PROPRANOLOL METABOLISM IN DOGS AFTER PORTACAVAL TRANSPOSITION

by

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DISSERTATION

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GLOSSARY

AUC	area under the drug concentration-time profile
C_b	drug concentration in blood
$C_{in,b}$,$C_{out,b}$	concentration of drug entering and leaving the liver, respectively
C_T	drug concentration in tissue
Cu_H	unbound drug concentration in liver
CL_e	elimination clearance
CL_f	formation clearance
CL_p	plasma systemic clearance
CL_T	clearance of propranolol from central to tissue compartment
CLu_{int}	intrinsic clearance of unbound frug
CNS	central nervous system
E	extraction ratio
F	bioavailability
fu_b	ratio of the unbound drug concentration in plasma water to the whole blood concentration
g	gravitational constant
Gly-G	propranolol glycol glucuronide
I.V.	intravenous
k	elimination rate constant
$K_{m,i}$	Michaelis-Menten constant of the ith enzyme
$K_{m_{f,M_i}}$	Michaelis-Menten enzyme constant for the formation of the metabolite M_i from propranolol
NAA	α-naphthoxyacetic acid
NEFA	nonesterified fatty acid
NLA	α-naphthoxylactic acid

4-OH-P-G	4-hydroxypropranolol glucuronide
Prop	propranolol
Prop-G	propranolol-O-glucuronide
Q_H	hepatic blood flow
SD	standard deviation
SE	standard error
SGOT	serum glutamic oxaloacetic transaminase
SGPT	serum glutamic pyruvic transaminase
ss	steady-state
t	time
v	velocity of a reaction
V	volume of distribution
V_C	volume of central compartment
V_T	volume of tissue compartment
$V_{max,i}$	maximum velocity of the ith enzyme
$V_{max_{f,M_i}}$	maximum enzyme velocity for the formation of the metabolite M_i from propranolol

INTRODUCTION

Propranolol is a beta-adrenergic blocking agent indicated for the treatment of hypertension, angina pectoris due to coronary atherosclerosis, cardiac arrhythmias, migraine, hypertrophic subaortic stenosis and pheochromocytoma. It is extensively metabolized in both man and animals and has a high first-pass effect. We became interested in the drug because it has been used as a model compound in validating the well-stirred perfusion model of Rowland, Benet and Graham (1973) by Shand and Wilkinson (Branch *et al*, 1973; Shand *et al*, 1975). These workers derived many equations from the basic model which have been used by other scientists in the field (Mackichan *et al*, 1980; Schneck *et al*, 1980). However, a basic assumption in the perfusion model which has often been neglected by researchers using the resultant equations is that to apply them there must be some assurance that the drug obeys linear kinetics. With propranolol there exists evidence that the disposition of propranolol is nonlinear. This evidence will be discussed below.

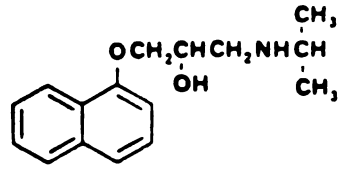
The present research was carried out to investigate in detail the nonlinear aspects of the first-pass metabolism of propranolol under controlled input conditions. We approached this using a special surgical technique by undertaking a portacaval transposition in the dog which allows us to differentiate gut-wall/intestinal metabolism from hepatic metabolism and which also serves as an in vivo liver perfusion model. This procedure has not been previously used in the study of drug metabolism and we will attempt to validate the method. The dissertation is divided into eight chapters. Chapter one deals with physicochemical aspects, structure-activity relationships and pharmacodynamic properties of propranolol. The fundamental pharmacokinetic properties and the perfusion model are discussed

in chapter two. In Chapter three the general surgical details of porta-caval transposition and the studies done to explore the effects of the surgery on hepatic blood flow and liver function are presented. Chapter four includes the special assay procedures for propranolol and metabolites in biological fluids which were developed in the course of this study. In Chapter five the influence of the use of indwelling heparinized cannula for blood sampling on the disposition of propranolol is dicussed. Chapters six and seven provide the experimental results on gut-wall/intestinal and hepatic metabolism of propranolol, respectively. The last chapter is an overall summary and conclusion.

CHAPTER I. CHEMICAL AND PHARMACOLOGICAL PROPERTIES OF PROPRANOLOL

I. PHYSICOCHEMICAL PROPERTIES

Chemically, propranolol is an aminoalcohol: (1-methylethyl)amino-3-(1-naphthalenyloxy)-2-propanol or 1-(isopropylamino)-3-(1-naphthyloxy)-2-propanol. Propranolol has an asymmetric carbon atom in the propranolamine side chain at the C-2 position.



The material used clinically is the hydrochloride salt of the racemate. The hydrochloride is an odorless white powder with a bitter taste and is soluble at 20^{o}C in 20 parts of water and in 20 parts of 95% ethanol (The Pharmaceutical Codex, 1979). Propranolol is a weak base with a pKa of 9.45 as determined by titration (Schurmann and Turner, 1978). Its partition between organic and aqueous solvents is obviously pH dependent. The apparent partition coefficient between n-octanol and phosphate buffer (0.16M) is 5.39 at pH 7 and 52.5 at pH 8 (Hennenbrecht *et al*, 1973). At the physiological pH of 7.4 its chloroform/water partition coefficient is 34.5 (Levy, 1968). The partition coefficients of propranolol with other organic solvents are summarized in Table I-1 (Schurmann and Turner, 1978).

2. HISTORY OF DEVELOPMENT OF PROPRANOLOL AS A BETA-BLOCKER AND STRUCTURE-ACTIVITY OF BETA-BLOCKERS

According to a proposal postulated by Ahlquist as early as 1948, sympathetic nerve stimuli are transmitted via two different types of

TABLE I-1. PARTITION COEFFICIENTS OF PROPRANOLOL BETWEEN VARIOUS ORGANIC SOLVENTS AND 0.1 N SODIUM HYDROXIDE[a].

Dielectric Constant	Organic Solvent	Partition Coefficient
1.92	n-Heptane	29.85
2.28	Benzene	309.6
2.38	Toluene	169.8
4.34	Dethyl ether	290.0
4.81	Chloroform	932.0
5.82	Amyl alcohol	391.0
b)	Octan-2-ol	110.9
6.02	Ethyl Acetate	2.98

a) From Schurmann and Turner, 1978
b) Value not available from literature.

TABLE I-2. CLASSIFICATION OF ADRENERGIC RECEPTORS[a].

Receptor	Effects Elicited by Stimulation
Alpha-adrenergic	Constriction of vascular smooth muscle Contraction of uterus, ureter.
Beta-adrenergic	Vasodilation Myocardial stimulation (contractility, O_2 consumption) Bronchodilation Relaxation of uterus Metabolic effects (increase in blood sugar, fatty acids)

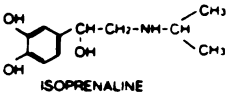
a) From deStevens and Wilheim, 1976

receptors designated alpha- and beta-adrenergic receptors. The effects induced by stimulation of these receptors are summarized in Table I-2. Beta-adrenergic blocking agents were not developed until 10 years after Ahlquist identified the so-called beta-receptors. This came about when dichlorisoprenaline (I), shown in Table I-3, was reported in 1958 to block the catecholamine-induced activation of beta-adrenoceptors (Powell and Slater, 1958; Moran and Perkins, 1958). However, the inherent sympathomimetic property of dichlorisoprenaline to stimulate beta-receptors made this compound unsuitable as a potential therapeutic agent.

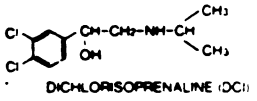
Pronethalol (II) was shown to be free of this liability, and subsequently proved to be an effective beta-adrenergic blocking agent when submitted to extensive clinical investigation (Black and Stephenson, 1962). An alleged carcinogenic effect observed in mice prompted the manufacturer of pronethalol to recommend restricted us of the drug (Paget, 1963). Fortunately, a few months later in 1963, Imperial Chemical Industries, Ltd., announced the development of a congenor, propranolol, with the pharmacological activity of pronethalol plus a 10-fold increase in potency and elimination of carcinogenicity as shown by long-term animal studies (Black *et al*, 1964). In the ten-year period that followed the discovery of propranolol, a total of 13 beta-adrenergic blockers have been launched as therapeutic entities in Europe. These have been subclassified into beta-1- and beta-2- adrenergic blocking agents as will be discussed below.

Compounds which interact with beta-adrenoceptors, either to stimulate or to block, show obvious structural similarities. From the structures given in Table I-3, it can be seen that the side chain of the antagonists is similar or for the most part identical to that of the agonist, isoprenaline; it is the side chain, with an isopropyl or teriary butyl substituted

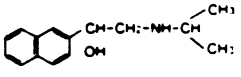
TABLE I-3. STRUCTURES OF ISOPRENALINE AND BETA-ADRENOCEPTOR BLOCKING AGENTS.



Beta-1 and Beta-2 Blockers



(I)



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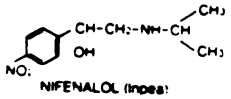
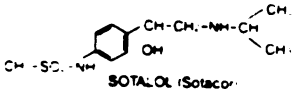
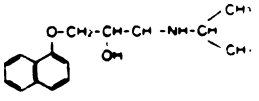
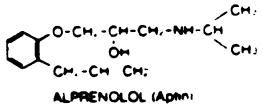
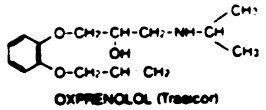
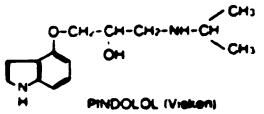


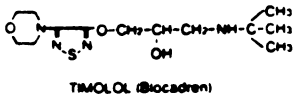




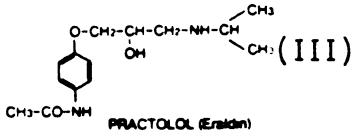








Beta-1 Blockers



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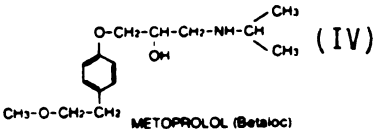

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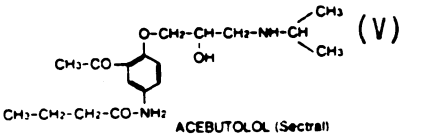

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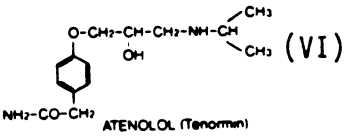

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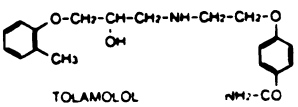
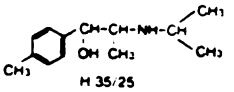


(VII)

Beta-2 Blockers



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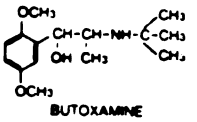


(IX)

secondary amine function, which determines interaction with beta-adrenoceptors. The asymmetric carbon atom gives rise to two optically active enantiomers. As with the catecholamines, the levo isomer has antagonist activity while the dextro isomer is virtually devoid of beta adrenergic activity (Howe and Shanks, 1966).

The nature of the substituents on the aromatic ring determines whether the effect of a compound will be predominantly activation or blockade. Hydroxyl groups in the 3-, 4- positions of the aromatic ring are optimal for stimulating activity (Innes and Nickerson, 1970). Replacement of these two hydroxyl groups with chlorine atoms (as in dichlorisoprenaline) results in a marked reduction in stimulant potency, a much longer duration of action and the emergence of receptor blocking properties. A fused aromatic ring in place of the chlorine atoms (as in pronethalol) brings about a further reduction in stimulant potency. Beta-adrenoceptor blocking potency is increased by the insertion of a methyleneoxy bridge ($O\text{-}CH_2$) between the aromatic ring and the asymmetric carbon atom (as in propranolol).

It has become evident that selective blockade of only a few of the pharmacological effects of beta-adrenoceptor activation can be achieved by specific structural modifications. Lands, in 1967, suggested that two types of beta-receptors existed: beta-1-receptors that predominantly regulate myocardial and intestinal functions and beta-2-receptors in the bronchi and vasculature (Lands, Arnold *et al*, 1967; Lands, Ludvena and Buzzo, 1967). The presence of a second asymmetric center resulting from the introduction of a methyl substituent (as in butoxamine (IX), alpha-methyl DCI, alpha-methyl INPEA, and H 35/25 (VIII)) alters receptor blocking characteristics in such a way that the ability of the compound to block the cardiac stimulant and intestinal inhibitory effects of isopren-

aline is diminshed, while the ability to antagonize other smooth muscle and metabolic effects of the agonist is retained (Levy, 1966; Levy, 1967; Somani, 1969; Van Deripe and Moran, 1965). Thus, these compounds are referred to as beta-2-blockers. Conversely, a number of compounds have a greater affinity for cardiac adrenoceptors than for those in other smooth tissues. The most significant feature common to all these compounds is a substituent group in the para position of the aromatic ring, as in practolol (III), metoprolol (IV), atenolol (VI), acebutolol (V) and tolamolol (VII). Thus, these compounds are beta-1-blockers. The nature of the substituent group appears to determine the degree of selectivity. Similar substitution in meta and ortho positions results in a progressive loss of selectivity (Ablad *et al*, 1970; Mylecharane and Raper, 1974; Vaughan Williams *et al*, 1973). Propranolol blocks both types of receptors equally well and is therefore classified as a non-specific beta-adrenoceptor blocking agent.

3. PHARMACOLOGICAL EFFECTS OF PROPRANOLOL

A. Beta Blockade Related Effects

Hemodynamic Effects: Propranolol (0.17 mg/kg i.v.) in normal supine subjects reduces heart rate, cardiac output, mean arterial pressure, and left ventricular work (Epstein *et al*, 1965). The degree of alteration depends on the extent of adrenoceptor activation; therefore, the greatest hemodynamic change is observed when propranolol is given during submaximal exercise. Under such conditions, the following average reductions are observed: heart rate, 20%; cardiac output, 20%, mean arterial pressure, 15%; left ventricular work, 34%; and myocardial consumption, 6%. Central venous pressure may rise slightly and the arteriovenous oxygen difference is increased by 12% (Epstein *et al*, 1965). Propranolol causes a reduction

in the indices of left ventricular contractility such as peak left ventricular $(dp/dt)_{max}$ and velocity of fiber shortening, resulting in a reduction in peak systolic pressure at rest and on exercise. It also alters the pattern of regional myocardial contractile activity (Coltart *et al*, 1975). In addition, propranolol reduces coronary blood flow by 15 to 25% secondary to the reduction in myocardial oxygen consumption. Blood flow in the splanchnic and renal vascular beds is also reduced secondary to the systemic hemodynamic changes (Price *et al*, 1967).

Renin release: The release of renin, which is formed in the granules in cells of the juxtaglomerular apparatus, is under the control of several mechanisms, including renal blood flow, the sodium concentration in the distal renal tubule, and sympathetic nervous activity mediated through the beta-adrenoceptor. Thus, propranolol reduces the renal release of renin resulting from sympathetic nerve stimulation or catecholamine infusion (Coote *et al*, 1972; Vandongen *et al*, 1973). Hypertensive patients with elevated plasma renin activity (PRA) show a satisfactory hypotensive response to propranolol but controversy exists concerning the relationship between the degree of reduction of PRA and the hypotensive response. In some studies a correlation is observed (Buhler *et al*, 1972; Castenfors *et al*, 1973), but not in others (Birkenhager *et al*, 1971; Hansson and Zweifler, 1974).

Respiratory activity: Propranolol increases bronchial tone in both animals and man. This is due to the unopposed action of the vagus when bronchial beta-adrenoceptors are blocked. The effect on bronchials becomes clinically significant in asthmatic subjects and in patients with allergic rhinitis. This increase in bronchial tone can be prevented by prior administration of atropine.

Hormonal interactions: Alpha-adrenoceptor activation reduces insulin release and because propranolol enhances the alpha-effects of endogenous catecholamines by blocking beta-adrenoceptors, it leads to a secondary depression of insulin release. However, the contribution of this effect to the overall change in metabolism caused by propranolol is not clear. Propranolol also increases circulating levels of growth hormone but does not alter levels of thyroid-stimulating hormone or thyroxine.

Metabolic effects: Stimulation of beta-adrenoceptors by catecholamines causes increased lipolysis and hence a rise in the circulating levels of free fatty acids and glycerol. This beta-1-mediated effect is blocked by propranolol, as is the rise in plasma lactate levels. Under resting conditions, propranolol does not alter circulating levels of glucose or lactate but may reduce free fatty acid levels. It does not alter serum levels of sodium, potassium, or chloride ions.

B. Non-Beta Blockade Related Effects

Propranolol has two additional pharmacological actions: membrane-stabilizing or local anesthetic activity and antagonism of 5-hydroxy-tryptamine (5-HT). The term membrane-stabilizing refers to the non-specific effects of a wide range of pharmacological agents on the trans-membrane flux of sodium and potassium ions, such that cellular function is depressed. In the case of sensory nerves, the result is local anesthesia, and in the heart it is depression of the rate of rise and overshoot of the transmembrane action potential. The membrane-stabilizing properties of propranolol are unlikely to be of clinical importance because the plasma concentration required for membrane-stabilizing activity is at

least 10 μg/ml (Coltart and Meldrum, 1971), which is 100-fold greater than that required for adequate beta-adrenoceptor blockade (approximately 100 ng/ml). The reduction in cardiac function caused by atenolol is identical to that caused by propranolol, yet only propranolol has membrane-stabilizing properties. Further, the dextro-isomer of propranolol, possessing minimal adrenoceptor actions, has membrane-stabilizing activity yet is ineffective in the management of angina pectoris and hypertension.

The central nervous system actions of propranolol are complex and have been reviewed elsewhere (Conway *et al*, 1978). Propranolol antagonizes both beta-adrenoceptors and serotonergic receptors within the CNS, but the clinical significance of these actions is currently unknown.

C. Mechanism of Action in Angina and Cardiac Arrhythmias

Propranolol acts in angina pectoris by decreasing heart rate, blood pressure and myocardial contractility on exercise, thereby diminishing myocardial oxygen consumption and increasing exercise tolerance (Pritchard, 1974). Its antiarrhythmic properties are considered to be principally the result of inhibition of adrenergic stimulation of the heart (Coltart *et al*, 1971). This results in decreased automaticity and conduction velocity, and refractoriness is increased, while the functional refractory period is prolonged relative to action potential duration.

D. Mechanism of Action in Hypertension

The model of action of propranolol in hypertension is not clear. The main possibilities are: a long-term reduction of cardiac output as demonstrated by Lund-Johansen and Ohm (1976), possibly with subsequent baroreceptor adjustments (Pritchard and Gillam, 1969); reduction of renin

release from the renal cortex (Buhler *et al*, 1972); a slow fall (following an inital rise) in peripheral vascular resistance (Ablad *et al*, 1976) possibly due to blockade of prejunctional beta-adrenoceptors mediating noradrenaline release from the sympathetic axon (Rand *et al*, 1976) and a possible effect mediated through the central nervous system (probably not important, Conway *et al*, 1978).

CHAPTER II. FUNDAMENTAL PHARMACOKINETIC PROPERTIES

Absorption: Propranolol is virtually completely absorbed after oral administration in man, rat and dog. After administration of a 40 mg single oral radioactive dose in 3 human subjects, 84-92% was recovered in the urine within 48 hours, while only 0.7 - 4.6% of radioactivity appeared in the feces in the three days following oral administration (Paterson *et al*, 1970). Data from rat and dog also showed complete absorption even though the excretion pattern is different (Hayes and Cooper, 1971). Thus urinary and fecal recoveries for the rat were reported to be 68.2% and 26.8%, respectively, and for the dog 79.5% and 21.5%, respectively, of orally administered radioactive propranolol. Very small amounts of free propranolol were found in urine and feces of both species. Peak plasma concentrations of propranolol were seen at approximately 2 hours (range 1 to 4 hours) in fasting patients (Paterson *et al*, 1970; Shand and Rangno, 1972; Lowenthal *et al*, 1974; Parsons *et al*, 1976; Castleden *et al*, 1975, 1978).

Administration of food increased the bioavailability of propranolol in 6 of the 7 healthy normal subjects studied by Melander *et al*, (1977) when the subjects were given 80 mg p.o. of propranolol on two separate occasions with and without food. The AUC ratio, nonfasting/fasting, ranged from 1.17 to 2.25. Peak plasma concentrations were increased in all but one of the subjects. However, the time to peak and the elimination half-life were not changed. Since the gastrointestinal absorption of propranolol is essentially complete, enhanced bioavailability can hardly be explained in terms of an improved tablet disintegration or a better drug dissolution subsequent to changes in gastrointestinal motility and

cholic acid production. The effect is probably related to other factors influencing the bioavailability of propranolol, such as changes in hepatic blood flow or in the metabolism of the drug during first passage through the liver. McLean *et al* (1978) studied, by computer simulation, changes in the absorption characteristics of a high first-pass drug (like propranolol) using the perfusion model with hepatic blood flow increased for 2½ hours over that observed during the control period. This mimics the in vivo transient increase of splanchnic blood flow due to the effect of food. What they found was that bioavailability is increased if blood flow is increased transiently. It thus appears that the increased bioavailability of propranolol with concurrent food intake is due to a temporary increase in liver blood flow.

Because the input rate can affect the bioavailability, investigators have examined the effect of gastric emptying on propranolol's bioavailability. Castleden *et al* (1978) studied six hypertensive patients after single oral doses of 80 mg and found no correlation between the rate of gastric emptying and peak plasma propranolol concentration. However, they did not analyze the propranolol concentration-time curve for absorption rate; rather, they based their conclusion on time to peak and peak concentration. These observations obviously vary among subjects due to differences in hepatic extraction ratio. They also studied four normal subjects on separate occasions by changing their gastric emptying times using metoclopramide and propantheline compared to a saline control. They found the peak propranolol plasma concentration was highest when metoclopramide was given and lowest when propantheline was given. The mean time to peak was 1.5 hours after metoclopramide, 2.8 hours after normal saline and 4.5 hours after propantheline. The authors concluded from their studies that individual variation in the rate of bioavailability

of propranolol is determined mainly by first-pass metabolism in the liver rather than by differences in gastric emptying. However, their results using metoclopramide and propantheline more convincingly point to the opposite conclusion.

Metabolism: Propranolol is eliminated almost completely by metabolism. More than 90% of an oral dose appeared in the urine of man in the form of metabolites and their conjugates (Paterson *et al*, 1970). Less than 1% of the dose was excreted as unchanged propranolol in urine (Walle 1978a). The biliary route is important in rat and dog in addition to urinary excretion in that up to 25% of the dose was recovered as metabolites in the feces (Hayes and Cooper, 1971).

The metabolic pattern of propranolol shown in Figure II-1 is quite similar in man, rat, and dog (Walle and Gaffney, 1972). There are three major pathways of metabolism:

a) O-glucuronic acid conjugation to form propranolol-O-glucuronide: The enzyme for this pathway is stereoselective such that it favors the formation of (-)propranolol-O-glucuronide over the (+)propranolol-O-glucuronide 4 to 1 in dogs when given a recemic mixture of propranolol (Walle and Walle, 1979).

b) Aromatic ring hydroxylation and subsequent conjugation: Propranolol forms a variety of ring-hydroxylated products. The picture is complex and species dependent. Whereas the dog only forms 4-hydroxypropranolol, the rat has been shown to form three other isomers (3-OH, 5-OH and 6-OH) and man 3-OH (12-16% of the total ring hydroxylation) (Walle *et al*, 1974; Tindell *et al*, 1978). Moreover, man also forms isomeric methoxyhydroxypropranolols (Walle *et al*, 1978b). The presence of only the 4-hydroxy isomer in dogs indicates a major species difference in ring hydroxylation. The dog may utilize a different

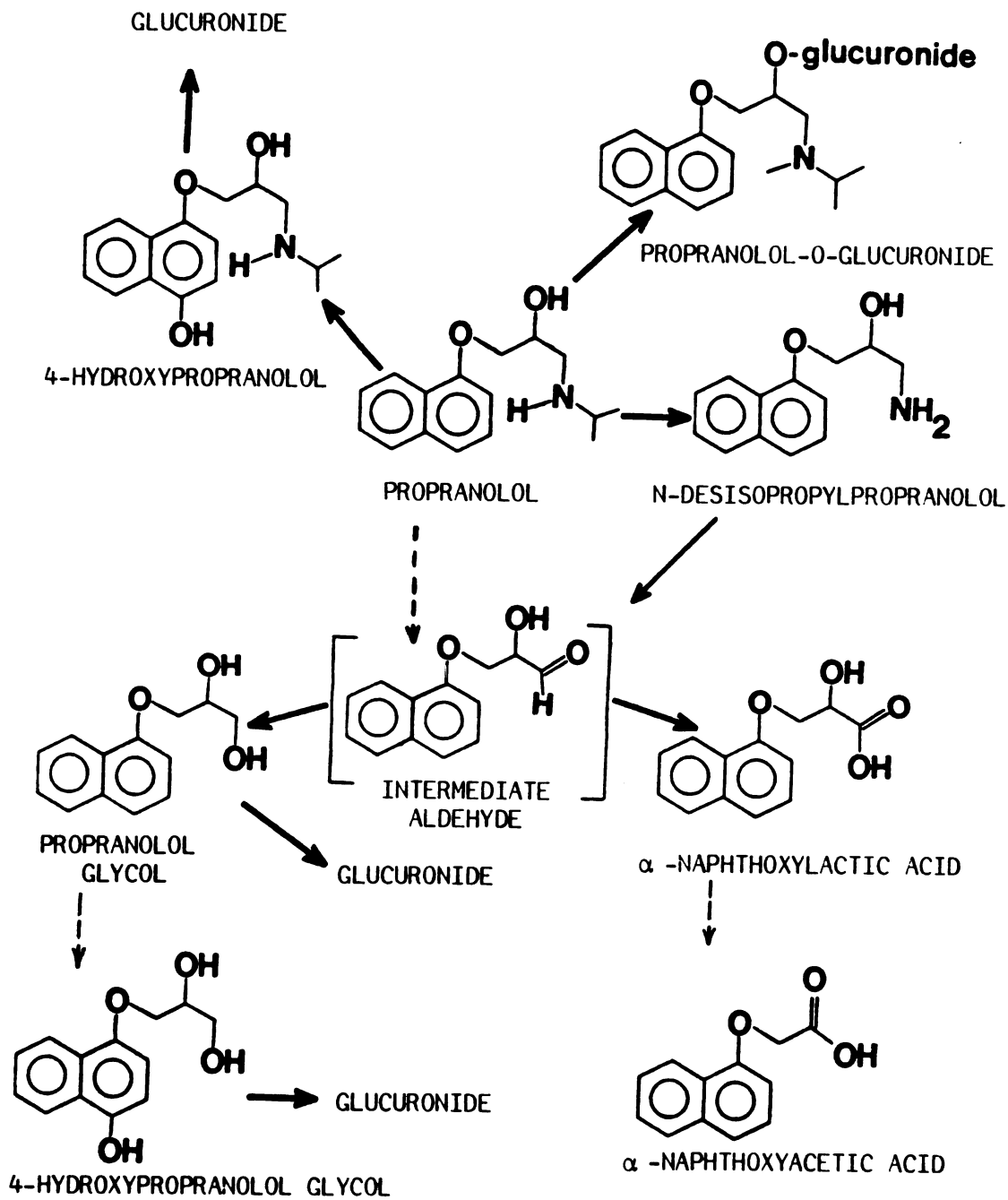


FIG II-1. Metabolic Scheme of propranolol.
Solid arrow represents a major pathway
Broken arrow represents a minor pathway

mechanism of hydroxylation involving the direct replacement of one nuclear hydrogen with an hydroxyl group; whereas rats and man may form an epoxide intermediate followed by non-enzymatic rearrangement to isomeric mononaphthols. It should be emphasized, however, the 4-hydroxypropranolol remains as the major ring hydroxylated metabolite observed in vivo. It occurs in the form of conjugates in both blood and urine of man as well as bile of dogs. Little unchanged 4-hydroxypropranolol exists in these fluids. Thus, even though it has been shown to have the same degree of beta-adrenergic blocking activity as propranolol (Fitzgerald and O'Donnell, 1971), the blood concentration of the unchanged form is so low that its clinical significance is questionable.

c) Side chain oxidation: Propranolol first undergoes oxidative dealkylation to N-desisopropylpropranolol which is then further metabolized by deamination to an intermediate aldehyde. The aldehyde either undergoes oxidation by aldehyde dehydrogenase to α-naphthoxylactic acid or reduction to propranolol glycol by an aldehyde reductase (see Figure II-1). Small amounts of α-naphthoxyacetic acid are formed from α-nephthoxylactic acid. The mechanism of the formation of the aldehyde has been studied and it has been shown to proceed via the N-desisopropylpropranolol intermediate instead of via a one-step oxidative deamination of propranolol (Nelson and Burke, 1978). Propranolol glycol and N-desisopropylpropranolol possess anticonvulsant activity but they do not contribute significantly to the anticonvulsant actions observed after propranolol administration since the anticonvulsant effect time course correlates significantly with the brain propranolol concentration-time profile rather than the brain concentration-time profiles of propranolol glycol and N-desisopropylpropranolol (Saelens *et al*, 1977).

Other minor pathways include O-dealkylation to α-naphthol and dihydroxynaphthalene (Walle and Gaffney, 1972) and the formation of methoxyhydroxypropranolol glycol and methoxyhydroxy α-naphthoxylactic acid (Walle *et al*, 1978b). These latter metabolites suggest propranolol can be metabolized to some degree via the cathechol-O-methyltransferase pathway.

Reports from the literature on extent of recovery of various metabolites are summarized in Table II-1. It appears that propranolol-O-glucuronide, 4-hydroxypropranolol conjugates, propranolol glycol conjugates and free α-naphthoxylactic acid represent the major known metabolites of propranolol. Since considerable biliary excretion also occurs in the dog it was necessary for us to develop an assay to quantitate the urinary and biliary recovery of these major metabolites (see Chapter IV).

In our studies in dogs, we have been able to account for approximately 70% of the metabolism of propranolol by measuring the major known metabolites. The rest of the metabolism, besides those minor ones that are mentioned in previous paragraphs, are still unknown. A reactive arene oxide intermediate has been proposed by Walle (1977) since methylthio-(CH_3S-) metabolites of propranolol have been isolated in vivo in urines of rat, dog and man. The arene oxide can presumably bind covalently to tissue protein or be converted to other chemical species. Pritchard *et al* (1980) have also postulated a reactive metabolite of propranolol which binds covalently to microsomal proteins, thus partially inhibiting the metabolism of propranolol.

Distribution: Propranolol is widely distributed throughout body tissues. By giving 10 mg i.v. to 6 normal subjects the apparent volume of distribution (Vd_β) was found to be 236 ± 51(SD) and 295 ± 53(SD) liters

TABLE II-1. URINARY EXCRETION (48 HR) OF PROPRANOLOL METABOLITES IN MAN AND DOG[a].

	Propranolol	4-Hydroxy-propranolol	Propranolol Glycol	α-Naphthoxy-lactic acid	α-Naphthoxy-acetic acid	α-Naphthol	Others[b]
Man[c]	8.3 - 15.	8.4 - 4.7	0.5 - 0.8	13.8 - 21.6	0.1 to 0.7	n.d.[d]	
Man[e]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2 - 2.5
Dog[f]	3	16	0.1	12	n.d.	0.4	

a) Results are expressed as percent propranolol dose. Free drug + conjugates are shown. The acid metabolites were not conjugated.

b) Methoxyhydroxypropranolols and methoxyhydroxypropranolol glycols.

c) Single oral doses from 20 to 320 mg to 6 normal adult males. From Schneck et al., 1980

d) n.d. = not determined.

e) Hypertensive patients on chronic propranolol therapy, 10 to 320 mg per day. 24 hr urine collection. From Walle et al., 1978b.

f) Single oral dose of 40 mg to one dog. From Vu and Abramson, 1978. The above results do not include biliary and fecal excretion. The latter was reported to be 22% (Hayes and Cooper, 1971).

by Shand and Rangno (1972) and McAllister (1976), respectively. This high value for volume of distribution suggests accumulation of the drug in tissues, as supported by experimental data in animals which indicated high concentrations of propranolol in lung, liver, kidney, brain, and heart after oral and i.v. administration (Hayes and Cooper, 1971; Myers *et al*, 1975; Street *et al*, 1979).

Propranolol is highly bound to proteins in plasma in man. At an equilibrium plasma concentration of 150-180 ng/ml after dialysis the per cent free was found to be 6.95±1.57(SD), ranging from 4.9 to 9.0 (Evans and Shand, 1973). Sager *et al* (1979) using sera from 21 healthy subjects found the per cent unbound to vary from 5.0 to 11.8 at an equilibrium serum concentration of 78 ng/ml. Piafsky *et al* (1978) and McDevitt *et al* (1976), using tritiated propranolol at concentrations of 8.8 and 1.08 ng/ml, found the per cent unbound to be 10.7 (range 8.3-15.4) and 6.8 ± 1.4(SD), respectively.

