreated guinea pigs (230).

Spirolactones affect both adrenal mitochondrial and microsomal cytochrome P-450 (29,230,229). Guinea pig and dog microsomal cytochrome P-450 decreased following administration of spironolactone (229). Spironolactone decreased

the content of mitochondrial cytochrome P-450 in male guinea pigs but not male dogs (229). In spironolactone treated guinea pigs adrenal microsomal and mitochondrial cytochrome P-450 levels decreased (230). In contrast, bovine adrenal mitochondria and human adrenal mitochondrial preparations showed no alteration in cytochrome P-450 (29) although spironolactone stimulated P-450 reduction. It has been suggested that in cytochrome P-450 systems spirolactones are converted to active metabolites which destroy the heme portion of the cytochrome P-450 molecule thereby decreasing enzyme activity (230,229).

II. TISSUE DISTRIBUTION OF TRITIATED CANRENOATE-K IN RABBITS

Abstract

Plasma and various organ concentrations of canrenone, canrenoate, and total ^{3}H-activity were measured following single IV dosing of 20 mg/kg ^{3}H-canrenoate-K to rabbits. Rabbits were used because in "pilot studies" rabbits were shown to have a very rapid rate of metabolism resulting in higher metabolite concentration. Organs studied included heart, lung, brain, kidney, liver, adrenal gland and spleen. Canrenoate was shown to be in rapid equilibrium with canrenone. Both canrenone and canrenoate were eliminated from plasma and other tissues with a half-life of about 1 hour. Plasma concentrations of canrenone and canrenoate were equal as early as 10 minutes after IV administration of the dose. Canrenone was found concentrated about 10 fold in organ tissues when compared to plasma, while no such preferential uptake was found with canrenoate. Total ^{3}H-activity declined slowly in all tissues with a half-life of approximately 15 hours, indicating extensive metabolism and metabolite retention in the rabbit.

Introduction

The pharmacokinetics of spironolactone, canrenone, and canrenoate have been investigated in the rat (209), the dog (208), and in man (199,200). Spironolactone is rapidly meta-

bolized via a 7- alpha-thiol derivative (207) to canrenone, which exists in equilibrium with canrenoate (Figure 4). The extensive metabolism of spironolactone in man leads to formation of sulfurous retaining metabolites which are chemically labile and potentially toxic. Therapetuic use of canrenone and canrenoate-K would eliminate these sulfurous metabolites. If canrenoate-K is to be used clinically, it must be shown to produce effective levels of canrenone in the body, as canrenone is presumably the active metabolite of spironolactone and canrenoate. Data on the kinetic disposition and further metabolism of canrenone and canrenoate are scant (207,221).

Bioavailability measurements for spirolactones have been based on canrenone plasma levels. Large differences between the tissue distribution of the lipophilic canrenone and the water soluble canrenoate can be expected. These differences in tissue distribution should be considered when interpreting the levels of canrenone and canrenoate in plasma. In this study the tissue distribution of canrenoate-K was investigated in rabbits.

Experimental

1. Materials

Pure crystalline samples of spironolactone and canrenoate (Boehringer Mannheim G.M.b.H.) were used. ^{3}H-canrenoate-K was prepared from ^{3}H-spironolactone (G.D Searle and Co.). This was accomplished by adding labelled spironolactone to methanol, water and NaOH and making sure

the pH was 13. After heating for 10 minutes at 100°C, .5 ml water was added followed by extraction with dichloromethane. .3 ml 1N HCl was then added and the pH adjusted to 1. This was followed by dichloromethane extraction and water wash. The dichloromethane layer was then washed with sodium sulfate removed and blown dry. .1 ml ethanol was added to the residue which contained canrenone. For at least 30 minutes prior to injection, .1 ml of .1N NaOH was added allowing for the conversion of canrenone to canrenoate. Finally the product ^{3}H-canrenoate-K with a specific activity of 860 uCi/mg was purified by solvent extraction and thin layer chromatography in dichloromethane:methanol (8:2) on Silica Gel K C F 254 Merck plates, prior to use. All reagents were of spectroquality and were used without further purification.

2. Animal Protocols

Serial Blood Sampling: Female New Zealand white rabbits weighing 2-2.5 kg were used. Each rabbit was prepared for blood sampling by insertion of a polyethylene 50 catheter about 4 cm into an ear vein. Doses of 5, 10 and 20 mg canrenoate-K/kg in aqueous solution were given by venous injections into the opposite ear. Blood samples of 2 ml each were taken at appropriate time intervals into heparinized syringes, centrifuged and the plasma was frozen for subsequent analysis.

Tissue Analysis: Doses of 20 mg ^{3}H-canrenoate-K/kg with a specific activity of .2-.5 uCi/mg ^{3}H-canrenoate-K were given IV into an ear vein. Rabbits were sacrificed by decapitation at the following times after administration of the dose; 5, 10, 20, 30, 60, 90 minutes, 2, 4, 8, 16 and 32 hours. One animal was used per time interval with the exception of the two hour experiments where 3 animals were used.

Blood was collected by exsanguination from the abdominal aorta. Organs removed were heart, lung, brain, kidney, liver adrenal gland and spleen. Tissue samples were homogenized using a tissue homogenizer (Polytron) in a five fold volume of methanol. After centrifugation at 2000 rpm the supernatant was removed and the tissue pellet washed with methanol and kept at 20°C for further analysis. The supernatant (.3 ml) or .1 ml of plasma was placed in a counting vial and ^{3}H counts determined by scintillation counting or measured by fluorescence (see 4) for canrenone and canrenoate. Tissue

pellets were analyzed to measure the extraction yield by methanol homogenation. Liquification of the methanol and water-washed pellet was achieved by using tissue solubilizer (NCS, Amersham-Searle) and an aliquot of the total volume was counted in 10 ml Aquasol counting solution (New England Nuclear). Pellet extractability was quantitative since all of the counts were found in the supernatants.

A portion of the kidney from one rabbit was homogenized using the Polytron and spun at 2000 rpm for 10 minutes. The supernatant was then counted directly and following evaporation to dryness in order determine if ^{3}H-activity might be accounted for by $^{3}H_2O$ exchange from the parent labelled compound.

3. Analytical Procedures

The fluorimetric determination of canrenone and canrenoate was performed using the procedure of Sadee et al., (217). This consisted briefly of a methanol:water extraction, addition of sodium bicarbonate, and dichloromethane. The organic layer was transferred to another tube. To the aqueous layer was added 2N HCl, and after 15 minutes at room temperature a dichloromethane extraction was performed. To both sets of tubes, organic and aqueous, was added .1N NaOH, the tubes were shaken and the bottom layer was pipetted out. 2 ml of 62% sulfuric acid was added to each tube, they were shaken for 20 minutes and then fluorometric readings were taken at 478-526 mu. A standard curve was performed to determine the F factor used in calculating canrenone and canrenoate values. A plot of the

standard curve for this procedure and "F" calculations are seen in Figure 5.

4. Calculations

Data reported are the results of scintillation counts corrected to disintegrations per minute by the channels ratio method, and ug/ml or gram tissue as measured by fluorescence and corrected by the method of Sadee et al., (217).

a. Formulas for converting fluorescence to ug canrenone or canrenoate/ml or gram tissue:

$$\frac{X \;\; 1.84 \;\; 0.3 \;\; F}{TV \;\; WT \;\; 1000} = \text{ug canrenone or canrenoate/ml or g (tissue)}$$

TV=Tissue Volume used from sample

WT=Wt of tissue

X=Fluorescence reading

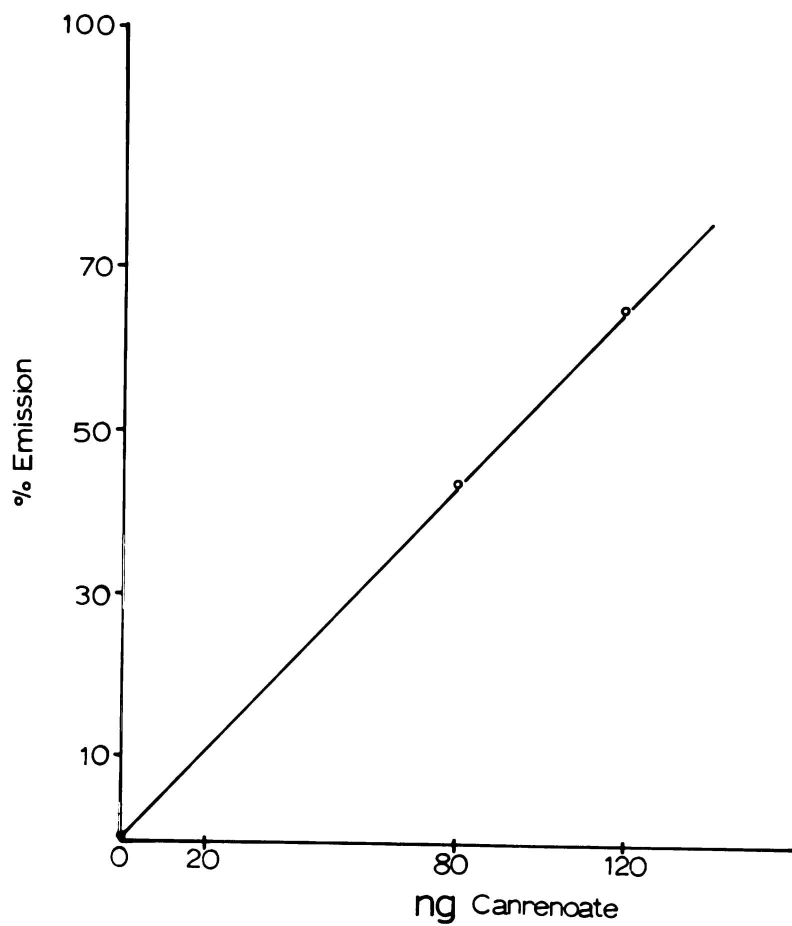
1.84 = ng (see Figure 5) constant

0.3 = volume used

F=Factor from previously determined conversion chart

FIGURE 5

Standard Curve for the Spirolactone Fluorescence Assay
(the factor "F" was calculated to be 1.84)



Results and Discussion

Plasma concentration versus time curves of canrenone and canrenoate were obtained by serial blood sampling after infusion of doses of 5, 10, and 20 mg canrenoate-K/kg given over 1-5 minutes. Equilibrium between canrenone and canrenoate was obtained as early as 10 minutes following administration of canrenoate. The plasma elimination half-life was about one hour, which is much faster than that observed in other species (207,208,199). The elimination half-life of the 25 mg and 50 mg doses was essentially the same, however the 12.5 mg dose appeared to exhibit variation, possibly due to individual variation within animals or assay error (Figure 6).

^3H-activity was not accounted for to a significant extent by ^3H_2O following possible ^3H exchange from ^3H-canrenoate or its metabolites, since the ^3H-activity of supernatants was the same when measured directly and following evaporation to dryness, Table 4.

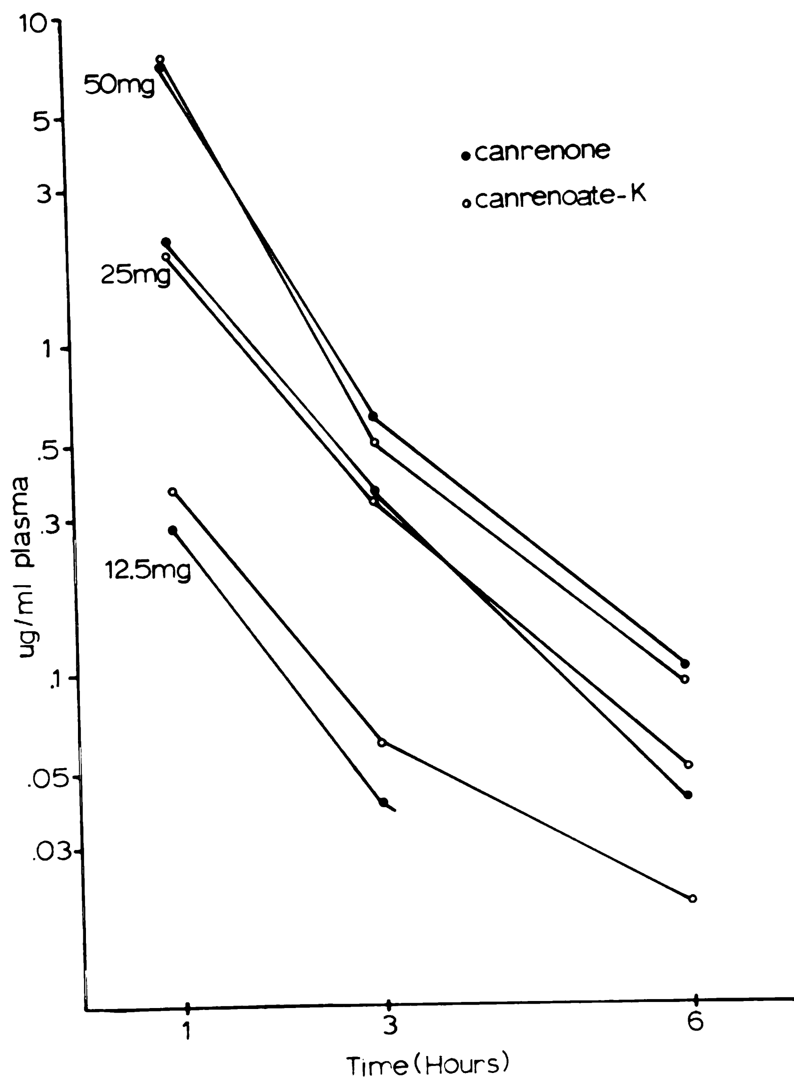
TABLE 4

Counts per minute from .1 ml, .3 ml, and .5 ml D (Dried down under nitrogen), S (Straight from the sample, no drying), and R (dried down and reconstituted in aqueous solution), supernatant from rabbit kidney homogenate.

.1 D	505	.1 S	773	.1 R	745
.3 D	2020	.3 S	2097	.3 R	2016
.5 D	3181	.5 S	3535	.5 R	3434

FIGURE 6

Plasma Elimination Time Curves for Canrenone and Canrenoate at various doses



Measurements of total radioactivity from all tissues analyzed are shown in Figure 7. Concentrations of total radioactivity in all organs analyzed show high concentrations and slow elimination of radioactivity with time. A rapid distribution into the tissues of canrenoate-K is indicated by the high levels of radioactivity seen even at the earliest time points.

Levels of total radioactivity, measured by scintillation counting and levels of canrenone and canrenoate measured by a specific fluorescence assay in rabbit liver tissue are seen in Figure 8. Liver appears to show a high uptake of canrenone and not canrenoate, which is apparent even at the earliest time point of 10 minutes after a dose of canrenoate-K where canrenone accounts for most of the radioactivity. This indicates a rapid conversion and slow elimination of ^{3}H-activity suggesting extensive metabolism.

Figure 9 shows total radioactivity measurements and measurements of canrenone and canrenoate in rabbit plasma. Here a faster metabolism of these compounds is seen than was reported in humans and a high concentration of metabolites. The half-life of canrenone and canrenoate was about 1 hour compared to about 20 hours in humans.

Figure 10 shows canrenone levels in plasma and all the tissues analyzed. Canrenone gets rapidly taken up and concentrated in tissues by a factor of 10 compared to plasma. The large differences in canrenone concentration after 10 minutes in various tissues may be attributed in part to perfusion differences of the various organs.

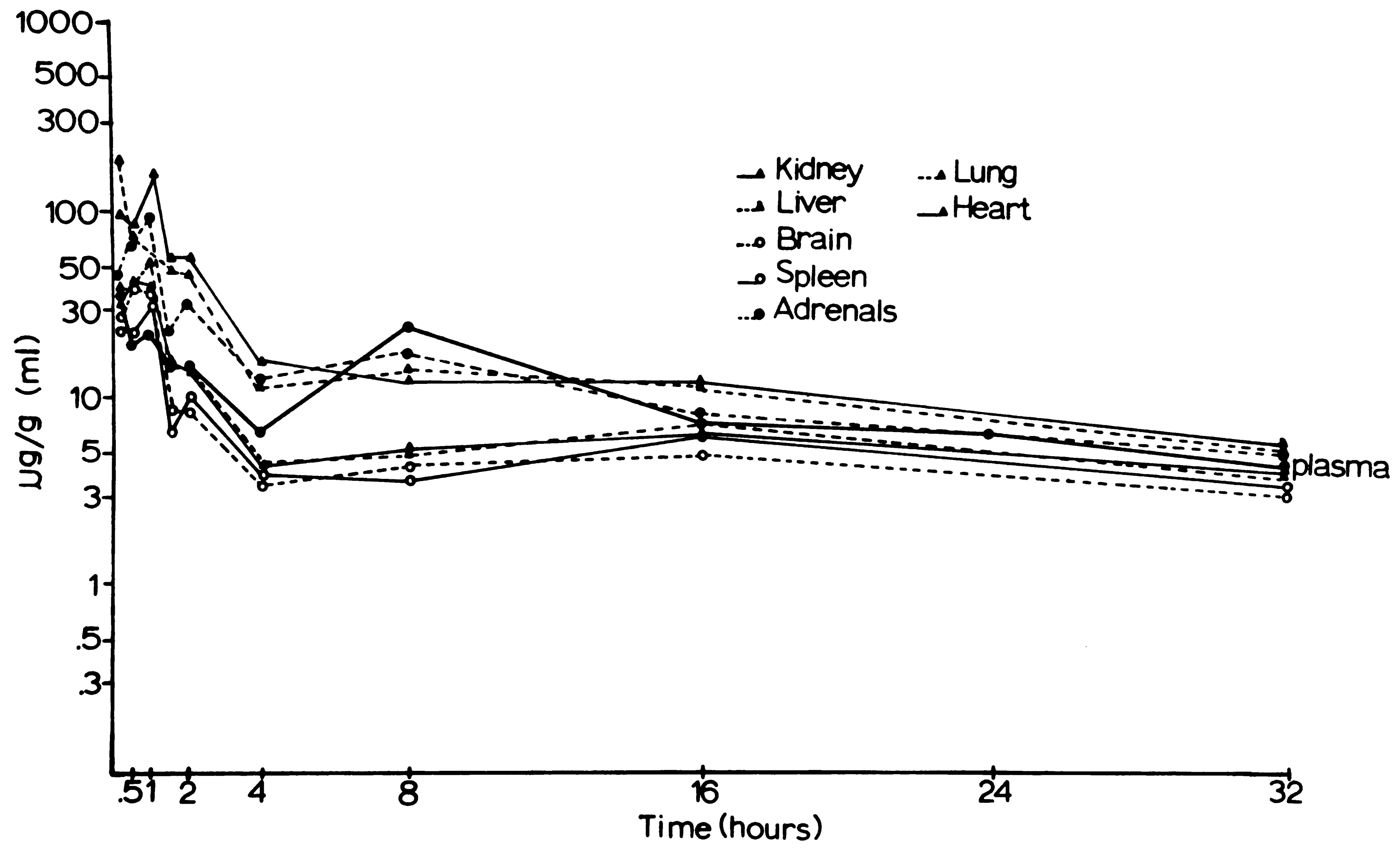


FIGURE 7: Levels of total ^{3}H-radioactivity measured by scintillation counting for all tissues analyzed and for plasma.