The question as to which protein(s) in plasma is (are) responsible for the binding of propranolol has been studied quite extensively. Both Piafsky *et al* (1978) and Sager *et al* (1979) found a correlation between the concentration of α_1 acid glycoprotein and the per cent free propranolol or the bound/free ratio of propranolol. The correlation coefficients were noted to be -0.77 and 0.849, respectively. However, there is no correlation between the albumin concentration and the bound/free ratio ($r=0.01$, $P > 0.1$, Sager *et al*, 1979). Results from binding experiments with serum lipoproteins showed that propranolol is not bound, rather it partitions into lipoprotein micelles independent of propranolol concentration (Sager *et al*, 1979). This differs from the observed binding of quinidine to lipoproteins (Nilsen and Jacobsen, 1975), but is similar to the interaction between tetracyclines and lipoproteins (Powis, 1974).

At therapeutic serum propranolol concentrations, partitioning into lipoproteins constitutes a minor fraction of that bound in serum, even in hyperlipemic sera. Thus, all the available data seem to suggest that about 75% of the propranolol in plasma is bound to α_1 acid glycoprotein, and the rest to other proteins of lower molecular weight than albumin (Sager *et al*, 1979). More direct evidence comes from the study of Scott *et al* (1979) who added ^{14}C propranolol to serum from controls and patients with inflammatory disease. Two dimensional immunoelectrophoresis was performed on these samples against anti-human whole serum (containing antibodies to 40 serum proteins). The plates were dried and radioautographed and it was found that the labelled drug was strongly associated with a single peak in the α_1 position.

The blood/plasma concentration ratio of propranolol in man has been determined by various workers. Kornhauser *et al* (1978) found a mean value of 0.78±0.12(SD) in 15 subjects by spiking whole blood with tritiated propranolol to give 2.7 ng/ml, centrifuging, and determining the plasma concentration. Weiss *et al* (1978) determined the blood and plasma concentrations by infusing propranolol to steady state and found the blood/plasma ratio to be 0.90±0.03 in 10 patients with suspected renal hypertension and normal liver function. The steady state blood concentrations achieved ranged from 67 to 92 ng/ml. Both the studies of Kornhauser and Weiss focused on propranolol concentrations below 92 ng/ml. In another study, Sawchuck *et al* (1974) studied 29 essential hypertensives on propranolol (20 to 480 mg orally per day). Blood samples were drawn when the subjects came to the clinic and the blood and plasma concentrations determined on the same sample. They found that the blood/plasma ratio varied widely from 0.6 to 1.8 with a mean of 1.33±0.28(SD). Even though Sawchuck *et al* did not report the concentration range studied we should expect that they

covered a much wider range of propranolol concentrations than previous workers. The discrepancy could be due to the fact that the subjects in Sawchuck's study were hypertensives and some of them were on multiple drug therapy. Also the α_1 acid glycoprotein concentration may have varied in these subjects. Another reason could be that the blood/plasma ratio is concentration dependent. Jellett and Shand (1973) in a short abstract addressed this problem by studying the red blood cell/buffer ratio of propranolol over a range of 50 to 1000 ng/ml and found no concentration dependency. But no definite answer can be obtained from this study because the study should have been done by spiking varying amounts of propranolol to whole blood and determining plasma concentrations. Recent studies in our laboratory (Silber *et al*, 1981) have indicated no evidence of concentration dependency in four normal healthy male subjects over a concentration range of 5 to 300 ng/ml. The knowledge of the blood/plasma ratio is important for a proper kinetic evaluation of plasma values because drug clearance should be computed in terms of whole blood (Rowland, 1972).

Disposition: After intravenous administration, racemic propranolol concentrations decline biexponentially and whole blood clearance was found to be 1.08±0.22(SD) L/min in six subjects given 10 mg intravenously (Shand and Rangno, 1972) and 0.90±0.27(SD) L/min in fifteen subjects given 8.9 micrograms of tritiated propranolol while the subjects were on chronic 240 mg orally of cold propranolol per day (Kornhauser *et al*, 1978). Recognizing that beta-adrenoceptor blockade lowers liver blood flow by approximately 20% in man (Weiss *et al*, 1978) and 33±5(SD)% in monkeys (Nies *et al*, 1973), these clearance values closely approach that of hepatic blood flow of 1.5 L/min (Mapleson, 1963), implying a high extraction efficiency. The

hepatic and systemic clearances of d-propranolol have been studied by Pessayre *et al* (1978) by administering 0.4 mg/kg of intravenous dextro-propranolol to 6 patients with fibrosis of the liver. Their hepatic veins were catheterized and samples were drawn from hepatic and peripheral veins at 3 hours to determine hepatic extraction ratio of propranolol. An indocyanine green infusion was given to the subjects to determine liver blood flow. The product of dextro-propranolol extraction ratio times liver blood flow yields hepatic clearance. This was estimated to be 18±6(SD) ml/min/kg which compares very favorably with the systemic clearance value of 18.2±7.7(SD) ml/min/kg, thus showing that the elimination of the d-isomer is essentially confined to the liver (Pessayre *et al*,1978). Weiss *et al* (1978) have suggested that dextro-propranolol be used to assess liver blood flow since it does not cause a decrease in liver blood flow.

Non-linear kinetics: Despite its complete absorption across the alimentary tract (Paterson *et al*, 1970), the kinetics of propranolol are more complex after oral administration than seen after intravenous administration. The avid hepatic extraction occurring during oral administration dictates low systemic bioavailability. However, the systemic bioavailability is dose dependent. Shand and Rangno (1972) first noted that when the area under the blood concentration/time curve (AUC) after single oral doses (6 subjects) was plotted against dose, a nonlinear curve was obtained. This was in contrast to the linear relationship which occurs with intravenous administration. With a 20 mg oral dose, only trace amounts of propranolol were detected. Over the range of 40 to 160 mg there was a linear relationship of AUC with dose with a slope of 5.41×10^{-6} $(ml/hr)^{-1}$. When this straight line was back extrapolated to the X-intercept, the authors estimated an apparent threshold dose of 30 mg. Gomeni *et al*,

(1977), in contrast, found a linear relationship between AUC (0 to 12 hr) and dose with a slope of 2.13 ± 10^{-6} $(ml/hr)^{-1}$ and an intercept not significantly different from zero in 3 subjects given 10, 20 and 40 mg oral doses of propranolol. When the AUCs from zero to infinity were plotted against dose, the curve was no longer linear and an unusually high AUC for the 10 mg data point was noted. This could be due to the fact that the AUCs for the 10 mg doses were overestimated because the extrapolated part of the area estimation is approximately 40% of the total area and the last three concentration time points were close to the limit of sensitivity of the gas chromatographic method, about 1 ng/ml. Mackichan *et al* (1980), using a more senstive HPLC-fluorometric method with an assay sensitivity of 0.5 ng/ml, confirmed a non-linear relationship between AUC and dose. The slope of the linear region at the higher doses was found to be 3.89×10^{-6} $(ml/hr)^{-1}$ with a threshold dose of 6.5 mg (data recalculated assuming a 70 kg man and using blood concentrations instead of plasma concentrations of propranolol) in 3 subjects receiving single oral doses of 10, 40, and 80 mg of propranolol. If one combines the data from all three studies, a gradual trend toward increasing slope is seen varying from 2.13 through 3.89 to 5.41×10^{-6} $(mg/hr)^{-1}$ when going from 10 to 160 mg. This indicates that the apparent oral clearance is dose dependent. Gomeni *et al* may have observed linearity due to the narrow dosage range studied. Another study by Walle *et al* (1978) in 46 patients on chronic propranolol with doses ranging from 40 to 960 mg/day also confirmed the nonlinear observation. The data, as analyzed by Muir *et al* (1981) using peak plasma concentrations, showed a threshold dose of 85 mg/day, which was considerably higher than that obtained by Shand and Rangno (1972) and Mackichan *et al* (1980).

Animal studies by Suzuki *et al* (1974) giving rats both intravenous and intraportal infusions of various doses of propranolol over 50 seconds also demonstrate nonlinearity (see Figure II-2).

The cause of the observed nonlinearity between AUC or $(Cp,ss)_{peak}$ and dose could be due to 1) saturation of some metabolic pathways of propranolol, or 2) saturation of a high affinity non-metabolizing binding site in the liver as proposed by Shand *et al* (1973) and Evans *et al* (1973).

The Perfusion Model and its Limitations as Applied to Propranolol Kinetics: For decades classical pharmacokinetic teaching has considered drug elimination in terms of compartmental models. A second approach is to develop models which incorporate physiology and anatomy of the system being studied as well as phenomena such as blood flow, membrane transport, reactions of the chemical species, and binding of the species in vivo. These models are physiologically based and have been recently reviewed by Himmelstein and Lutz (1979). One of the first physiologically based models is the model of Teorell (1937). It was a simple five-compartment scheme representing the circulatory system, a drug depot, fluid volume, kidney elimination, and tissue inactivation. The next significant development was the work of Jacques *et al* (1960). Their major contribution was to note that while many tissue regions seemed homogeneous when drug concentrations were measured, for normal tissue there are in fact intercellular, intracellular and capillary regions which may possess vastly different drug concentrations. Mass transport between these various regions occurs by diffusional processes. Bischoff and Brown (1966) combined the various ideas of their predecessors into a comprehensive model for drug distribution. The general model included

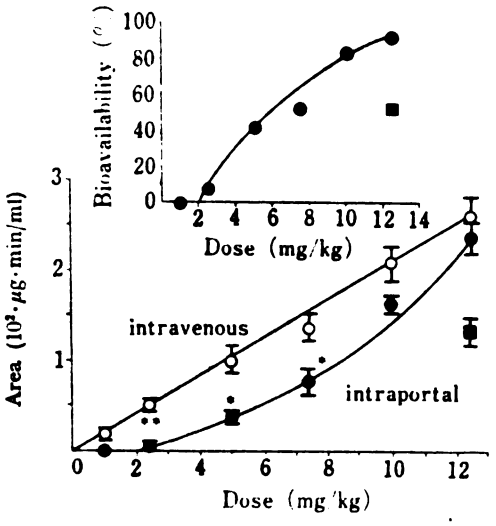


FIG II-2. Mean areas under the blood concentration-time curves after intravenous and intraportal infusion of various doses of propranolol in rats. Insert: relationship between administered dose and bioavailability. Intravenous doses were given over 30 sec into the femoral vein, and intraportal doses were given at constant rates over 50 min into the hepatic portal vein. Vertical bars represent standard errors of the estimation from 3 rats after intravenous infusion or from 5 rats after portal vein infusion. Statistically significant at $P<0.01$ (**) and $P<0.05$ (*) when compared with the area after intravenous administration. A solid square (■) represents the area or the bioavailability of propranolol in a dose of 12.5 mg/kg at the same infusion rate as that of 5 mg/kg (Suzuki et al., 1974).

a multiplicity of local regions that could be characterized as capillary, interstitial and intracellular regions. The various tissues were arranged in a flow network based on anatomical considerations.

The kind of information required for the development of a physiological model can be categorized as 1) anatomical, e.g., organ volumes and tissue sizes, 2) physiological, e.g., blood flow rates and enzyme reactions, 3) thermodynamic, e.g., drug-protein binding isotherms, and 4) transport, e.g. membrane permeabilities. There are two limiting cases in which these models can be treated. A flow-limited model can be developed if the cell membrane permeability for a particular drug is much greater than the blood flow rate per unit volume of tissue. In such a case, the rate of drug uptake by a tissue is limited by the rate at which it arrives at the tissue in the blood, since the transmembrane transport is relatively rapid. Propranolol, being a very lipophilic compound, falls into this category. The membrane-limited model represents the opposite case. The rate of drug uptake by a tissue is limited by the rate at which it crosses the cell membrane. Tetraethylammonium ion fits into this category; a detailed physiological model which incorporates active transport of this compound across membranes has been published by Mintun *et al* (1980).

In 1973, Rowland, Benet and Graham proposed a well-stirred perfusion-limited model describing a drug's clearance across an eliminating organ and its relationship with blood flow to the organ and the "clearing capacity" of the organ. This model has been modified and expanded by Shand and associates (see Wilkinson and Shand, 1975). A derivation of the model, using the liver as an example of an eliminating organ (Figure II-3), is outlined below following the format of Pang and Rowland (1977).

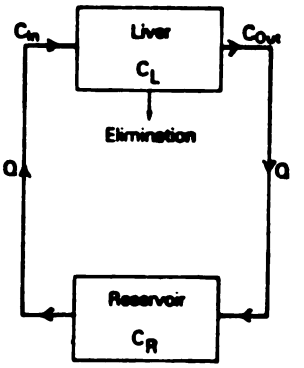


FIG II-3. Diagrammatic representation of the liver, an eliminating compartment, and the reservoir, a noneliminating compartment. The arrow indicates the direction of blood flow (Q). C_{in} , C_{out} , C_L , and C_R are the concentrations of drug in the blood entering and leaving the liver, in the liver, and in the reservoir, respectively. (Pang and Rowland, 1977)

At any moment, the velocity of removal of drug from blood by the liver (v) is the difference between the influent ($C_{b,in}$) and effluent ($C_{b,out}$) concentration of drug in blood times the hepatic blood flow (Q_H). This loss of drug from blood is matched by uptake of drug into the liver, by metabolism, and by biliary excretion:

$$v = Q_H \cdot (C_{b,in} - C_{b,out}) = \begin{array}{l}\text{rate of change of drug in the liver + metabolism + rate of biliary excretion}\end{array} \qquad \text{(II-1)}$$

At steady-state, the net rate of change of drug in the liver is zero, and v equals the sum of the rates of metabolism and biliary excretion. Hence, by definition, at steady-state

$$\text{hepatic drug blood clearance, } CL_{H,b} = \frac{v}{C_{b,in}} = \frac{Q_H \cdot (C_{b,in} - C_{b,out})}{C_{b,in}}$$

$$= Q_H \cdot E \qquad \text{(II-2)}$$

where E is the extraction ratio. An additional concept which needs to be defined is the intrinsic hepatic drug clearance of the unbound drug, $CL_{u,int}$. This concept was developed in an attempt to measure hepatocellular enzymatic activity, independent of hepatic blood flow and binding within the vascular system. The intrinsic unbound clearance, which relates the rate of hepatic elimination to the concentration of drug surrounding the hepatic enzymes; thus

$$CLu_{int} = \frac{v}{Cu_H} \qquad \text{(II-3)}$$

may be defined as the volume of liver water that is effectively cleared of drug per unit time. (Cu_H is the unbound drug concentration in the liver).

Assuming that the liver is a single well-stirred compartment and that distribution equilibrium is achieved so rapidly that drug in the emergent venous blood is in equilibrium with that in the liver, it then follows that the concentrations of unbound drug in venous blood ($Cu_{b,out}$) and in liver (Cu_H) are equal. Then the steady-state mass balance equation is

$$v = Q_H \cdot (C_{b,in} - C_{b,out}) = \sum_{i=1}^{n} \frac{V_{max,i} \cdot Cu_H}{K_{m,i} + Cu_H} \qquad \text{(II-4)}$$

so that

$$CL_H = Q_H \cdot E = \frac{1}{C_{b,in}} \cdot \sum_{i=1}^{n} \frac{V_{max,i} \cdot Cu_H}{K_{m,i} + Cu_H} \qquad \text{(II-5)}$$

($V_{max,i}$ and $K_{m,i}$ are the maximum velocity and Michaelis-Menten constant of a variety of eliminating enzymes (designated from i to n) within the liver, respectively).

Also

$$CLu_{int} = \sum_{i-1}^{n} \frac{V_{max,i}}{K_{m,i} + Cu_H} \qquad \text{(II-6)}$$

Substituting Equation II-6 into II-5, and realizing that $Cu_H = Cu_{b,out} = fu_{b,out} \cdot C_{b,out}$, where $fu_{b,out} = Cu_{b,out}/C_{b,out}$, yields

$$E = \frac{fu_{b,out} \cdot C_{b,out} \cdot CLu_{int}}{Q_H \cdot C_{b,in}} \qquad \text{(II-7)}$$

which, upon substituting (1-E) for $C_{b,out}/C_{b,in}$ and appropriate rearrangement, gives

$$E = \frac{fu_{b,out} \cdot CLu_{int}}{fu_{b,out} \cdot CLu_{int} + Q_H} \qquad \text{(II-8)}$$

so that

$$CL_H = Q_H \cdot \left[\frac{fu_{b,out} \cdot CLu_{int}}{fu_{b,out} \cdot CLu_{int} + Q_H} \right] \qquad \text{(II-9)}$$

According to Equation II-9, the biological determinants of hepatic clearance are liver blood flow, fraction unbound of drug in blood, and intrinsic clearance of unbound drug. Under conditions of constant drug binding and constant intrinsic unbound clearance, the relationship of hepatic clearance and liver blood flow is a curvilinear one, which depends on the magnitude of the intrinsic clearance of the drug in question. The hepatic clearance of a drug with a high intrinsic clearance is limited by flow when the extraction ratio approaches unity. For a drug with low intrinsic clearance (low extraction ratio) its hepatic clearance is limited by enzyme activity.

For propranolol, the above assumptions seem to be valid when one gives the drug intravenously. In this case propranolol behaves like a drug with a high extraction ratio. Thus one expects hepatic clearance to be dependent on liver blood flow. This relationship has been substantiated by in vivo data in dog and man (Branch and Shand, 1976; Weiss *et al*, 1978) by measuring liver blood flow using flow probes or by the indocyanine green method (see Figures II-4 and II-5).

However, if higher doses of propranolol are given such that the metabolism of propranolol becomes capacity limited, the extraction ratio of propranolol should decrease and a linear relationship between hepatic clearance and liver blood flow would no longer be observed. This was demonstrated in one study (Branch *et al*, 1973) in which 1 and 5 mg doses were added to the reservoir of an isolated rat liver perfusion system.

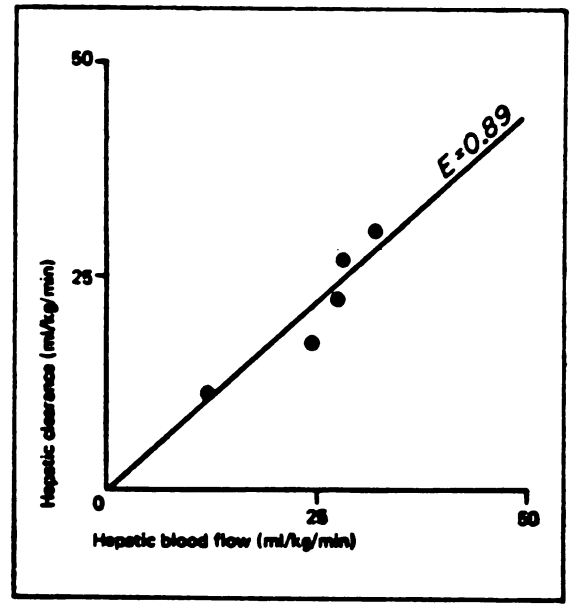


FIG II-4. The relationship between propranolol clearance measured at steady-state and liver blood flow measured with flow probes in the portal vein and hepatic artery in 5 anesthetized dogs (Branch and Shand, 1976).

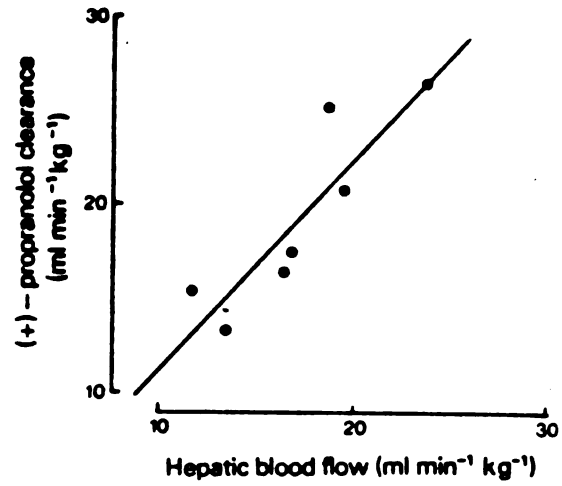


FIG II-5. Relationship between hepatic blood flow and (+)-propranolol total plasma clearance. The equation is y = 1.05 x + 1.16. r = 0.86, $P < 0.01$. The intercept value is not significantly different from zero (Weiss et al., 1978).

These authors found that, with the 1 mg dose, hepatic clearance varies linearly with hepatic blood flow, just as in the study in the intact dog. However, when they gave the 5 mg dose, hepatic clearance no longer varied linearly with flow rate. Instead it approached a limiting value. When the dose is increased, the liver extraction ratio of propranolol decreased due to a decrease in intrinsic clearance of the drug (see Figure II-6). This is not surprising if one examines the definition of intrinsic clearance (Equation II-6). The relative magnitudes of Cu_H and $K_{m,i}$ within the dosage range studied determine whether CLu_{int} remains constant or continually changes.

If applied correctly the perfusion model is a powerful tool since it allows prediction of hepatic clearance if one of the other variables is changed, for example, blood flow to the organ. However, one has to be aware of the assumptions one is making when using Equation II-9. The most common assumption that investigators have used is that intrinsic clearance is constant for drugs like propranolol and lidocaine. From the previous discussions it appears that the intrinsic clearance of propranolol is not constant, especially upon oral multiple dosing.

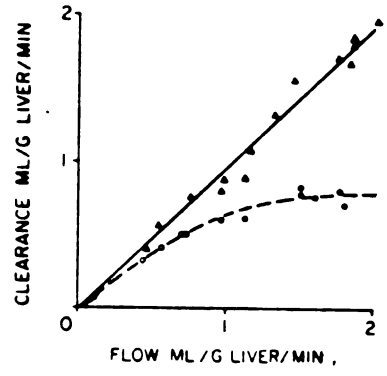


FIG II-6. The relationship between hepatic blood flow and the clearance of propranolol by the perfused rat liver when 1 mg (▲) and 5 mg (O) of propranolol was added to the reservoir. Each data point represents a single experiment (Branch et al., 1973).

CHAPTER III. ANIMAL MODELS TO STUDY FIRST PASS METABOLISM

1. PREVIOUS TECHNIQUES USED

When a drug is given orally, it has to be absorbed through the gastrointestinal tract and is then delivered to the liver via the portal circulation. Metabolism of the drug can occur in both sites. Enzyme systems reported to occur in the human intestinal wall include monoamine oxidase (Floch *et al*, 1967), glucuronyl transferase (Hoffmann and Breuer, 1968), β-glucuronidase (Jodl *et al*, 1968), aryl sulfatase (Pulkkinen, 1961), phenol and steroid sulfokinase (Bostrom and Wengle, 1964; Bostrom *et al*, 1968) and acetylase (Jenne, 1965; Moore *et al*, 1946).

Evidence for gastrointestinal drug metabolism include hydrolysis of aspirin (Levy and Angelino, 1968), conjugation of benzoic acid with glycine (Strahl and Barr, 1971), glucuronidation of salicylamide (Barr and Riegelman, 1970a,b), thyroxine, triiodothyronine, estradiol, testosterone and androstenedione (Herz *et al*, 1961; Kreek *et al*, 1963; Smith *et al*, 1963). Most of these studies involved the in vitro everted intestinal sac (Wilson and Wiseman, 1954; Crane and Wilson, 1958; Kaplan and Cotler, 1972), ring and slice techniques (Crane and Mandelstam, 1960; Stadie and Riggs, 1944). A more realistic approach is to use in situ ligated single or multiple intestinal loops (Levine *et al*, 1955; Levine and Pelikan, 1961). An elegant chronic approach involves the use of a Thiry-Vella loop in which a segment of the small intestine is isolated with its blood, nerve and lymph supplies intact. The proximal and distal end of the intestinal segment are then exteriorized through the abdominal wall and sutured in place. The surgical procedure for the Thiry-Vella loop in dogs is described by Streeten and Williams (1951) and Markowitz *et al* (1964). The bioavailability of acetaminophen following its

administration in an oral solution and in gelatin capsules has been studied in Thiry-Vella loop dogs (Sample *et al*, 1968).

While the gastrointestinal tract plays a minor role, the liver remains the major organ of drug metabolism. The in vitro isolated perfused liver is the most popular technique used to study hepatic metabolism. An extensive discussion of perfusion apparatuses and the surgical preparation of the liver is present by Miller (1973). If carefully prepared, the organ appears to be viable for 2-4 hours. It has advantages over homogenate incubation techniques in that it avoids cellular disorganization and allows for measurement of metabolic rates in addition to elucidation of the metabolic pathway of the drug. In both the liver perfusion and the homogenate incubation methods non-specific uptake of the drug by tissues occurs and can make it difficult to achieve material balance. Since the perfused liver is an isolated system, it permits detection and quantitation of metabolites that may be present in too low a concentration and/or excreted too rapidly via the kidneys to be detected in vivo. However, the accumulation of a metabolite can be a disadvantage since it could act as an end product inhibitor (in a recirculating liver perfusion system only). An additional advantage is that metabolism and distribution occurring at other sites do not mask the effects occurring in the liver per se. Shand *et al* (1975) and Branch *et al* (1973) have extensively used the isolated perfusion technique to study the well-stirred perfusion model using propranolol as the model compound. With the isolated system it is easy to alter the perfusion rate and the protein concentration in the perfusate. Despite the above advantages, the technique remains an acute preparation and suffers from limited viability.

The perfused liver is also adaptable for use as an in situ technique (Benzi *et al*, 1967). A more realistic approach is to cannulate the

portal vein. The surgical procedure has been described by Cotler *et al* (1976) and Boyes *et al* (1970). The catheter, once in place, is exteriorized for use. This catheter allows for rapid administration of drug into the hepatic portal vein, assuring 100% passage of the dose through the liver prior to entering the systemic circulation, and allows for complete bypass of the gastrointestinal tract. Following intravenous, oral and hepatoportal administration of levodopa, Cotler *et al* (1976) noted incomplete oral availability in three dogs. However, following hepatoportal administration, the plasma level curves indicated complete availability. These studies thus distinguish between potential hepatic and intestinal "first pass" metabolism, and indicate that the reduced bioavailability upon oral dosing of levodopa is due to gastrointestinal lumen and intestinal-wall metabolism.

A major disadvantage of this technique lies in maintaining the hepatoportal catheter patent for a long period of time for chronic studies and the need to keep the animal from biting the catheter.

An alternate technique developed to isolate gastrointestinal metabolism from first pass liver metabolism involves end-to-side portacaval shunt surgery. Gugler *et al* (1975) have used this technique to study the first pass metabolism of antipyrine, lidocaine and salicylamide. From their study, salicylamide is shown to undergo 42% gut-wall metabolism and 36% hepatic metabolism. Since the portal blood flow involves 72% of total hepatic blood flow (Kreuzer and Schenk, 1971), hepatic blood flow is vastly reduced and normal liver function could be seriously impaired. Also this model does not allow the investigator to study liver metabolism after the surgery, and one has to compare pre- and post-surgery data and assume that the animal has not changed physiologically after the surgery.

2. PORTALCAVAL TRANSPOSITION

Portacaval transposition, first described by C.G. Child *et al* in 1953, is a surgical technique that involves an end-to-end transposition of the inferior vena cava and hepatic portal vein beneath the liver as pictured in Figure III-1. This technique was modified for our purposes. The detailed procedure as drafted and performed by Dr. David Effeney, Department of Surgery, Veterans Administration Hospital, San Francisco is reproduced below:

> The dog is anesthetized using intravenous phenobarbital. Following intubation and ventilation with air via Harvard Animal Respirator, the dog is placed supine on the operating table and the entire abdomen shaved, prepared with 1% iodine and draped with sterile towels.
>
> INCISION
>
> A long midline incision is used extending from the xiphisternum to the tip of the penis in the male dog and in the female, if available, is extended to half way between the umbilicus and the pubis. The incision is deepened using diathermy and the abdomen entered. Wound towels are placed and the abdominal musculature retracted utilizing a self-retaining retractor.
>
> PROCEDURE
>
> A super mesenteric artery is dissected free in the base of the mesentery, isolated close to the aorta and cleaned over a length of approximately one half inch of its investing facia. This facilitates clamping of the super mesenteric artery at the time of portal cross clamping. When the artery has been isolated tape is placed around the vessel for easy identification. Following this the duodenum is mobilized by division of the peritoneum along its lateral border. The duodenum and pancreas are mobilized medially. This exposes the portal vein and inferior vena cava both of which vessels are cleaned of the posterior peritoneum and investing fascia. The portal vein is cleaned from the portahepatis down to the entrance of the superior mesenteric vein. The inferior vena cava is cleaned from the renal veins as far approximal as possible. Both vessels are circumferencely mobilized. The only vessels to be sacrificed are the right supra renal vein, possibly one pair of lumbar veins and several small pancratic veins which drain into the portal. Once these vessels have been isolated the spleen

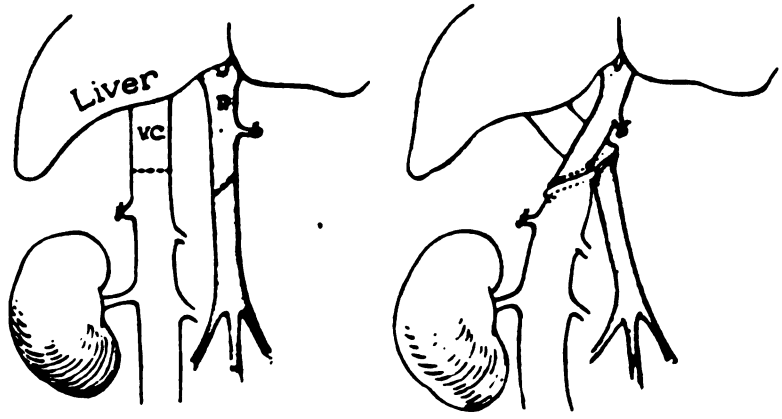


FIG III-1. Diagram of the method used to transpose the portal vein and vena cava in a dog (Silen et al., 1957).

is mobilized, subcapsular adrenaline injected into the spleen to cause it to contract to autotransfuse and a splenectomy is then performed. At completion of splenectomy the superior mesentery artery is cross clamped, the intestines and duodenum again retracted to the left and the portal vein and inferior vena cava occluded with non-crushing vascular clamps. Both vessels are divided and the first anastomosis of the distal portal vein to proximal inferior vena cava is completed using a running suture of 60 Prolene or silk. At the completion of this anastomosis, clamps are removed and circulation is restored to the small intestine by removing the clamp on the super mesenteric artery. Hemostasis is secured and any leaks repaired using interrupted sutures of 70 silk. The second anastomosis of the distal inferior vena cava to proximal portal vein is then constructed utilizing, again, a continuous suture of 60 Prolene or silk. The clamps are released and any obvious leaks repaired and the entire operative field checked for hemostasis.

CLOSURE

When satisfactory hemostasis has been insured the abdomen is closed in layers of 0 chromic gut to the peritoneum and posterior rectus sheath. Interrupted sutures of tycron to the fascia and an interrupted nylon suture to the skin and subquetaneous tissue complete the closure. An occlusive dressing is placed by means of a body binder and the dog returned to the postoperative observation area.

During the procedure the dogs are hydrated using normal saline and are covered by perioperative antibiotics extending for 72 hours of the post-operative period. The animals are checked on the evening of surgery and then daily until recovery. As soon as practicable after the operation the animals are returned to their normal conditioning routine, low protein diet and regular exercise.

Reported series of animals undergoing this operation have indicated that there is from 20 to 50 per cent mortality in unconditioned dogs having this procedure performed on them. The mortality is usually early, related to the development of sepsis following the period of cross clamping the portal vein. This time of shock has been obviated by the technique of cross clamping the superior mesentery artery which significantly reduces the damage to the gut vasculature due to marked venous hypertension. The only other major complication is wound complications and every effort is made to ensure satisfactory closure and appropriate prophylaxis of infection. Strict adherence to sterile technique is enforced. If these provisions are carefully adhered to a chronic model can be produced with a mortality of less than 20%.

By this procedure gastrointestinal metabolism is separated from hepatic metabolism. A drug now given orally will be absorbed through the gastrointestinal tract, but instead of going to the liver it enters the upper segment of the inferior vena cava and into the systemic circulation, thereby obviating the liver during the first-pass. Whereas a dose given via a peripheral hind-limb vein enters the lower segment of the inferior vena cava and thence is delivered to the liver via the portal vein. We have accomplished setting up an in vivo liver perfusion animal model that allows for repeated use of the same animal.

Silen *et al* (1957) have examined the health of four transposed dogs over a period of three months and the laboratory and histological findings were compared with normal animals. There was no significant loss of body weight. The livers and kidneys were of normal weight, and there were no gross or microscopic abnormalities. The fat and glycogen contents of their livers were normal. However, the total circulating albumin increased from 10 to 40% in 3 of the 4 dogs studied and hepatic clearance of bromosulfophthalein increased 2½ fold in the 3-month period after the operation, probably reflecting an increase in liver blood flow. More recently, Kreuzer and Schenk (1971) studied seven transposed dogs for three months after surgery. Hemodynamically they found total liver blood flow was increased 27% above control. Blood flow via the caval source showed a 50% increase over the control value ($P < 0.05$). Hepatic artery flow was decreased 32% from the control value ($P < 0.01$). Systemic arterial pressure was normal. Pressure in the hepatic venous inflow tract was decreased below that seen in the portal vein of the control animals, being almost that found in the cava of the control animals. There was a slight decrease in total serum proteins, in contrast

to the results found by Silen *et al* (1957). The serum glutamic pyruvic transaminase (SGPT) level was elevated; however, serum bilirubin, alkaline phosphatase and sulfobromophthalein retention values were all within normal ranges. The liver appeared to be moderately enlarged showing soft contours and edges. Liver specimens obtained 2 and 14 days post-transposition appeared entirely normal. In specimens obtained at six weeks, minimal fatty liver-cell metamorphosis was seen which became more pronounced at 12 weeks.

We have monitored several of these clinical values for three of our dogs: two for a period of three months and one for seven months and the results are shown in Table III-1. The overall health of the animals is excellent. Their body weights are maintained and they remained active.

3. EFFECT OF PROPRANOLOL ON LIVER BLOOD FLOW IN PORTACAVALLY TRANSPOSED DOG

Since propranolol is known to decrease liver blood flow (Weiss *et al*, 1978; Nies *et al*, 1973) we would like to know the magnitude of decrease in the dogs that have undergone portacaval transposition. This of course requires base line data in the absence of propranolol.

Theory: Methods to measure liver blood flow have been reviewed by Ohnhaus (1979). Direct methods include electromagnetic flowmeter and heat exchange method. Indirect methods are less invasive and do not require opening the abdomen. The most common method is the application of the clearance concept to a substance that is completely and solely extracted by the liver. Then the following equation can be used:

TABLE III-1. CLINICAL VALUES IN THREE DOGS BEFORE AND AFTER PORTACAVAL TRANSPOSITION SURGERY.

	Dog A[a)]	Dog B[b)]	Dog C[b)]	Normal Range
Total Serum Proteins(gm%)				
Before	n.d.[c)]	6.2	6.3	5.4-7.8
After	6.5	6.4	6.0	
Albumin (gm%)				
Before	n.d.	2.9	2.9	2.3-3.4
After	2.5	3.0	3.1	
Serum Glutamic Oxaloacetic Transaminase (SGOT, IU/L)				
Before	n.d.	23.	17.	5.-25.
After	n.d.	15.	12.	
Serum Glutamic Pyruvic Transaminase (SGPT, IU/L)				
Before	n.d.	n.d.	n.d.	5.-55.
After	13.	117.	16.	
Total Bilirubin (mg%)				
Before	n.d.	0.3	0.2	0.1-0.6
After	n.d.	0.1	0.1	
Alkaline Phosphatase (IU/L)				
Before	n.d.	41.	22.	20.-150.
After	15.	77.	19.	

a) 7 months after surgery
b) 3 months after surgery
c) not determined

$$Q_H = \frac{\text{Hepatic Blood Clearance}}{\text{Liver Extraction Ratio}} = \frac{\text{Systemic Blood Clearance}}{\text{Liver Extraction Ratio}} \quad \text{(III-1)}$$

Various dyes have been used, the most common ones being bromosulfophthalein and indocyanine green. Bromosulfophthalein has the disadvantage of having a small extra-hepatic clearance component, thus liver blood flow tends to be overestimated (Winkler *et al*, 1965). Also it undergoes enterohepatic circulation (Lorber *et al*, 1953). Indocyanine green, in contrast to bromosulfphthalein, is cleared exclusively by the liver and excreted in unconjugated form into bile without significant enterohepatic circulation (Hunton *et al*, 1960; Cherrick *et al*, 1960). Recoveries of 97% (Wheeler *et al*, 1958) and 91% (Ketterer *et al*, 1960) of the infused dye from the bile duct were obtained in dogs. The only disadvantage of using indocyanine green in dogs is that it has a small extraction ratio. Since indocyanine green is confined exclusively to plasma, equation III-1 can be modified to calculate liver blood flow using plasma indocyanine green data:

$$Q_H = \frac{\text{Plasma Systemic Clearance}}{\text{Liver Extraction Ratio} \cdot (1 - \text{Hematocrit})} \quad \text{(III-2)}$$

The plasma clearance of indocyanine green is usually obtained by

$$CL_p = k \cdot V$$

where k is the first order elimination constant and V is the volume of distribution of indocyanine green.

Experimental: A male mongrel dog weighing 23.3 kg was studied. Eight months after the portacaval transposition the dog was anesthetized with sodium pentobarbital and the right hepatic vein was catheterized

using a fluoroscopic procedure. Ten mg indocyanine green was given as a bolus dose in one forelimb vein. Serial blood samples were taken every 5 minutes for 30 minutes from both hepatic vein and another forelimb vein. After a wash-out period of 50 minutes from the last indocyanine green sample, a propranolol hind-limb infusion was initiated (0.56 mg/min). After one hour of infusion a second 10 mg dose of indocyanine green was given to the dog and blood samples again were taken as above from both veins for 30 minutes. The blood samples were collected into heparinized tubes, the plasma separated and the concentration of indocyanine green determined spectrophotometrically by measuring the absorbance of the plasma at 804 nm.

Results and Discussion: The peripheral and hepatic venous concentration-time profiles of indocyanine green are tabulated in Table III-2 and plotted semi-logarithmically in Figure III-2. The curves are log-linear. They were back extrapolated to zero time to obtain the initial peripheral and hepatic concentrations. The first order elimination rate constants were estimated from the peripheral venous curves. The extraction ratio was calculated from the equation:

$$E = \frac{\text{Initial Peripheral Venous Conc.} - \text{Initial Hepatic Venous Conc.}}{\text{Initial Peripheral Venous Conc.}} \quad \text{(III-3)}$$

The volume of distribution of indocyanine green = dose/(initial peripheral venous concentration). Hepatic blood flow is then calculated according to Equation III-2. The results are shown in Table III-3.

At a peripheral propranolol concentration of approximately 200 ng/ml hepatic blood flow is seen to drop from a control value of 1426 ml/min to 524 ml/min, a decrease of 63%. This probably reflects the maximum

TABLE III-2. INTRAVENOUS DISPOSITION OF INDOCYANINE GREEN IN DOG AS MEASURED FROM PERIPHERAL AND HEPATIC VEINS.

Before Propranolol Administration			
Peripheral Venous Conc		Right Hepatic Venous Conc	
Time (min)	μg/ml	Time (min)	μg/ml
6.3	3.16	3.1	3.38
18	1.43	8.2	2.42
25.7	0.82	24.8	0.78
34.5	0.50	34.7	0.41
After Propranolol Administration[a)]			
Peripheral Venous Conc		Right Hepatic Venous Conc	
Time (min)	μg/ml	Time (min)	μg/ml
8.6	3.26	3.3	2.70
13.8	1.99	13	1.39
19.8	1.26	20.3	0.88
29.5	0.73	29.9	0.59

a) The peripheral propranolol concentration was about 200 ng/ml.

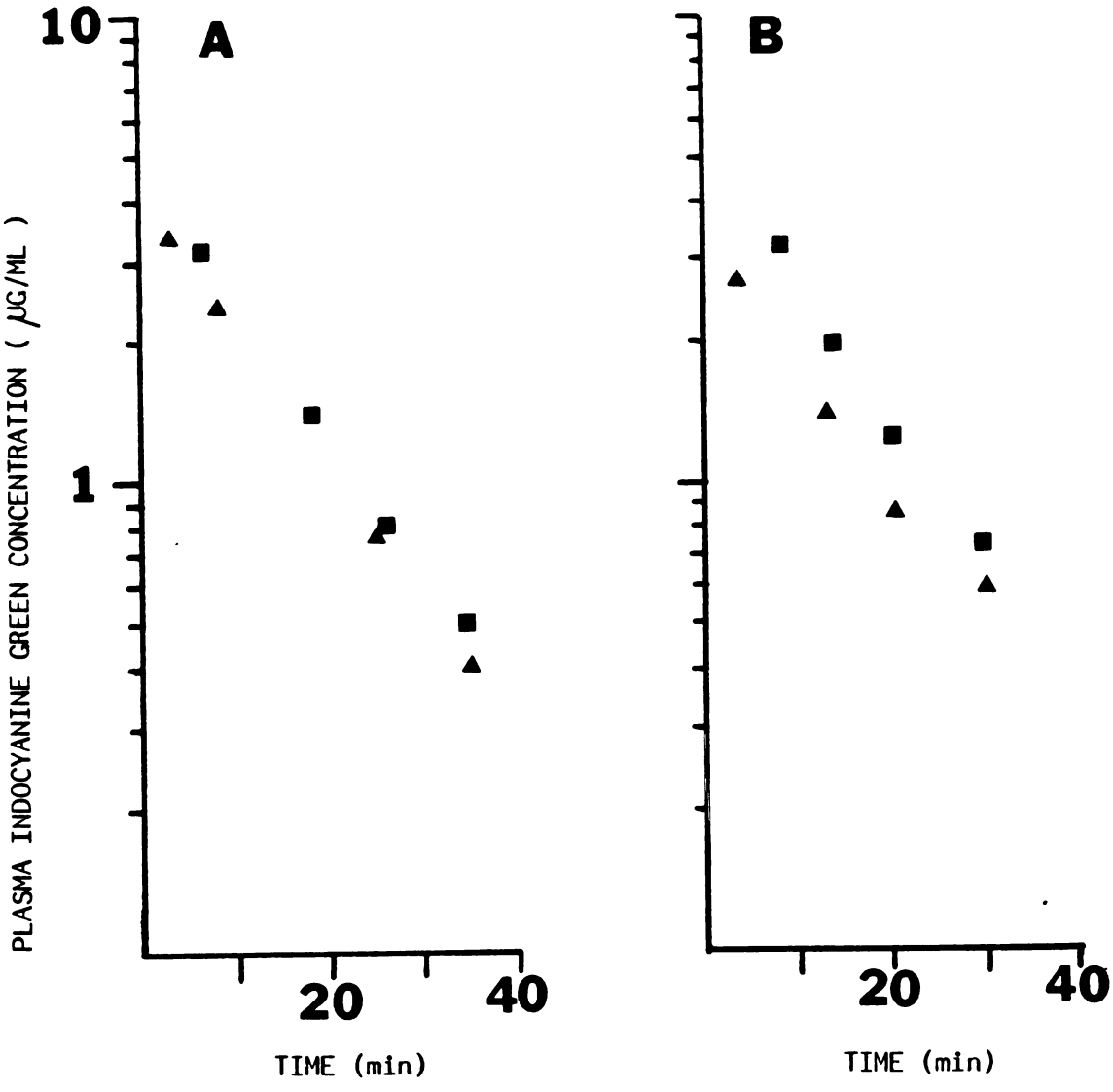


FIG III-2. Data illustrating indocyanine green disposition when a bolus dose (10 mg) is given in the absence (A) and presence (B) of a steady-state propranolol infusion (0.56 mg/min) via a hind-limb vein in a surgically transposed dog. Symbols: Peripheral vein data (■); hepatic vein data (▲).

TABLE III-3. ESTIMATION OF THE EFFECT OF PROPRANOLOL ON INDOCYANINE GREEN HEPATIC CLEARANCE AND LIVER BLOOD FLOW.

	Before Propranolol	After[a] Propranolol	Percent change
Extraction ratio	0.146	0.368	+ 152
Volume of Distribution (ml)	2083	1802	- 13
Elimination Rate Constant (min^{-1})	0.066	0.071	+ 7
Plasma Systemic Clearance (ml/min)	137.4	127.4	- 7
Liver Blood Flow[b,c] (ml/min)	1426	524	- 63

a) The peripheral propranolol concentration is about 200 ng/ml.
b) The hematocrit is 34% in the dog.
c) Calculated from Equation III-2.

decrease of liver blood flow which is physiologically possible. At a concentration of 200 ng/ml of propranolol all the beta-receptors should be completely blocked and the blood flow decreased maximally (Nies and Shand, 1975). George *et al* (1976) measured hepatic blood flow in 8 dogs with ^{198}Au colloidal gold while the dogs were on propranolol intravenous infusions yielding steady-state propranolol peripheral concentrations between 100 to 200 ng/ml. The value of liver blood flow they found when corrected for body weight was 21.1 ± 5.8(SD) ml/min/kg. This value agrees very well with the present value we found in our dog of 22.5 ml/min/kg after administration of propranolol.

Indocyanine green is a compound possessing low extraction ratio in the dog. Therefore its clearance is not expected to be dependent on hepatic blood flow. As shown in Table III-3, even though there is a 63% decrease of liver blood flow, its clearance remains relatively constant (a decrease of 7%) since the extraction ratio of indocyanine green increases to an extent that compensates for the decreased blood flow. The biological determinant of indocyanine green clearance in the dog is the intrinsic clearance of indocyanine green which is unchanged by propranolol.

4. BILIARY DRAINAGE

During the course of the study it was found necessary to collect bile since a significant portion of the metabolites of propranolol is excreted into the biliary system. Several methods have been described for bile collection. The balloon-occludable Baldwin T-tube method (Soloway *et al*, 1972) is suitable for monkeys that are set in chairs

and in man, but not for chronic unrestricted dogs since they tend to bite the catheters even if protected with a dog jacket. The simplest method for chronic use in the dog is that of Thomas (1941) who devised a cannula that allows one to get access from outside of the animal to the ampulla of Vater, where the common bile duct enters the duodenum. Since then many variations of the basic technique have been published. In 1960, Lester *et al* gave a detailed description of the preparation of the cannula and the complete surgical procedure. Jones *et al* (1971) devised the simplest Thomas cannula made from a 2-inch Teflon rod. This single-unit construction eliminates turning multiple pieces and the time-consuming threading process.

One disadvantage in the use of the Thomas cannula is that it requires some degree of skill and manipulation to find the ampulla and insert the catheter. There is also a possibility of bile leakage around the catheter.

An alternative method is to permanently catheterize the common bile duct with a short piece of silastic tubing, without ligation of the duct. Normally the tube stays in the duodenum to maintain enterophepatic circulation of bile. When bile collection is needed, the tubing is brought outside through a Thomas cannula. Marshall *et al* (1964) prepared 5 dogs according to this method. The shortest length of survival was 3 months and three animals had patent catheters for over 6 months. However, according to Wyman *et al* (1968), they would elect to use the Thomas cannula alone in preference to the permanently implanted catheter method because of potential complications in the latter.

Another modification is that of DenBesten (1971). His technique involves quite extensive surgery. A segment of the duodenum where the common bile duct enters is isolated and ligated. Duodenal continuity is

restored by end-to-end anastomosis. The separate segment of duodenum is closed at both ends to form a small pouch into which bile is constantly draining. A Gregory pouch cannula is inserted into the midpoint of the pouch, which drains into the duodenum. A Thomas cannula is then placed in the duodenum exactly opposite the bile Gregory cannula. During experiments, a balloon catheter is placed in the Gregory cannula for complete bile collection.

We elected to use the procedure of Lester *et al* (1960) for making the Thomas cannula (see Figure III-3). When bile samples were to be collected, the Thomas cannula was opened, the duodenal contents were removed with warm saline and gentle section until the ampulla of Vater was visualized with the help of a direct focusing headlight. The common bile duct was then catheterized using a disposable 6 Fr olive-tip ureteral catheter. The catheter was advanced about 4 to 5 cm into the common duct. The opening of the Thomas cannula was then closed with a screw-cap which had a hole in the center allowing the ureteral catheter to pass through. The catheter was anchored securely with cotton thread fastened to holes in the flange of the Thomas cannula.

If the Thomas cannula was surgically inserted directly across from the ampulla of Vater and positioned correctly, it became relatively easy to catheterize the common duct. However, if the cannula was placed off-center then locating the ampulla was no longer an easy task. In two out of the three dogs the position of the cannula was less than ideal which made cannulation much more difficult. In conclusion, the Thomas cannula technique remains the simplest method of bile collection. However, if enough surgical expertise and the Gregory cannula are available, then the use of the Gregory bile pouch cannula method is preferred. The DenBeston group had dogs that had patent cannulas for more than 15 months.

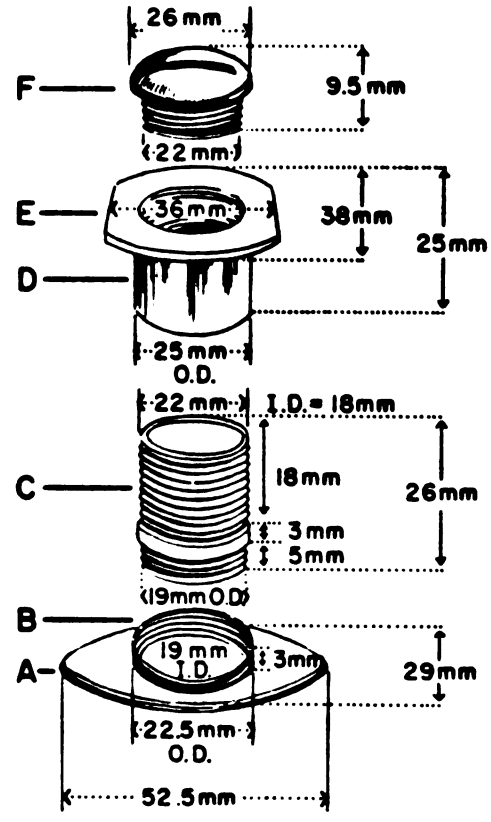


FIG III-3. Diagram of Thomas' duodenostomy cannula separated into into its several parts (Lester et al., 1960).

A. Intraluminal phlange-elliptical (hard rubber).
B. Circular collar extending from A; inside thread.
C. Male part of cannula; detachable extension of B with outside thread (hard rubber).
D. Female part of cannula with inside thread (stainless steel).
E. Exterior phlange on D.
F. Screw-button to close D (hard rubber).

CHAPTER IV. ASSAY PROCEDURE FOR PROPRANOLOL AND ITS MAJOR METABOLITES IN BIOLOGICAL FLUIDS

As outlined in Chapter II, the major metabolites identified in man include conjugates of propranolol and 4-hydroxypropranolol, and free α-naththoxylactic acid in plasma and urine. Preliminary results in the dog show that propranolol glycol is another metabolite excreted mainly in the bile as its glucuronide metabolite.

The assay procedure for these metabolites has been revised several times. There are two major approaches to the problem.

The first approach is the direct injection method in which protein is precipitated with acetonitrile and the clear supernate is injected onto the high-pressure liquid chromatographic column. The eluant system is such that all the different acidic, neutral and basic metabolites elute separately. The drawbacks of this method are: 1) the metabolites have different fluorescent properties, and in order to detect them all in one chromatographic run, the operator has to switch the fluorescent maximum. This precludes the use of the method in the automation mode; 2) the method is not sensitive enough to detect some of the metabolites if their concentrations are below 20 ng/ml.

A second approach uses differential solvent extraction to separate the acidic, neutral and basic metabolites and subsequent quantitation by HPLC. No wavelength change is necessary, thus the procedure can be automated. However, the work up of the samples is somewhat more time-consuming but is mitigated by the opportunity to automate the total procedure.

In this chapter both methods will be presented and a recommended

overall procedure described at the end of the chapter. The direct injection method has already been published (Lo and Riegelman, 1980).

1. DIRECT INJECTION METHOD

To date, this is the only direct injection assay available for determination of propranolol and metabolites. The assay method for unchanged propranolol alone in blood or plasma will be presented first. This method offers advantages because there are times one needs to know only the free concentration of propranolol and not the metabolites. The assay description for drug and metabolites follows.

EXPERIMENTAL

Standards and Reagents: Propranolol HCl, 4-hydroxypropranolol HCl, α-naphthoxylactic acid, propranolol glycol and N-desisopropylpropranolol were kindly supplied by Imperial Chemical Industries, Macclesfield, Great Britain. α-naphthol and α-naphthoxyacetic acid were obtained from Aldrich, Milwaukee, Wisc., U.S.A. and Trans World Chemicals, Inc., Washington, D.C., U.S.A., respectively.

The internal standard N-ethylpropranolol was prepared according to the method of Wood *et al* (1978). However, the crude product contained an impurity that interfered with the assay and could not be separated by solvent extraction. Therefore it was necessary to purify the product by reverse phase HPLC and solvent extraction. The recovered N-ethylpropranolol was stored in methanol, which was used to make up three concentrations for use as internal standards: one in acetonitrile, for unchanged propranolol in plasma and two in dilute phosphoric acid, for hydrolyzed plasma and at a five fold higher concentration for use in the urine assay.

Acetonitrile (UV grade) and methanol were supplied by Burdick and Jackson Labs, Muskegon, Mich., U.S.A. All other chemicals were of analytical grade.

Instrumentation: A Varian Model 8500 high-performance liquid chromatograph equipped with a Perkin-Elmer 650-10 LC fluorescence spectrophotometer, a Lichrosorb RP-8 column (25 cm X 4.6 mm I.D.; 10 μm particle size; Altex Associate, Berkeley, CA, U.S.A.) and a Lichrosorb RP-2 precolumn (4 cm X 3.2 mm I.D.; 10 μm particle size; Altex) were used. The fluorescence output was recorded on a dual channel recorder (Linear Instruments Corp., Irvine, CA, U.S.A.). Injections were made with a 100 μl Hamilton syringe through a Valco CV-6-UHPa-N60 sweep-flow injector equipped with a 100 μl loop.

Method 1: Measurement of Unconjugated Propranolol in Plasma: Daily standard curves were prepared as follows: A 1.5 μg/ml propranolol (free base) solution in water was prepared from a 50 μg/ml propranolol HCl aqueous stock solution. A 0.5 ml quantity of this standard solution was added to 2 ml of drug-free human plasma to make up a 300 ng/ml standard. It was then serially diluted with plasma to yield concentrations of 150, 75, 30, 20, 10 and 5 ng/ml.

Plasma samples were processed by transferring a 0.2 ml quantity into an Eppendorf polypropylene 1.5 ml micro test tube (Brinkmann No. 2236411-1, Brinkmann Instruments, Inc., Westbury, N.Y., U.S.A.), and a 0.4 ml of the N-ethyl-propranolol solution in acetonitrile was added. After the sample was vortexed for 15 seconds it was centrifuged for 2 minutes at 12800 g using an Eppendorf Microcentrifuge, Model 5412 (Eppendorf, West Germany). The clear supernatent was transferred to a disposable glass culture tube (13 X 100 mm) and evaporated to an approximate volume of 0.1 to 0.2 ml

under a gentle stream of nitrogen. After adding 0.2 ml of 0.05M phosphoric acid and briefly vortexing, a 50-90 µl aliquot was injected onto the column. The mobile phase was composed of a mixture of 360 ml of acetonitrile, 180 ml methanol, and 70 ml of 0.0871 M phosphoric acid which was diluted to one liter with glass distilled water. The flow rate was 100 ml/hr. The fluorometer was set an an excitation wavelength of 230 nm and an emission wavelength of 340 nm, respectively. Both slit openings were set at 20 nm. The fluorometer was operated at a sensitivity range of 0.1 and normal power gain. The output was 1 volt and sensitivity varied by changing the voltage spans on the dual pen recorder. One pen was always fixed to measure the internal standard.

Method 2: Measurement of Propranolol, 4-hydroxypropranolol and α-Naphthoxylactic acid in Plasma and Urine after Enzymatic Hydrolysis: The plasma and urine standards were made up as follows: the most concentrated plasma standard containing 2 µg/ml of α-naphthoxylactic acid and 1 µg/ml of propranolol and 4-hydroxypropranolol was prepared by evaporating to dryness 0.5 ml of a 20 µg/ml α-naphthoxylactic acid in methanol. Then 0.5 ml of propranolol (10 µg/ml in water) and 0.25 ml of 4-hydroxypropranolol (20 µg/ml in 0.1 M phosphoric acid including 5 mg/ml ascorbic acid to minimize oxidation) were added. These were diluted with 4.25 ml of human (drug-free) plasma. Additional standards were prepared by serial dilution with more blank plasma. Urinary standards were prepared slightly differently because of the instability of 4-hydroxypropranolol in urine. A urine sample containing 20 µg/ml of propranolol and α-naphthoxylactic acid was first prepared and then serially diluted with more blank urine. A series of disposable culture tubes were prepared each containing 0.1 ml of a 200 mg/ml solution of ascorbic acid. Then 0.025, 0.05, 0.1 or 0.2 ml

of 4-hydroxypropranolol (20 μg/ml protected by ascorbic acid) was added. To each of the tubes was added 0.2 ml of the appropriate propranolol α-naphthoxylactic acid mixture and a 0.2 ml quantity of the 5-fold concentrated internal standard solution.

The assays were performed as follows: a 0.2 ml quantity of urine or a 0.4 ml quantity of plasma were mixed with 0.2 ml aqueous internal standard in a disposable glass culture tube. The urine sample was diluted with an addition of 0.2 ml of water. A 0.1 ml quantity of ascorbic acid (200 mg/ml), 0.04 ml of acetate buffer (1.4M, pH 5.5) and 25 mg of β-glucuronidase/aryl sufatase (400 units/mg, Sigma G 0751, St. Louis, MO, U.S.A.) were added. The mixture was incubated at 37°C for 90 minutes. After precipitating the protein with 0.8 ml of acetonitrile, the resulting mixture was transferred to a 1.5 ml microcentrifuge tube and centrifuged for 3 minutes at 12800 *g*. A 0.6 ml quantity of the clear supernate was removed and 0.3 ml of 0.05 M phosphoric acid was added. It should be noted that no evaporation was necessary. A 40-50 μl aliquot was injected onto the column. The eluant was composed of a mixture of 300 ml of acetonitrile, 90 ml of methanol, and 66 ml of 0.0871 M phosphoric acid which was diluted to one liter with glass distilled water. The flow rate was 100 ml/hr. Since 4-hydroxypropranolol fluoresces differently when compared to propranolol and α-naphthoxylactic acid, the fluorometer's emission wavelength was first set to 430 nm to measure 4-hydroxypropranolol. The excitation wavelength was fixed at 310 nm. After the elution of 4-hydroxypropranolol (about 6 minutes), the emission was then changed to 350 nm for the detection of propranolol and α-naphthoxylactic acid. Slit widths were set at 20 nm and the sensitivity range set at 0.1. The recorder output was set at 1 volt and a normal power gain was used. The spans

of the recorder were used to vary sensitivity.

By omitting the enzymatic hydrolysis procedure one can directly measure α-naphthoxylactic acid and of unconjugated propranolol. For these assays the fluorescent excitation wavelength was set at 230 nm and the emission at 340 nm.

RESULTS AND DISCUSSION

Figure IV-1 includes chromatograms of blank plasma and a typical patient plasma obtained by the direct injection assay. The total elution time per assay is 8 minutes. All the known metabolites of propranolol elute before propranolol and do not interfere with the assay. The limit of detection using 0.2 ml of plasma is 2 ng/ml. The precision and accuracy of the assay are shown in Table IV-1. Intra- and inter-assay coefficients of variation (C.V.) were 2.2% and 5.5%, respectively, over the concentration range of 10-300 ng/ml.

The direct injection method was compared to the extraction technique discussed below. The latter involved alkalinizing a plasma sample containing propranolol with sodium carbonate buffer and extracting it with ether. The propranolol was then extracted into phosphoric acid and injected onto the column. Comparison of the value obtained using the two techniques to assay three samples from angina patients differed from one another by only 6.4%, -3.7% and 1%, respectively, even though the analyses were done over a six-month period.

Drugs tested for interference by direct injection onto the column included hydralazine, hydrochlorothiazide, triamterene, furosemide, procainamide and quinidine. The only compound found to interfere was quinidine.

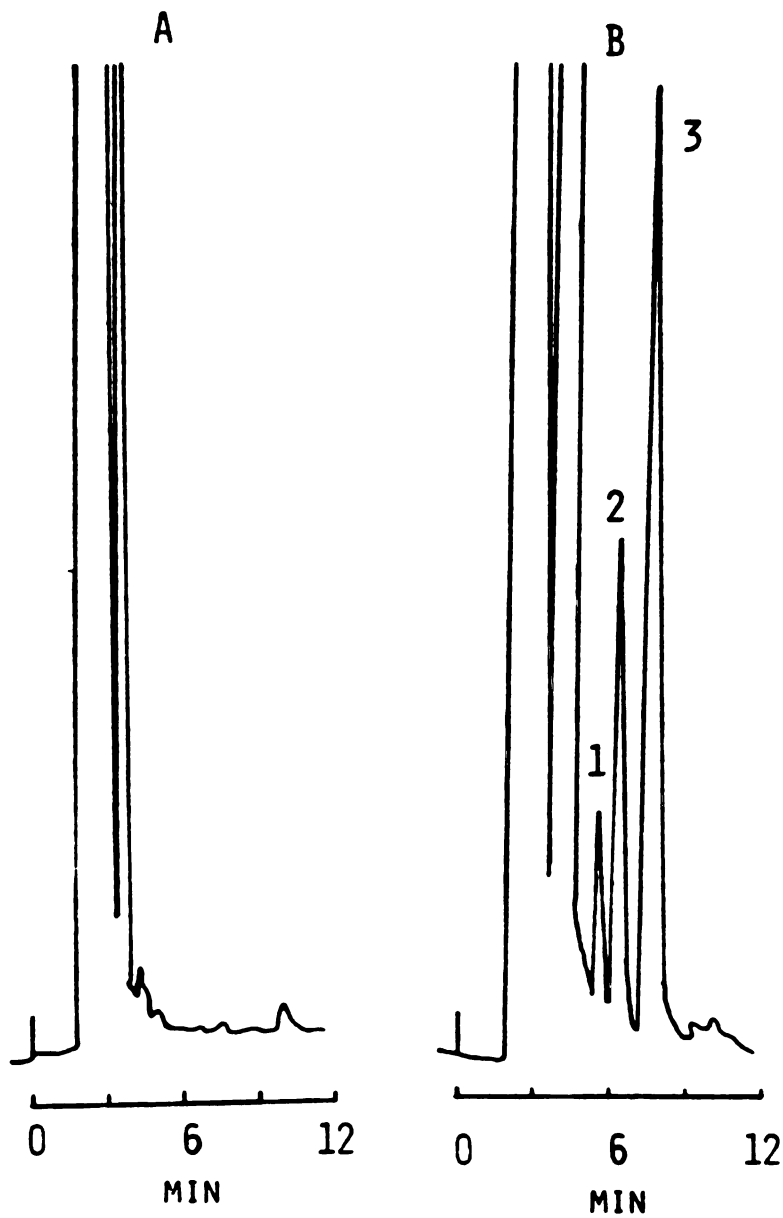


FIG IV-1. Assay of unchanged propranolol in plasma (see method 1). A. Drug free human plasma. B. 2 hr plasma sample obtained from an angina patient taking 20 mg of propranolol every 6 hr. 1= α-naphthoxyacetic acid; 2= propranolol 34 ng/ml; 3= N-ethylpropranolol (internal standard). Fluorometer settings: Excitation = 230 nm; Emission= 340 nm.