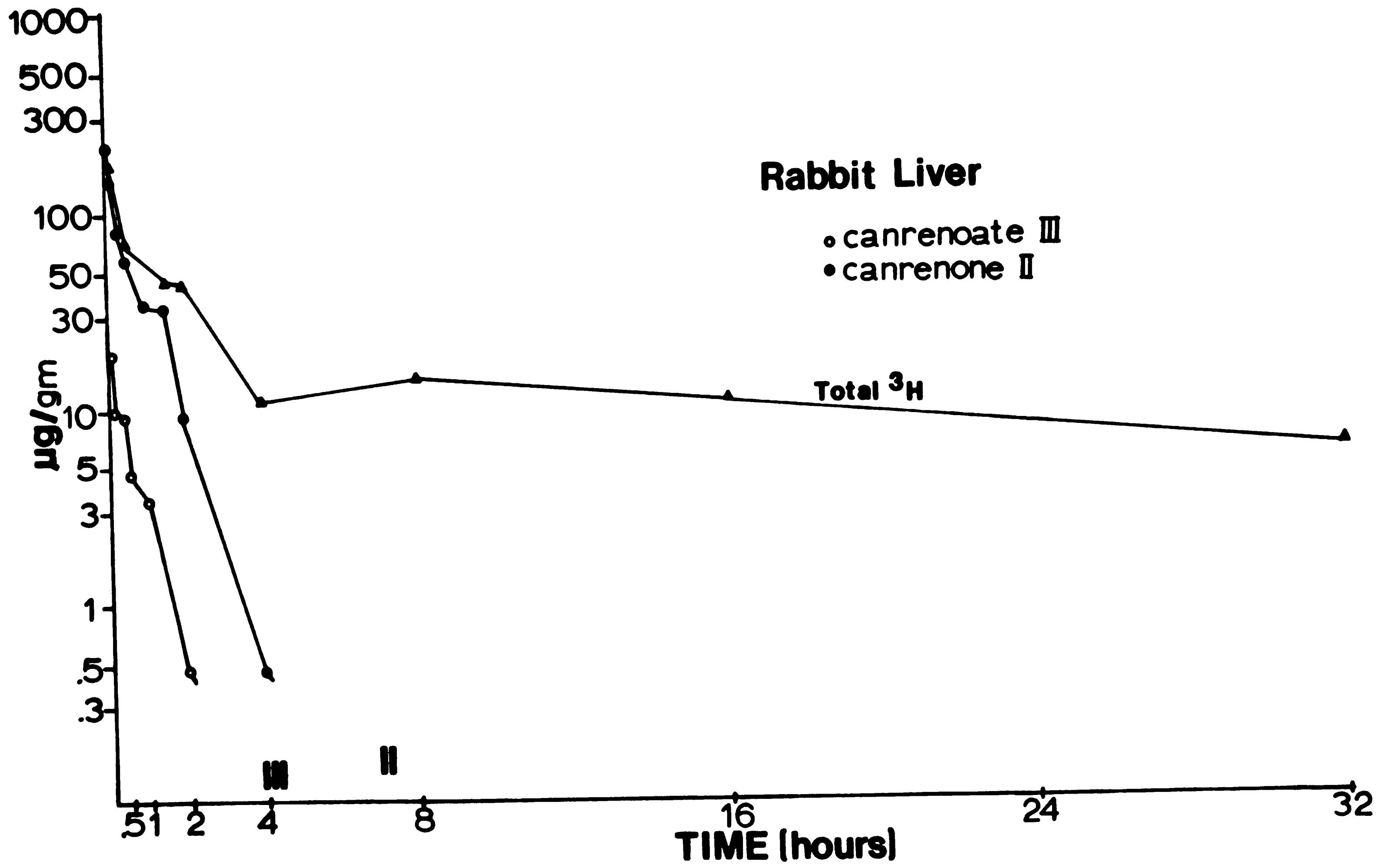


FIGURE 8: Concentrations of total ^3H-radioactivity measured by liquid scintillation counting, and concentrations of canrenone (II), and canrenoate (III) measured by a specific fluorescence assay in rabbit liver.

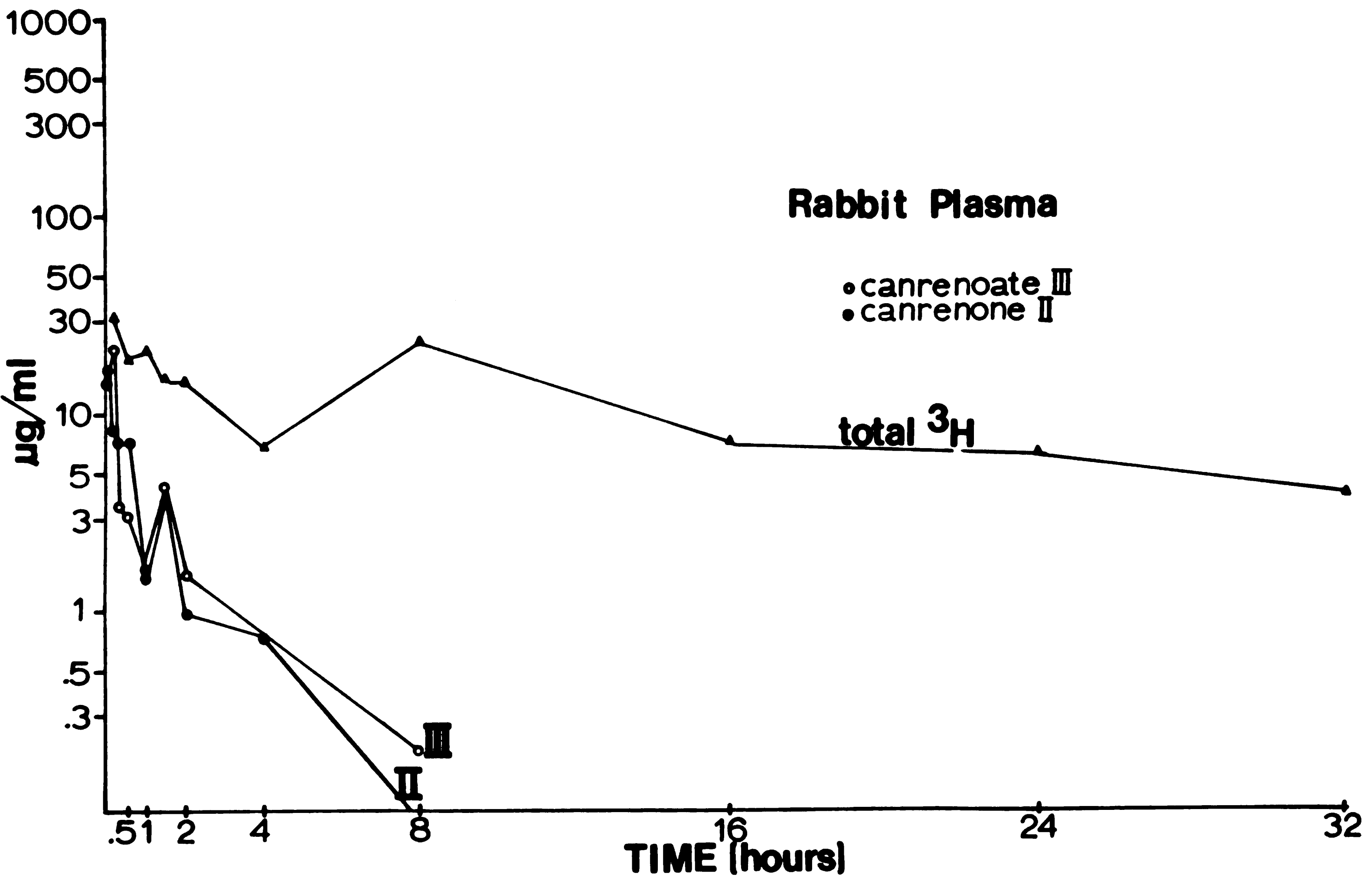


FIGURE 9: Concentrations of total ^3H-radioactivity measured by liquid scintillation counting, and concentrations of canrenone (II), and canrenoate (III) measured by a specific fluorescence assay in rabbit plasma.

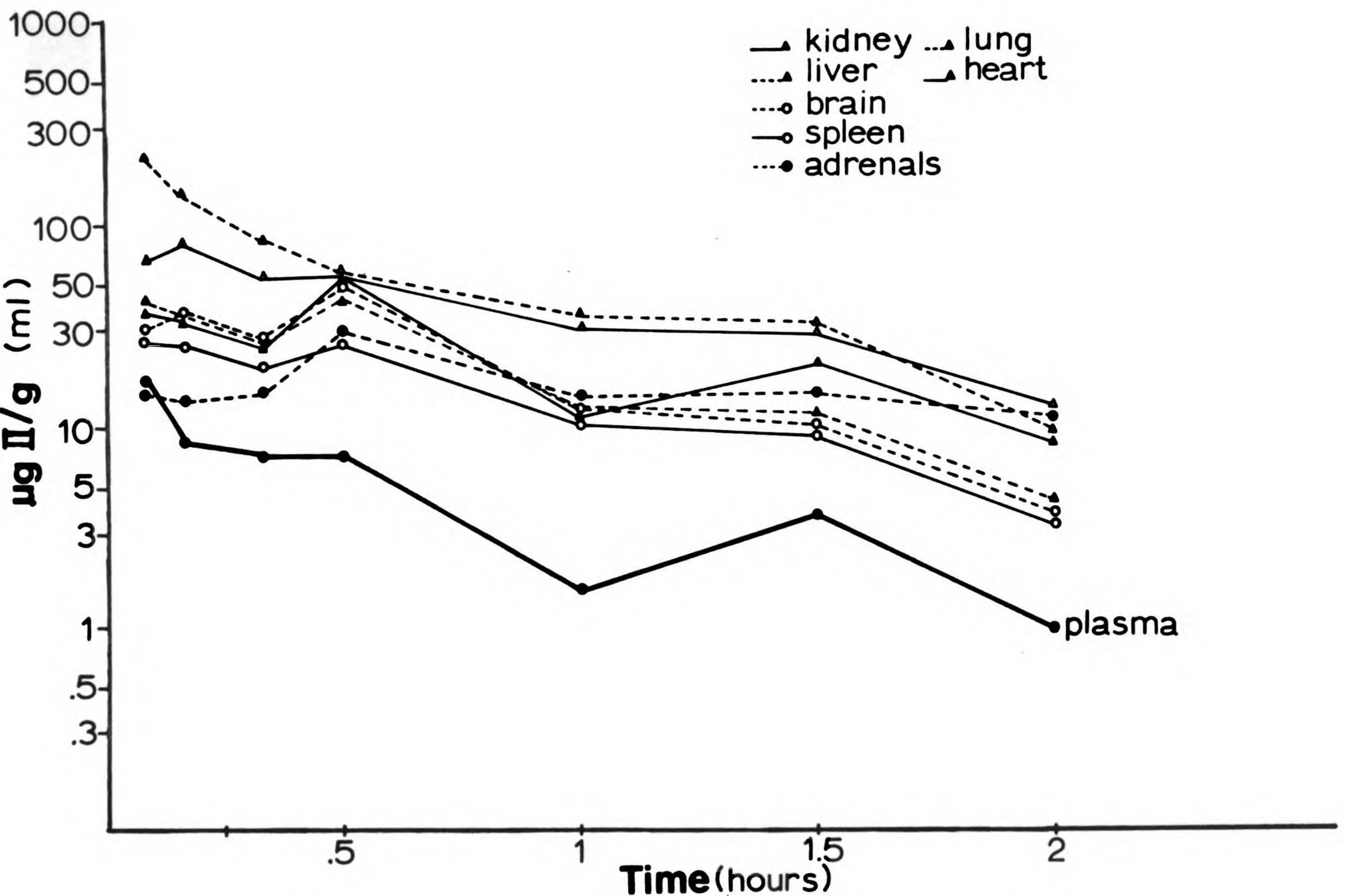


FIGURE 10: Plasma and organ concentrations of canrenone (II).

Levels of canrenoate in plasma and tissues is shown in Figure 11, and are of similar magnitude indicating a lower tissue affinity of canrenoate when compared to canrenone. Levels of canrenone and canrenoate past the 2 hour time point, from 4 hours to 32 hours were measured and found to be approximately equal to background.

In order to examine the variability between animals, 3 animals were analyzed at the two hour time point. All other points are represented by single animal experiments. Table 5 shows the averaged values and ranges for the 2 hour experiments in rabbit plasma and liver.

Table 5

Levels of Canrenone and Canrenoate in plasma and liver at 2 hours following administration of ^{3}H-canrenoate-K to 3 rabbits.

Mean (range) in ug/ml or gram

	Total ^{3}H-activity	Canrenone	Canrenoate
Plasma	18[12-25]	1.16[.6-1.5]	1.3[.4-1.8]
Liver	68[35-119]	10[4-16]	1[.6-1.2]

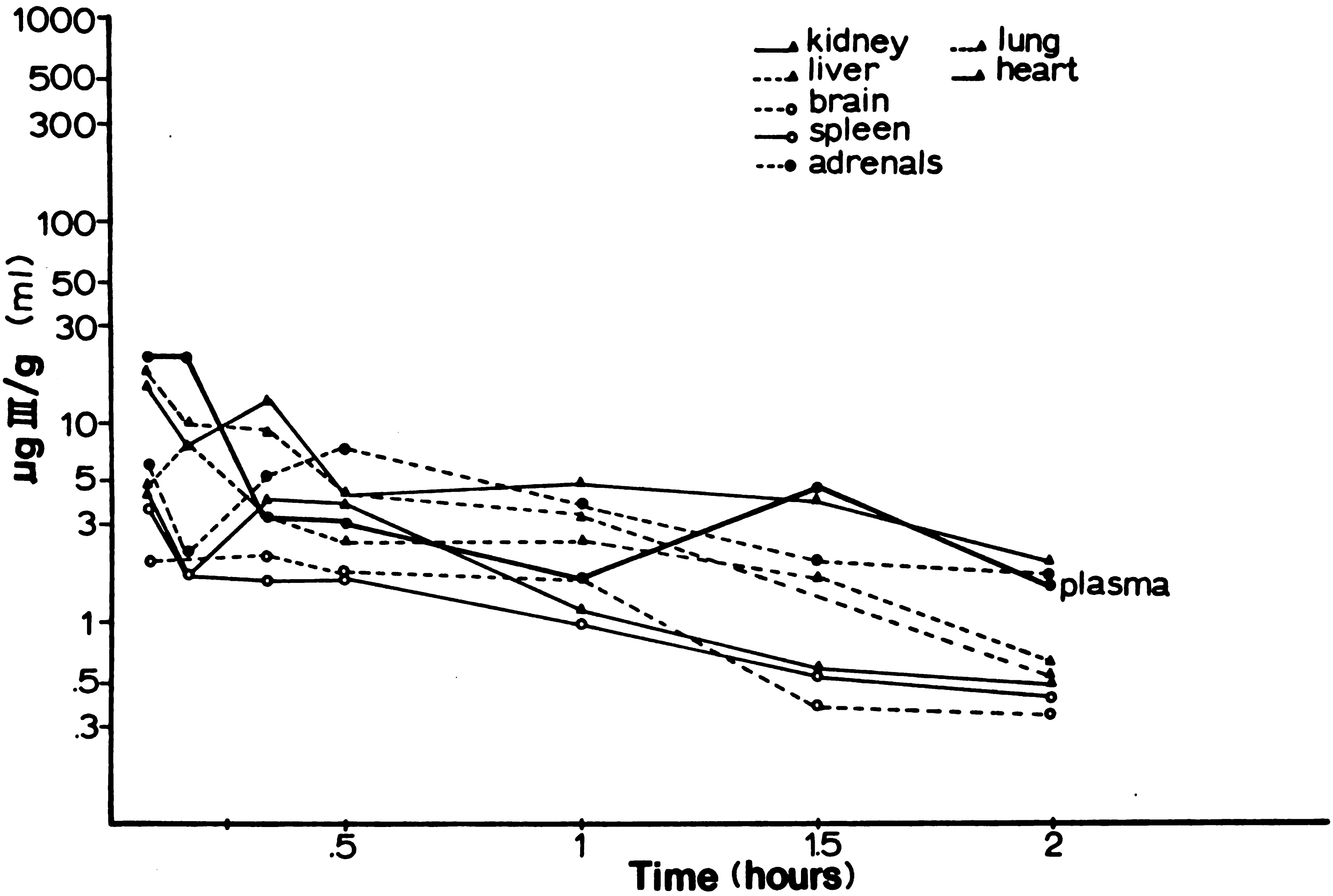


FIGURE 11: Plasma and organ concentrations of canrenoate (III).

These tissue distribution studies have demonstrated that canrenone is immediately formed after a dose of canrenoate-K and is rapidly concentrated in organ tissues. Therefore canrenoate-K is an effective agent to introduce canrenone, presumably the principal active metabolite of the spirolactones, into the body. Sadee et al., (199) have shown that canrenoate-K is well absorbed from the gastrointestinal tract and other investigators have demonstrated its water solubility.

The assays available for measurement of spirolactone levels are specific for the fluorigenic compounds canrenone and canrenoate. Plasma concentrations of canrenone represent a larger amount of this metabolite in the body when compared to plasma levels of canrenoate. It seems therefore justified to consider canrenone as a major metabolite upon which pharmacokinetic studies such as bio-availability should be based. This result can most likely also be applied to species other than the rabbit.

Extentive transformation to metabolites of unknown structures and unknown pharmacological activity occurs, and needs to be investigated. Therefore, a study of the further metabolism of canrenoate-K was undertaken (Section III of this work).

III. CHEMICAL STRUCTURE AND ALDOSTERONE RECEPTOR AFFINITY OF CANRENOATE-POTASSIUM METABOLITES IN RABBITS

Abstract

Two new major metabolites of canrenoate-K were isolated in sufficient quantities for structural elucidation, from rabbit livers, in addition to the known metabolite canrenone. One metabolite (M_A) contains two additional oxygen atoms of unknown location. The second metabolite (M_B) was identified as 20-hydroxy-canrenone. Canrenone, and to a lesser extent metabolite M_B showed affinity to rat kidney cytosol aldosterone receptors. Both of these metabolites might contribute to the pharmacological effects of canrenoate-K.

Introduction

The steroidal spirolactones, spironolactone, canrenoate-K and their common major metabolite canrenone are reversible competitive antagonists of mineralocorticoids at the renal and at the adrenal level. Sadee et al., (199) reported that the plasma radioactivity half-life following an oral dose of ^{3}H-canrenoate-K in humans exceeded 50 hours. Half-lives of canrenone and canrenoate, measured by a specific fluorescence assay, were less, about 20 hours. Fluorescence measurements yielded about 20% of a dose of canrenoate excreted into the urine within 5 days (199). Thus, unknown metabolites appear to account for the long half-life of these compounds in humans.