TABLE IV-1. INTRA- AND INTER-ASSAY VARIATION OF PROPRANOLOL IN PLASMA

Spiked Conc (ng/ml)	Intra-assay % C.V. (n=5)	Inter-Assay % C.V. (n=3, over 2 weeks)
10.05	1.5	3.9
30.14	n.d.*	3.8
75.35	0.9	3.9
150.69	2.2	n.d.*
301.38	1.1	5.5

*not determined

Approximately 75% of propranolol in plasma, virtually all propranolol in urine and 4-hydroxypropranolol in plasma and urine appear as their conjugates. Enzymatic hydrolysis is therefore required to liberate the compounds. Figure IV-2A shows how 4-hydroxypropranolol, α-naphthoxylactic acid, propranolol and the internal standard N-ethylpropranolol are separated from the minor metabolites of propranolol, namely N-desisopropylpropranolol, propranolol glycol, α-naphthol and α-naphthoxyacetic acid. Chromatograms of patient plasma and urine samples and blanks after enzymatic hydrolysis are shown in Figure IV-2B, C and Figure IV-3. It can be seen that there is no interference from normal biological constituents. The same patient plasma sample was analyzed before hydrolysis for α-naphthoxylactic acid and unconjugated propranolol. The chromatogram is shown in Figure IV-2E; again there is no interference from blank plasma (Figure IV-2D). The concentration of propranolol conjugates can thus be determined by the difference of the propranolol level measured before and after hydrolysis (Figure IV-2C and IV-2E). The total chromatographic analysis time is 15 minutes. This method allows determination of all three compounds even though they have widely different acid-base characteristics. A kinetic study examining the rate of enzymatic hydrolysis revealed that 90 minutes was sufficient for complete hydrolysis of the conjugates using 10,000 units of enzyme. Pritchard *et al* (1979) used sodium metabisulfite as an antioxidant during hydrolysis. In contrast, the present method utilizes ascorbic acid. A comparison of these two antioxidants as well as sodium bisulfite was made using plasma and urine spiked with the three compounds. Duplicate plasma standards containing 1 μg/ml of α-naphthoxylactic acid, 0.5 μg/ml of propranolol and 4-hydroxypropranolol, and urine standards containing 5 μg/ml of all three compounds were incubated with enzyme for 90 minutes using 20 mg of each of the

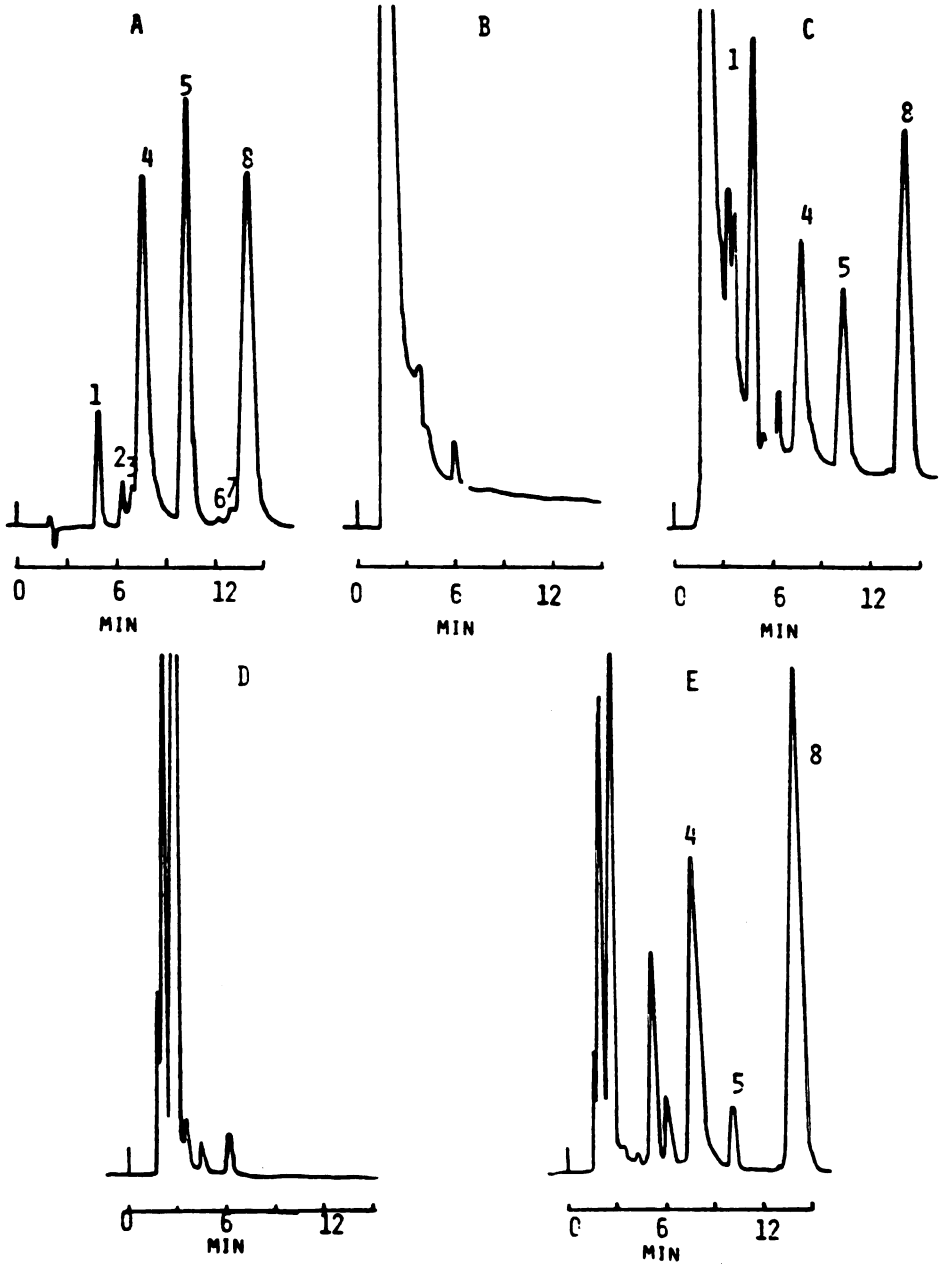


FIG IV-2. Assay of propranolol and metabolites in plasma (see method 2).
A. Standard mixture. 1= 4-hydroxypropranolol; 2= N-desisopropylpropranolol; 3= propranolol glycol; 4=α-naphthoxylactic acid; 5= propranolol; 6=α-naphthol; 7=α-naphthoxyacetic acid; 8= N-ethylpropranolol, the internal standard.
B. Hydrolyzed human plasma blank.
C. Hydrolyzed 1 hr plasma sample from an angina patient taking 80 mg of propranolol every 6 hr. 1= 490 ng/ml; 4= 770 ng/ml; 5= 490 ng/ml.
D. Unhydrolyzed human plasma blank (enzyme omitted).
E. Unhydrolyzed patient sample same as C. 4=770 ng/ml; 5= 128 ng/ml.
Fluorometer settings: B and C, excitation=310 nm; emission= 430 nm for the first 6 min, then changed to 350 nm. D and E, excitation = 230 nm and emission = 340 nm.

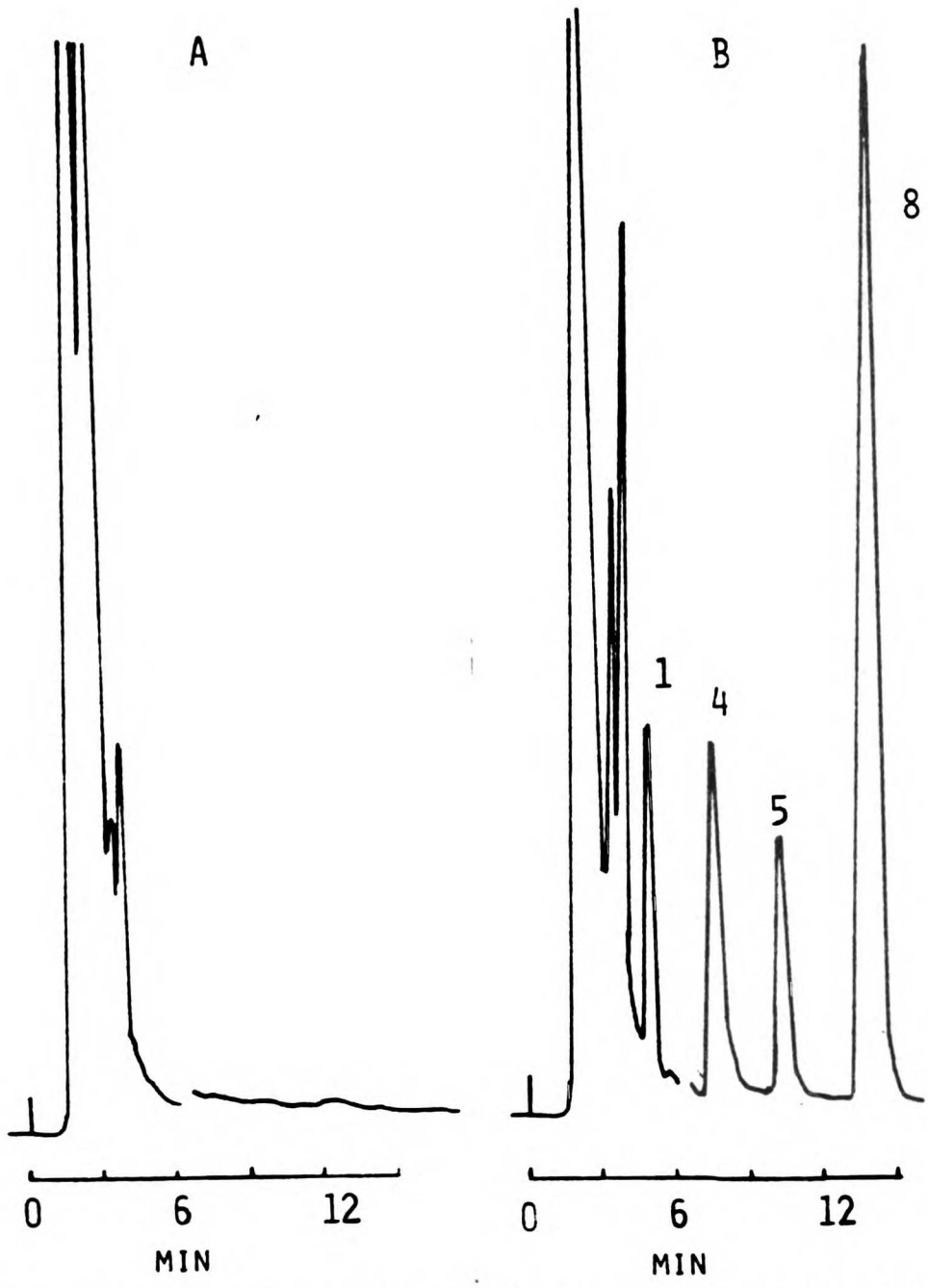


FIG IV-3. Assay of propranolol and metabolites in urine (see method 2)

A. Hydrolyzed human urine blank

B. Hydrolyzed 2-3 hr urine collection from a hypertensive patient taking 10 mg of propranolol every 6 hr. 1= 3.3 μg/ml; 4= 3.27 μg/ml; 5= 2.36μg/ml

Fluorometer settings: Excitation=310 nm; Emission=430 nm for the first 6 min, then changed to 350 nm

three antioxidants. The percent of each compound remaining was calculated by comparison to an unincubated standard. The results shown in Table IV-2 suggest that ascorbic acid is the best antioxidant for the protection of all three compounds.

Intra- and inter-day assay variation of propranolol and its metabolites determined by assaying patient plasma and urine samples over a period of two weeks for plasma and two months for urine are shown in Table IV-3. The limits of detection of the three compounds are about 20 ng/ml using 0.4 ml plasma and 100 ng/ml using 0.2 ml urine. Lower levels can be measured by use of larger volumes of samples.

We have improved the direct-injection method since it was published by using a more efficient column. The Altex Ultrasphere RP-8, 5 micron, 15 cm X 3.2 mm column gives a better separation of the metabolites and has a much higher plate count than the Lichrosorb RP-8 column. We have also applied the method to the analysis of bile specimens.

ALTEX ULTRASPHERE RP-8: The work up procedure for the samples remains the same as previously described. The elution systems are different and also a higher excitation wavelength is used in method 1. The new chromatographic conditions are as follows:

Unchanged Propranolol (Method 1): 39% acetonitrile, 17% methanol in 0.012 M phosphoric acid at a flow rate of 100 ml/hr. Excitation wavelength is 290 nm and emission wavelength held at 340 nm. See Figure IV-4.

Propranolol and Metabolites After Enzymatic Hydrolysis (Method 2): 28% acetonitrile, 14% methanol in 0.014 M phosphoric acid. 100 ml/hr. Excitation 310 nm; emission 430, 350 nm. See Figure IV-5.

ANALYSIS OF BILE: The procedure is essentially the same as for urine. However, no α-naphthoxylactic acid needs to be added to the standard curve because no α-naphthoxylactic acid is found in bile. Instead, one adds

TABLE IV-2. PERCENT OF PROPRANOLOL AND METABOLITES REMAINING IN PLASMA AND URINE FOLLOWING ENZYMATIC HYDROLYSIS* USING ASCORBIC ACID, SODIUM METABISULFITE, AND SODIUM BISULFITE AS ANTIOXIDANTS.

COMPOUND	ANTIOXIDANT+		
	Ascorbic Acid	Sodium Metabisulfite	Sodium Bisulfite
Propranolol			
Plasma (0.5 μg/ml)	99.4	95.9	94.6
Urine (5.0 μg/ml)	96.7	88.3	88.6
4-Hydroxypropranolol			
Plasma (0.5 μg/ml)	99.9	76.1	72.5
Urine (5.0 μg/ml)	99.2	39.2	46.3
α-Naphthoxylactic acid			
Plasma (1.0 μg/ml)	99.6	91.3	91.5
Urine (5.0 μg/ml)	91.9	71.6	66.3

*10,000 units of enzyme, 90 min, 37 C

+20 mg of each antioxidant used

TABLE IV-3. INTRA- and INTER-ASSAY VARIATION OF PROPRANOLOL AND ITS METABOLITES IN PATIENT PLASMA AND URINE SAMPLES* FOLLOWING ENZYMATIC HYDROLYSIS+.

	Coefficient of Variation (%)		
	Propranolol	4-Hydroxyprop-ranolol	α-Naphthoxy-lactic acid
PLASMA			
Intra-day (n=5)	1.6	2.2	2.3
Inter-day (n=3)	1.3	7.1	1.9
URINE			
Intra-day (n=5)	1.4	1.4	0.9
Inter-day (n=3)	1.3	3.9	4.2

*Determinations were made over a 2 week, and 2 month period for plasma and urine samples, respectively.

+10,000 units of enzymes, 37°C, 90 min

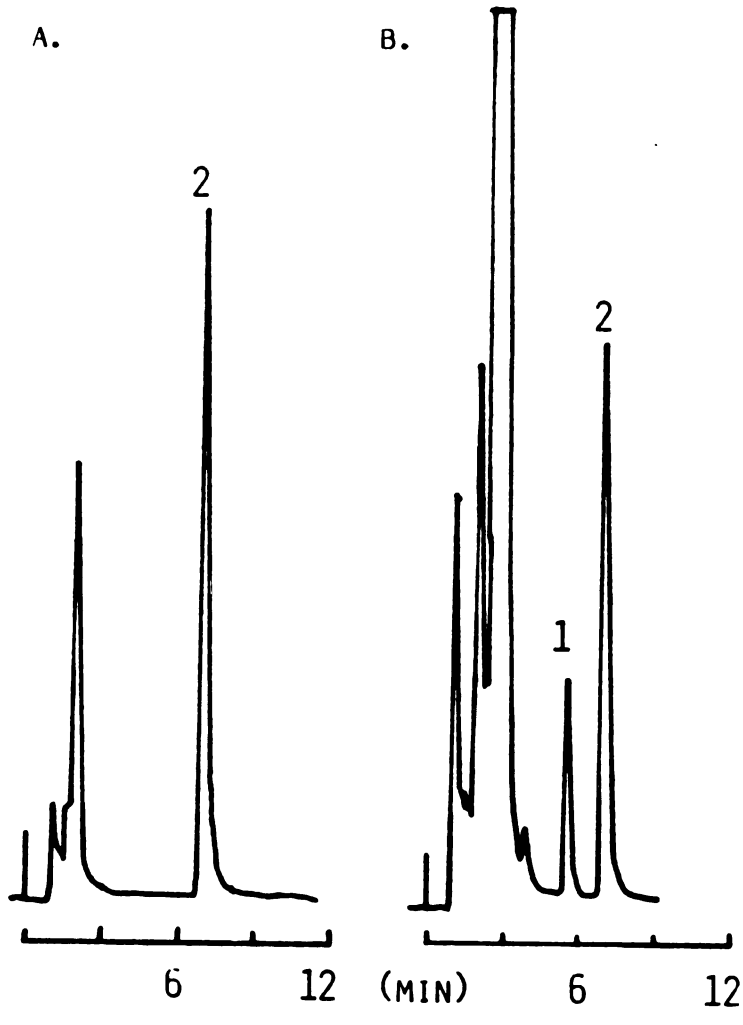


FIG IV-4. Assay of unchanged propranolol in plasma by direct injection using a more efficient column.

A. Drug free dog plasma.

B. Steady state plasma sample from a dog given 1.4 mg/min of propranolol via a hind-limb vein.

1= propranolol 479 ng/ml; 2= N-ethylpropranolol (internal standard)

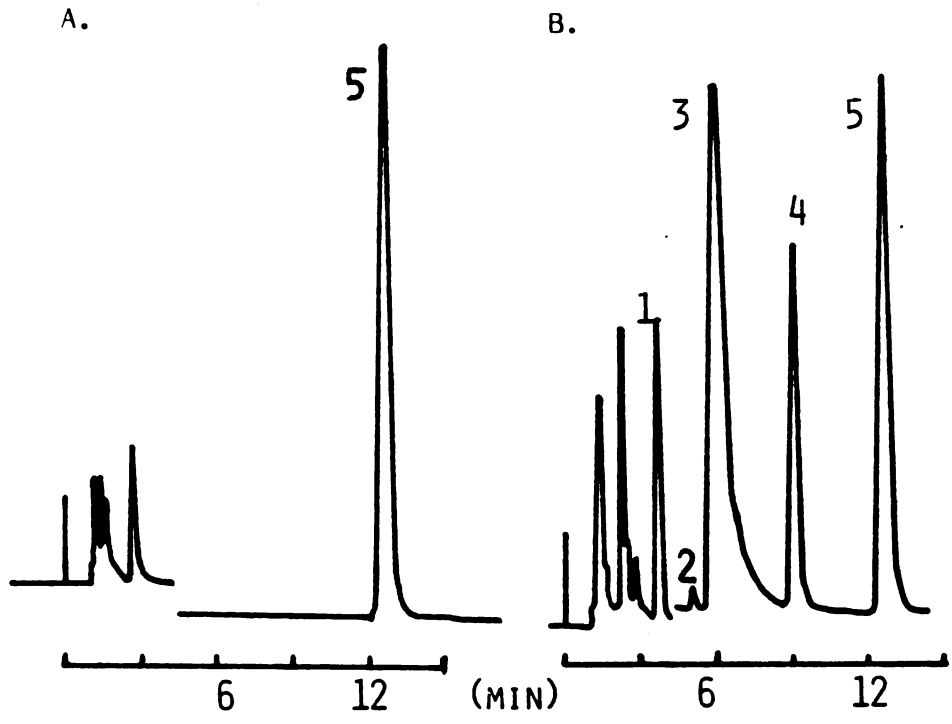


FIG IV-5. Assay of propranolol and metabolites in urine by direct injection using a more efficient column.

A. Hydrolyzed dog urine blank

B. Hydrolyzed steady state urine collection from a dog given 1.4 mg/min of propranolol via a hind-limb vein.

1= 4-hydroxypropranolol 395 μg/ml; 2= propranolol glycol 12 μg/ml; 3= α-naphthoxylactic acid 660 μg/ml; 4= propranolol 232 μg/ml; 5= N-ethylpropranolol (internal standard).

propranolol glycol because it is found to be a major metabolite in bile. Only the conjugated form is found in bile. Therefore an enzyme hydrolysis step is necessary to liberate the free compound. The eluent system is 31% acetonitrile, 5% methanol in 0.0056 M potassium hydroxide and 0.015 M phosphoric acid. The flow rate is 100 ml/hr. The excitation wavelenth is 310 nm and the emission wavelengths are 430 nm and 350 nm. See Figure IV-6.

2. EXTRACTION METHOD

Even though the sensitivity for measuring propranolol alone is quite adequate (2 ng/ml using 0.2 ml of plasma) with the direct injection method, it is not possible to measure 4-hydroxypropranolol below 20 ng/ml due to interference from normal plasma constituents. In addition, the method is not adaptable to automation because the fluorescence emission maxima for 4-hydroxypropranolol and propranolol are 430 nm and 350 nm, respectively. The excitation and emission wavelengths of the Perkin-Elmer 650-10 LC detector are monochromator controlled; therefore, one must change the emission wavelength setting during the assay to measure both compounds. However, in the Schoeffel FS 970 fluorescence detector, one can insert a filter in the emission pathway which is transparent above a certain wavelength, thereby allowing both compounds to be detected without necessitating a change of wavelength during each assay. However, a problem arises using the direct injection method with the Schoeffel fluorometer since normal plasma constituents now interfere with the detection of 4-hydroxypropranolol. These endogenous compounds can be removed using a double extraction technique after alkalinizing the sample. Furthermore, the sensitivity of this method can be increased by extracting a larger volume of sample.

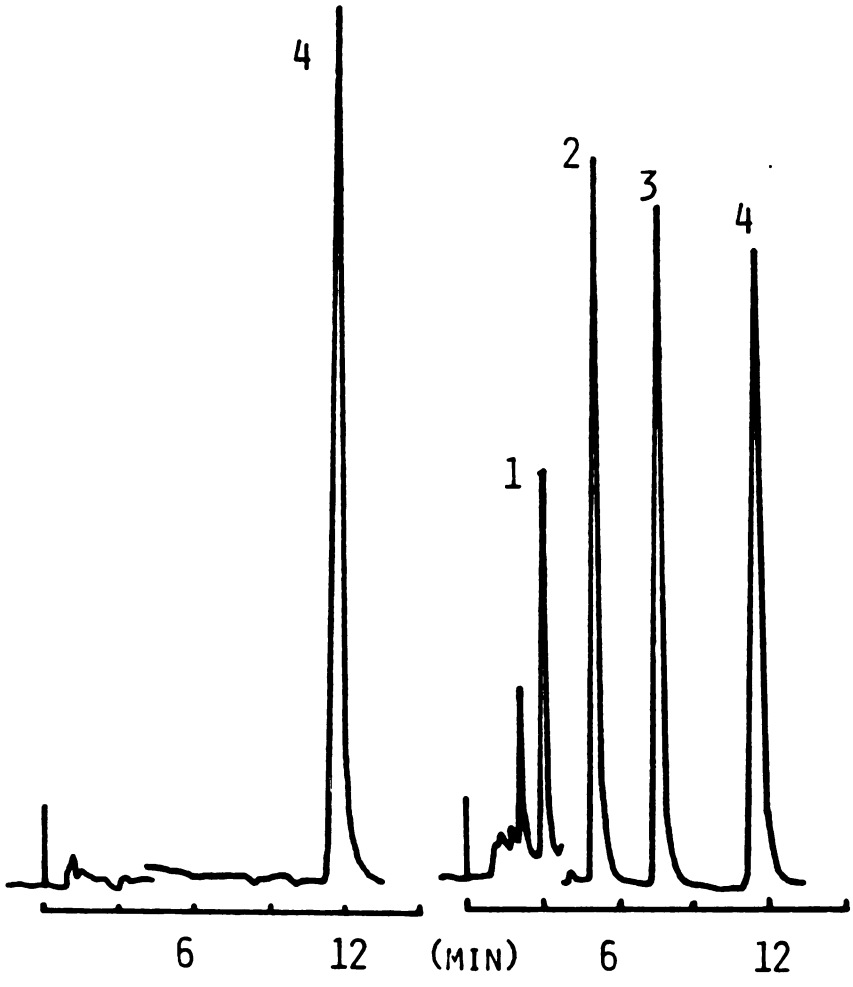


FIG IV-6. Assay of propranolol and metabolites in bile by direct injection using a more efficient column.

A. Hydrolyzed dog bile blank.

B. Hydrolyzed steady state bile collection from a dog given 1.4 mg/min of propranolol via a hind-limb vein.

1= 4-hydroxypropranolol 520 μg/ml; 2= propranolol glycol 324 μg/ml; 3= propranolol 422 μg/ml; 4=N-ethylpropranolol.

Other HPLC methods that measure 4-hydroxypropranolol and propranolol together in plasma have appeared in the literature but these do not include N-desisopropylpropranolol (Mason *et al*, 1977; Taburet *et al*, 1979; Schneck *et al*, 1979; Nation *et al*, 1978). Two of these methods require separate injections and detect two compounds at different emission wavelengths (Mason *et al*, 1977; Taburet *et al*, 1979). The method of Schneck *et al* (1979) involves a time consuming evaporation step after a single basic extraction step. The method of Nation *et al* (1978) is most similar to the method reported below, but once again was not adapted to the assay of metabolites in urine nor was N-desisopropylpropranolol determined.

EXPERIMENTAL

Standards and reagents: Propranolol HCl, 4-hydroxypropranolol HCl, N-desisopropylpropranolol, and the two internal standards pronethalol HCl and 1-(methylamino)-3-(1-naphthyloxy)-2-propanol (ICI 45837) were all generously supplied by Imperial Chemical Industries, Ltd., Macclesfield, Great Britain.

Acetonitrile and methanol (both UV grade) were from Burdick and Jackson Labs, Muskegon, MI. Absolute diethyl ether (analytical reagent grade), was from Mallinckrodt Inc., St. Louis, Missouri. All other reagents were of analytical grade.

Apparatus: An Altex 100A high-performance liquid chromatograph equipped with a Schoeffel FS 970 LC fluorometer and a Waters WISP 710A automatic injector were used. The fluorescence output was recorded on a Spectra-Physics Model 4100 integrator. The fluorometer was set at an excitation wavelength of 216 nm and the fluorescence emission monitored

by a 340 nm cut-off filter (Hoya Glass Company, available through Schoeffel).

HPLC columns and chromatographic conditions: Three different columns were utilized in various phases of the study. The optimal conditions for the separation of the three compounds and internal standard are as follows:

1. Waters μBondapak phenyl column (30 cm X 3.9 mm I.D.: 10 μm particle size). The elution solvent included 27% acetonitrile in 0.0019 M phosphoric acid at 90 ml/hr, ambient temperature, pronethalol as internal standard.

2. Altex Lichrosorb RP-8 column (25 cm X 4.6 mm I.D.; 10 μm particle size). The chromatographic conditions are: 1) 34% acetonitrile in 0.0026 M phosphoric acid, 110 ml/hr, and 2) 38% acetonitrile, 12% methanol in 0.00348 M phosphoric acid, 70 ml/hr for separating the compounds before and after enzyme hydrolysis, respectively. In both cases, the separation is done at ambient temperature and uses pronethalol as internal standard.

3. Altex Ultrasphere ODS (15 cm X 4.6 mm I.D.: 5 μm particle size). 28% acetonitrile, 16% methanol in 0.021 M phosphoric acid, 150 ml/hr, 30°C water-jacketed. ICI 45837 as internal standard.

Method 1. Measurement of free propranolol, 4-hydroxypropranolol and N-desisopropylpropranolol in plasma: Daily standard curves were prepared as follows. A 0.4 ml quantity of 10 μg/ml propranolol in methanol and 0.3 ml of 4 μg/ml N-desisopropylpropranolol also in methanol were added to an empty 16 mm X 100 mm disposable culture tube and evaporated to dryness under nitrogen. A 3.6 ml quantity of drug-free human

plasma was then added and the tube vortexed briefly. A 0.4 ml aliquot of 4-hydroxypropranolol (10 µg/ml in 0.01 M phosphoric acid including 5 mg/ml ascorbic acid to minimize oxidation) was added to the plasma to yield the highest plasma standard (1000 ng/ml propranolol, 1000 ng/ml 4-hydroxypropranolol and 300 ng/ml N-desisopropylpropranolol). The standard was then serially diluted 1000-fold to lower concentrations with more drug-free plasma.

Plasma samples were processed as follows. Depending on the columns used, either 0.05 ml of a methanolic internal standard solution (2 µg/ml ICI 45837) or 0.1 ml of an aqueous 0.3 µg/ml pronethalol HCl solution was added to a 16 X 150 mm disposable screw-cap culture tube (Kimble 73750-16150). After evaporating the methanolic standard, 1.0 ml of plasma along with 0.05 ml of ascorbic acid (100 mg/ml in water) were added and the solution vortexed. The plasma was then alkalinized with 0.5 ml of carbonate buffer (0.25 N sodium sydroxide in 0.25 M sodium carbonate) yielding a final pH of 10.0 and the mixture was extracted with 10 ml of absolute diethyl ether by vortexing for 2 minutes using a Thermolyne Maxi Mix (M-16715, Sybron Corporation, Dubuque, Iowa). After centrifuging at 500 X g for 5 minutes, most of the organic layer was transferred to a 15 ml conical glass centrifuge tube (Kimble 45161-15) to which had been added 0.25 ml of a 0.01 M phosphoric acid solution stabilized with 5 mg/ml of ascorbic acid. The sample was vortexed for 1 minute and centrifuged at 500 X g for 5 minutes. After discarding the upper organic layer, an aliquot of the acid layer was injected onto the HPLC.

Method 2. Measurement of propranolol, 4-hydroxypropranolol and N-desisopropylpropranolol in plasma and urine after enzymatic hydrolysis:

The plasma standards were prepared as in method 1. Much higher urine standards were needed. To 1 ml of aqueous propranolol (80 μg/ml) was added 1 ml of aqueous 4-hydroxypropranolol (40 μg/ml in 0.01 M phosphoric acid with 5 mg/ml of ascorbic acid). The mixture was serially diluted up to 100-fold with phosphoric acid-ascorbic acid solution. A 0.05 ml aliquot of each standard and 0.05 ml of drug-free urine were added to each culture tube.

Either 0.1 ml of 0.15 μg/ml pronethalol HCl or 0.2 ml of methanolic ICI 45837 (4 μg/ml) was added to an empty disposable screw-cap culture tube and the methanol evaporated. After adding 0.1 ml of a 200 mg/ml ascorbic acid solution and 0.05 ml of urine, the sample was incubated with 0.2 ml of β-glucuronidase/aryl sulfatase (10,000 units/0.2 ml, Sigma G 0751) in 0.09 M sodium acetate buffer (pH 5.0) for 90 minutes at 37°C. For plasma samples, a 0.2 ml aliquot of plasma and the same amount of internal standard was used as in method 1.

After incubation, 0.75 ml of carbonate buffer (0.25 N sodium hydroxide in 0.25 M sodium carbonate) was used to alkalinize the mixture to pH 10.0 and the sample was then extracted with ether as described in method 1 except that 1 ml of the phosphoric acid/ascorbic acid solution was used to re-extract the compounds from ether.

RESULTS AND DISCUSSION

Figures IV-7 through 9 include chromatograms of blank and typical patient plasma, respectively, obtained by method 1 in the various columns tried. Excellent separation of the four components is achieved in all cases. The shortest elution time for the whole assay is 8 minutes using

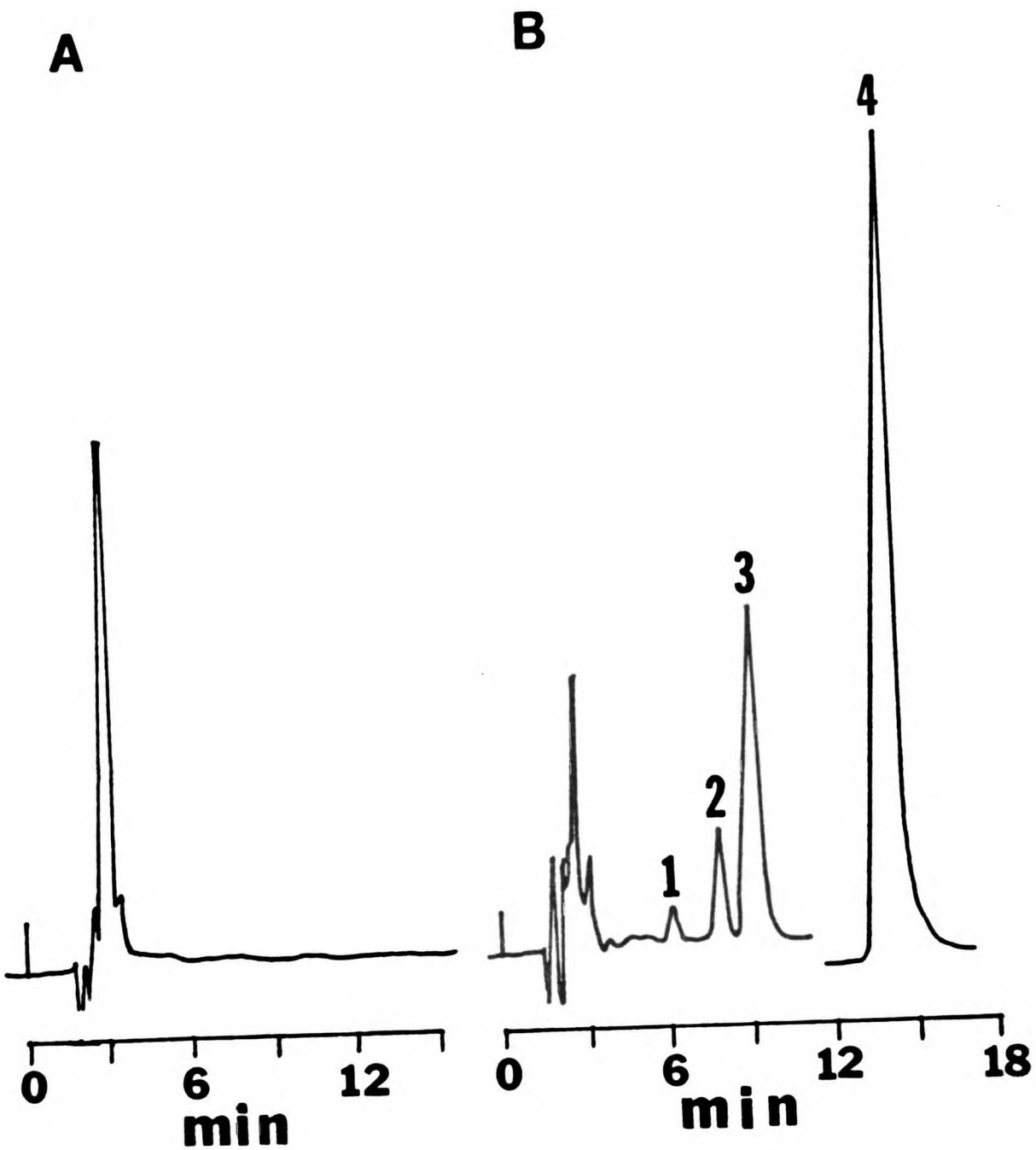


FIG IV-7. Assay of free propranolol and metabolites in plasma by extraction using a Waters phenyl column (see method 1).

A. Drug-free human plasma.

B. Plasma sample obtained from a hypertensive patient. Peaks: 1= 4-hydroxypropranolol, 1.7 ng/ml; 2= N-desisopropylpropranolol, 1.9 ng/ml; 3= pronethalol (internal standard); 4= propranolol, 182.4 ng/ml.

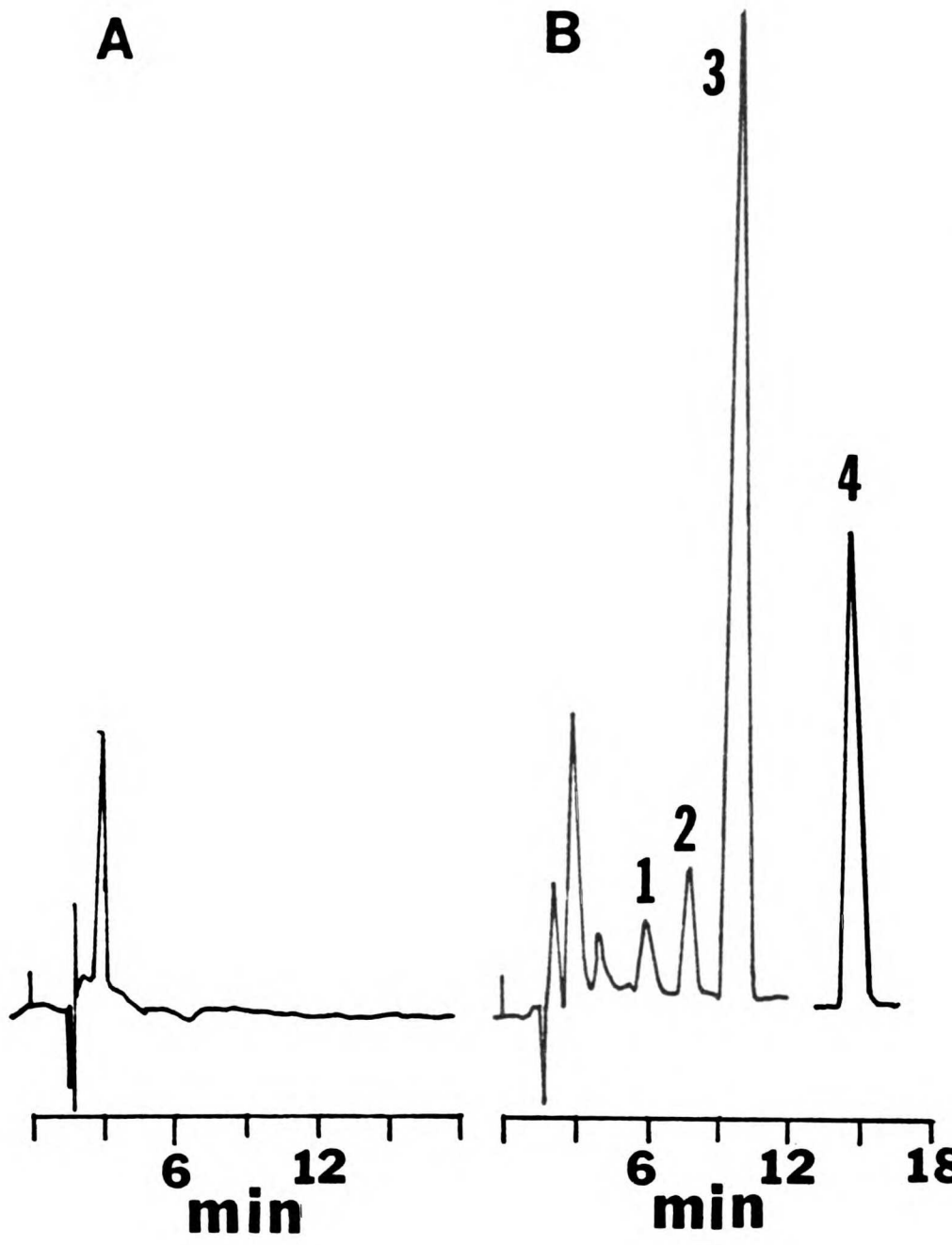


FIG IV-8. Assay of free propranolol and metabolites in plasma by extraction using an Altex Lichrosorb RP-8 column (see method 1).

A. Drug-free human plasma.

B. Plasma sample obtained from an angina patient. Peaks: 1= 4-hydroxypropranolol, 1.79 ng/ml; 2= N-desisopropylpropranolol, 0.85 ng/ml; 3= pronethalol (internal standard); 4= propranolol, 132.7 ng/ml.

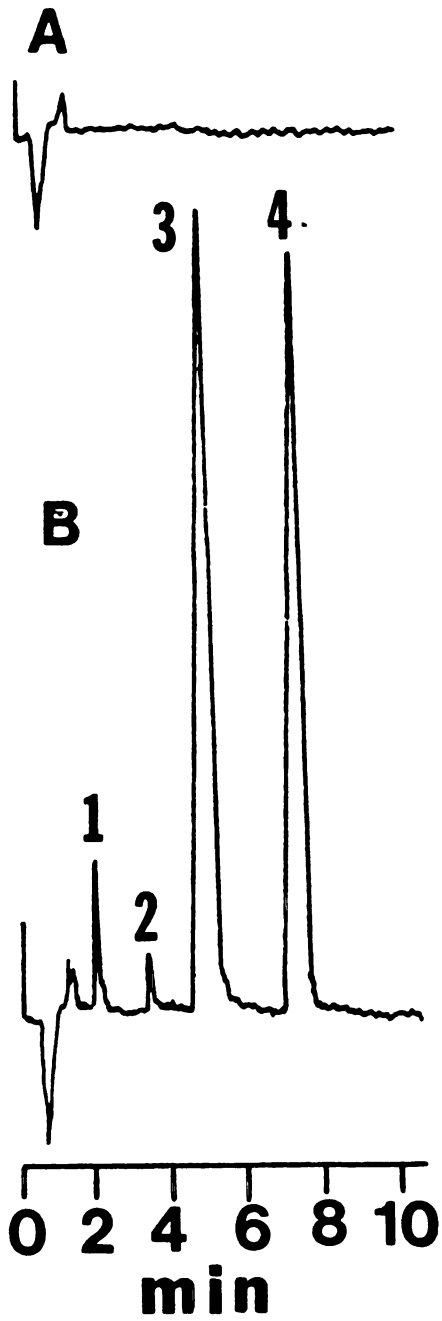


FIG IV-9. Assay of free propranolol and metabolites in plasma by extraction using an Altex Ultrasphere ODS column (see method 1).

A. Drug-free human plasma.

B. Plasma sample obtained from a normal volunteer on propranolol. Peaks: 1= 4-hydroxypropranolol, 8.7 ng/ml; 2= N-desisopropyl-propranolol, 1.8 ng/ml; 3= I.C.I. 45837 (internal standard); 4= propranolol, 43.5 ng/ml.

the Altex Ultrasphere ODS column. The limits of detection using 1 ml of plasma are 0.2 ng/ml, 1 ng/ml and 0.2 ng/ml for propranolol, 4-hydroxypropranolol and N-desisopropylpropranolol, respectively.

Figures IV-10 through 13 depict chromatograms of hydrolyzed plasma and urine samples using method 2. It can be seen that enzymatic hydrolysis does not introduce any significant interference.

The double extraction technique is superior to a single extraction step not only in saving the time which would be required in evaporation but also in being more specific for measuring basic compounds. Various extraction solvents have been tried, viz. benzene, methylene chloride, hexane/isoamyl alcohol (98.4:1.6 v/v), hexane/n-butanol (80:20 v/v), ether/methylene chloride combination and ether alone. None of these offer any advantage over the use of ether alone.

The extraction efficiencies of the three compounds from plasma are as follows: propranolol 68 to 82% (4 to 500 ng/ml), 4-hydroxypropranolol 66 to 81% (2 to 40 ng/ml) and N-desisopropylpropranolol 59 to 73% (1 to 20 ng/ml). The precision and accuracy for both methods for plasma and urine are summarized in Tables IV-4 through 6. Excellent reproducibility and accuracy of the assay method can be seen from the data summarized in the above tables.

One of the metabolites, 4-hydroxypropranolol, is unstable in aqueous solution but is stable in plasma for at least 2 months if kept frozen. It is also stable in acidified aqueous solution for up to 36 hours, if protected with ascorbic acid and stored at $4^{o}C$. This has been validated by testing the final acidic extract immediately after extraction and 36 hours later; no degradation of 4-hydroxypropranolol was found.

As noted earlier, the direct injection technique is not adequate to measure very low concentrations of free 4-hydroxypropranolol and N-des-

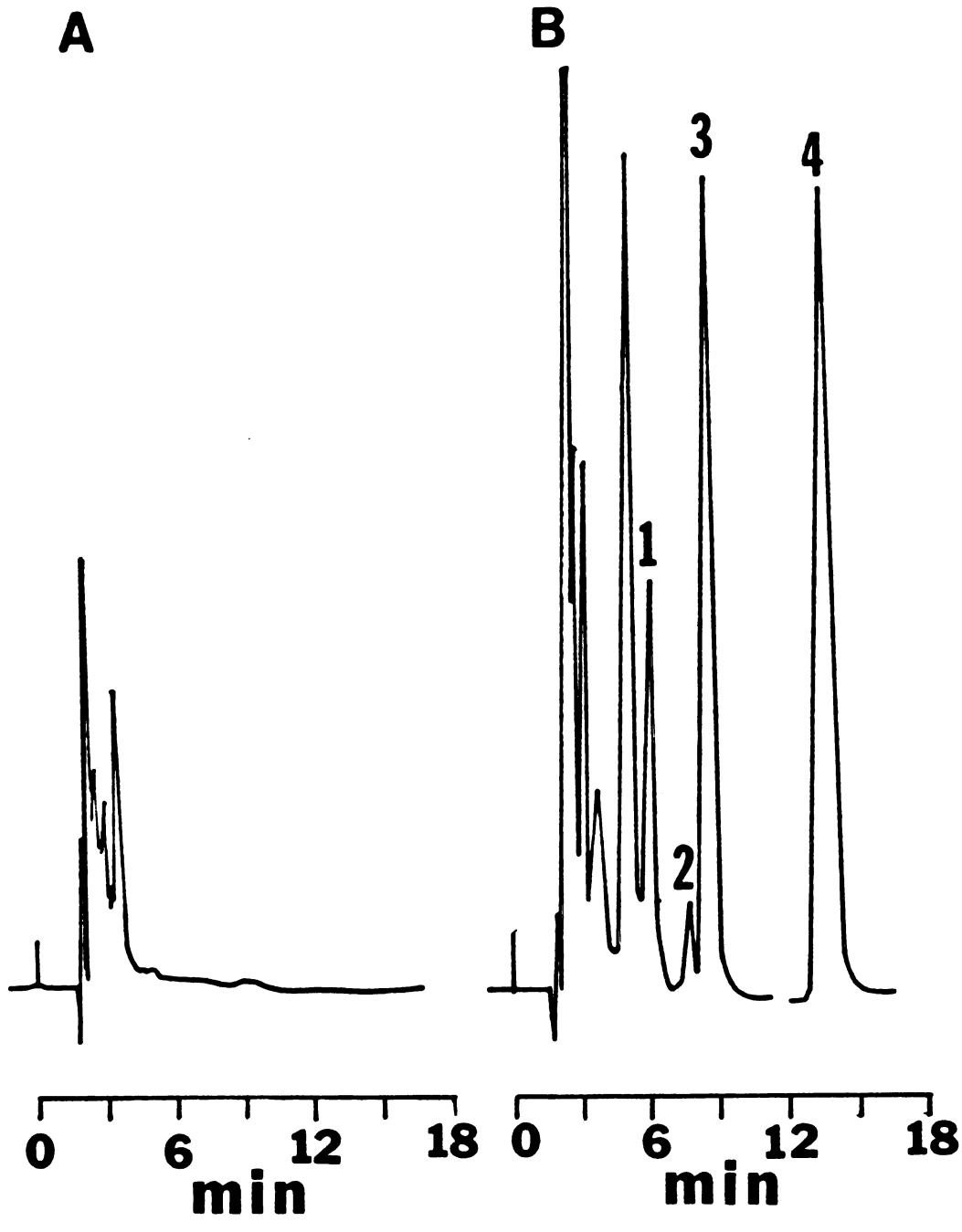


FIG IV-10. Assay of propranolol and metabolites in urine after enzymatic hydrolysis by extraction using a Waters Phenyl column (see method 2).

A. Drug-free human urine.

B. Urine sample obtained from a hypertensive patient on propranolol. Peaks: 1= 4-hydroxypropranolol, 194 ng/nl; 2= N-desisopropyl-propranolol, 5 ng/ml; 3= pronethalol; 4= propranolol, 633 ng/ml.

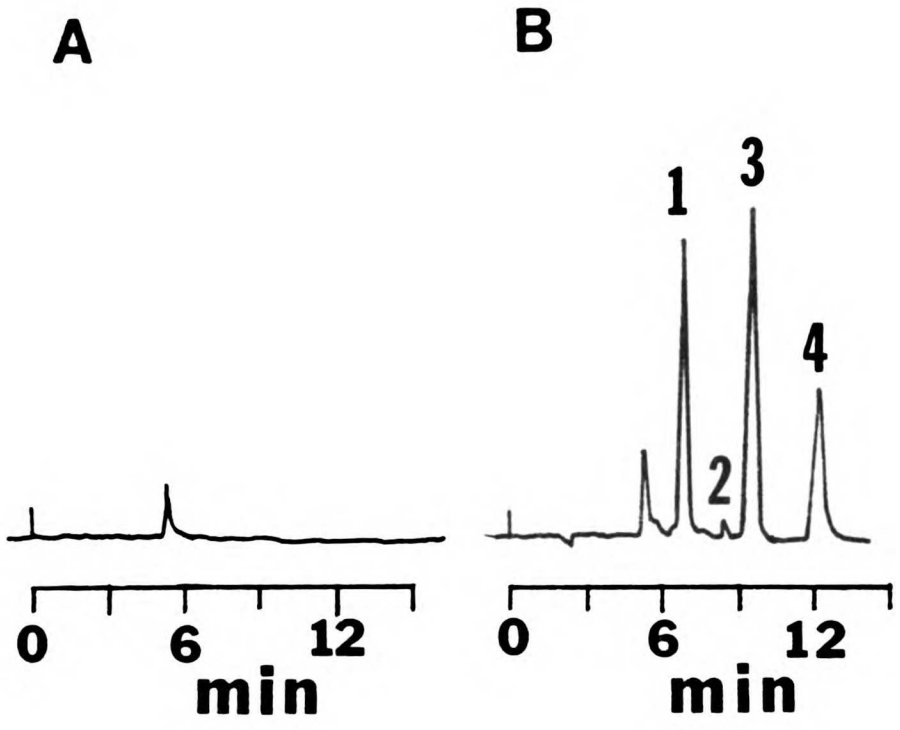


FIG IV-11. Assay of propranolol and metabolites in urine after enzymatic hydrolysis and extraction using an Altex Lichrosorb RP-8 column (see method 2).

A. Drug-free human urine.

B. Urine sample from an angina patient on propranolol. Peaks: 1= 4-hydroxypropranolol, 13.9 μg/ml; 2= N-desisopropylpropranolol, 0.4 μg/ml; 3= pronethalol (internal standard); 4= propranolol, 3.7 μg/ml.

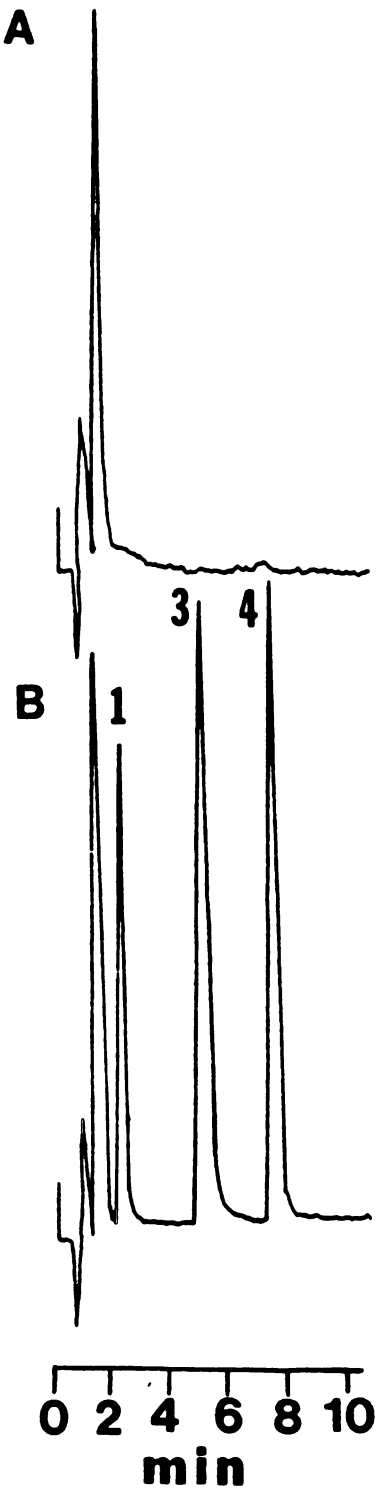


FIG IV-12. Assay of propranolol and metabolites in plasma after enzymatic hydrolysis and extraction using an Altex Ultrasphere ODS column (see method 2).

A. Drug-free human plasma.

B. Plasma sample from a normal volunteer on propranolol. Peaks: 1= 4-hydroxypropranolol, 415 ng/ml; 3= I.C.I.45837 (internal standard); 4= propranolol, 519 ng/ml.

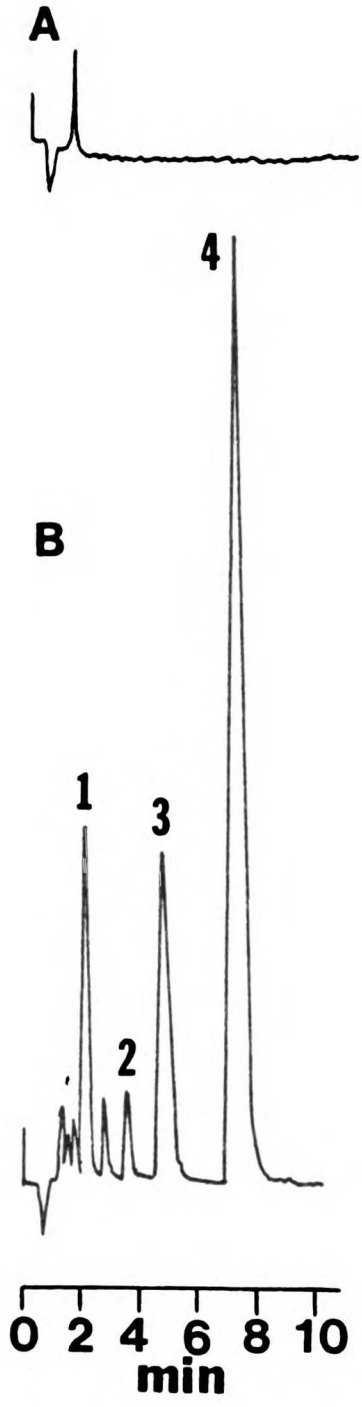


FIG IV-13. Assay of propranolol and metabolites in urine after enzymatic hydrolysis and extraction using an Altex Ultrasphere ODS column (see method 2).

A. Drug-free human urine.

B. Urine sample from a normal volunteer on propranolol. Peaks: 1= 4-hydroxypropranolol, 8.7 μg/ml; 2= N-desisopropylpropranolol, 1.1 μg/ml; 3= I.C.I. 45837 (internal standard); 4= propranolol, 21.2 μg/ml.

TABLE IV-4. INTRA-ASSAY VARIATION OF PROPRANOLOL, 4-HYDROXYPROPRANOLOL AND N-DESISOPROPYLPROPRANOLOL IN PLASMA. METHOD 1.

Spiked Concentration (ng/ml)	% C.V. (n = 3)
Propranolol	
1.0	8.0
3.9	8.3
15.5	8.7
62.0	8.3
248.0	1.9
4-Hydroxypropranolol	
1.0	10.0
3.9	10.1
15.6	2.4
62.5	8.8
250.0	6.6
N-Desisopropylpropranolol	
0.6	8.8
2.3	10.0
9.4	8.3
37.5	6.0
150.0	5.9

TABLE IV-5. INTER-ASSAY VARIATION OF PROPRANOLOL, 4-HYDROXY-PROPRANOLOL, AND N-DESISOPROPYLPROPRANOLOL IN PLASMA. METHOD 1[a)].

	Propranolol		4-Hydroxy-propranolol		N-Desisopropyl-propranolol	
Spiked Conc (ng/ml)	56.8	227.8	49.5	197.8	20.5	163.6
Average Conc Found(ng/ml)	59.9	236.6	50.2	202.9	21.4	173.7
% C.V.	6.5	4.3	11.2	5.1	15.0	5.9
Bias %	+5.5	+3.9	+1.4	+2.6	+4.4	+6.2

a) Determinations were made over a 7 week period, n= 13.

TABLE IV-6. INTRA- AND INTER-ASSAY VARIATION OF PROPRANOLOL AND 4-HYDROXYPROPRANOLOL IN URINE FOLLOWING ENZYMATIC HYDROLYSIS. METHOD 2[a].

Spiked Conc (μg/ml)	Intra-Assay Variation[b] % C.V.	Inter-Assay Variation[c] Average Conc Found (μg/ml)	% C.V.
Propranolol			
0.30	8.3	-[d]	-
1.18	4.6	-	-
2.37	3.5	2.46	7.7
9.46	3.7	-	-
18.92	4.5	-	-
37.84	6.6	38.1	3.4
4-Hydroxypropranolol			
0.73	7.5	-	-
1.45	6.7	-	-
1.69	-	1.76	8.5
5.80	2.6	-	-
11.60	6.7	-	-
23.20	6.1	-	-
26.96	-	27.67	6.9

a) Each sample was incubated with 10,000 units of enzyme for 90 min at 37°C.

b) n = 3.

c) Inter-day variation study was done over a 4-week period. n= 4.

d) Not determined.

isopropylpropranolol. In addition, if one has many hydrolyzed samples to assay (plasma or urine) the wavelength change becomes a hindrance to automation because no fluorometer is yet available which can automatically switch the emission wavelength during a chromatographic run. Yet in spite of the fact that the present extraction technique requires more time for sample preparation, the analyst ends up saving time because of automation in HPLC analysis. In our laboratory two technicians can process approximately 80 samples pre- and post-enzymatic hydrolysis per day.

Of the three columns that were tested over a three-year period, the Altex Ultrasphere ODS is the most reproducible. Both the Waters phenyl and the Altex Lichrosorb RP-8 require elution solvent adjustment of the phosphoric acid strength to yield acceptable separation of the compounds when a new column was put in use. However, these two columns do have the advantage of being suitable to measure a third major metabolite of propranolol, viz. α-naphthoxylactic acid. When chromatographed on the Altex Ultrasphere ODS an unacceptable degree of tailing which did not allow quantitation of α-naphthoxylactic acid was observed.

Analysis of Propranolol Glycol in Plasma

To measure the low levels of propranolol glycol present in plasma, a different extraction approach is needed. This assay will be important to researchers who would like to correlate the anticonvulsant activity of this metabolite with concentration, similar to the studies of Saelens *et al* (1977). See also the discussion in Chapter II, p 17.

Experimental: A 0.5 ml aliquot of 0.44 M phosphoric acid was added to 0.2 ml of plasma. The sample was vortexed with 10 ml of ether for 1 minute and centrifuged for 5 minutes at 500 X *g*. A 9.5 ml quantity of the ether layer was transferred to a graduated conical tube and evapor-

ated to dryness under a gentle stream of nitrogen. The residue was reconstituted in a 0.2 ml of a 25% acetonitrile in water mixture. After vortexing for 30 seconds, a 100 µl aliquot was injected onto the HPLC using a 100 µl sample loop.

A Waters µ-Bondapak alkyl phenyl column was used for the separation of propranolol glycol from plasma constituents. The elution was carried out using a mixture of 25% acetonitrile in water at a flow rate of 2 ml/min. The HPLC effluent was monitored by a Schoeffel FS 970 LC fluorometer operating at an excitation wavelength of 216 nm and the emission was monitored using a narrow band pass filter with a transmission maximum at 358 nm (Corning 7-60 filter, Corning, N.Y.).

Results and Discussion: Chromatograms illustrating the applicability of the assay to blank and patient plasmas are shown in Figure IV-14. Normal plasma constituents did not interfere with the analysis. The choice of an acidic extraction step eliminates the co-extraction of basic metabolites of propranolol. By using a solvent system near neutral pH, the acidic metabolites (α-naphthoxylactic acid and α-naphthoxyacetic acid) elute early in the chromatographic run. No suitable internal standard has been found as yet for this procedure. Therefore it is important to transfer the extraction solvent accurately. Due to the high fluorescence of propranolol glycol, very low concentrations of this metabolite can be monitored (down to subnanogram levels).

3. OVERALL RECOMMENDATION

Depending on what apparatuses are available, the analyst can tailor the assay procedure to his needs.

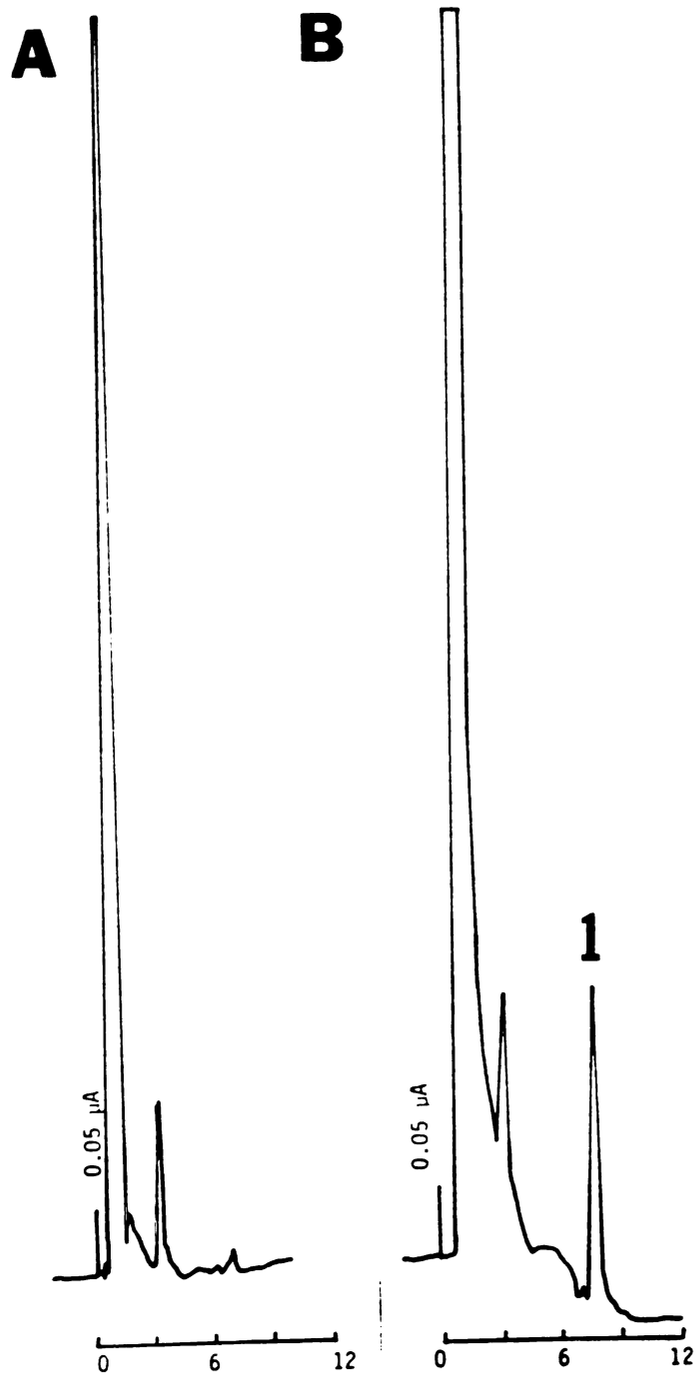


FIG IV-14. Assay of propranolol glycol in plasma by extraction using a Waters Phenyl column.

A. Blank human plasma.

B. Plasma sample from a patient on propranolol.

Peak: 1= propranolol glycol, 12.6 ng/ml.

1. Measurement of unchanged propranolol alone in blood or plasma: If the Perkin-Elmer detector is available the direct injection method is the simplest and the fastest. One can measure down to 2 ng/ml using 0.2 ml of plasma and the method can be automated. However, if subnanogram levels are anticipated, then the double extraction method is preferred. More plasma can then be used to increase assay sensitivity.

2. Measurement of 4-hydroxypropranolol, propranolol and α-naphthoxy-lactic acid in plasma and urine: If a limited number of samples are to be quantitated, the direct-injection assay is the method of choice. All three compounds can be measured in one run after hydrolysis. However, if very low concentrations are anticipated and there are many samples to be analyzed, then one can process the sample using both the direct-injection and the extraction methods. Firstly, one can measure free α-naphthoxy-lactic acid before enzyme treatment by the direct injection method. Automation is possible. Secondly, the sample is hydrolyzed and the basic metabolites measured after double solvent extraction. Automation is again possible.

3. Measurement of metabolites in bile: The direct injection method is preferred.

4. OTHER METHODS

If an HPLC system is not available then the analyst has to use other techniques to measure propranolol and metabolites. Previous published methods include:

1. Spectrofluorometry: Rao *et al* (1978) measured propranolol and 4-hydroxypropranolol using a spectrofluorometer under two different sets of excitation and emission conditions.

2. Thin-layer fluorometry: Garceau *et al* (1978a) measured all three major metabolites using this technique with high sensitivity (propranolol, 4-hydroxypropranolol and α-naphthoxylactic acid).

3. Gas-chromatography: Propranolol and N-desisopropylpropranolol can be measured to 0.1 ng/ml using electron-capture gas chromatography after basic extraction of plasma and subsequent back extraction (Walle, 1974). Similarly propranolol glycol and N-desisopropylpropranolol have been quantitated in brain tissue using a single step basic extraction (Saelens *et al*, 1976). α-Naphthoxylactic acid, following acidic extraction, is quantitated with electron-capture detection at levels as low as 2 ng/ml in plasma (Easterling *et al*, 1979).

4. Gas-chromatography-Mass-spectrometry: Walle *et al* (1975) measured both propranolol and 4-hydroxypropranolol after a single basic extraction with G.C.M.S. The minimum detectable concentration of propranolol is 1 ng/ml and of 4-hydroxypropranolol 5 ng/ml using 1 ml of plasma. Vu *et al* (1978) describe a method that can measure most of the metabolites in a single G.C.M.S. run. But these workers only describe the assay for urine.

CHAPTER V: THE LACK OF INFLUENCE OF THE USE OF INDWELLING CANNULAS (HEPARIN LOCK) FOR SAMPLING ON THE PLASMA PROTEIN BINDING AND DISPOSITION OF PROPRANOLOL

Heparin is an anticoagulant given parenterally for direct therapeutic purposes. It is also used to maintain function of indwelling catheters (heparin lock). Recently, reports have indicated that heparin injection or infusion decreases the plasma protein binding of propranolol (Wood *et al*, 1979), digoxin and digitoxin (Storstein and Janssen, 1976), triiodothyronine (Thomson *et al*, 1977), bilirubin and salicylate (Wiegand and Levy, 1979). However, warfarin plasma protein binding was found to increase after heparin administration (Nilsen *et al*, 1977 and Routledge *et al*, 1979).

Since the unbound or free concentration of drug at the receptor is believed to be responsible for drug action and to control drug clearance, compounds affecting plasma protein binding may cause significant perturbations in drug disposition and pharmacological response. This is particularly important with highly bound drugs where small changes in percent bound cause large changes in the fraction free.