The clinically observed toxicity of spironolactone (178,163,116,115,159,184) may be caused in part by metabolites retaining sulfur groups in the C-7 position. The side effect of gynecomastia has prompted studies on the effects of spirolactones on testosterone metabolism (232,188, 179). Spironolactone and not canrenone, was shown to cause inhibition of testosterone production by destruction of testicular cytochrome P-450 in laboratory animals (187,188). Presumably, intermediary metabolites formed through metabolism of the thioacetyl moiety of spironolactone, which is not present in canrenone are responsible for this action (229,230). Therapeutic use of canrenone or canrenoate would eliminate chemically labile and potentially toxic sulfur containing spironolactone metabolites.

The tissue distribution studies (section II) have shown extensive metabolite formation and retention in the rabbit. It is the purpose of this study to present the physical-chemical characterization of two major metabolites in rabbits of canrenoate-K, and their relative affinities for renal cytosol aldosterone receptors using the method of Marver et al., (93). This parameter may indicate antimineralocorticoid activity, since the spirolactones are thought to act by competition for specific aldosterone receptors (binding sites) in target tissues.

Experimental

1. Chemicals and Radiochemicals

The sources and purification procedures for the chemicals and radiochemicals used in these studies are mentioned in sections II and IV of this report. Compound IV, 3-(3-oxo-17β-hydroxy-4,6 androstadiene 17α-yl) propanoic acid lactone, was obtained from G.D. Searle and Co., Chicago, Ill.

2. Apparatus

Ultra violet spectra were taken on a Beckman UV spectrophotometer. Fluorescence was determined on an Aminco-Bowman Spectrophotofluorometer. Scintillation counting was done on a Searle Mark III counter and counts per minute were corrected to disintegrations per minute by the channels ratio method. Gas chromatography was performed on a Varian 2740 gas chromatograph, using a 6' x1/4" OD, 2 mm i.d. column packed with 3% OV 1 on Chromosorb W. Mass Spectra were taken by direct insertion on a Varian CH-7 Mass Spectrometer using 70eV electron impact, and on an AEI MS 902 high resolution mass spectrometer using chemical ionization with isobutane. Accurate mass measurements were performed using perfluoro-tri-butyl-amine as standard. Gas Chromatography-Mass Spectrometry (GC-MS) was done on a Varian 2740 GC coupled to a CH-7 MS in the EI mode. The GC column was the same as that used for GC alone. Fourier transform proton nuclear magnetic resonance spectra were taken on a Varian XL 100-15 at 100 MHz and on a Bruker-360 MHz in deuterochloroform.

3. Analytical Procedures

Rabbits weighing up to 4 kg were injected IV with single doses of 20 mg 20,21-^{3}H-canrenoate-K/kg with a specific activity of 10 Ci/20 mg canrenoate-K and sacrificed after two hours. The entire liver was removed and homogenized in a five fold volume of methanol using a Waring blender, centrifuged and the pellet then washed with additional methanol. The methanolic extracts were combined, concentrated *in vacuo*, diluted with water, dried over sodium sulfate and evaporated *in vacuo*. To the residue 20 ml of methanol and 30 ml of 1N NaOH were added and the mixture was washed with dichloromethane. The aqueous layer was then acidified with 20 ml 5N HCl, extracted with dichloromethane, and the organic layer was dried. This extraction procedure results in an effective purification of spirolactones without changing the chromatographic profile of metabolites (3). The extraction residue was chromatographed on a silica gel column using increasing concentrations of methanol in dichloromethane. Column fractions were evaporated and chromatographed on thin layer plates (Silica Gel KG Merck F 254) using dichloromethane:methanol (95:5) as solvent system. Tritium containing bands were scraped off the plates and the metabolites eluted using methanol.

One column chromatography fraction contained 80% of the total radioactivity and resulted in three major radioactive bands on thin layer chromatography, ie: M_A (R_f 0.21), M_B (R_f 0.18), and canrenone (R_f 0.56). Following elution from thin layer chromatography plates, M_A, M_B and canrenone were again

subjected to extraction at pH 1 and pH 13 with solvent on thin layer plates using dichloromethane:methanol (90:10) eluted and purified by water-dichloromethane solvent extraction. The metabolites were then analyzed by tlc, GC, MS, GC-MS, NMR, UV and fluorescence techniques.

4. Aldosterone Cytosol Receptor Binding Assay

The method of Marver et al., (93) was utilized. Adrenalectomized rat kidney slices are incubated in the presence of dexamethasone , buffer, radioactive aldosterone and varying concentrations of canrenone, M_A, and M_B. An outline of the procedure is shown in Table 6 .

TABLE 6

KIDNEY SLICE EXPERIMENT-Aldosterone Receptor Rat Kidney Cytosol Binding Assay

1. Adrenalectomize rats-wait 48-72 hours, maintain on normal saline.
2. Prior to sacrificing animals flush kidneys out to remove blood (this is done either by perfusion in situ or by cutting the abdominal aorta).
3. Remove kidneys
4. Decapsulate
5. Cut kidneys in half and place in ice-cold incubating solution awaiting slicing (incubating solution is a standard buffer solution).
6. Slice kidneys using a tissue slicer to a thickness of 275uM, place in a petri dish after slicing and slices are separated.
7. Decant off buffer and separate slices into small petri dishes for distribution to incubation flasks.
8. Add slices from 1-2 kidneys to 10 ml STD buffer solution and flasks which contain controls, unlabelled d-aldosterone, and varying concentrations of spirolactone metabolites to be tested as well as canrenone concentrations to be tested.*
9. Incubate flasks 20 minutes at $25^{\circ}C$.
10. At the end of the incubation time remove flasks from the bath. The contents are poured through Nytex material to remove excess buffer and to wash the slices.
11. Homogenize slices in 2 ml 0.25M sucrose, with 0.3mM $CaCl_2$.
12. Pour homogenates into centrifuge tubes and spin first at 600 x g for 10 minutes leaving a soft pellet.
13. Decant off supernatant. Respin supernatant at 10,000 x g for 10-15 minutes.
14. Supernatant from the second spin is used for measurement of the cytosol receptor content. This supernatant is then filtered through G-50 Sephadex columns (to determine the content of bound ^3H-aldosterone).
15. Count a fraction of the void volume from the columns and do a protein determination on an additional fraction.

* ^3H-aldosterone is added to all tubes ($5.9 \times 10^{-9}M$)

Results

Following a total dose of 80 mg ^{3}H-canrenoate-K to a rabbit weighing 4 kg, the liver was removed and extracted and resulted in purified metabolic fractions of 574 ug M_A, 351 ug M_B, and 480 ug canrenone expressed in ^{3}H-canrenone equivalents which represent 33% of total methanol extractable ^{3}H liver content.

Characterization of Metabolites

Canrenone: The ultra violet spectrum (Figure 12) was superimposable on that of authentic canrenone, with a ultra violet maximum in methanol at 284 nm and a molecular extinction coefficient of 27,000, theoretical extinction coefficient of canrenone is 27,500, based on ^{3}H measurement of the quantity of the metabolite, canrenone. Thus, no exchange of ^{3}H-label occurred in canrenone during animal treatment and isolation procedure. Proof of identity with authentic canrenone was obtained by comparison of identical electron impact mass spectra .

Metabolite M_A: The electron impact (EI) mass spectrum is summarized in Table 7. M_A showed a molecular ion at m/e 372, indicating the incorporation of two oxygen atoms into canrenone. Loss of water from the molecular ion, suggested the presence of one hydroxyl function of unknown location. Loss of oxygen to give m/e 356 indicated that the second oxygen function may not be a hydroxyl. The UV spectrum of M_A (Figure 12) did not

TABLE 7

Table 7: Electron impact mass spectra of II, IV, M_B and M_A. Numbers in parentheses are intensity relative to the base peak in each spectrum.

Fragment		II	IV	M_B-GC-MS	M_B	M_A
M^+	m/e	340(100%)	338(100%)	338(100%)	356(100%)	372(100%)
M^+-CH_3	m/e	325(28%)	323(20%)	323(23%)	341(17%)	357(20%)
M^+-O	m/e	------	------	-------	------	356(30%)
M^+-H_2O	m/e	------	------	-------	338(65%)	354(20%)
M^+-O-CH_3	m/e	------	------	-------	------	341(16%)
$M^+-CH_3-H_2O$	m/e	307(81%)	305(8%)	305(10%)	323(21%)	-------
M^+-C-17ring	m/e	267(89%)	267(12%)	267(10%)	267(87%)	267(25%)

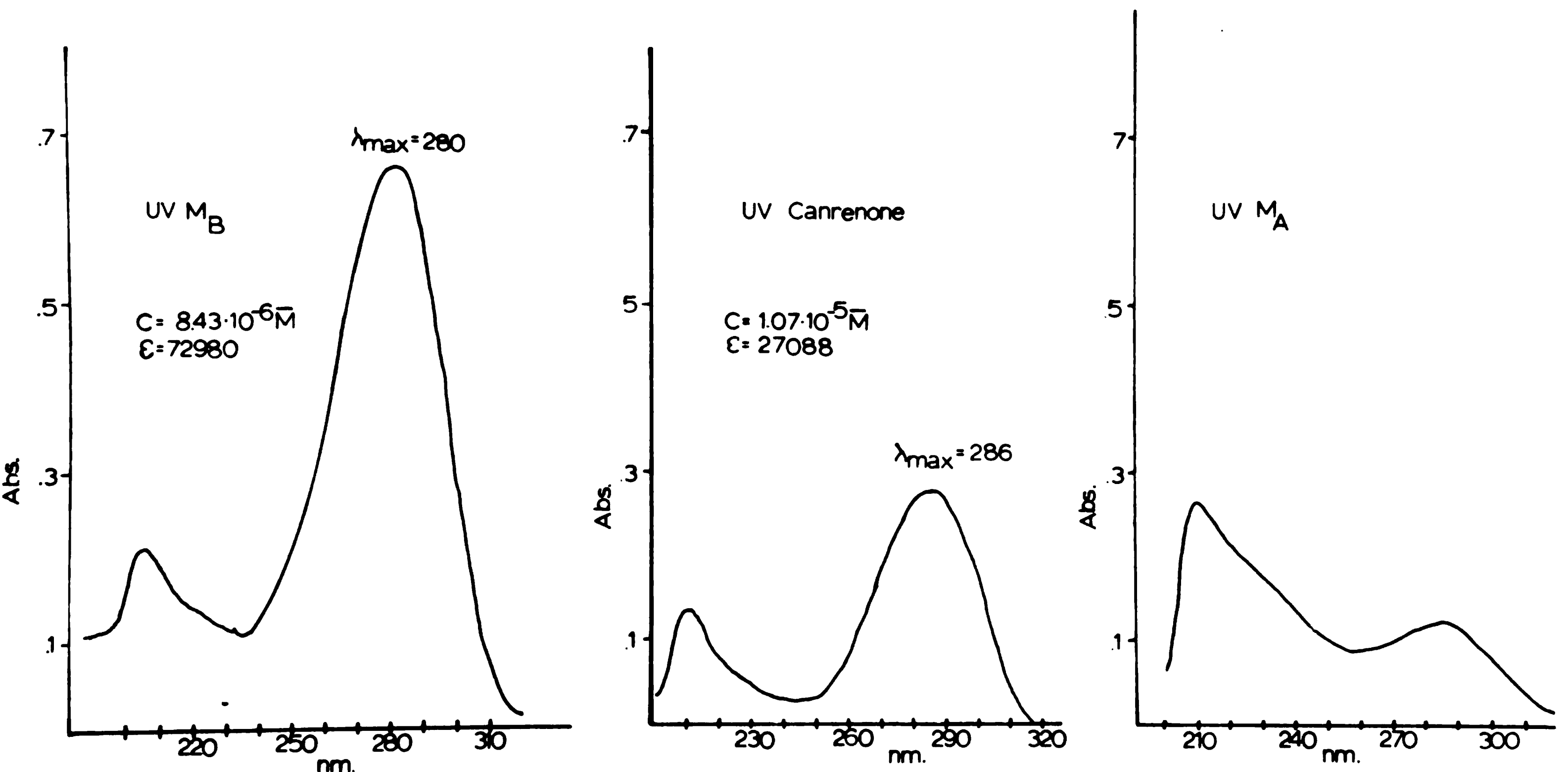
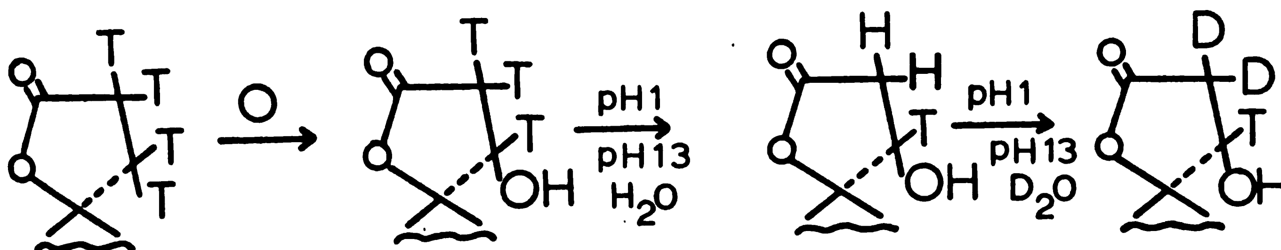


FIGURE 12: Ultra Violet Spectra of Metabolite M_B, canrenone and Metabolite M_A. (Absorbance vs nm.)

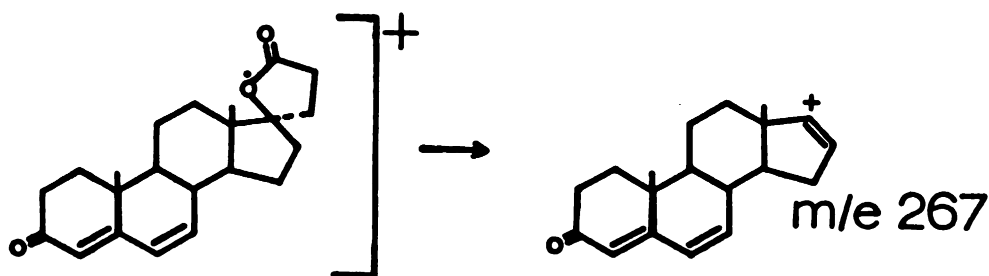
Metabolite M_B: The UV spectrum of M_B was identical to that of canrenone (Figure 12), however with a calculated extinction coefficient of ϵ=75,000 assuming a specific tritium activity identical to that of the parent drug. Therefore about 2/3 to 3/4 of the ^{3}H-label of M_B has been exchanged during body passage and/or isolation. The correct amount of M_B was about 1 mg assuming that the molar extinction coefficient of canrenone did not change as a result of metabolism. Since the ^{3}H-label was randomly scrambled between the C-20 and C-21 positions, these findings can best be explained by hydroxylation at the C-20 position resulting in loss of one ^{3}H-label and subsequent exchange of another two 3H's at the activated position C-21 during isolation. The observed loss of ^{3}H-label was less than expected [3/4] which could be a result of an isotope effect in the C-20 hydroxylation. (Figure 13).

FIGURE 13



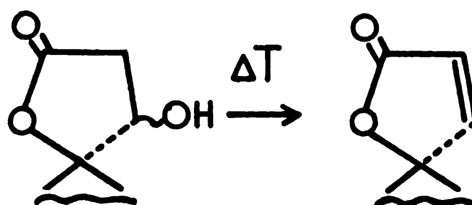
Electron Impact mass spectrometry confirmed the presence of one hydroxyl function which is readily lost resulting in an $M^+ - H_2O$ peak at m/e 338. Chemical ionization high resolution accurate mass measurement (<3ppm) of m/e 357 $(M + H)^+$ gave the correct empirical formula for M_B as a hydroxylated canrenone metabolite (C_{22} H_{28} O_4). Major fragmentation of canrenone during EI involves loss of the C-17 side chain in addition to one proton which gives rise to an abundant fragment of m/e 267. Since this fragment is also present in the spectrum of M_B, further support is given to the location of the hydroxyl function in the gamma lactone ring (Figure 14, Table 7).

FIGURE 14



Gas chromatography of M_B at 250°C resulted in one peak with a retention time similar to that of canrenone. Mass spectral analysis of this GC peak (Table 7) indicated thermolytic dehydration to IV (Figure 15).

FIGURE 15



Identity of GC-MS data with those obtained from authentic IV proved the hydroxyl position in the gamma-lactone ring, while facile dehydration again supported its position for the C-20 position.

The loss of tritium label of M_B, but not canrenone, during isolation is also consistent with the C-20-hydroxyl. Canrenone, M_B and IV were treated with D_2O at pH 13 and pH 1 and the products analyzed by EI-MS in order to directly demonstrate exchangeability of the C-20,21 protons (Table 8).