In this investigation, the effect of heparin administration on the plasma protein binding and disposition of propranolol was determined in the dog. This work had already been published (Silber, Lo and Riegelman, 1980). A previous investigation (Wood *et al*, 1979) on the effect of heparin administration on the plasma protein binding of propranolol revealed a correlation between the change in the free fraction and either the log dose of heparin, or the change in non-esterified fatty acid (NEFA) concentrations. The decreased plasma protein binding of bilirubin and salicylate (Wiegand and Levy, 1979) after the administration of heparin

was reversed when the plasma was treated with activated charcoal, a procedure known to remove most of the NEFA from albumin. It has been proposed that NEFA concentrations are elevated as a result of heparin's lipolytic activity (Storstein and Janssen, 1976). Since NEFAs are extensively protein bound, the result is a competitive reaction leading to displacement of the drug from the plasma proteins. Because studies of propranolol disposition were underway in dogs using heparin locks, it became important to examine this reaction, since drug disposition might be critically affected by changes in the free fraction.

METHODS

Propranolol was administered in a 5 mg intravenous (I.V.) bolus dose to a 24 kg male mongrel dog on two separate occasions, one week apart. In the first experiment, each blood sample was obtained by separate venipuncture. In the second experiment, 1 ml of heparinized saline (10 U/ml) was given after each blood sample was obtained by separate venipuncture. A total of 130 U of heparin was given over a 500 minute period.

The effect of intermittent heparin administration on the plasma protein binding and disposition of propranolol in the same dog was also examined at steady-state. Following an 8 mg I.V. loading dose, propranolol was infused at a rate of 0.035 mg/min using a Harvard^{R} pump through an indwelling catheter in a right fore-limb vein. Blood samples (6 ml) were withdrawn from the left fore-limb vein through an indwelling Butterfly catheter (Abbot Laboratories) whose function was maintained by infusion of physiological saline at a rate of 0.02 ml/min. Blood samples were collected into plastic syringes and immediately transferred to disposable screw cap glass test tubes containing 100 U of heparin. After gentle

mixing and centrifugation for 10 minutes, plasma was transferred to glass screw cap vials and stored at -20°C. Nine blood samples were obtained from 309 to 530 minutes after the start of the infusion. During the next period, the rate of propranolol administration was maintained and the saline infusion in the contralateral vein was discontinued. Nine additional blood samples were obtained between 644 and 744 minutes; 1 ml of heparinized saline (10 U/ml) was injected after each blood sample was obtained.

In order to establish appropriate conditions for determining protein binding of propranolol in plasma using equilibrium dialysis, we dialyzed 700 µl of plasma containing 50 ng/ml propranolol against an equal volume of Krebs-Ringer buffer, pH 7.4. From these experiments, we chose to dialyze experimental samples for 8 hours at 37°C with plexiglas cells rotated in a water bath. The cell components were separated by a cellulose membrane having average pore radius of 24 Å.

Propranolol concentrations on both sides of the membrane were determined after equilibration by the extraction method. NEFA concentrations were determined by colorimetric titration (Dole and Meinertz, 1960).

RESULTS

Intravenous bolus studies: The disposition of propranolol following a 5 mg I.V. dose was unaffected by the administration of 130 U of heparin. As shown in Figure V-1, the two curves are virtually superimposable. The terminal half-life was 120 minutes.

Protein binding: Equilibration of free propranolol between plasma and buffer chambers was complete by six hours (Figure V-2). Since cells

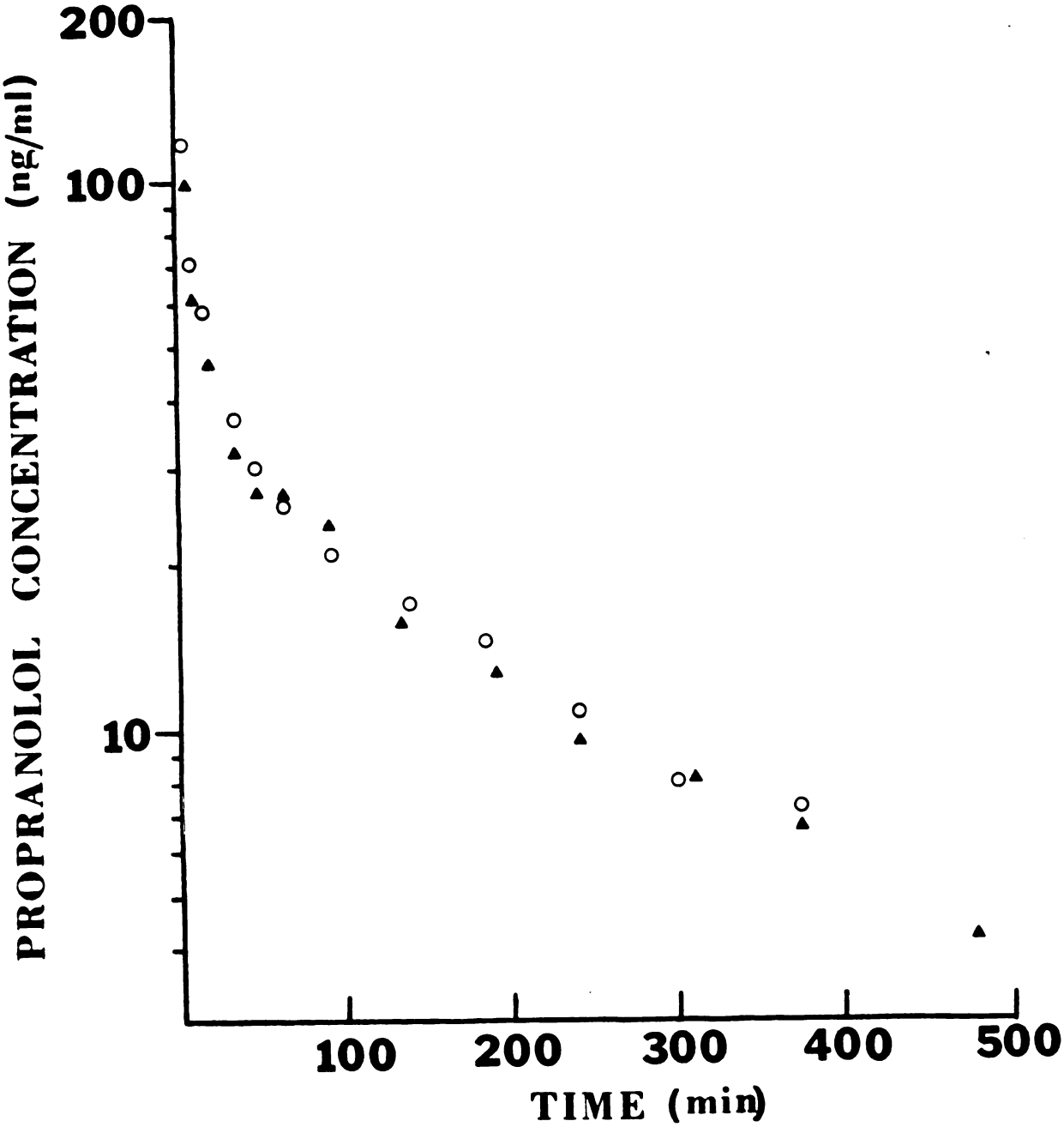


FIG V-1. . Plasma propranolol concentration after a 5 mg I.V. dose in a 24 Kg dog before (O) and after (▲) heparin administration. The studies were separated by a period of one week.

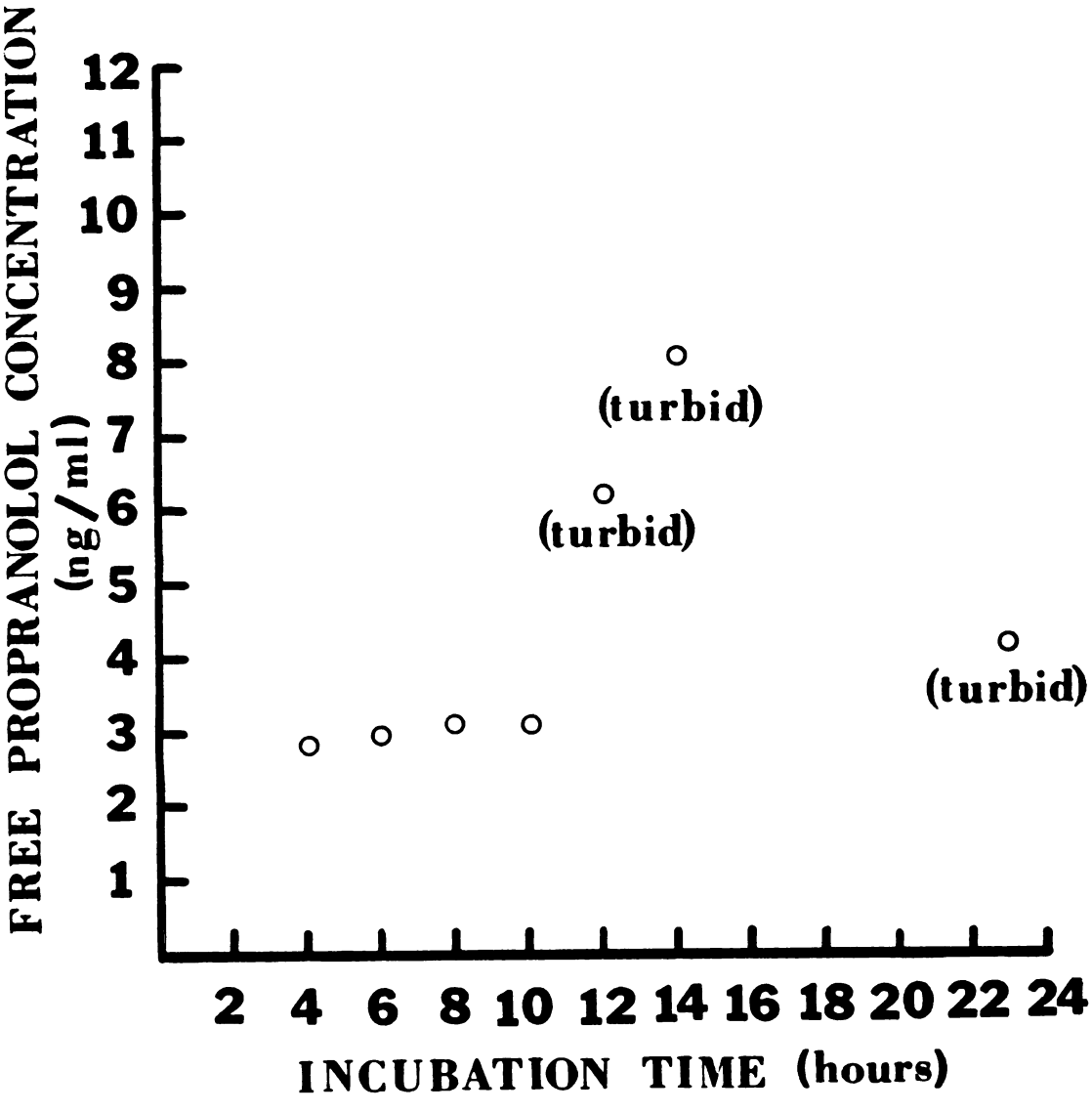


FIG V-2. Plasma protein binding of propranolol using equilibrium dialysis at 37°C.

became turbid if they were allowed to equilibrate for longer than 10 hours, we assessed plasma protein binding after 8 hours.

Intravenous infusion studies: Figure V-3 and Table V-1 summarize the results of the dog infusion experiment. After an 8 mg I.V. loading dose of propranolol, the drug was infused at 0.035 mg/min for 530 minutes. Since the terminal half-life was 120 minutes, eight blood samples were taken after 3 half-lives (360 minutes) between 309 and 530 minutes and resulted in average plateau free and total propranolol concentrations of 5.93 ± 0.43 (mean ± SD), and 46.58 ± 2.27 ng/ml, respectively, during the heparin-free period. The average fraction free was 12.71 ± 0.47 percent. The infusion of propranolol was continued and nine additional blood samples were obtained from 644 to 774 minutes. After each blood sample, 1 ml of heparinized saline (10 U/ml) was injected. Analysis of these samples led to the data summarized in Table V-1. One can, therefore, conclude that there is no change in propranolol clearance following the administration of heparin. In addition to the above assays, NEFA concentrations were determined in each sample.

The average NEFA concentrations were found to significantly increase ($p < 0.001$) after heparin administration (Table V-2) but apparently the increase in NEFA concentrations did not cause changes in the plasma protein binding of propranolol in the dog.

DISCUSSION

The results of this investigation show that administration of heparin in doses necessary to maintain the function of indwelling catheters (Holford *et al*, 1977) has no apparent effect on either the plasma

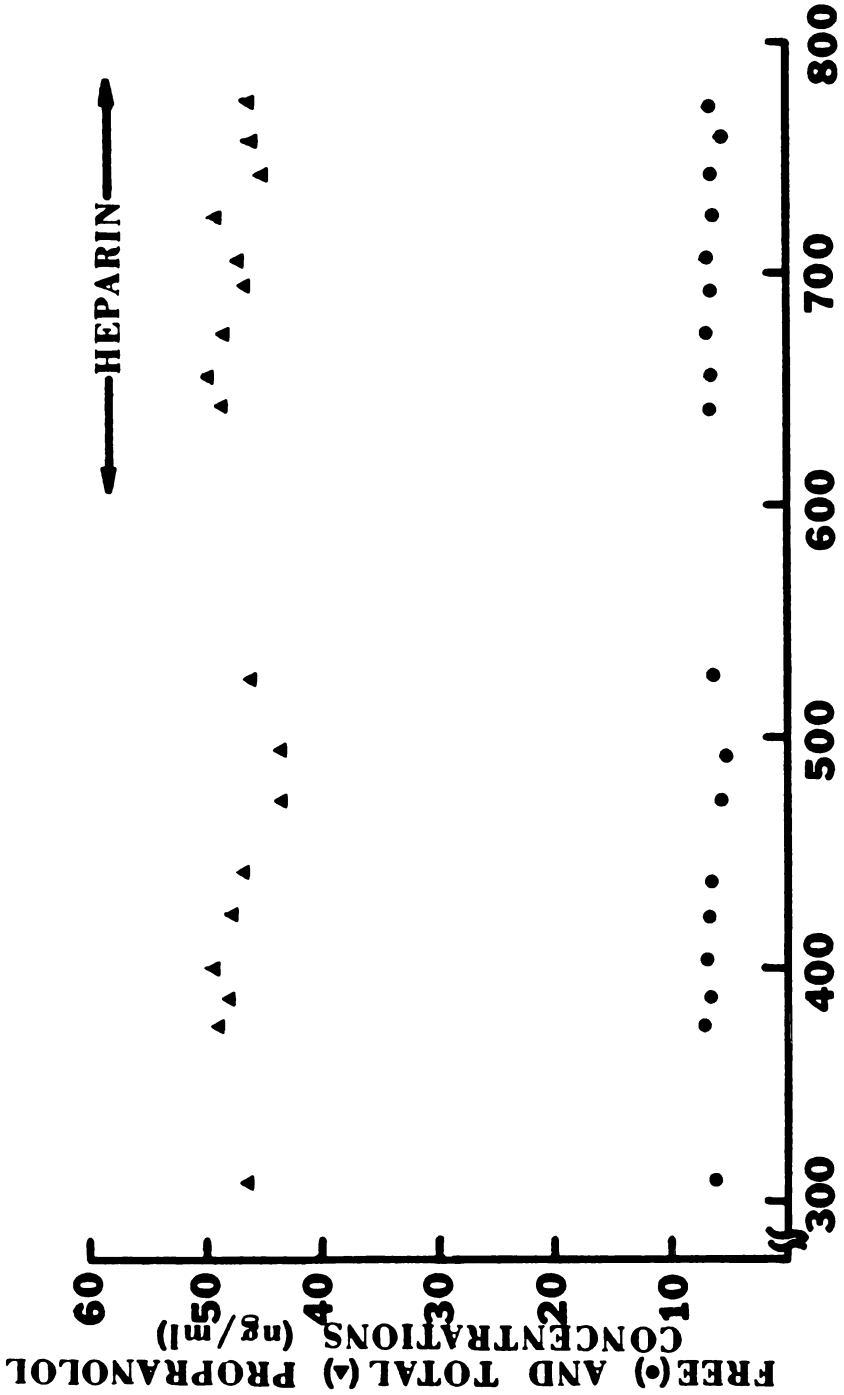


FIG V-3. Free (●) and total (▲) propranolol concentrations following an 8 mg loading dose along with an infusion at 0.035 mg/min for up to 772 min. 1 ml of heparinized saline (10 U/ml) was given after each blood sample was obtained between 644 and 772 min.

TABLE V-1. MEAN PLATEAU FREE AND TOTAL PROPRANOLOL CONCENTRATIONS, AND PERCENT FREE PROPRANOLOL BEFORE AND AFTER HEPARIN ADMINISTRATION IN THE DOG.

Parameter	Before Heparin	After Heparin
N	9	9
Total Propranolol Concentration (ng/ml)	46.58± 2.27*	47.29 ± 2.21
Free Propranolol Concentration (ng/ml)	5.93± 0.43	6.42 ± 0.39
Percent Free Propranolol	12.71 ± 0.47	13.52 ± 1.06

*Standard deviation

There were no significant differences ($P > 0.05$) between pre- and post heparin values.

TABLE V-2. NONESTERIFIED FATTY ACID (NEFA) CONCENTRATIONS BEFORE AND AFTER HEPARIN ADMINISTRATION IN THE DOG DURING AN INFUSION OF PROPRANOLOL.

PERIOD	TIME OF SAMPLE (MIN)	NEFA CONCENTRATION (μM/ML)
HEPARIN-FREE	309	0.35
	379	0.47
	393	0.43
	408	0.36
	425	0.40
	441	0.44
	476	0.58
	494	0.51
	530	0.61
	MEAN±S.D.	0.46 ± .09
HEPARIN	644	0.59
	659	0.67
	677	0.60
	693	0.70
	710	0.62
	725	0.61
	743	0.72
	758	0.71
	772	0.55
	MEAN±S.D.	0.64 ± .06*

*NEFA CONCENTRATIONS WERE SIGNIFICANTLY GREATER ($P < .001$) AFTER HEPARIN ADMINISTRATION.

protein binding (Figure V-3 and Table V-1) or disposition of propranolol (Figures V-1 and V-3) even when non-esterified fatty acid (NEFA) concentrations are significantly increased ($p < 0.001$) as a result of heparin's lipolytic activity (Table V-2).

At least in the case of propranolol, we have shown that heparinized catheters can be used to sample blood during chronic studies without altering the protein binding or disposition of the drug.

Our results, however, differ from those obtained by others (Wood *et al*, 1979). In these studies, blood was obtained from subjects given accumulated doses of heparin. Propranolol was added and the fraction free was determined using equilibrium dialysis. The change in the free fraction correlated with the log dose of heparin and with the change in NEFA concentrations. However, these workers gave doses of heparin that far exceed the amounts required in heparin locks, covering a range from 5 to 1200 U administered over 48 minutes. In addition, no pre-heparin NEFA concentrations were reported. However, our mean NEFA concentrations for the dog prior to heparin were 0.46 ± 0.09 μm/ml (mean ± SD), virtually identical to that reported in man (Wood *et al*, 1979) after 5 U of heparin, 0.45 ± 0.05 μm/ml. This group saw an exponential increase in NEFA concentrations as the heparin was injected. The rate at which heparin was administered exceeds the usual anticoagulating regimen, and far exceeds what is necesseary to maintain catheter patency.

A previous study (Wiegand and Levy, 1979) reported that NEFA displaces both bilirubin and salicylate from albumin. NEFA binds to albumin and lipoproteins, but <u>not</u> to α_1-acid glycoprotein (Skipski *et al*, 1967 and Shafrir, 1958). Since propranolol is bound in plasma predominantly (>75%) to α_1-acid glycoprotein (Sager *et al*, 1979), elevated

concentrations of NEFA resulting from heparin's increased lipoprotein lipase activity would not be expected to significantly increase the free fraction of propranolol in plasma.

The paradoxical observation of increased warfarin plasma protein binding after heparin administration (Nilsen *et al*, 1977 and Routledge *et al*, 1979) and the lack of a relationship between changes in NEFA concentrations and warfarin binding (Routledge *et al*, 1979) suggest that mechanisms other than competetive binding need to be considered.

CHAPTER VI. THE LACK OF GASTROINTESTINAL METABOLISM OF PROPRANOLOL IN DOGS THAT HAVE BEEN PORTACAVALLY TRANSPOSED

Since propranolol is extensively metabolized during absorption it is important to assess whether any metabolism occurs in the lumen or during transit through the gastrointestinal wall. The present dog model offers an opportunity to address this question more directly.

The first indication that propranolol may undergo extrahepatic metabolism came from the study of George *et al* (1976) who gave I.V. infusion of dextro-propranolol to 2 dogs and levo-propranolol to 3 dogs. Hepatic blood flow was measured independently by the colloidal gold method. By sampling the hepatic venous blood for propranolol they were able to obtain estimates of the hepatic and total systemic clearances of propranolol. For dextro-propranolol the contribution of liver to the total clearance averaged 74% and for levo-propranolol, 91%. It thus appears for dextro- and for levo-propranolol that 26% and 9% of their metabolism, respectively, may be extrahepatic. The possibility of lung metabolism of propranolol was ruled out by the same authors using an isolated dog lung preparation. However, intestinal mucosal metabolism was not investigated.

In 1978, Garceau *et al* (1978) studied the bioavailability of a pro-drug of propranolol, the hemi succinate ester of propranolol (on the side chain hydroxyl group). They compared it with the parent drug in 8 beagle dogs and reported an eight fold increase in AUCs of unchanged propranolol when equivalent doses were given. Since propranolol is glucuronidated to the O-glucuronide as one of the major metabolic pathways, it is possible that a portion of this glucuronidation occurs in the gastrointestinal tract. Since the pro-drug blocks the potential site for glucuronidation, the observed increase in the bioavailability

of propranolol could be due to a reduction in gut wall O-glucuronidation.

However, data suggesting that gastrointestinal metabolism is relatively insignificant are also available in the literature. Shand *et al* (1971) infused propranolol intravenously into 4 dogs and sampled blood from the aorta and the portal vein. There was close agreement between arterial (149 ± 26 ng/ml) and portal vein (161 ± 66 ng/ml) unchanged propranolol concentration (mean ± SD) indicating that drug transitting the intestines and later sampled in the portal vein did not show significant evidence of metabolism. In another study in man, Shand and Rangno (1972) studied a patient with portacaval anastomosis. The patient was given 80 mg orally and 30 mg I.V. on two separate occasions. After correction for the different doses used, the ratio of the areas under the blood concentration/time curves after oral and I.V. administration was 1.10, showing that the drug was fully available and that absorption was complete. This study suggests that first pass metabolism of propranolol occurs predominantly in the liver. Pessayre *et al* (1978) found that the total systemic clearance, 18.2 ± 7.7 (SD) ml/min/kg of dextro-propranolol, was the same as the hepatic clearance, 18.6 ml/min/kg, in 6 patients with liver fibrosis without cirrhosis. They concluded that dextro-propranolol is eliminated mainly by the liver in man.

Theory: A drug, administered orally to a dog that had undergone portacaval transposition, transits through the gastrointestinal tract and then enters the systemic circulation directly, bypassing hepatic metabolism during absorption. The same drug when given via a fore-limb vein is like a conventional intravenous dose. Provided the drug is absorbed completely (no dissolution problem, etc.) one can estimate

the extent of gastrointestinal metabolism by comparing the availabilities of equivalent oral and fore-limb I.V. doses. The bioavailability obtained by comparing the two routes of administration will be the fraction of the dose that has escaped gastrointestinal metabolism, as shown by Equation VI-1.

$$F_{\substack{\text{fraction escaping} \\ \text{g.i. metabolism}}} = \frac{AUC_{oral}}{AUC_{iv,\ forelimb}} \cdot \frac{Dose_{iv,\ forelimb}}{Dose_{oral}} \qquad \text{(VI-1)}$$

Experimental: Five male mongrel dogs with weights ranging from 23 to 28 kg were selected for the study. Their physical health and blood picture were monitored weekly for at least one month by the Vivarium at the University of California, San Francisco. Before performing the transposition, they were studied on two separate occasions (one week apart) by giving a 20 mg oral dose and a 5 mg intravenous bolus dose of propranolol via a forelimb vein. Blood samples (4 ml each) were collected from the other forelimb vein for seven hours through a 21-G indwelling needle (Abbott, North Chicago) which was kept patent by flushing with one ml of heparinized saline (10 U/ml) after each venous blood sampling. For the oral study, the sampling schedule was as follows: 15, 30, 45, 60, 90, 120, 180, 240, 300, 360 and 420 minutes. For the I.V. study additional samples were taken at 3 and 7 minutes. The blood was collected in an empty 18 ml disposable glass culture tube (Kimble 73750-16150) with teflon screw-cap containing 50 U of heparin. The blood was centrifuged for 10 minutes at 500 X *g* and the plasma separated and stored at -20°C in a glass vial until assayed. After at least one week had elapsed from the last experiment, a portacaval transposition surgery was performed

on the dog. The gallbladder was first removed, the inferior vena cava and the portal vein were then transposed, and lastly, a Thomas cannula put in the duodenum opposite the ampulla of Vater and exteriorized through the abdominal wall. The Thomas cannula was used in subsequent studies of hepatic metabolism. The animals were then allowed to recover from surgery for at least a month. All animals were fed a low protein diet (Prescription Diet k/d, Hill's, Topeka, Kansas) except the week after surgery during which they received an extra protein rich diet to help them recover faster from surgery. After a month the animals were then studied as before by giving a 20 mg oral dose of propranolol and a 5 mg intravenous bolus dose via a forelimb vein. The plasma was assayed for propranolol using the direct injection method.

Calculations: The area under the plasma concentration-time curve of propranolol (AUC) was estimated by the linear trapezoidal method from 0 to 7 hours. The extrapolated portion was obtained by dividing the last measured concentration by the terminal rate constant. The bioavailability before and after portacaval transposition was computed by Equation VI-1. The systemic plasma clearance was obtained by dose/AUC_{iv}.

Results: Tables V-1 through 5 include a tabulation of the collection times and the resultant propranolol concentrations in all the 5 dogs before and after portacaval transposition. Figure V-1 and 2 are semi-logarithmic plots of the data from dogs B and C. The various pharmacokinetic parameters estimated for the dogs are summarized in Table V-6. Whereas the bioavailability ranged from 2 to 20% before surgery, it increased to virtually 100% after surgery (95 to 115%) in all the dogs studied. Systemic plasma clearance increased in all but one of the 5 dogs (+30 to +44%). However, the observed terminal half-lives do not

TABLE VI-1. PLASMA PROPRANOLOL CONCENTRATION IN DOG A BEFORE AND AFTER PORTACAVAL TRANSPOSITION.

Before Transposition

5 Mg Forelimb I.V.		20 Mg P.O.	
Time(min)	Cp (ng/ml)	Time(min)	Cp (ng/ml)
3.6	92.25	16.6	3.76
7.1	69.93	30.5	4.52
15.2	56.23	46	3.77
31.7	44.67	77.9	3.34
45.1	36.5	103	2.74
60.8	32.12	125.7	2.66
88.9	29.38	190.6	2.03
123	23.21	247	1.59
180.8	16.91	303	1.13
233.8	12.43	367	0.72
304.6	8.13	422	0.92
368.2	5.28		
421.6	3.67		

After Transposition

5 Mg Forelimb I.V.		20 Mg P.O.	
Time (min)	Cp (ng/ml)	Time(min)	Cp (ng/ml)
3.9	83.4	16.8	10.5
8.7	70.0	31.7	28.4
15.3	63.8	46.2	34.8
24.6	43.1	61.2	51.5
36	33.9	91.8	99.0
49.2	35.2	131.9	92.5
64.9	30.6	184.2	87.5
90.5	20.1	252	66.3
124.8	17.6	306.1	48.9
181.8	8.6	363.7	38.4
247.8	3.8	408.3	27.9
302.9	2.2		
417	1.46		

TABLE VI-2. PLASMA PROPRANOLOL CONCENTRATION IN DOG B BEFORE AND AFTER PORTACAVAL TRANSPOSITION.

Before Transposition

5 Mg Forelimb I.V.		20 Mg P.O.	
Time(min)	Cp(ng/ml)	Time(min)	Cp(ng/ml)
3.3	135.7	20.5	31.73
6.7	85.9	47.4	33.55
15.4	56.1	67.6	30.9
30.5	38.2	95	25.6
43.9	34.5	122.9	18.8
60.3	28.6	176	12.5
91.9	22.1	245	7.2
123	17.2	306.5	5.2
180.3	13.4	362	3.7
241.4	7.7		
300	5.2		
360	3.4		
423	2.5		

After Transposition

5 Mg Forelimb I.V.		20 Mg P.O.	
Time(min)	Cp(ng/ml)	Time(min)	Cp(ng/ml)
3.1	131.3	15	55
7	48	30.3	89.9
14.5	41.4	47.1	82.6
29.1	23.2	60.4	78.3
44.8	20.4	90.5	64
62.1	16.1	119.2	51.8
91.6	11.2	181.6	44.8
121	10.3	239.4	32.2
182	7.1	301.1	24.9
242.3	5.1	359	19.1
300.9	3.6	425.8	15.1
363.8	2.9		
426.9	2.1		

TABLE VI-3. PLASMA PROPRANOLOL CONCENTRATION IN DOG C BEFORE AND AFTER PORTACAVAL TRANSPOSITION.

Before Transposition

5 Mg Forelimb I.V.		20 Mg P.O.	
Time(min)	Cp(ng/ml)	Time(min)	Cp(ng/ml)
6.7	48.3	18.5	6.4
13.7	30.6	38.8	3.9
29.5	18.1	61.7	2.2
44.2	17.1	91	1.5
85.7	12.3	120.5	0.96
127	10.6	182	0.57
184.6	6.9		
270	5.2		
350	4.0		
486	2.3		

After Transposition

5 Mg Forelimb I.V.		20 Mg P.O.	
Time(min)	Cp(ng/ml)	Time(min)	Cp(ng/ml)
3.2	42.1	14	22.3
7.3	24.1	28.9	41.2
15	22.7	47.5	53.3
29.9	17.2	61.2	60.1
47.5	15.4	90.4	55.9
61.1	12.6	120.8	39.1
90.8	9.9	182	28.9
119.6	8.6	240.3	25.1
181.4	5.8	299.9	16.5
239.2	5.1	367	10.9
301.8	3.5	426.3	8.8
359.8	2.5		
422.6	2.3		

TABLE VI-4. PLASMA PROPRANOLOL CONCENTRATION IN DOG D BEFORE AND AFTER PORTACAVAL TRANSPOSITION.

Before Transposition

5 Mg Forelimb I.V.		80 Mg P.O.	
Time(min)	Cp(ng/ml)	Time(min)	Cp(ng/ml)
6.5	93.4	15	36.6
16.5	55,2	30	56.5
31.5	36.2	45	57.2
45	32.6	60	55.7
60.5	26.5	90	55.7
90	18.1	120	42.9
120	18	150	37.5
150	15.6	170.5	31.3
180	9.8	240	13.6
240	6.8	285	8.2
300	4.6		

After Transposition

5 Mg Forelimb I.V.		20 Mg P.O.	
Time(min)	Cp(ng/ml)	Time(min)	Cp(ng/ml)
6.5	68.4	20.5	10.02
25	35.0	31.5	17.1
48.3	23.8	54.5	119.9
60	19.6	90	101
95.3	17.8	120	100.3
126.3	11.0	180	72.8
186.4	6.75	240	46.5
270.5	2.42	301.5	20.8
366	1.6	362.5	13.3
419.5	1.24	446	9.4
483.5	1.07		

TABLE VI-5 PLASMA PROPRANOLOL CONCENTRATION IN DOG E BEFORE AND AFTER PORTACAVAL TRANSPOSITION.

Before Transposition

5 Mg Forelimb I.V.		20 Mg P.O.	
Time(min)	Cp(ng/ml)	Time(min)	Cp(ng/ml)
5	85.6	16.7	0.77
15.4	59.3	30	1.92
29.6	36.2	45.6	2.35
44.4	31.1	60.7	2.96
59.6	26.0	90.4	2.29
93.3	18.5	123	2.03
128.9	14.2	196.1	1.24
181.6	10.1	243.2	0.58
246	6.3	301.1	0.30
300.4	4.6	366.6	0.03
359.6	4.3		
421.5	2.9		
483.5	2.1		

After Transposition

5 Mg Forelimb I.V.		20 Mg P.O.	
Time(min)	Cp(ng/ml)	Time(min)	Cp(ng/ml)
2.4	159.4	10.1	17.4
6.8	84.9	19.2	60.2
16.1	56.1	29.7	104.1
29.5	46.6	40.5	127.9
45.8	30.1	52.9	160.3
63.3	27.2	60.6	149.9
93.2	25.7	75.2	131.9
128.2	18.7	86.7	121.4
180.9	11.2	105.5	109.9
248.1	7.1	137.5	97.7
302.1	4.3	179.8	70.7
360.7	4.0	241.5	46.0
404.7	2.5	305	27.8
		359.9	20.1
		426.5	13.9

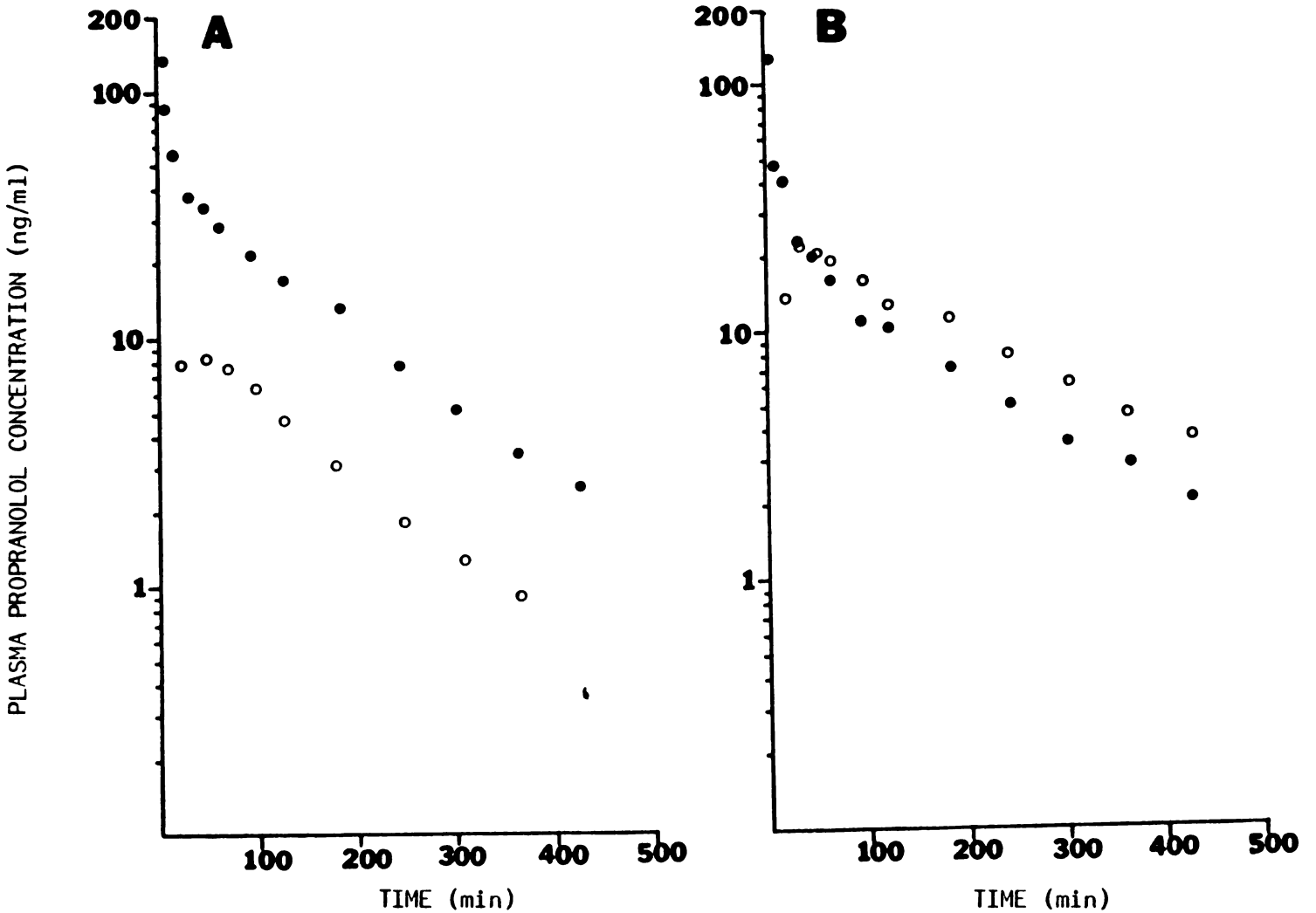


FIG VI-1. Plasma propranolol concentration-time curves for dog B before surgery (A) and after portacaval transposition (B). i.v.= (●); p.o.= (○); i.v. dose= 5 mg; p.o. dose= 20 mg. For comparison, the p.o. values are reduced four-fold as if 5 mg dose had been given.

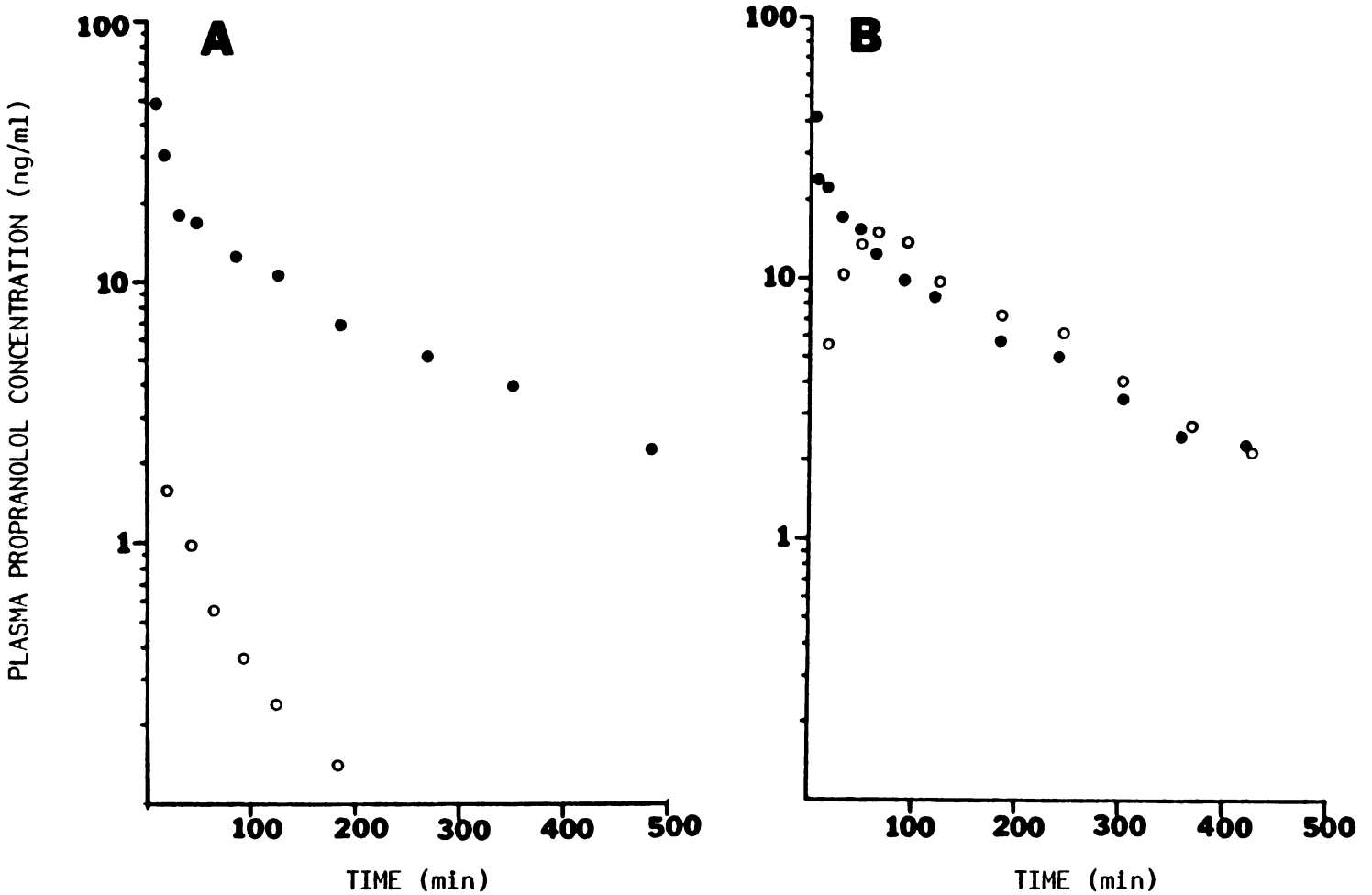


FIG VI-2. Plasma propranolol concentration-time curves for dog C before surgery (A) and after portacaval transposition (B). i.v.= (●); p.o.= (O); i.v. dose= 5 mg; p.o. dose= 20 mg. For comparison, the p.o. values are reduced four-fold as if 5 mg dose had been given.

TABLE VI-6. SUMMARY OF ESTIMATED PHARMACOKINETIC PARAMETERS OF PROPRANOLOL IN DOGS BEFORE AND AFTER PORTACAVAL TRANSPOSITION.

	DOG A	DOG B	DOG C	DOG D	DOG E
Weight (Kg)	27.7	25.6	23.4	23.3	24.5
$(AUC)^{a)}_{i.v.}$ (ng.min/ml)					
Before	8760	7318	4611	6595	6798
After	6323	5101	3556	4865	7418
$(AUC)^{b)}_{p.o.}$ (ng.min/ml)					
Before	904	5895	488	11234	461
After	27453	19714	13489	22399	28458
Bioavailability, F(%)					
Before	3	20	3	11	2
After	109	97	95	115	96
Systemic Plasma Clearance (ml/min)[c)]					
Before	571	683	1084	758	736
After	791	980	1406	1028	674
Terminal Half-life (min)[c)]					
Before	112	95	142	90	115
After	66	133	131	79	95

a) Zero to infinity; all dogs were given 5 mg of propranolol, forelimb

b) Zero to infinity; all dogs were given 20 mg of propranolol orally except dog D which was given 80 mg before transposition.

c) From forelimb intravenous data.

show a consistent trend. This may be partially due to assay error at the low plasma concentrations.

Discussion: The fact that the post surgical bioavailability was virtually 100% (see Table V-6) is quite convincing evidence that propranolol does not undergo gastrointestinal metabolism to any significant extent. However, it is surprising for a drug that has such a high first pass effect and extensive metabolism by glucuronidation to not undergo at least some gastrointestinal metabolism.

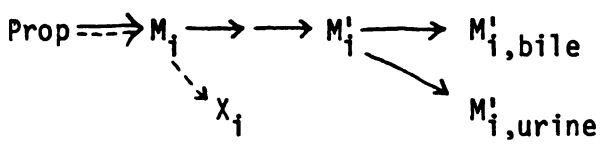
For a perfusion rate-limited drug the I.V. clearance is a reflection of hepatic blood flow. The post-surgical increase is to be expected from such an operation since the higher volume of blood carried by the inferior vena cava, now flows to the liver. Kreuzer and Schenk (1971) also found that total liver blood flow was increased 27% over control animals after portacaval transposition in 7 dogs using flow-probes to measure blood flow. We observed a mean change of 37 ± 6 (SD%) in 4 of the dogs that showed an increase of liver blood flow. For some unexplained reason, dog E was found to have a decreased plasma clearance.

CHAPTER VII. NONLINEAR HEPATIC METABOLISM OF PROPRANOLOL IN DOGS THAT HAVE BEEN PORTACAVALLY TRANSPOSED

From the results of Chapter VI, it is evident that propranolol does not undergo significant gastrointestinal metabolism. Thus most of the metabolism of propranolol is hepatic. Using dogs that have been portacavally transposed, the drug can be administered directly to the liver via a peripheral hind-limb vein. Studies are reported wherein propranolol was infused to different steady-states and the measureable metabolites excreted in the bile and urine were assayed. Using these data, estimates of the various kinetic parameters that describe the metabolism of propranolol were obtained.

1. THEORY

Three major pathways of metabolism of propranolol are followed, namely: 1) O-glucuronidation to propranolol glucuronide; 2) ring oxidation to 4-hydroxypropranolol; and 3) N-dealkylation to N-desisopropylpropranolol and subsequent conversion to propranolol glycol glucuronide and α-naphthoxylactic acid. Each pathway may undergo capacity-limited kinetics. A gernal model hypothesizing the sequential processes of pathways 2 and 3 is:



where Prop = Propranolol in the body

M_i = Amount of the ith metabolite of propranolol formed at time t

M_i' = A conjugate or further metabolized form of M_i

X_i = Unknown species formed from M_i

$M_{i,urine}', M_{i,bile}'$ = Amount of compounds M_i' recovered in urine and bile, respectively

In this scheme, the special arrow ($\dashrightarrow$) is used to indicate a capacity-limited process. There is evidence, reviewed in Chapter II, pointing to nonlinear metabolism of propranolol. Thus, one must propose that the first step in pathways 2 and 3 may undergo Michaelis-Menten kinetics. The remaining step(s) in the sequence indicates additional processes which may occur before the ultimate metabolite (M_i') derived from the metabolic sequence is detected in the bile or urine. As we are primarily interested in monitoring the conversion of propranolol to M_i, we hydrolyzed the glucuronides, e.g. (M_i'), and thus measured the precursor, (M_i). It is possible a portion of metabolite, M_i, is converted to an additional (unknown) species, X_i, which would cause an underestimation of the extent of metabolism by this pathway. The secondary metabolic processes between the formation and ultimate detection of M_i are assumed to be first order processes.

The rate of change of the amount of the ith metabolite in the body, dM_i/dt, is

$$dM_i/dt = CL_{f,M_i} \cdot C_{b,prop} - CL_{e,M_i} \cdot C_{b,M_i} \qquad \text{(VII-1)}$$

and the rate of change of the amount of the conjugate of the ith metabolite, dM_i'/dt, is

$$dM_i'/dt = CL_{e,M_i} \cdot C_{b,M_i} - CL_{e,M_i'} \cdot C_{b,M_i'} \qquad \text{(VII-2)}$$

where

$C_{b,prop}$ = Blood concentration of propranolol

C_{b,M_i} = Blood concentration of M_i

$C_{b,M_i'}$ = Blood concentration of M_i'

CL_{f,M_i} = Formation clearance of M_i from propranolol

CL_{e,M_i} = Total elimination clearance of M_i

$CL_{e,M_i'}$ = Total elimination clearance of M_i'

When steady-state is achieved, $dM_i/dt = dM_i'/dt = 0$. It then follows from Equation VII-1 and 2 that

$$CL_{f,M_i} \cdot C_{b,prop,ss} = CL_{e,M_i} \cdot C_{b,M_i,ss} = CL_{e,M_i'} \cdot C_{b,M_i',ss} \qquad \text{(VII-3)}$$

where $C_{b,prop,ss}$, $C_{b,M_i,ss}$, and $C_{b,M_i',ss}$ are steady-state blood concentrations of propranolol and the metabolites M_i and M_i'. Equation VII-3 is only correct if M_i is completely converted to M_i'; i.e., no parallel reaction to form an unknown metabolite X_i occurs. If parallel (first order) metabolism does occur, the last term in Equation VII-3 would be a constant fraction of the total formation rate of M_i, the first term.

Equation VII-3 can be rearranged to Equation VII-4:

$$CL_{f,M_i} = \frac{CL_{e,M_i'} \cdot C_{b,M_i',ss}}{C_{b,prop,ss}} \qquad \text{(VII-4)}$$

The product $CL_{e,M_i'} \cdot C_{b,M_i',ss}$ is the total excretion rate of M_i' by the biliary and urinary routes. Equation VII-4 can be rewritten as

$$(\text{Total Excretion Rate of } M_i')_{ss} = CL_{f,M_i} \cdot C_{b,prop,ss} \qquad \text{(VII-5)}$$

which of course directly relates to the process presumed to follow Michaelis-Menten enzyme kinetics. The formation clearance CL_{f,M_i} can be

expressed in Michaelis-Menten terms with $V_{max_{f,M_i}}$ being the maximum enzyme activity for the formation of M_i from propranolol and $K_{m_{f,M_i}}$ being the concentration of propranolol at half $V_{max_{f,M_i}}$. Thus:

$$(\text{Total Excretion Rate of } M_i')_{ss} = \frac{V_{max_{f,M_i}} \cdot C_{b,prop,ss}}{K_{m_{f,M_i}} + C_{b,prop,ss}} \qquad \text{(VII-6)}$$

According to Equation VII-6, if the formation of M_i is a linear process (e.g. $C_{b,prop,ss} << K_{m_{f,M_i}}$), then the steady-state excretion rate of M_i' is linearly related to $C_{b,prop,ss}$. However, if the process of the formation of M_i from propranolol is saturable, then a hyperbolic relationship exists between the two variables.

The meaning of M_i formation clearance depends on the route of administration. If the intravenous route is chosen, then this clearance is the fractional contribution to systemic metabolic clearance of propranolol as related to the formation of M_i. The limiting value of the systemic clearance of propranolol is liver blood flow. The liver cannot clear the drug any faster than the rate of presentation. It may be difficult to study the nonlinear aspects of the hepatic metabolism of propranolol using the intravenous route, because the propranolol concentration in the body would have to be elevated to a toxic value to saturate the metabolizing enzymes.

If one were to administer the drug directly to the liver either orally or via a portal vein infusion, the apparent oral clearance obtained will be much greater than the systemic clearance. In terms of the per-

fusion model, this clearance is called the total intrinsic drug clearance (when referred to whole blood concentrations). This is a direct measure of hepatic enzyme activity and its value is not limited by liver blood flow.

The glucuronides of propranolol and 4-hydroxypropranolol were each measured in both bile and urine to yield information on the O-glucuronidation and aromatic ring hydroxylation pathways. For metabolites arising from one common metabolic pathway, it is important to sum up all the metabolites to obtain estimates of the kinetic parameters for that pathway. Since propranolol glycol and α-naphthoxylactic acid both arise from dealkylation, their excretion rates were summed together.

The elimination clearances of the metabolites were also measured to make sure that their elimination into urine or bile did not follow saturation kinetics.

As only about 70% of the dose of propranolol was accounted for in the study, a modelling procedure was devised to estimate the characteristics of the unknown pathway. At steady state, the rate of propranolol infusion equals the rates of metabolism by the measured metabolites and by the unknown metabolite(s).

$$\begin{aligned} \text{Rate of infusion of propranolol} &= \text{Rate of elimination by all processes} \\ &= \text{Rate of metabolism by KNOWN processes} + \text{Rate of metabolism by UNKNOWN processes} \end{aligned} \qquad \text{(VII-7)}$$

For simplicity, the unknown pathway(s) is assumed to be a single process.

Using the results from the steady-state experiments and the derived pharmacokinetic parameters of the known metabolic pathways, the unknown is fitted either as a first-order or a Michaelis-Menten process.

Additional experiments were run to test the model in the nonsteady-state condition. A short infusion of propranolol was administered via the hind-limb femoral vein. Blood, urine and bile were collected. Differential equations describing the concentration-time profiles of propranolol and the metabolites in the blood were tested for goodness of fit utilizing the parameter estimates obtained from the steady-state studies. The equations are shown below:

$$\frac{dC_{b,prop}}{dt} = \frac{C_{b,prop}}{V_{c,prop}} \cdot \left(CL_{linear} + \sum_{i=1}^{n} \frac{V_{max_{f,M_i}}}{K_{m_{f,M_i}} + C_{b,prop}}\right) +$$

Distribution into and out of a tissue compartment (VII-8)

$$\frac{dC_{b,M_i}}{dt} = \frac{1}{V_{c,M_i}} \left(CL_{f,M_i} \cdot C_{b,prop} - CL_{e,M_i} \cdot C_{b,M_i}\right) \quad \text{(VII-9)}$$

where $C_{b,prop}$ and C_{b,M_i} represent blood concentrations of propranolol and metabolite M_i and $V_{c,prop}$ and V_{c,M_i} represent the volumes of distribution of propranolol and metabolite M_i in the central compartment.

2. EXPERIMENTAL

Of the five dogs that were used in the study of gastrointestinal metabolism, one died of infection shortly after initiation of the hepatic study and another died of electrolyte imbalance due to the accidental loss of the bile cannula plug. The remaining three male mongrel dogs were monitored by a veterinarian weekly. Blood samples were taken to monitor their liver enzymes and complete blood count. The dogs weighed between 23.4 and 27.7 kg. A day before the study the dogs were fed very early in the morning with half their normal food intake. On the day of the study their bile cannula caps were opened and the duodenal contents removed with warm saline flush and gentle suction. The entry of the common bile duct to the duodenum was located with the help of a direct-focusing headlight (Welch Allyn 46003, Skaneateles Falls, N.Y.). The bile duct was cannulated with a disposable no. 6 French olive tip polyurethane ureteral catheter (Cat. no. H8011-136506, Bard Inc., Murray Hill, N.J.) extending 4 to 5 cm into the common bile duct. The catheter was then passed through a screw cap with a centered hole and anchored with cotton thread to the stainless steel flange of the Thomas cannula. The bladder of the dog was catheterized with a 7 Fr Swan-Ganz double lumen flow-directed monitoring balloon-tipped catheter (Cat. no. 93-111-7F, Edwards Lab, Santa Ana, CA) following the technique of Garner and Laks (1976). This technique was found to be superior to others including using a Foley catheter and polyethylene tubing. Periodic flushing, with warm saline, of the Swan-Ganz catheter was necessary to clear the catheter lumen of sediment-type material produced by the dog. The bile and urine of the dog were collected into glass graduated cylinders. After

taking the necessary amount of bile, the unused bile was mixed with the dog's food and eaten by the dog at the end of the experiment. Propranolol hydrochloride in water (approximately 25 mg/ml) was made up the night before in a laminated hood and filtered using a 0.22 μm Millipore disposable filter unit (Cat. no. SLGS 0250S, Bedford, Mass.). It was stored in an evacuated sterile 250 ml bottle (Cat. no. S9902, McGaw Lab, Irvine, CA). On the day of the experiment the propranolol solution was transferred aseptically into a 5% dextrose and 0.45% sodium chloride solution (Travenol Lab, Deerfield, Ill.) and mixed thoroughly to yield final concentrations below 3 mg/ml. A hind-limb vein of the dog was catheterized with a 20 gauge 2 inch Angiocath (Cat. no. 2818, Deseret Co., Sandy, Utah) and connected to the propranolol solution via an I.V. solution administrations set (Cat. no. 2C0700, Travenol Lab, Deerfield, Ill.) and a 150 ml in-line burette (Cat. no 2C0916, Travenol Lab, Deerfield, Ill.) to monitor the flow rate. A loading dose of propranolol was usually administered as a fast infusion at 2 ml/min for one hour which was then followed by the maintenance infusion of 1 ml/min for 9 hours. In some studies the loading dose was given as a single hind-limb bolus. A peristaltic pump was used for infusion purposes (Model 1201, Harvard Apparatus Co., Millis, Mass.). Different propranolol concentrations were used to achieve infusion rates between 1 and 6 mg/min. Dog A was studied at six infusion rates: 0.92, 1.42, 2.09, 3.03, 4.09 and 6.27 mg/min. Dogs B and C were each studied four times. In dog B the infusion rates were 1.01, 1.89, 2.66 and 3.45 mg/min and in dog C 1.00, 1.93, 3.15 and 4.28 mg/min. The chronological sequence of the study in dog A was not in the order of increasing dose since dog A was our pilot study and we therefore did not know how high a dose would be

required. The sequence in this dog was 0.92, 3.03, 2.09, 1.42, 4.09 and 6.27 mg/min. The chronological sequence in dog B was 1.01, 2.66, 1.89 and 3.45 mg/min. The time span for completion of the studies varied from 6 to 9 weeks. Each experiment lasted about 10 hours and the dogs were kept awake in a torso-sling (Alice King Chatham Medical Arts, Los Angeles, CA) on a table. Blood, urine and bile samples were collected at 60 and 90 minute intervals, starting approximately 3 hours after the initiation of the infusion. The blood samples were collected into disposable glass culture tubes (Kimble 73750-16150) containing 50 units of heparin. The plasma was separated upon centrifugation at 500 X *g* and stored at -20°C until assayed. Urine and bile were stored at -20°C with 10 mg/ml of ascorbic acid added.

Blood/plasma ratio determinations: In order to assess the blood/plasma ratio in dogs B and C, their heparinized blood was spiked with propranolol at final concentrations ranging from 0.11 to 3.17 μg/ml. After equilibrating for an hour at room temperature the plasma was separated and the concentration of propranolol determined. In dog A the study range was from 0.08 to 4.5 μg/ml. These studies are done to allow conversion from plasma concentration to blood concentration values.

Nonsteady-state studies: Dogs A and B were given 1.17 gm and 0.73 gm of propranolol, respectively, via a hind-limb vein over 100 minutes. Blood, urine and bile were collected at frequent intervals for the next 460 minutes. The blood samples were stored in glass vials containing EDTA at -20°C. From past experience it was found that EDTA was a better anticoagulant for whole blood than heparin.

2. RESULTS AND DISCUSSION

Blood/plasma ratio: The results shown in Table VII-1 indicate a definite concentration dependence of blood/plasma ratio with increasing propranolol concentration. In dog A the ratio varied from 0.76 to 1.25; in dog B from 0.86 to 1.30; and in dog C from 1.22 to 1.88. The blood/plasma ratio increased an average of 1.5 fold over the blood propranolol concentration from 0.1 to 4 μg/ml. Graphs of the relationship between blood and plasma concentrations are shown in Figure VII-1. One can explain these increases by presuming that we have exceeded the linear binding region of propranolol to the α_1 acid glycoprotein. This would result in a disproportionate increase in the free concentration and a subsequent increased uptake by the red blood cells.

Steady-state infusion: Figures VII-2 and 3 depict plasma concentration-time profiles of propranolol and its major metabolites for two of the six steady-state studies in dog A. The others are similar. Figures VII-4 and 5 are composite graphs of propranolol steady-state levels in dogs B and C during two of the four steady-state infusions. The metabolites were not measured in plasma in dogs B and C. It can be seen in the figures that propranolol and its metabolites approached steady-state after 400 minutes. The biliary and urinary excretion rates versus time are shown in Figures VII-6 through 11. The last few data points were averaged to give estimates of the steady-state excretion rate. A summary of the infusion rates, the resultant steady-state propranolol plasma concentrations and the various metabolite steady-state excretion rates into bile and urine are presented in Table VII-2. It is interesting to note the propranolol glycol glucuronide is primarily excreted into the bile whereas α-naphthoxylactic acid is found predominantly in urine.

TABLE VII-1. PROPRANOLOL BLOOD/PLASMA CONCENTRATION DEPENDENCE IN DOGS.

Spiked Blood Conc (ng/ml)	Plasma Conc Determined (ng/ml)	Blood/Plasma Ratio
	DOG A	
75.9	100.3	0.76
227.7	306.7	0.74
500.3	636.5	0.79
1001	1244	0.80
2001	2111	0.95
3002	2769	1.08
4502	3606	1.25
	DOG B	
113.8	132.3	0.86
527	563	0.94
1055	1105	0.95
2110	1936	1.09
3165	2432	1.30
	DOG C	
113.8	93.4	1.22
527	381	1.38
1055	686	1.54
2110	1241	1.70
3165	1688	1.88

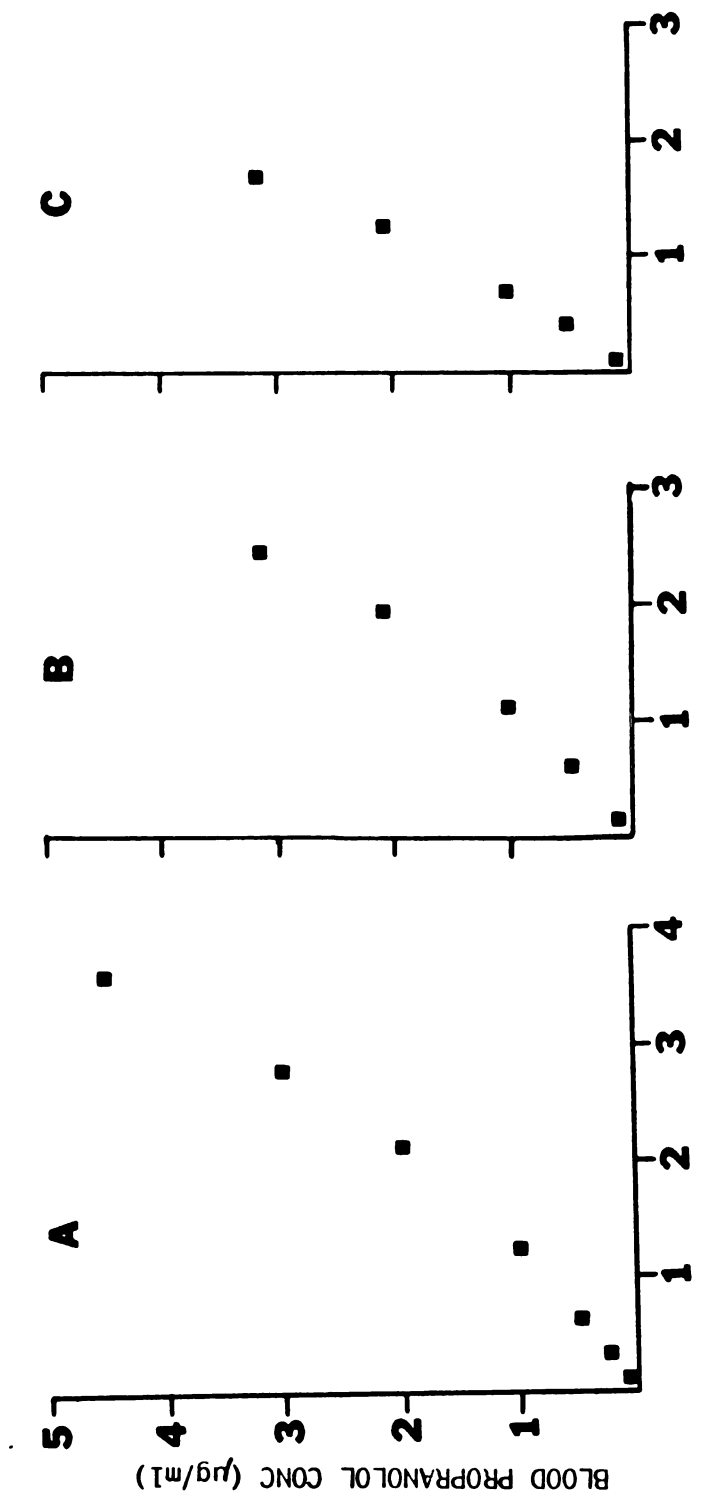


FIG VII-1. Equilibrium blood plasma concentration ratios for dogs A, B, and C.

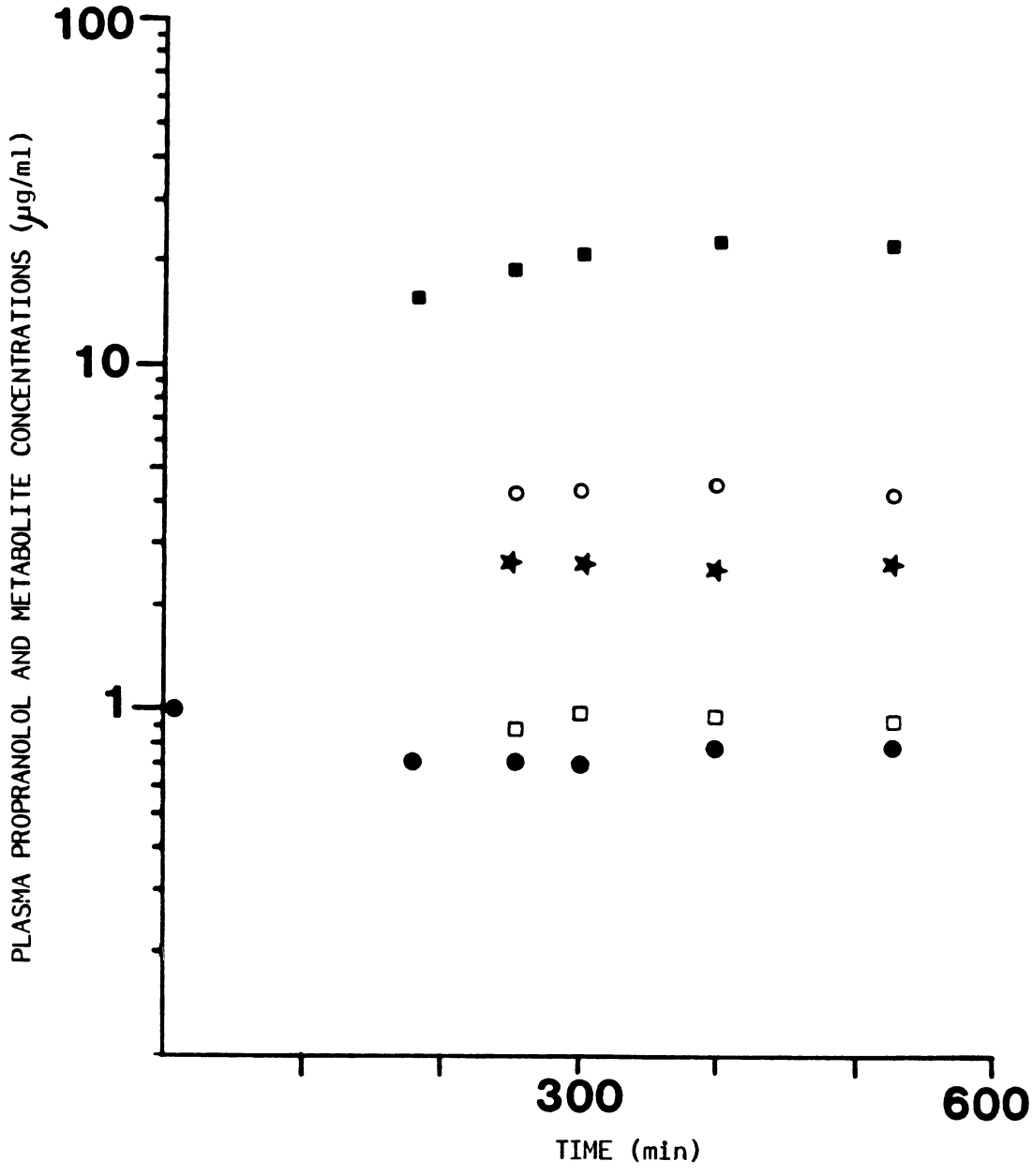


FIG VII-2. Plasma concentration-time plots of propranolol and its metabolites in dog A during a hind-limb propranolol infusion (2.09 mg/min).