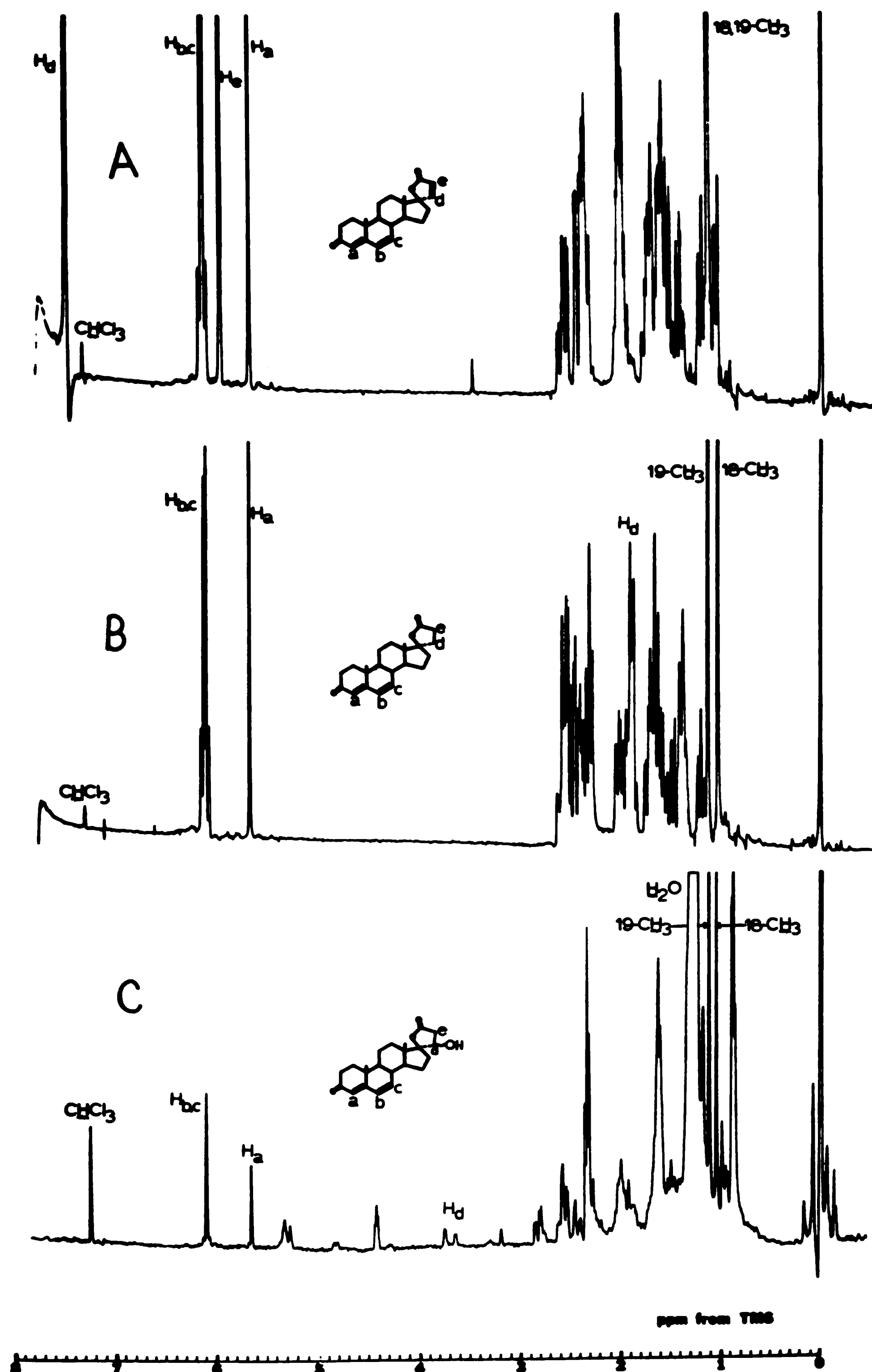
TABLE 8

Table 8. **Molecular ion cluster in the E.I. mass spectra of II, IV, and M_B prior to and after deuterium exchange. The normotopic parent ion is normalized to 100%.**

	M^+	M^++1	M^++2	M^++3
II	**100%**	**24%**	**4%**	**<1%**
II deut.	**100%**	**76%**	**27%**	**6%**
IV	**100%**	**25%**	**4%**	**<1%**
IV deut.	**100%**	**76%**	**29%**	**6%**
M_B	**100%**	**27%**	**5%**	**<1%**
M_B deut.	**100%**	**300%**	**250%**	**100%**

This treatment resulted in incorporation of zero to one deuterium atoms into canrenone and compound IV. The deuterium was not located in the gamma-lactone ring, since the fragment generated by loss of this ring retained the deuterium enrichment; two to three deuterium atoms were incorporated in M_B, two of these presumably in the C-21 position (Figure 13).

High resolution (360 MHz) proton nuclear magnetic resonance spectra were taken of M_B, canrenone and IV in order to further establish the position of the hydroxyl function (Figure 16). The alpha and beta protons in the spectrum of butyrolactone are in a broad multiplet located between δ1.9 and δ2.75 ppm (234). Methyl substitution of the gamma position to give methyl-butyrolactone shifts the beta proton signals to δ1.88 and δ2.25 ppm, while the alpha protons are found at δ2.41 ppm. Analogously, one can assign the multiplet between δ1.8 and δ1.95 to the C-20-H resonance in the spectrum of canrenone. The C-21-H resonance should then be found within the range of δ2.2-2.6 ppm. The signal for the C-20-H of canrenone is absent in the spectrum of IV, which supports assignment of the C-20-H of canrenone multiplet at δ1.8-1.95, and is also missing in the spectrum of M_B, which is consistent with a C-20 position of the OH function of M_B. The C-21 protons of M_B could not positively be identified within the multiplets at δ2.5-2.9 ppm.



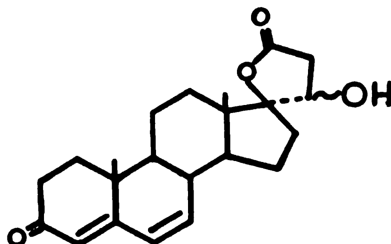
<u>FIGURE 16</u>

360 MHz Nuclear Magnetic Resonance Spectra of Canrenone (B), Compound IV (A) and Metabolite M_B (C).

One can therefore assign the structure of 20-hydroxy-canrenone to metabolite M_B, (Figure 17). to M_B, (Figure 17).

FIGURE 17

Metabolite M_B



More evidence is needed to establish the configuration at C-20. The NMR spectrum suggests that two configurations are present in M_B since the C-20 alpha and the C-20 beta protons might be associated with the two doublets at δ3.64-3.76 ppm (J 7Hz) obtained at 360 MHz Figure 16. A 100 MHz NMR spectrum of M_B taken under otherwise identical conditions gave two doublets at δ3.7 and δ 3.8 ppm with a coupling constant of J = 7Hz, demonstrating that these two doublets cannot be considered a quadruplet and indeed represent two identical protons.

Results of Renal Cytosol Binding Studies

Canrenone, M_A, and M_B at concentrations between 100 and 1000 times the concentration of ^{3}H-aldosterone were incubated with adrenalectomized rat kidney slices (93) to determine their relative affinities to the renal aldosterone receptor. The 50% displacement of ^{3}H-aldosterone was obtained at a canrenone concentration 300 times that of the aldosterone concentration, which is comparable to previous results (235). The available concentrations of M_A and M_B were insufficient

to bracket the 50% displacement value. A M_A concentration of 1000 times the concentration of ^{3}H-aldosterone caused displacement of 10% ^{3}H-aldosterone from the receptor. At this displacement level, M_A demonstrated less than 10% the activity of canrenone in the receptor assay. Metabolite B at 1000 times ^{3}H-aldosterone concentration caused 40% aldosterone displacement in two separate assays, making its affinity 20% that of canrenone.

Discussion

Metabolic hydroxylation as a minor metabolic pathway was shown to occur in positions C-6 and C-15 (222). This report adds hydroxylation of canrenone in the C-20 position as a major metabolic pathway in rabbits. Although a number of metabolites of the spirolactones have been reported, anti-mineralocorticoid activity has been reported for very few, eg., the 7 α-SH intermediate in the dog, and the 7α-SCH_3 intermediate in the rat. These metabolites have affinity for the aldosterone binding sites in *in vitro* experiments approximately 4 times higher than that of canrenone (211).

Chemical substitution into various positions of the parent spirolactone moiety including hydroxylation can critically alter its antimineralocorticoid activity (235,120, 122). There appears to be various structural requirements in the spirolactone moiety for activity (126,235,24). Gantt reported that the lactone is the active form (126). In the rat kidney cytosol receptor binding assay, alterations in the gamma-lactone ring produced a reduction in the affinity for aldosterone receptors. Canrenone was shown to have ten times greater affinity for these receptors than the water soluble canrenoate-K (235). Ring opening reduced the affinity for binding sites in other pairs of spirolactones tested as well. Unsaturation in the gamma-lactone ring and in the C 6-7 position seemed to decrease affinity for these receptors (235).

The 20-hydroxy canrenone metabolite might be expected to

have affinity to the rat kidney cytosol mineralocorticoid receptor due to its structural similarity to aldosterone (Figure 1). This _in vitro_ assay system measures the first step in the proposed mechanism of steroid action, that of binding to the cytosol mineralocorticoid receptor. It is a measure of the affinity of a compound to the receptor and does not strictly imply pharmacological activity. However affinity to this receptor might indicate pharmacological activity, either agonistic or antagonistic. Should substantial affinity to the cytosol receptor be shown, further biological testing is indicated to determine pharmacological properties. In our studies 20-hydroxy-canrenone (M_B), was shown to be 20% as effective as canrenone in reducing the ^3H-aldosterone binding in this assay, while the activity of M_A, a lesser metabolite of unknown structure, was lower than 10% relative to canrenone. Since it was not possible to bracket the 50% ^3H-displacement value with the concentrations of M_A and M_B used, these figures should be considered only indications of relative receptor affinity. The moderate activity shown by M_B in this test system was not sufficient to further pursue its potential biological activity.

Results of recent studies (157) raise the question of spirolactone metabolites other than canrenone contributing to the efficacy of spironolactone and canrenoate-K. Metabolite M_B is also present in human plasma after spironolactone administration since it caused a measurable interferance with a radioimmunoassay procedure for aldosterone as reported in

section IV of this work. The concentrations of this metabolite in human plasma is low, however, and because of this it was decided that additional studies with this metabolite were not warranted at this time.

III. THE EFFECTS OF SPIROLACTONES ON CONCENTRATIONS AND PHARMACOKINETICS OF ALDOSTERONE

Abstract

The plasma aldosterone radioimmunoassay developed by Ito et al., (236) was found to be non-specific for aldosterone following administration of the spirolactones, spironolactone and canrenoate-K, in rabbits, dogs and humans. The assay interfering principle was identified as a hydroxylated derivative (M_B) of canrenone, which itself is a metabolite common to both spironolactone and canrenoate-K. The metabolite M_B, possessed a high cross-reactivity to the 21-hemisuccinyl BSA aldosterone antibody relative to other spirolactones. A modified procedure was developed specific for plasma aldosterone in the presence of this metabolite. Following single doses of spironolactone, and canrenoate-K plasma aldosterone levels were unchanged in humans and in dogs and decreased in rabbits.

Introduction

The aldosterone antagonists spironolactone, canrenoate-K and their common major metabolite canrenone (Figure 18) can affect endogenous aldosterone biosynthesis and secretion in several ways. Early work with these drugs showed increases in aldosterone *in vivo* after treatment with spirolactones (53, 237,128).

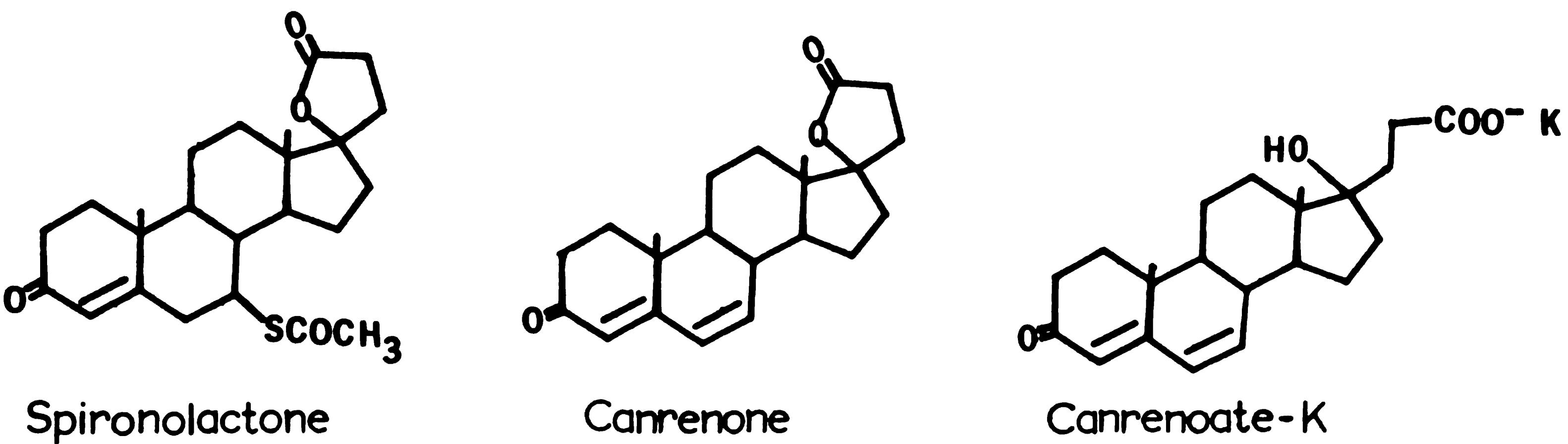


FIGURE 18: Structures of Spironolactone, Canrenone, and Canrenoate-K.

Davidson et al., (238) showed that spirolactone administration in man failed to affect aldosterone secretion rate, urinary aldosterone levels or the urinary aldosterone metabolite level. Kittinger et al., (237) showed increases in *in vitro* production of aldosterone in male and female rat adrenals when the animals were pre-treated with spirolactone (SC 8109) for 12 weeks. Friis (239) reported increased aldosterone production during spironolactone treatment in patients with severe cardiac insufficiency. Erbler has done a number of studies showing inhibition of aldosterone production by spirolactones (225,226,227,240,241,233,242). Sundsfjord et al., (228) provided evidence in support of Erbler's data by showing that spironolactone when administered in therapeutic doses decreases aldosterone secretion *in vivo* in man. In a case of primary aldosteronism these authors report a decrease in aldosterone secretion rate during spironolactone treatment with maintenance of a normal metabolic clearance rate of aldosterone (228).

"Spirolactone bodies" have been reported (227,31,243) in the adrenal cortex. Davis et al (243) described them as "concentric lamellar formations". Davis suggested that these bodies represent an attempt on the part of the zona glomerulosa cells to produce increasing amounts of mineralocorticoid. Janigan (244) reported "round acidophilic cytoplasmic inclusions in the zona glomerulosa of the adrenal cortices" of patients treated with spironolactone. Rohrschneider et al (245) could not show "spironolactone bodies" by light microscopy, however the zona glomerulosa width was increased in

animals treated with spironolactone compared with untreated animals (245). Jenis and Hertzog, also noted "distinctive laminated intracytoplasmic inclusions" in the human adrenal gland following prolonged spironolactone therapy (246), and suggested that these bodies may have a relationship to aldosterone biosynthesis. Spironolactone may play a direct role in endoplasmic reticulum turnover (247). These "spirolactone bodies" may reflect effects by spironolactone on endoplasmic reticulum of adrenal zona glomerulosa cells, the site of aldosterone production (247).

The conflicting results of spirolactone effects on endogenous aldosterone levels reported in the literature may be explained in part by the different methodology used to measure aldosterone. Plasma aldosterone levels have been measured by a number of different methods including bioassay (12), double-isotope dilution (248,249,250), gas-chromatography (251,252,253,254,255,256,257,258), and finally by radioimmunoassay (56,57,259,236,260,261,262,263,264,265, 266,267,268,269). Presently the radioimmunoassay is preferred for routine measurements due to its high sensitivity and specificity. Ito, et al., (236) published in 1972 an aldosterone radioimmunoassay, using LH-20 Sephadex column chromatography. Samples run by this method compared with those run by double-isotope dilution and radioimmunoassay using paper chromatography yielded results in good aggrement. Radioimmunoassays have been developed which use simplified separation techniques (269), or more highly specific antisera which allows the assay to be run without the use of separation procedures (268,270).

Aldosterone antibodies have been produced by several methods yielding the aldosterone C-3 oxime (57,262), the C-18,21 dihemisuccinate BSA antibody (236), the C-21 hemisuccinate antibody (262), and the gamma-lactone antibody (264).

Possible interference of the aldosterone radioimmunoassays by administration of drugs has to be ruled out for each individual drug. The spirolactones deserve special attention as a possible source of erratic aldosterone determinations. These drugs interfer with radioimmunoassays of other drugs (271,272,228,273).

The studies of Baethmann et al., (138,102) showing that both spirolactones and aldosterone were useful in treating brain edema led to a collaborative effort between Baethmann and this laboratory on the possible mechanism of the aldosterone-like action of spirolactones on the brain. This study deals with aldosterone kinetics during spirolactone treatment in several species using the Ito et al., (236) radioimmunoassay. We report interference of this aldosterone radioimmunoassay by a yet unknown spirolactone metabolite, and a modified specific procedure to correct this interference. Preliminary data indicate no increases in aldosterone plasma levels following short term spirolactone treatment.

Experimental

1. Aldosterone Plasma Radioimmunoassay

The radioimmunoassay procedure of Ito et al., (236)

consisted essentially of the following steps: Plasma extraction with dichloromethane, column chromatography on LH-20 Sephadex columns with dichloromethane:methanol (98:2), as eluent, incubation with an aldosterone 21-hemisuccinyl BSA antibody and measurement of the bound fraction of aldosterone by Florisil separation. From 0.5-1.5 ml of plasma could be analyzed by this method. To the extraction vessel was added a tracer amount of radioactive aldosterone which allowed recoveries to be calculated from the columns. This value was used in the final calculations. Tne pre-packed, pre-calibrated LH-20 Sephadex columns were used to separate endogenous steroids and other compounds such as metabolites of aldosterone from the aldosterone fraction. Pre-calibration of the columns was done by placing a small quantity of labelled aldosterone on the pre-washed columns, eluting with 98:2 and collecting 2 ml fractions from 50-80 ml. This enabled the fraction containing radioactive aldosterone to be precisely determined by scintillation counting. This fraction was generally represented by a 7-8 ml fraction volume. A column calibration curve in seen in Figure 19. When samples were run through the procedure this fraction, containing the aldosterone, was dried down and brought up in 2.5 ml of methanol. 0.5 ml of the 2.5 ml was prepared for liquid scintillation counting. The additional 2 ml were used for the radioimmunoassay. One ml was pipetted in duplicate into two labelled 13 x 100 mm test tubes which were either stored or immediately placed through the assay procedure.

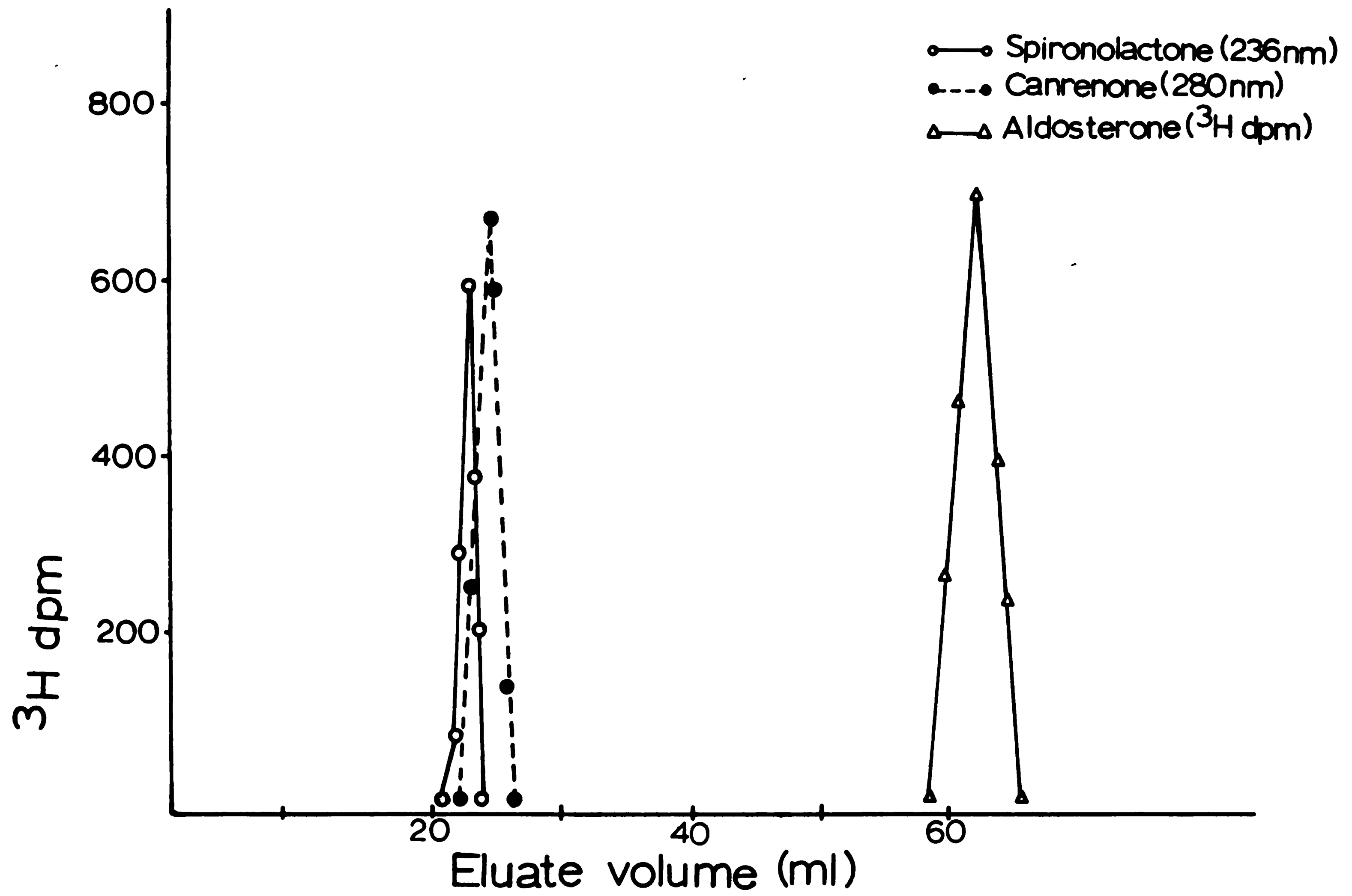


FIGURE 19: Column Retention (LH-20) Sephadex of Spirolactones and Aldosterone.

Standard curves were run each time the radioimmunoassay was performed. An example of a standard curve is seen in Figure 20. Following incubations, with the working solution, free aldosterone was separated from bound aldosterone at 4-6°C using activated Florisil. Bound aldosterone was counted by liquid scintillation and calculations to determine percentage bound were performed according to the method of Ito et al., (236). All samples were dried down and brought up in 0.6% bovine serum albumin and a cocktail prepared consisting of PPO-POPOP^R, absolute ethanol, toluene and NCS^R solubilizer.

Recoveries from the Sephadex LH-20 columns ranged from 30-60% and the sensitivity limit was about 1-2 pg/incubation. Aldosterone standards of 100 pg

were run through the procedure. The average amount of aldosterone found was 101 pg with a standard deviation of 17% (n = 6).

The titer of the 21-hemisuccinyl- BSA antibody was determined prior to beginning these experiments. Serial dilutions of concentrated antibody were made from $1:10^2$ to $1:10^8$. The dilution of antibody which yielded a Bound/Free ratio of 1 was sought, Figure 21. The titer was determined to be 50,000. The final dilution used in the assay system was 1:250,000, which was capable of binding ^3H-aldosterone (3 pg) to about 60-65%.

The working solution used in this radioimmunoassay consisted of the suitable antibody dilution, phosphate buffer, bovine serum albumin, and ^3H-aldosterone.

FIGURE 20

Standard Curve for the Aldosterone Radioimmunoassay

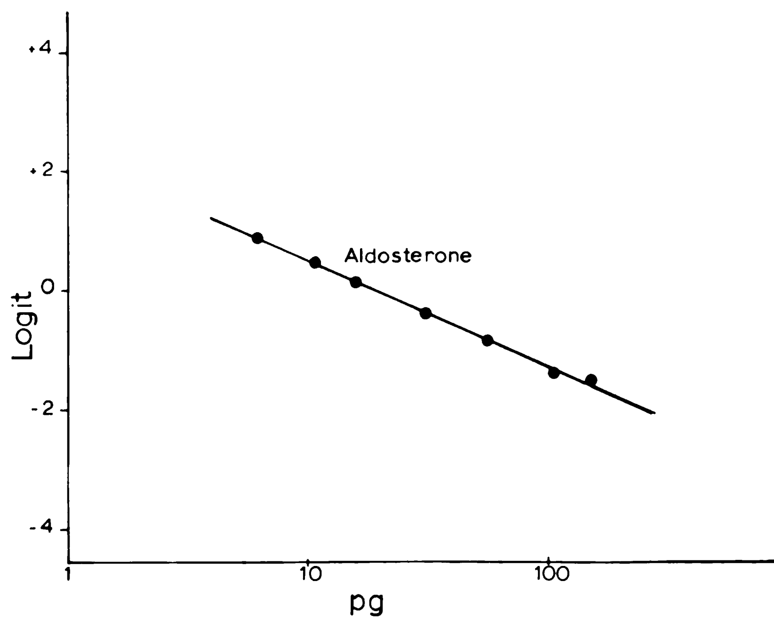
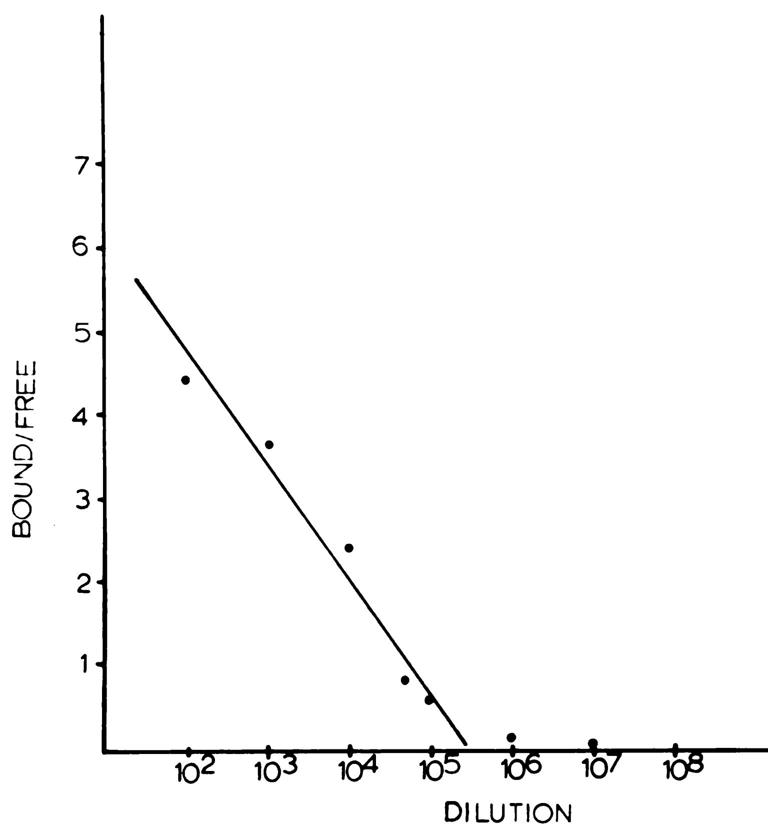


FIGURE 21

Antibody Dilution Curve for the
21-hemisuccinyl-BSA antibody for
Aldosterone.



2. Modified Aldosterone Plasma Radioimmunoassay in the Presence of Spirolactone Metabolites

To 1 ml plasma, 0.2 ml of 1N NaOH was added to adjust the pH to approximately 13, and the samples were kept at room temperature for 10 minutes. This resulted in hydrolysis of the gamma-lactone ring of all steroidal spirolactones to yield gamma-hydroxycarboxylic acids which are not extractable from aqueous medium into organic solvents above pH 6. After 10 minutes the pH was readjusted to about 8 with the addition of 1 ml of 0.4 M pH 7.4 phosphate buffer to prevent decomposition of aldosterone . The aldosterone was then extracted with 15 ml dichloromethane and carried through the same procedure as described by Ito et al., (236). Aldosterone standards of 100 pg were run through the procedure. The average amount of aldosterone was found to be 94 pg with a standard deviation of 11% (n =5).

3. Administration of Spironolactone and Canrenoate-K

a. Human Subjects

400 mg spironolactone (Aldactone^{R}) was given in tablet form in a single dose. Heparinized plasma samples were obtained at 0, 3, 8, 24, and 48 hours following administration. Three patients received rapid intravenous infusions over 10 minutes of 1 gram of canrenoate-K (Soldactone^{R})/day at 9 a.m. on three consecutive days as part of their medical treatment prior to neurosurgery and plasma samples were collected at 9, 12, 15 and 18 hours each day.

b. Dogs

Doses of 25 to 150 mg per day of canrenoate-K were infused intravenously over 1-5 minutes. Heparinized plasma samples were obtained from 45 minutes prior to administration until 100 minutes following administration in 15-30 minute intervals. Two dogs were adrenalectomized and corticosteroids were substituted by daily intramuscular injection of 0.4 mg aldosterone and 10 mg prednisolone-21-hemisuccinate sodium for 3 days. A dose of 100 mg/kg canrenoate-K was given intravenously two days after termination of corticosteroid substitution and again plasma samples were collected as above.

Cerebrospinal fluid samples were analyzed from dogs who had been given spirolactones in an attempt to determine if changes in aldosterone levels could be seen after administration of the drug. A number of samples were taken at time points coincident with the removal of plasma samples.

c. Rabbits

40 mg spironolactone/kg were given orally in aqueous suspension and 20 mg canrenoate-K/kg was given intravenously in aqueous solution. Heparinized plasma samples were obtained 24 hours prior to administration until 72 hours following administration at appropriate intervals.

4. Administration of ^{3}H-canrenoate-K to rabbits and isolation of metabolites

20,21-^{3}H-Canrenoate-K with a specific activity of 860 uCi/mg was provided by G.D. Searle and Company. 20 mg ^{3}H-canrenoate-K/kg with a tritium activity of 50 uCi was

injected intravenously. The rabbits were sacrificed at 2 hours following administration, and total plasma was obtained for metabolite analysis. The procedure for isolation of large quantities of metabolites is described in section III of this work. Radioactive bands were found to consist of the previously discussed metabolites canrenone, M_A and M_B.

5. Cross-reactivity of Spirolactones to Aldosterone Antibody

Tritiated aldosterone (3 pg) with a specific activity of 300 dpm/pg (Amersham-Searle Corportation, Chicago) was added to solutions of its aldosterone 21-hemisuccinyl- BSA antibody sufficiently concentrated to bind 60-65% of the ^3H-aldosterone. Displacement of ^3H-aldosterone from its antibody was measured with concentrations of spirolactones ranging from 1-200 ng. The following spirolactones were used: spironolactone, canrenone, canrenoate-K and the two newly identified metabolites M_A and M_B.

6. Fluorescence Measurements of Canrenone and Canrenoate

The fluorescence procedures used were recently described by Sadée et al., (217). The spirolactone metabolites M_A and M_B did not interfer with the assay of canrenone and canrenoate.

7. Apparatus

Ultra violet spectra were taken on a Beckman UV spectrophotometer. Fluorescence was read on an Aminco-Bowman Spectrophotofluorometer. Mass spectra were obtained by direct insertion on a Varian CH-7 Mass Spectrometer using 70eV electron impact and on an AEI MS 902 high resolution mass spectrometer using chemical ionization with isobutane.

8. Materials

Spironolactone: 17-hydroxy-7αacetylthio-3-oxo-androsten-17α-yl -propionic acid lactone. Canrenone: 17-hydroxy-3-oxo-4,6-androstadien-17 α -yl β-propionic acid lactone. Canrenoate-K: Potassium 17-hydroxy-3-oxo-4,6-androstadien-17α -yl -propionate. The amounts of metabolites M_A and M_B were calculated from their ^3H-activity and the secific activity of the parent ^3H-canrenoate-K. All chemicals were of spectro-quality (Matheson, Coleman, Bell) and were used without further purification. The 21-hemisuccinyl- BSA antibody was supplied by Research Plus Laboratories, Inc., New Jersey and was from Anti-Aldosterone Ewe Serum Batch 141-Serial No. 0196-09.

Results

Following single doses of spironolactone and canrenoate-K in rabbits, dogs and man, we have found increased aldosterone plasma values using the radio-immunoassay of Ito et al., (236). However, high aldosterone values after canrenoate-K administration were also observed in adrenalectomized dogs, which suggests that the spiro-lactones or their metabolites interfer with the aldosterone radioimmunoassay.

Spirolactone, its major metabolite canrenone, and canrenoate did not contribute to this assay interference, since canrenoate was not extractable by dichloromethane from aqueous medium above pH 6 and spironolactone and canrenone were completely separated from aldosterone during Sephadex

LH-20 column chromatography, even in a 10^6 fold excess. The erratic aldosterone values were equally not dependent upon sulfur-retaining metabolites specific to spironolactone, since spironolactone as well as canrenoate-K without the 7 alpha-thioacetyl substituent led to equivalent increases in false aldosterone readings in rabbits. Figure 22 shows results of an experiment in which rabbits were dosed with both canrenoate and spironolactone with the high aldosterone readings following treatment. Thus, high aldosterone readings were caused by yet unidentified metabolite(s) of spironolactone and canrenoate-K.

Spirolactone metabolites were isolated from rabbit plasma and liver 2 hours following intravenous administration of ^{3}H-canrenoate-K at which point the highest aldosterone assay interference was observed. The spirolactones were separated from aldosterone and other corticosteroids by differential extractions at pH 13 and pH 1 and purified by chromatography. Figure 23 shows the ^{3}H-activity of ^{3}H-canrenoate-K metabolites eluted from a Sephadex LH-20 column and the retention volume of aldosterone. One ^{3}H-fraction consists of canrenone. Two further fractions consisted of the metabolites previously identified (section III of this work), M_A and M_B. Metabolite B had a retention volume similar to that of aldosterone.

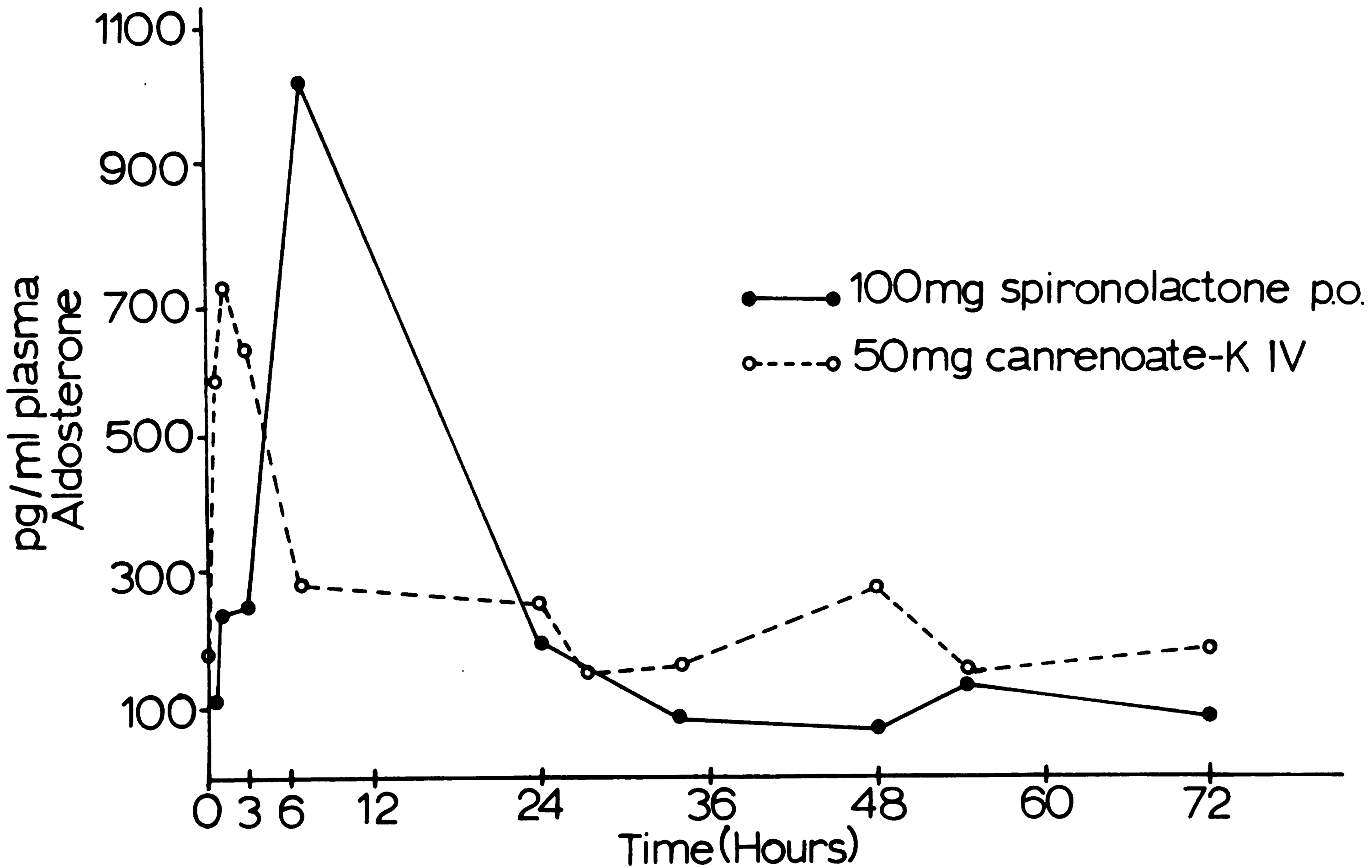


FIGURE 22: Aldosterone Plasma Levels in Rabbits Dosed with Spironolactone and Canrenoate-K.

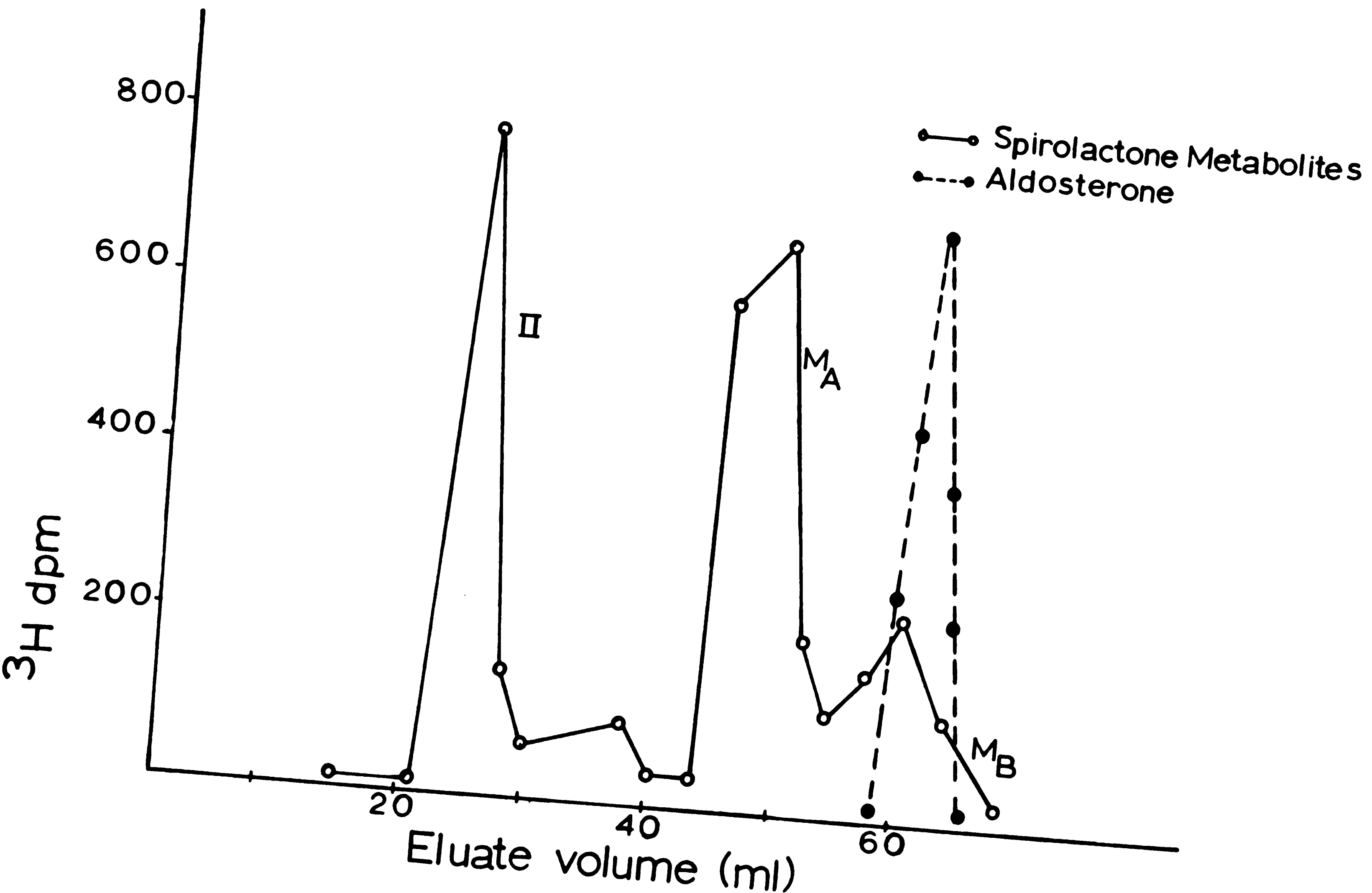
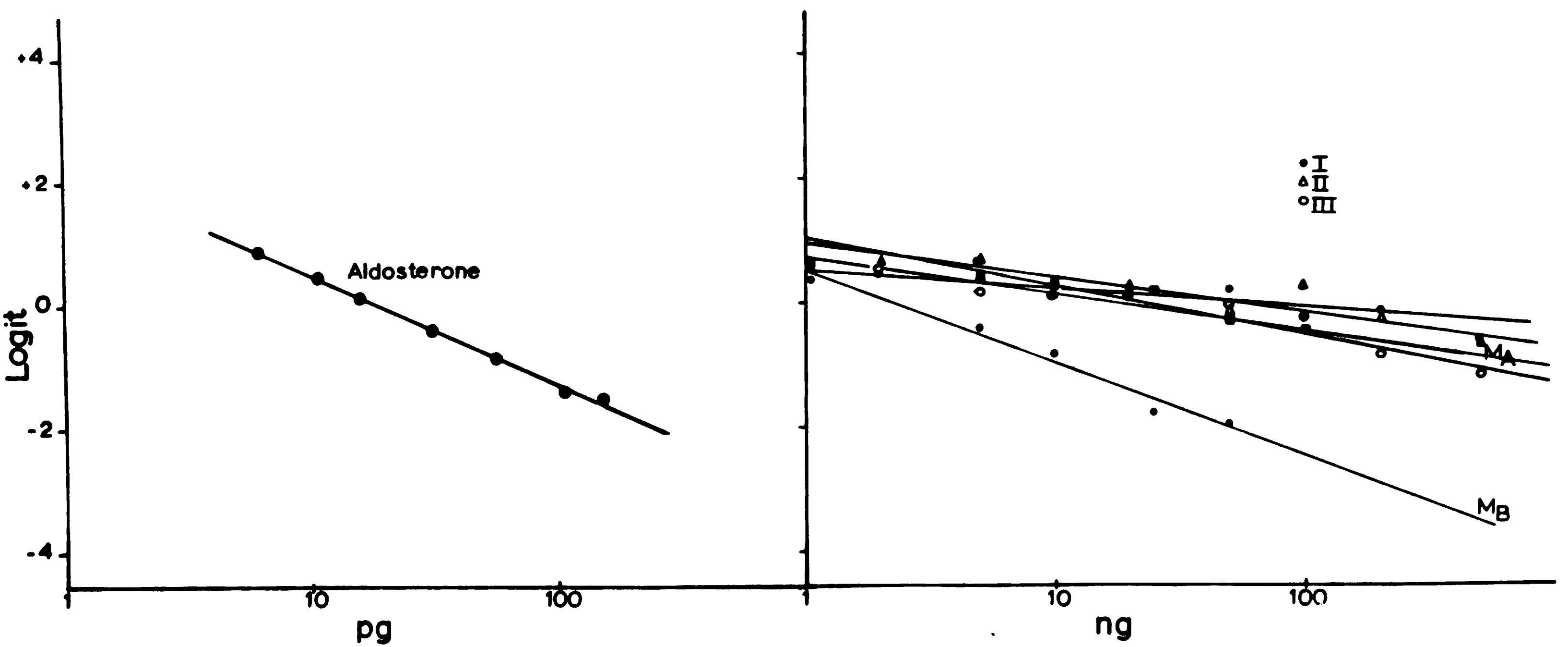


FIGURE 23: LH-20 Sephadex Column Retention for Spirolactones, Aldosterone and the New Spirolactone Metabolites.

Spirolactone Cross-Reactivity to Aldosterone Antibody

Logit-logs plots were prepared to linearize the data of ^{3}H-aldosterone displacement from the antibody by aldosterone and by several spirolactones and resulted in straight lines of varying slopes (Figure 24). The cross reactivity was calculated relative to aldosterone at 50% displacement of bound ^{3}H-aldosterone and amounted to < 0.01% for spironolactone, <0.01% for canrenone, 0.04% for canrenoate-K, 0.03% for M_A and 0.3% for M_B. Cross-reactivity of M_B to this antibody was found to be markedly higher than that of other spirolactones. Such a small percentage of cross-reactivity might be negligible; however this assay is performed at such small aldosterone concentrations that even low cross affinity is significant.



<u>FIGURE 24</u>: Cross Reactivity Curves for the spirolactones, spironolactone (I), canrenone (II), canrenoate (III), Metabolites M_A and M_B.

Modified, Specific Aldosterone Radioimmunoassay

The metabolic fraction M_B, the 20-hydroxy-canrenone metabolite, can be separated from aldosterone by liquid-liquid extraction following hydrolysis at pH 13. The modified procedure described in this section resulted in zero blank aldosterone values in adrenalectomized dogs prior to and following canrenoate-K administration.

Figures 25, 26, and 27 show plasma levels of aldosterone following spirolactone administration in rabbit, dog and humans measured by the procedure of Ito et al., (236) and by the modified procedure described in this section. The method of Ito et al., (236) resulted in an over-estimation of aldosterone plasma levels of 100-500 pg/ml in patients receiving 1 gram canrenoate-K/day for three consecutive days, which represents an extreme in therapeutic dosage levels of spirolactones. The high antibody cross-reactivity of metabolite M_B conversely allows a rough estimation of M_B concentrations by aldosterone replacement analysis. Peak levels of M_B in rabbits were approximately 100 ng/ml and were considerably lower in dogs and humans.

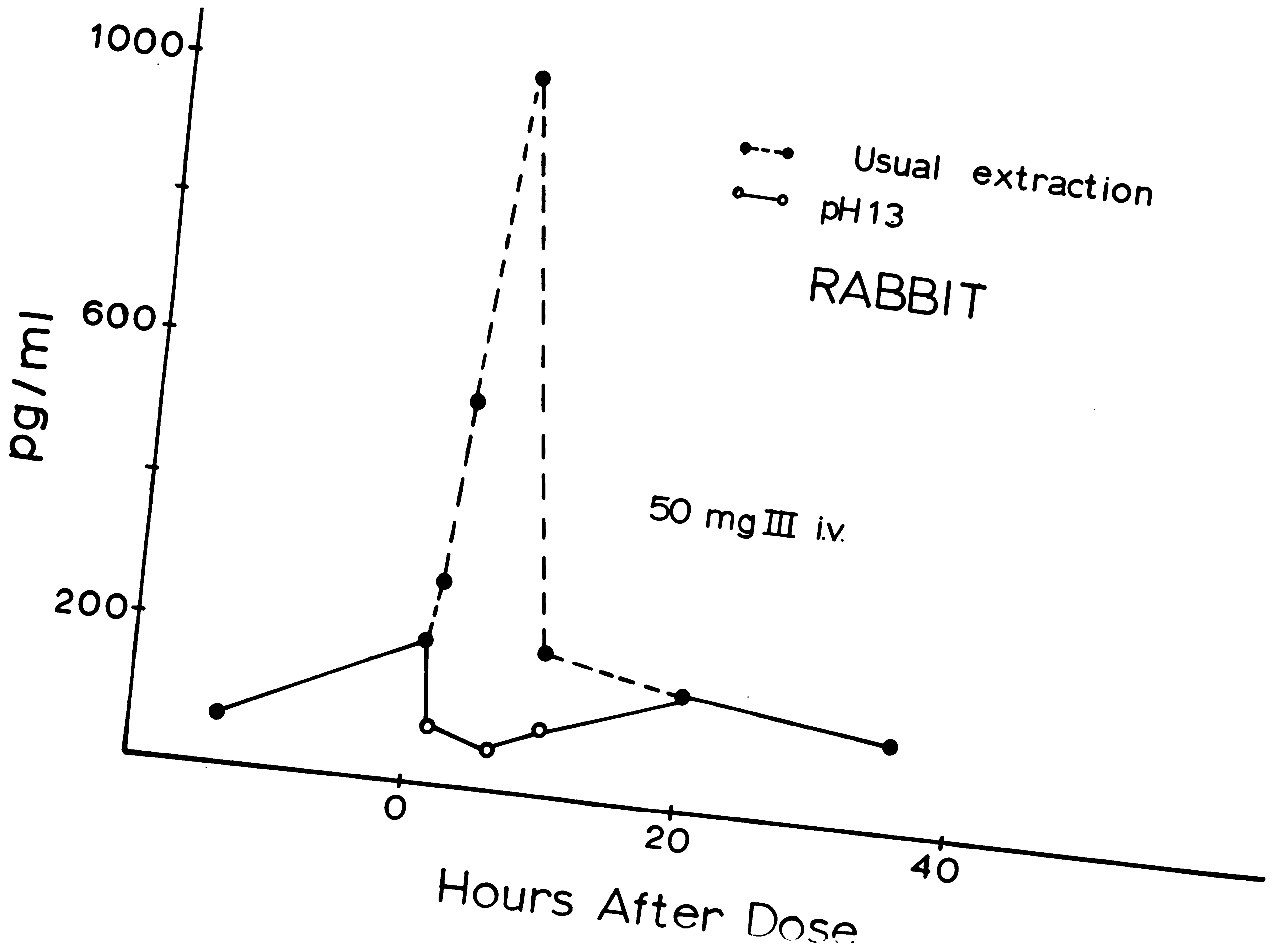
The modified assay for aldosterone in the presence of spirolactones indicated no significant changes in plasma aldosterone concentrations following single spirolactone administration in dogs and humans (Figures 26 and 27). In the rabbit aldosterone levels were decreased following spirolactone administration, Figure 25. Large differences in spirolactone plasma levels in the three species were caused by differences in the dose as well as by species differences

in metabolism. The plasma half-lives of canrenone and canrenoate were 1 hour, 5 hours (208) and 20 hours (199) in the rabbit, dog and human respectively (Figures 28,29, and 30).

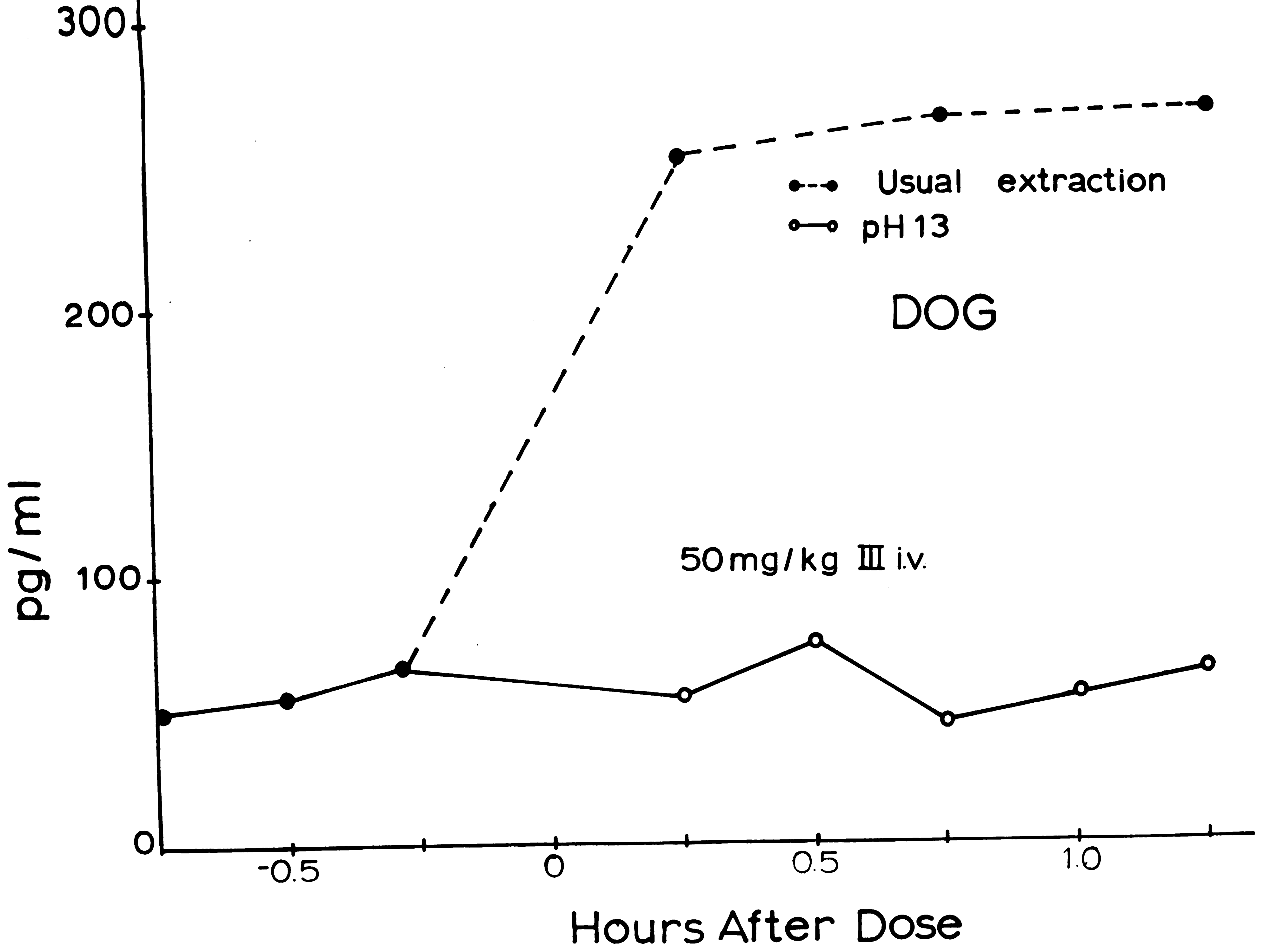
FIGURE 25: Aldosterone Plasma Levels in a Rabbit dosed with 50 mg canrenoate-K (III) IV as measured by the Ito et al., method and by the modified radio-immunoassay procedure

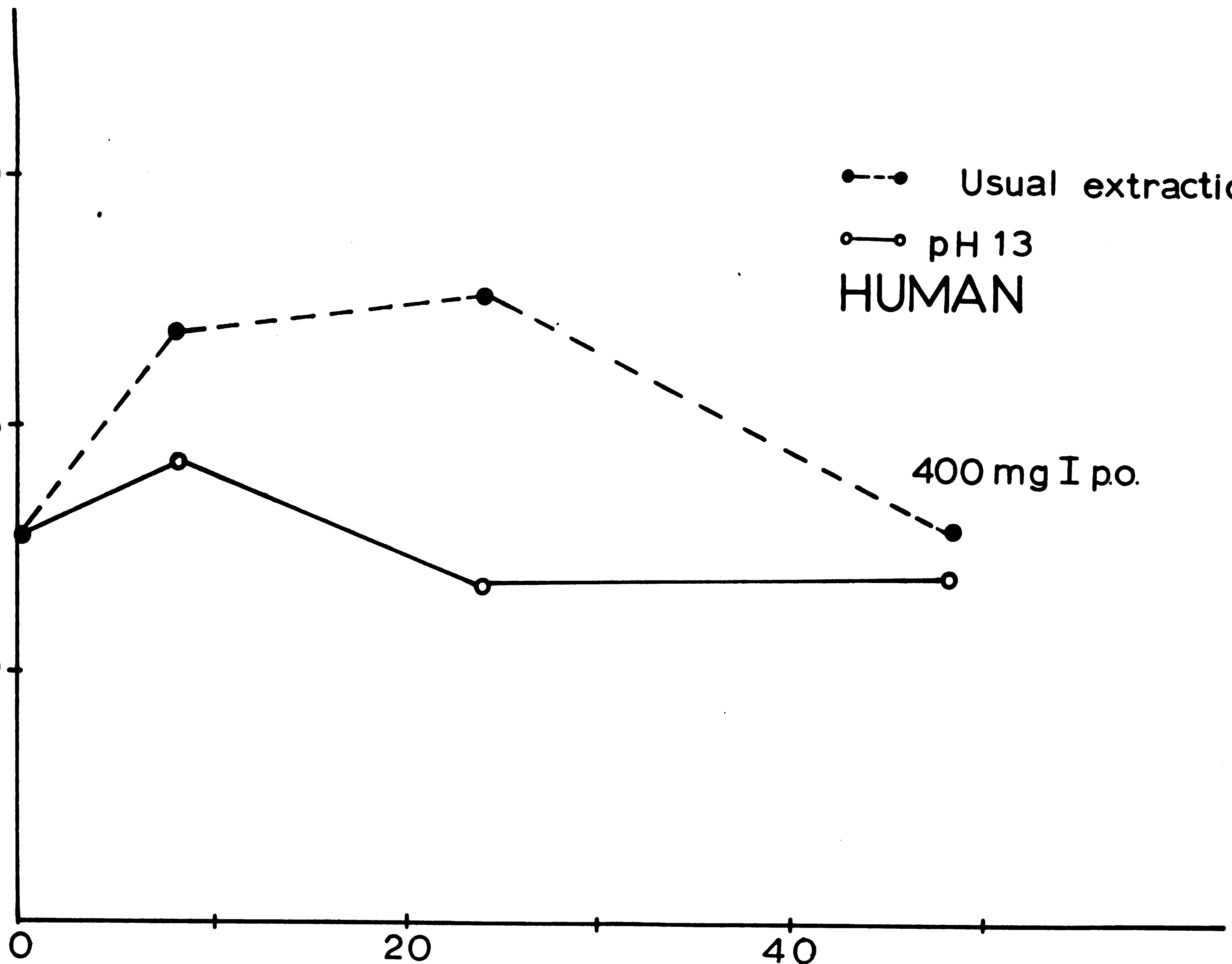
FIGURE 26: Aldosterone Plasma Levels in a Dog dosed with 50 mg/kg canrenoate-K (III) IV as measured by the Ito et al., method and by the modified radio-immunoassay procedure

FIGURE 27: Aldosterone Plasma Levels in a Human dosed with 400 mg spironolactone orally as measured by the Ito et al., method and by the modified radio-immunoassay procedure



1000
600
200
pg/ml
Usual extraction
pH 13
RABBIT
50 mg III i.v.
0
20
40
Hours After Dose


300
200
100
0
pg/ml
Usual extraction
pH 13
DOG
50mg/kg III i.v.
-0.5
0
0.5
1.0
Hours After Dose


Usual extraction
pH 13
HUMAN
400 mg I p.o.
150
100
50
pg/ml
0
20
40
Hours After Dose

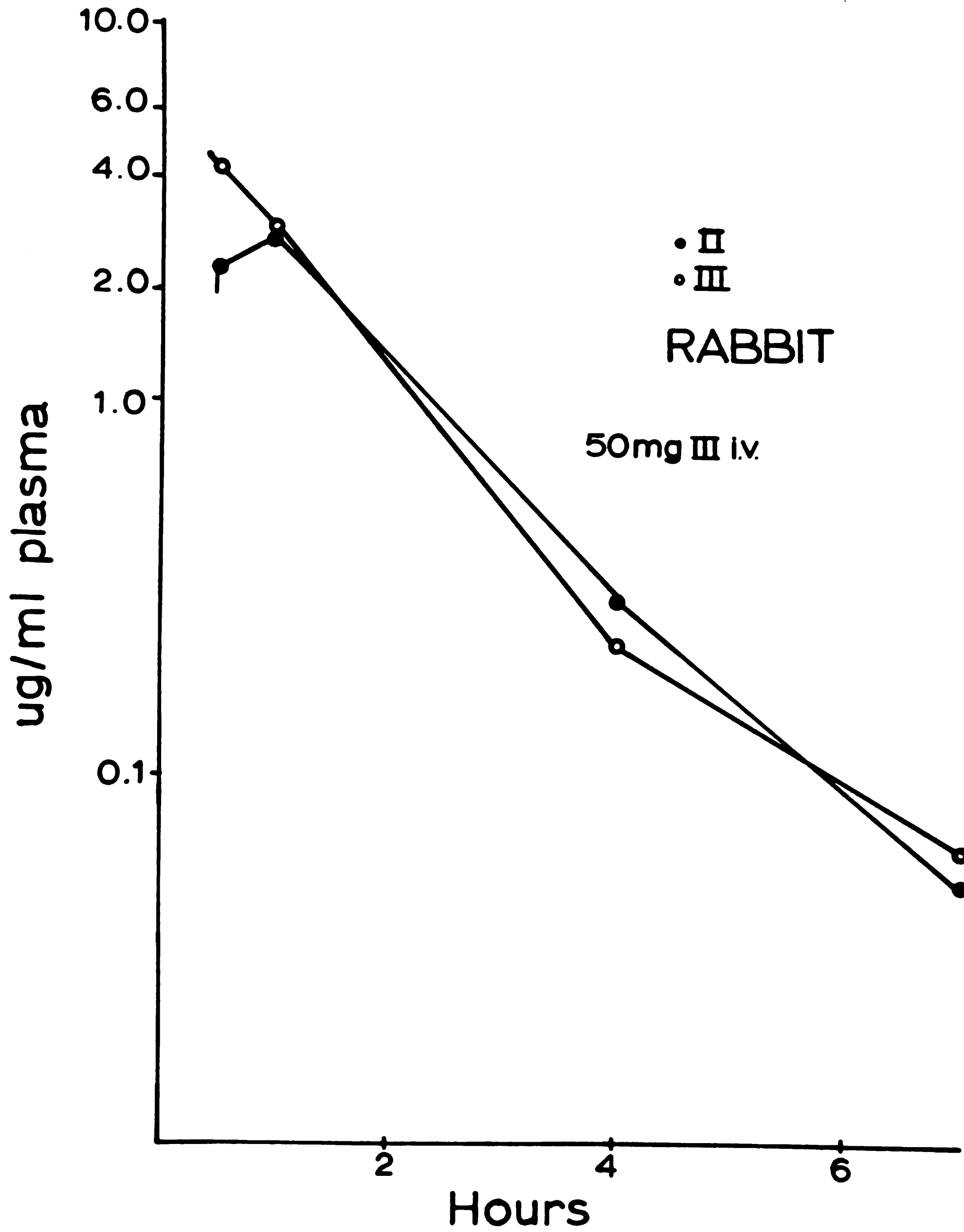


FIGURE 28: Canrenone (II) and Canrenoate (III) levels in the rabbit.

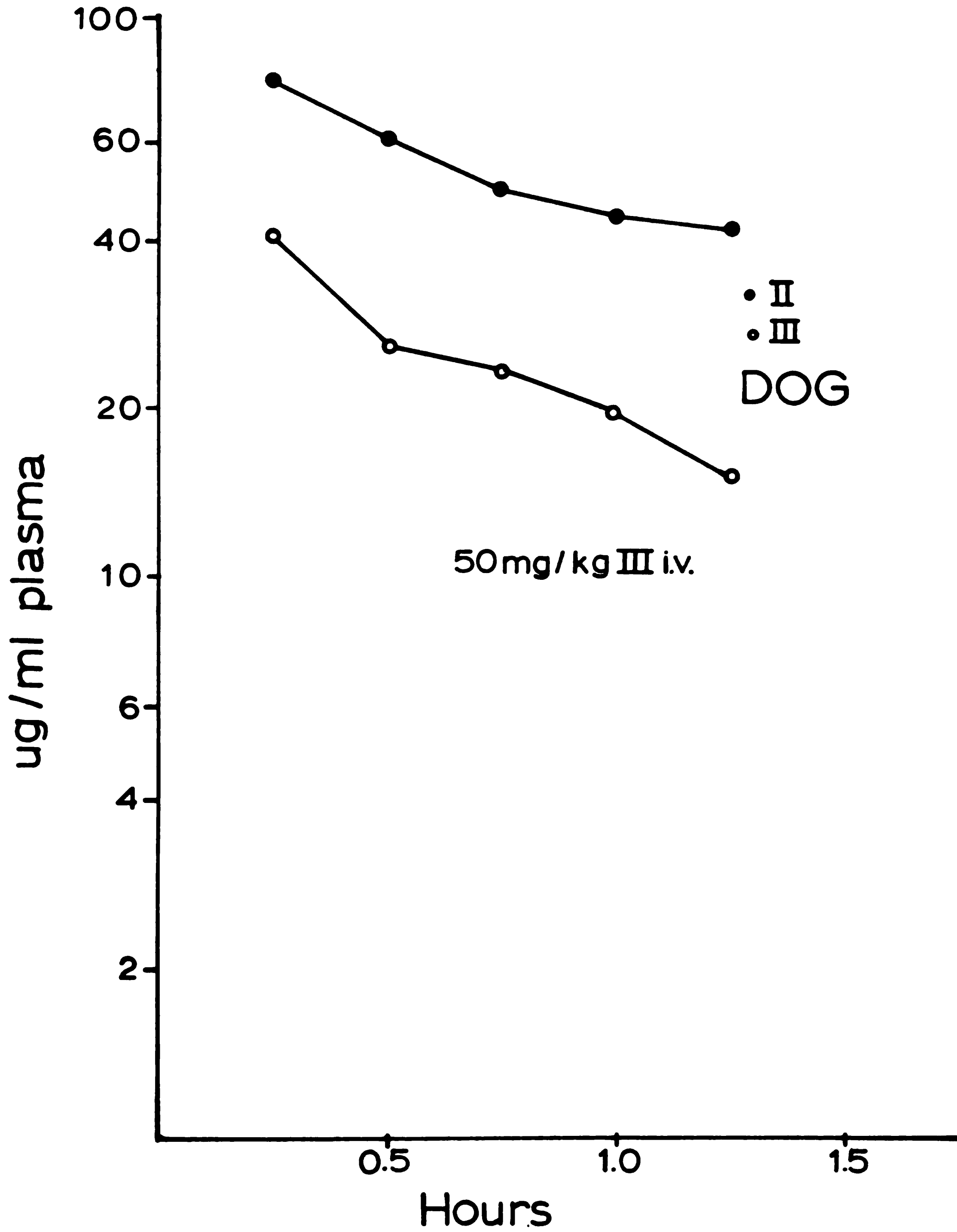


FIGURE 29: Canrenone (II) and Canrenoate (III) levels in the dog.

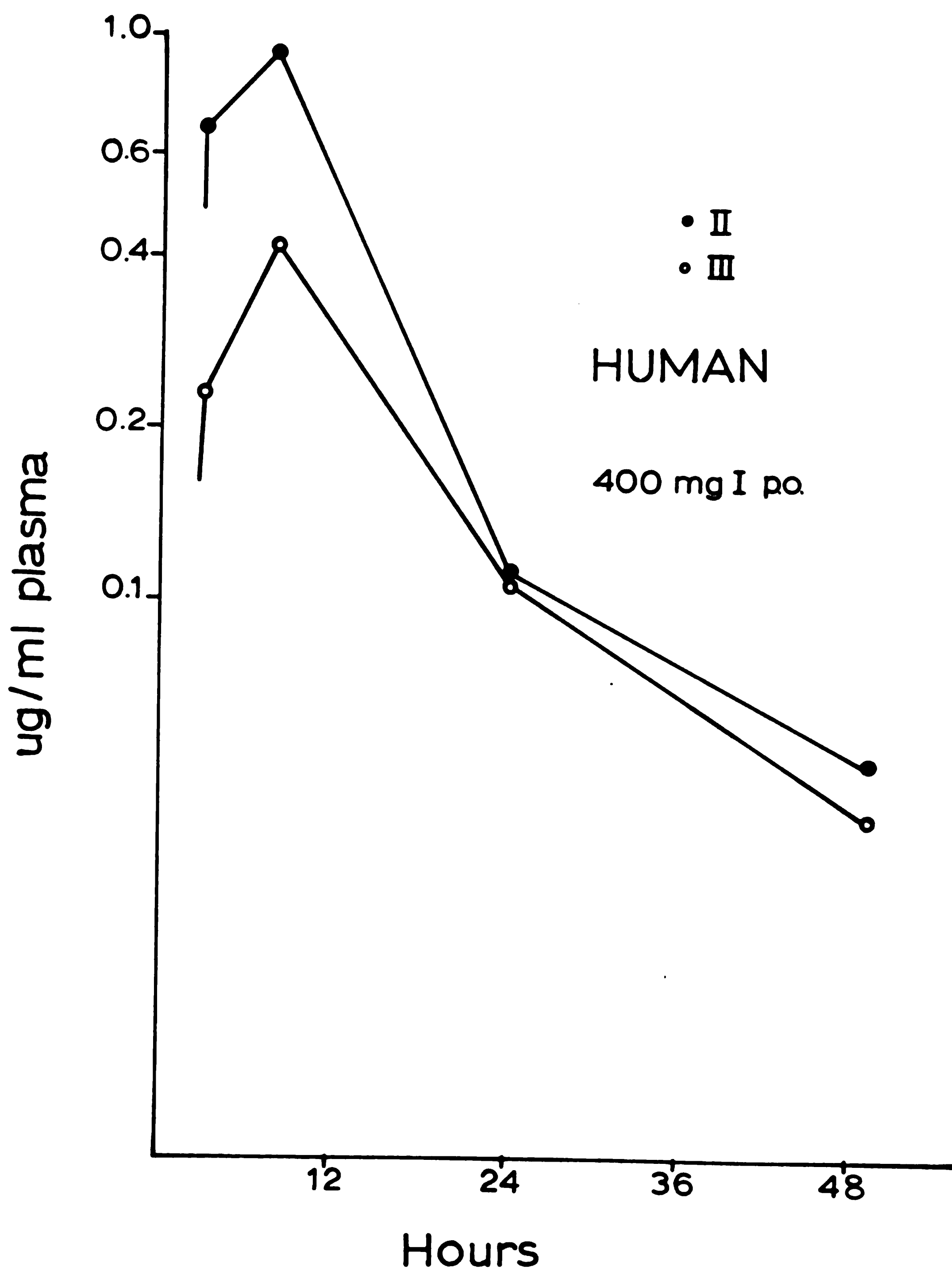


FIGURE 30: Canrenone (II) and Canrenoate (III) Levels in man.

Cerebrospinal Fluid Analysis

Cerebrospinal fluid samples from dogs given canrenoate-K were analyzed for aldosterone.

Dog cerebrospinal fluid samples and plasma samples taken at the same time points showed that cerebrospinal fluid aldosterone concentration is lower than plasma aldosterone concentration. This might be expected due to aldosterone protein binding. Concentrations of cerebrospinal fluid aldosterone are approximately 50-70% those of plasma aldosterone.

Discussion

This study was designed to answer the question of effects of short term administration of spirolactones on endogenous aldosterone levels. Interference by a drug metabolite was observed in the aldosterone radioimmunoassay procedure. This interference is of concern to other investigators using antibodies to aldosterone and samples of this metabolite have been requested by other laboratories to be tested in assay systems for aldosterone. There is a need for investigation of drug metabolism to insure specificity of radioimmunoassay methods.

Administration of spironolactone and canrenoate-K resulted in a hydroxylated metabolite, identified previously as 20-hydroxy-canrenone, which interfered with the plasma aldosterone radioimmunoassay of Ito et al., (236). Therefore a modified procedure for plasma aldosterone determination was developed which was specific in the presence of spirolactone metabolites.

Plasma levels of spirolactones and aldosterone were measured in rabbits, dogs and humans following single doses of spironolactone and canrenoate-K. While canrenone and canrenoate plasma levels in rabbits and dogs (Figures 28 and 29) were high enough to potentially inhibit aldosterone synthesis at the adrenal level (227), a drop in aldosterone plasma levels was observed only in the rabbit. Spirolactone levels in the dog were sufficient to displace aldosterone from unspecific binding sites, eg., plasma or tissue proteins (38), which could have resulted in no detectable net changes in

aldosterone plasma concentrations in this species. In humans, a single dose of 400 mg of spironolactone did not significantly alter aldosterone plasma levels. The canrenone and canrenoate levels obtained (Figures 28-30) may have been too low to inhibit aldosterone production _in vivo_. A more detailed study is necessary to understand the relation between single dose spirolactone administration and aldosterone kinetics. Also, these results do not exclude the possibility that aldosterone plasma levels may be elevated during long-term treatment with spironolactone and canrenoate-K.

Cerebrospinal fluid samples were analyzed for aldosterone to measure the effects of short term administration of spirolactones on aldosterone levels. Since both aldosterone and the spirolactones, which are aldosterone antagonists, were shown to reduce peri-focal brain edema, the hypothesis that the spirolactones were acting in the central nervous system by causing an increase in aldosterone production needed to be examined. Results of these studies merely showed the presence of aldosterone in the cerebrospinal fluid in dogs. The possibility that spirolactones act independently in the central nervous system of aldosterone antagonism is still open to question.

Methodology may in part explain the differences in reported effects of spirolactones on aldosterone kinetics _in vivo_. In order to clarify the effects of these drugs on aldosterone levels, and to investigate their effects on aldosterone production and secretion it is necessary to measure

aldosterone with specificity and accuracy. Attention of investigators interested in these studies has been drawn to the fact that aldosterone radioimmunoassays need to be checked for specificity.

PILOT STUDY

V. ELIMINATION KINETICS OF ALDOSTERONE DURING LONG TERM AND SHORT TERM ADMINISTRATION OF SPIROLACTONES

Abstract

The elimination kinetics of aldosterone were studied in rats using infusions of tritiated aldosterone in control rats and in rats receiving spirolactones. In short term studies, rats received a bolus injecion of canrenoate-K in solution prior to surgery which was followed by infusion of labelled aldosterone. In long term studies, rats were pre-treated with daily injections of 100 mg/kg of canrenoate-K for 7 days. 7 days. Preliminary results showed no changes in the elimination kinetics of aldosterone as determined by clearance calculations in long term and control rats. Short term rats had clearance values equal to one-half those of control and long term pre-treated rats.

Introduction

Concentrations of aldosterone in plasma are the result of aldosterone production, secretion and elimination. Spirolactones affect aldosterone production and secretion in a number of ways as reported in part IV of this thesis section. However little is known concerning the effect of spirolactones on the elimination kinetics of aldosterone. Sex differences in the metabolism of aldosterone were shown in

rats (65,66). Differences in the effective concentration of Δ^4-reductases in female rat livers when compared to those of male rat livers have been found to exist for aldosterone (19,70). Spirolactones might effect aldosterone elimination kinetics, possibly by affecting enzymes which metabolize aldosterone. These effects may also show differences with the sex of the species studied.

As enzyme inducing agents the spirolactones might affect the metabolism of aldosterone. The mechanism by which spirolactones produce increased resistance or catatoxic effects against many drugs is at least in part due to this enzyme inducing capacity (149,274,275,144,145,6,150,151). Taylor et al., (276) showed that spironolactone is a weak enzyme inducer in man. This may be significant because spironolactone is used clinically in combination with other drugs known to undergo oxidation in the liver. However, Leber, reported that Aldactone[R] does not affect renal microsomal enzymes. In mice, spironolactone pretreatment was shown to decrease the toxicity of furosemide, a drug which is excreted unchanged into the urine thus ruling out the possibility of enzyme induction as a mechanism (148).

This study was designed to investigate the effects of the spirolactone, canrenoate-K on aldosterone elimination kinetics in male rats. An infusion procedure of aldosterone was followed by calculation of total body clearance values.

Experimental

1. Materials

Radioactive 1,2-d-^{3}H-aldosterone and ^{14}C-aldosterone were ordered from New England Nuclear. Pure crystalline canrenoate-K was obtained from Boehringer Mannheim G.M.b.H. All solvents were of spectroquality (Matheson, Coleman, Bell) and were used without further purification. Columns used for the separation of ^{3}H-aldosterone from its metabolites are those described in section IV of this work for use with the aldosterone radioimmunoassay. Scintillation counting was done in 10 ml Aquasol on a Searle Mark III counter. A Sage Instruments infusion pump was used for infusion studies (Model 352-syringe pump).

2. Animal Procedures

a) Male Sprague- Dawley rats were used ranging in weight from 250-350 grams. They were stored in an animal facility for at least 3 days prior to use. Animals were divided into 3 groups; control animals, single-injection (short-term) animals and long term (pre-treated) animals. Long term animals were injected daily with doses of 100 mg/kg canrenoate-K over 7 days. Short term animals were given bolus injections of 100 mg/kg canrenoate-K prior to beginning the infusion experiments. Controls were given no drug prior to the infusion studies.

b) Cannulation procedure: This surgery was performed on all groups of animals. The rats were anesthetized with chloral hydrate solution, (Merck Pharmaceuticals). From a

3.6% solution the dose equalled 1% of body weight and was injected intraperitoneally. A small incision was made across the area of the femoral artery and vein. The fat pad was torn away exposing the vessels. Artery and vein were separated from the nerve and tied off. They were individually cannulated with Polyethylene 50 tubing. The cannulas were flushed with saline and clamped off. A small incision was made in the animals' back and the cannulas were run out through this opening. Wound clips were applied to the cannulation site to prevent disturbance by the animal. The animal was allowed to fully awaken prior to beginning the infusion.

c) Blood Sampling: ^{3}H-aldosterone for the infusion experiments was drawn up and placed in a syringe connected to the pump. A series of control animals were used to establish the time necessary to reach steady state and the preliminary steady state levels of labelled aldosterone. These animals had 0.5-1ml of blood removed from the arterial line at various time periods, while the infusion proceeded via the venous line. All other animals had 3 blood samples withdrawn of 0.5-1 ml at 1, 1.5 and 2 hours during the infusion to establish steady state levels of aldosterone.

3. Preparation of Doses

500 mg of Canrenoate-K was weighed out and placed in a beaker. Five to six ml of sterile water was added along with 1% Na bicarbonate solution dropwise until the canrenoate-K went into solution. The drug solution was then drawn up into a syringe and put through a millipore filter to insure

sterility and forced into an empty sterile vial for storage. Daily injections were given at approximately the same time each day for long term animals and were between 0.3-.4 ml. The animals did not seem to show any signs of infection at the intraperitoneal injection site.

^{3}H-Aldosterone for the infusion experiments was dried down under nitrogen and taken up in normal saline. A pre-calibrated infusion pump was used to deliver the dose which was generally delivered in a 2 hour time period at a rate of 1 ml/hr (.017 ml/min). Doses for control animals, short term and chronics were 10 x 10^{6} dpm ^{3}H-aldosterone (about 30 ng aldosterone), specific activity = 57 Ci/mmole.

4. <u>Analytical Procedures</u>

a) Specific ^{3}H-aldosterone measurements were obtained by chromatography and ^{14}C-aldosterone was used as an internal standard to check recoveries. Known amounts of ^{3}H-aldosterone counted on the tritium program were pipetted into counting vials with known amounts of ^{14}C-aldosterone. A volume of 10-15 ml of 98:2 (dichloromethane:methanol) was added and the vials were dried under nitrogen. 98:2 was added to allow for adjustments of any quench which might occur in column fractions, since the column samples were run in 98:2. The double-isotope ratio ^{3}H/^{14}C was plotted vs. dpm of ^{3}H-aldosterone to give standard curves, (Figures 31 and 32).

FIGURE 31

Standard Curve for the $^{3}H/^{14}C$ Double-Isotope Assay with ~300 dpm (25λ) ^{14}C added.

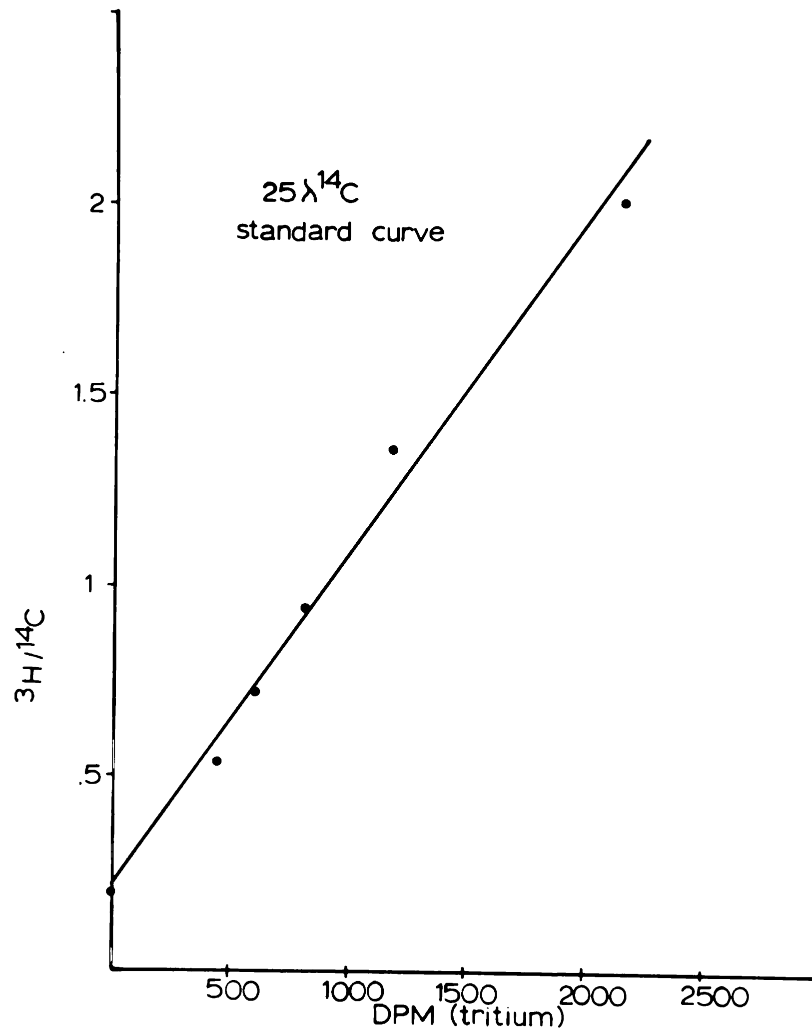
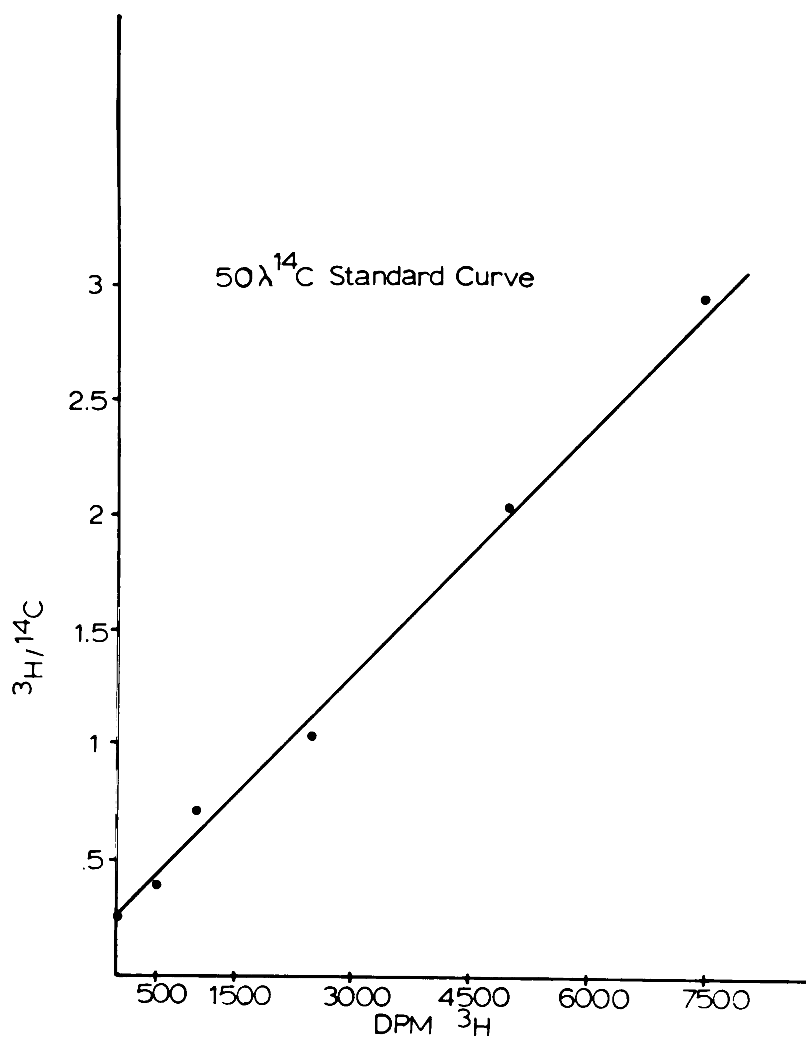


FIGURE 32

Standard Curve for the $^{3}H/^{14}C$ Double-Isotope Assay with ~600 dpm (50λ) ^{14}C added.



b) Extraction of 0.25-0.5 ml of plasma was performed in test tubes with the addition of either 300 dpm or 600 dpm of ^{14}C-aldosterone (internal standard). 5 ml Dichloromethane was added and the tubes shaken, dried down and reconstituted with 1 ml of 98:2 (dichloromethane:methanol).

c) Column Step: Samples from (b) above were placed on pre-calibrated, pre-washed LH-20 Sephadex columns. A 10-15 ml fraction containing aldosterone was collected, dried down under nitrogen and dissolved in in 10 ml Aquasol. Double-isotope ratios were counted on the $^{3}H/^{14}C$ cap in the counter and dpm of aldosterone per sample was obtained by comparing ratios to those on standard curves.

Results and Discussion

Male and female rats injected intravenously with doses of aldosterone were found to have a biexponential decline of aldosterone in fall off curves. The half-life of aldosterone in male and female rats is 4 minutes for the first component and 12 minutes and 16 minutes for male and female rats respectively for the second component (65). Figure 33 shows ^{3}H-aldosterone plasma level curves during infusion indicating that steady state levels are reached. Total body clearances were calculated.

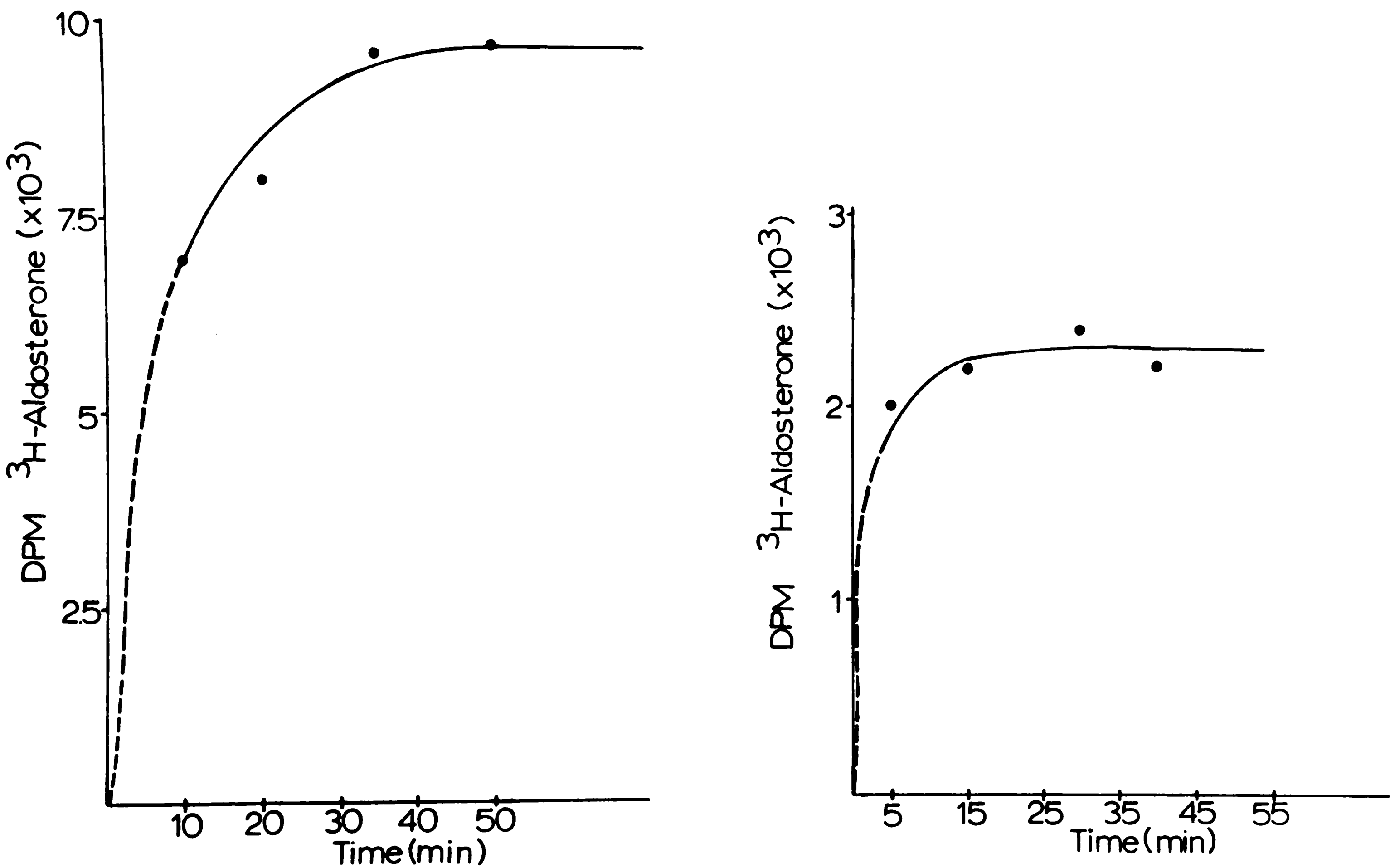


FIGURE 33: Steady State Curves observed Following Infusions of tritiated aldosterone in control experiments in rats.

Three blood samples were taken each from control rats, short-term rats and long-term animals and these values were averaged to give the $C_{p_{ss}}$ value. Reproducibility for this assay was very good as exemplified by 3 replicate assays of the same sample. Values for these were 900, 1050, and 900 with an average value of 950 dpm/ml of aldosterone. Measurements of total radioactivity in plasma at this time point gave 2950 dpm/ml which means that 32% of the radioactivity is aldosterone.

Total radioactivity was counted for all samples in addition to the specific measurement of aldosterone. For control animals steady state measurements showed that aldosterone accounted for between 30-60% of radioactivity. Pre-injected short-term animals showed about 45% of the measurements were aldosterone and pre-treated animals showed about 50% of the radioactivity counted at steady-state was aldosterone.

Preliminarly results are shown in Table 9 . Control animals gave a clearance value (corrected to 100 grams weight) of 11 ml/min/100g. Animals pre-treated with canrenoate-K over 7 days had clearance values for aldosterone of 9 ml/min/100g. These values are not statistically different. The indication here is that the spirolactones do not affect the elimination kinetics of aldosterone, however further experiments are indicated to confirm this. In the animals pre-treated with canrenoate-K, the normal dose of chloral hydrate used for anesthesia did not produce sufficiently deep sleep at times to perform the surgery and

more compound had to be given. Chloral hydrate was chosen as an anesthetic because it is known that spirolactone pretreatment can reduce barbiturate sleeping time consistent with the idea that spironolactone increases the activity of liver microsomal drug metabolizing systems (274,149,143,148). Canrenoate-K pretreatment apparently effected chloral hydrate sleeping time, however, this effect was not pursued further in these studies.

TABLE 9

Clearance Values normalized to 100 g body weight. Values reported are in ml/min/100 g.

Control Rats	Short-Term Rats	Rats Pre-Tx For 7 days
10.2	4.6	5.6
9.5	5.2	9.6
11.4		7.5
9.0		13.2
15.0		
$\bar{X}$ = 11	$\bar{X}$ = 5	$\bar{X}$ = 9

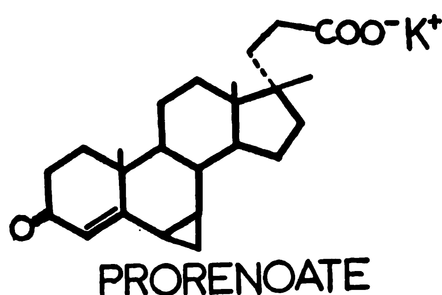
In the two animals treated as short-term experiments the clearance values for aldosterone are about 50% those of control, 5ml/min/100 g.

The data for this study are not by any means complete and further experiments are needed to confirm these results. However, it appears that canrenoate-K pre-treated rats have aldosterone clearance values similar to those of control animals. Further experimentation in this area should include pre-treated animals dosed with spironolactone by stomach tube, and female rats to test for sex differences.

VI. Concluding Statement

In an attempt to find more potent and less toxic spirolactone analogues new spirolactones have been recently synthesized (277,278,279,210). In 1975, potassium prorenoate, Figure 34, was announced and reported to possess greater antimineralocorticoid activity than spironolactone (279,224). Studies in dogs indicate that following oral administration of prorenoate, sodium diuresis occurred within 100 minutes, peaked in 5-6 hours and the effects were reported to last 7 hours (279).

FIGURE 34



Side effects possibly related to prorenoate in human studies were minimal and were possibly related to fludrocortisone employed in the bioassay tests (210).

Canrenoate-K may be indicated in various clinical situations although it is currently unavailable in the United States. Elsewhere it is given both orally and by injection. Canrenoate-K appears to be a good therapeutic agent for introducing canrenone, presumably the principle active metabolite of the spirolactones, into the body.

It is important to recognize that the search for new steroidal antimineralocorticoid spirolactones may be prompted by the current re-evaluation of spironolactone (280,281).

Recently it was reported that an additional warning be placed on labels of Aldactone[R] and Aldactazide[R] (281). This was prompted by rodent studies which indicate the tumor producing ability of spironolactone (281). The Food and Drug Administration and its cardiovascular and renal advisory committee indicated the addition of warning labels and also sought to restrict use of these two products to "use in essential hypertension, congestive heart failure, and hypokalemia, in patients in whom other therapy is inadequate or is considered inappropriate." (281).

Future work in the areas encompassed in this dissertation would include a continuation of the work on spirolactone metabolism, and testing of the toxicology in order to establish safer spirolactone preparations. Also, the question of spirolactone effects on aldosterone elimination kinetics needs to be studied in more detail.

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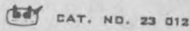

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