Key: propranolol (●); propranolol glucuronide (★); 4-hydroxypropranolol glucuronide (○); α-naphthoxylactic acid (■); propranolol glycol glucuronide (□).

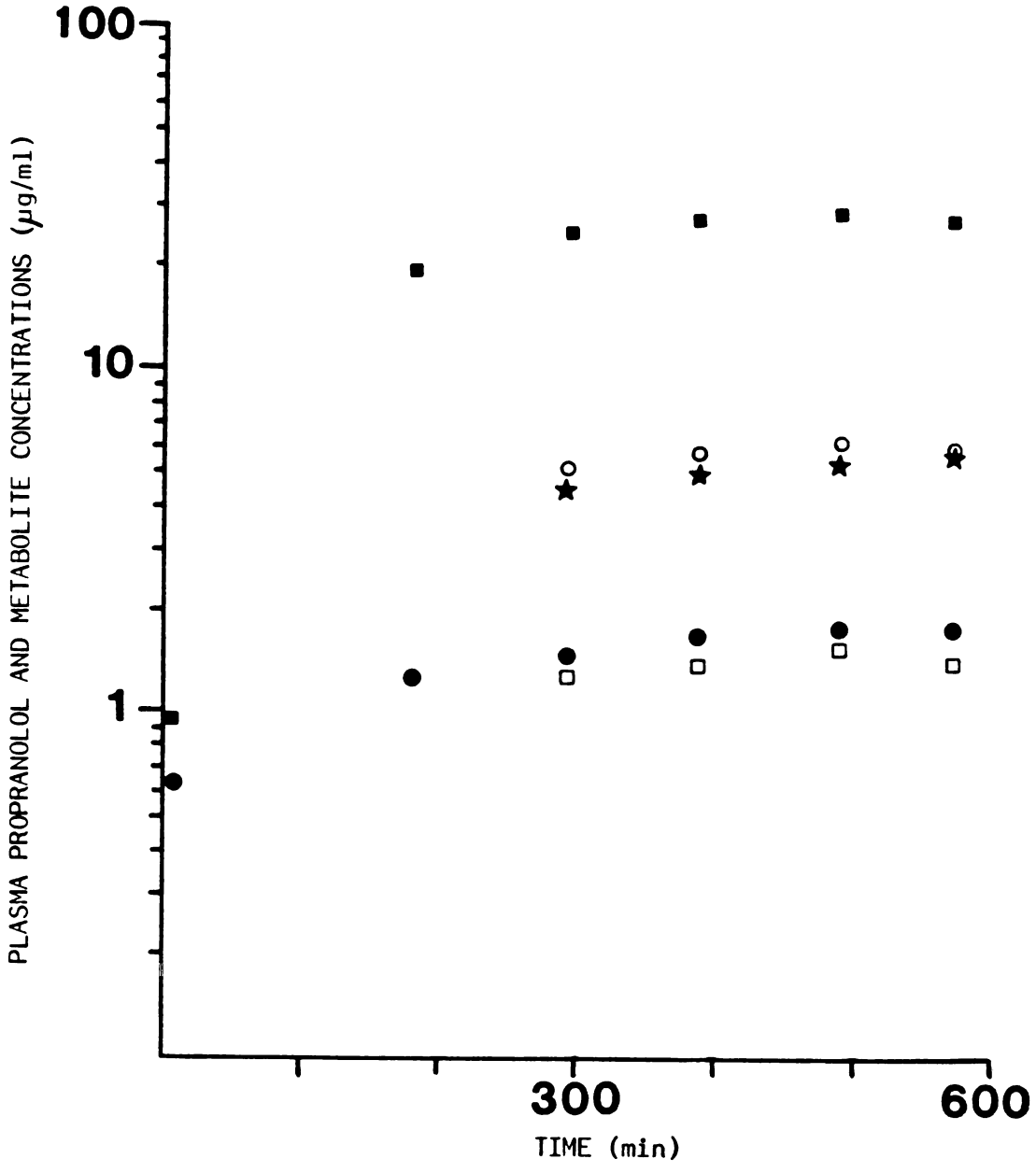


FIG VII-3. Plasma concentration-time plots of propranolol and its metabolites in dog A during a hind-limb propranolol infusion (4.085 mg/min).

Key: propranolol (●); propranolol glucuronide (★); 4-hydroxypropranolol glucuronide (○); α-naphthoxylactic acid (■); propranolol glycol glucuronide (□).

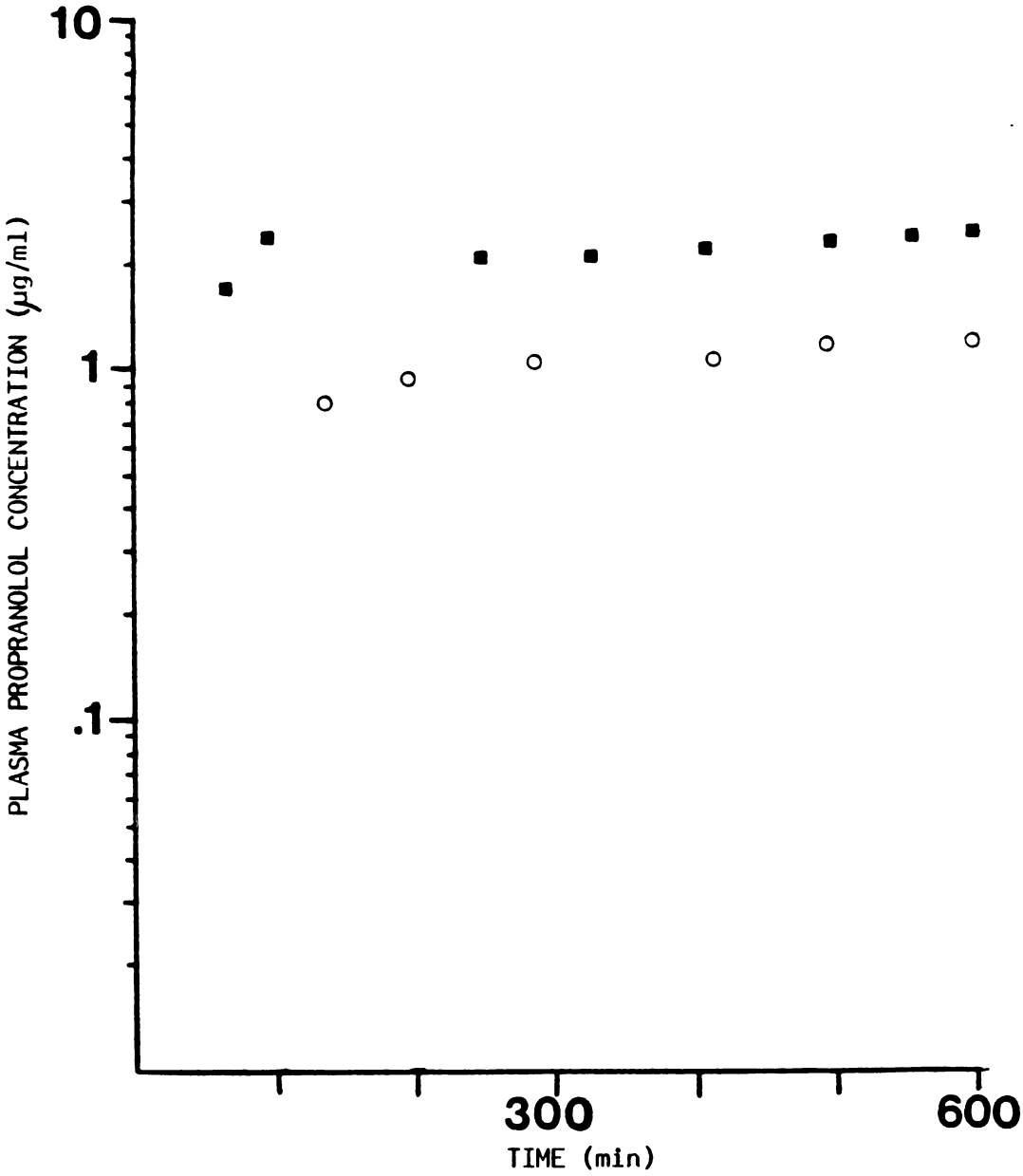


FIG VII-4. Plasma concentration-time plots of propranolol in dog B during two hind-limb propranolol infusions: 1.89 mg/min (O) and 3.45 mg/min (■).

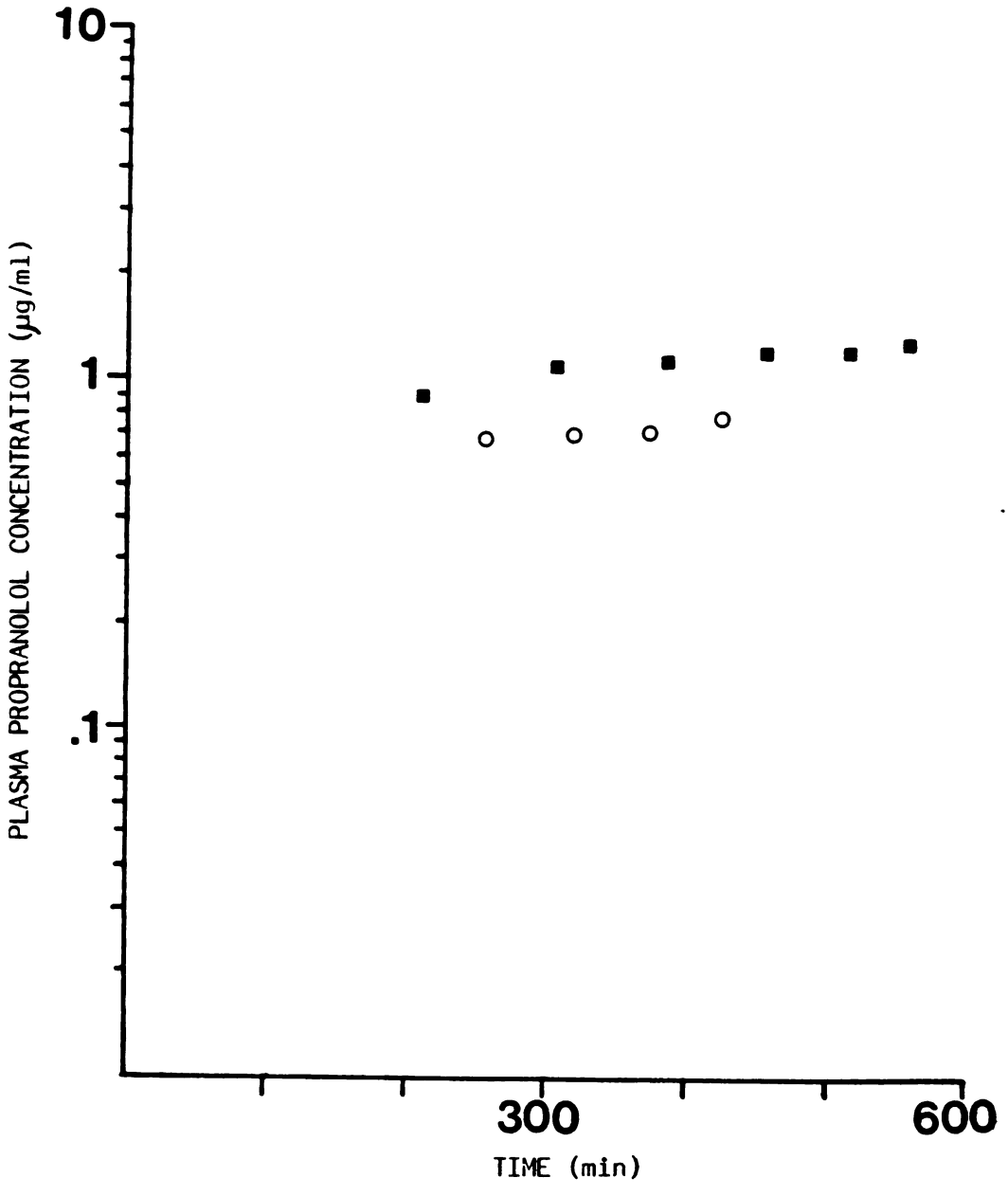


FIG VII-5. Plasma concentration-time plots of propranolol in dog C during two hind-limb propranolol infusions: 1.927 mg/min (O) and 3.129 mg/min (■).

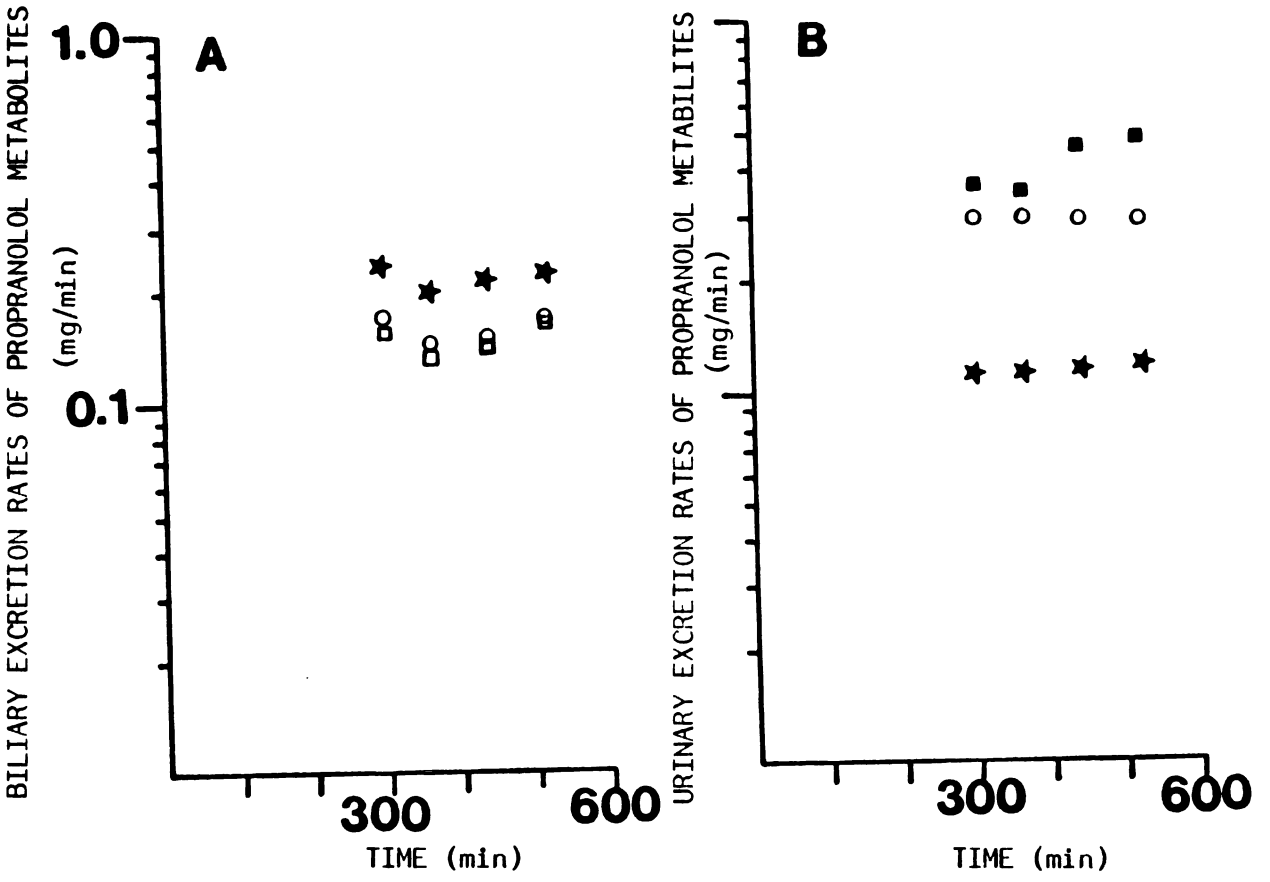


FIG VII-6. Biliary (A) and urinary (B) excretion rate-time plots of propranolol metabolites in dog A during a hind-limb propranolol infusion (2.088 mg/min) Key: Propranolol glucuronide (★); 4-hydroxypropranolol glucuronide (○); α-naphthoxylactic acid (■); propranolol glycol glucuronide (□).

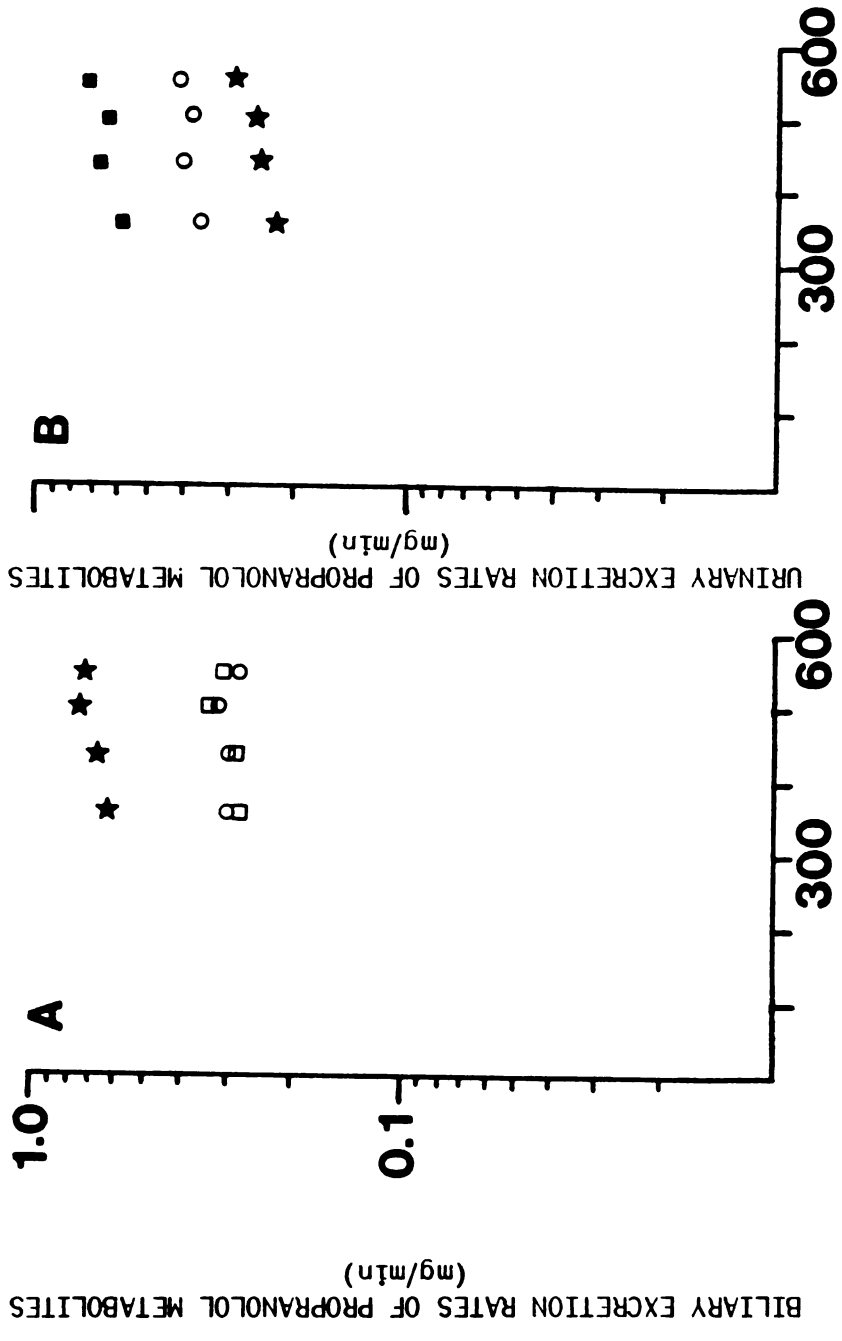


FIG VII-7. Biliary (A) and urinary (B) excretion rate-time plots of propranolol metabolites in dog A during a hind-limb infusion of propranolol (4.085 mg/min) See key of FIG VII-6 for details.

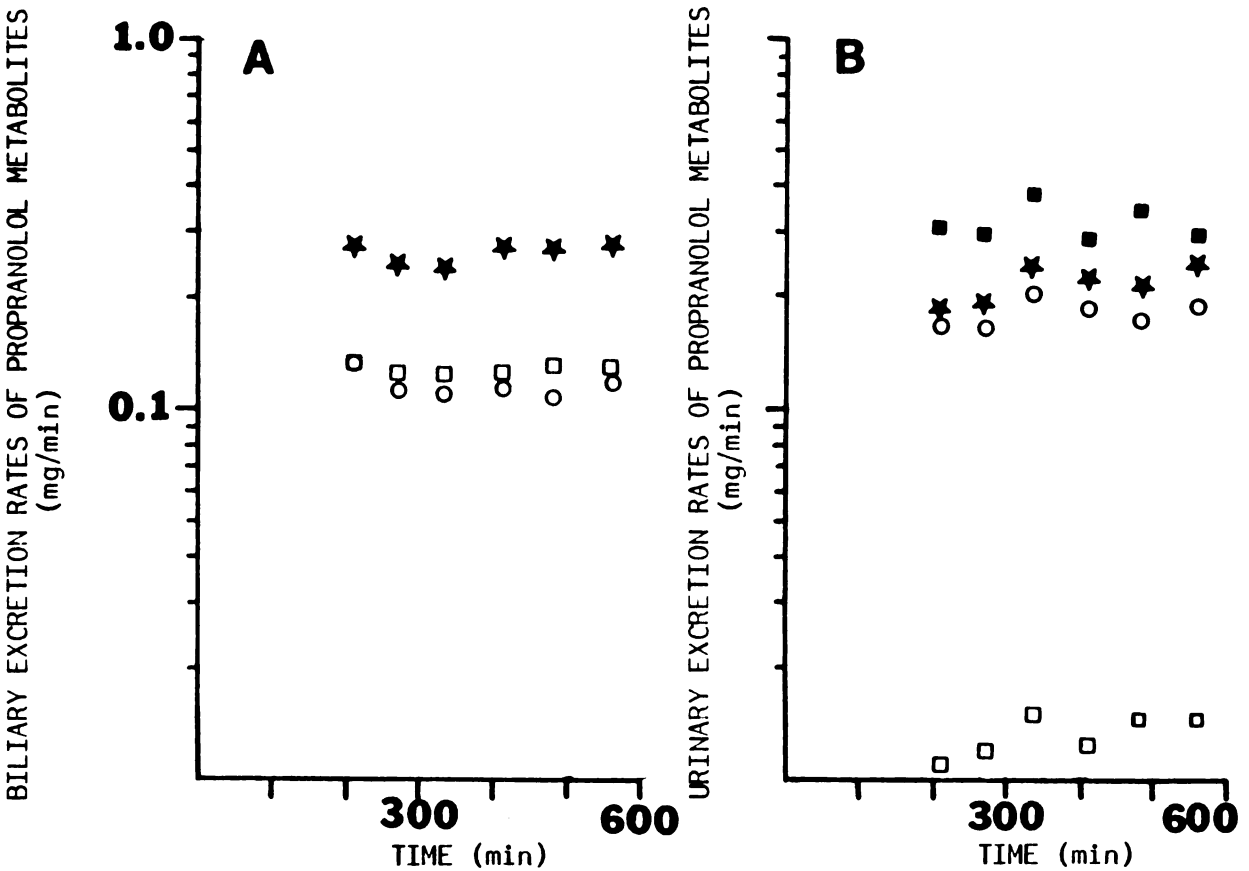


FIG VII-8. Biliary (A) and urinary (B) excretion rate-time plots of propranolol metabolites in dog B during a hind-limb infusion of propranolol (1.89 mg/min). See key of FIG VII-6 for details.

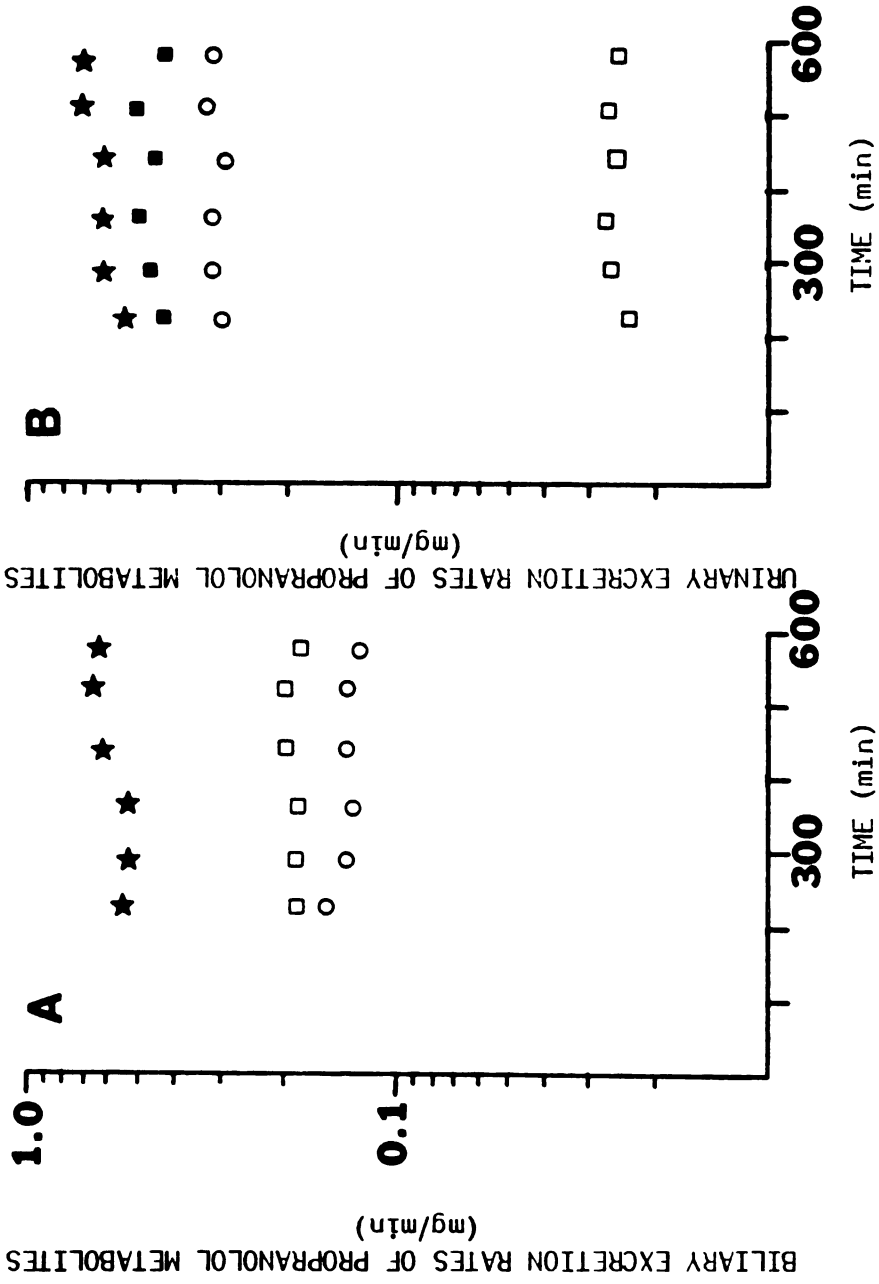


FIG VII-9. Biliary (A) and urinary (B) excretion rate-time plots of propranolol metabolites in dog B during a hind-limb propranolol infusion (3.454 mg/min). See key of FIG VII-6 for details.

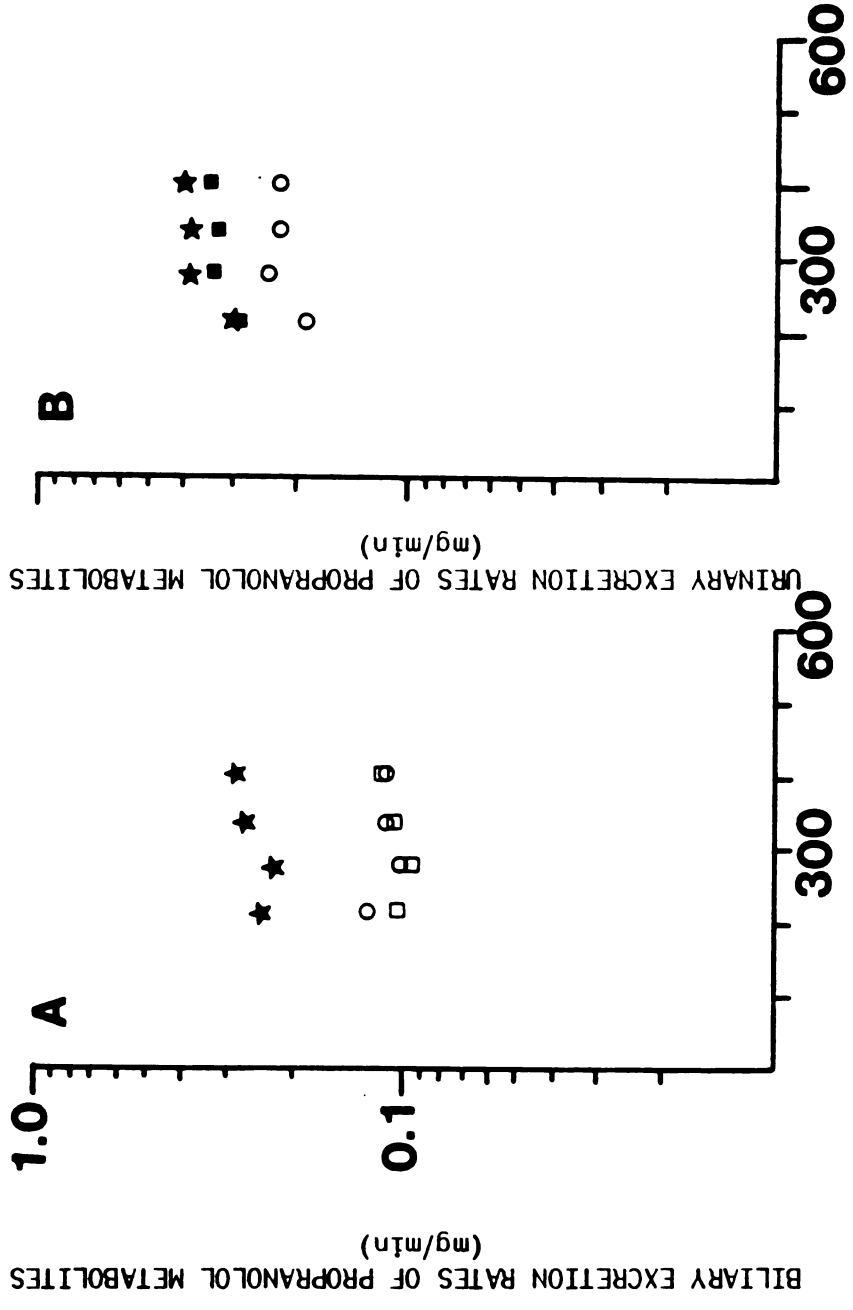


FIG VII-10. Biliary (A) and urinary (B) excretion rate-time plots of propranolol metabolites in dog C during a hind-limb propranolol infusion (1.927 mg/min). See key of FIG VII-6 for details.

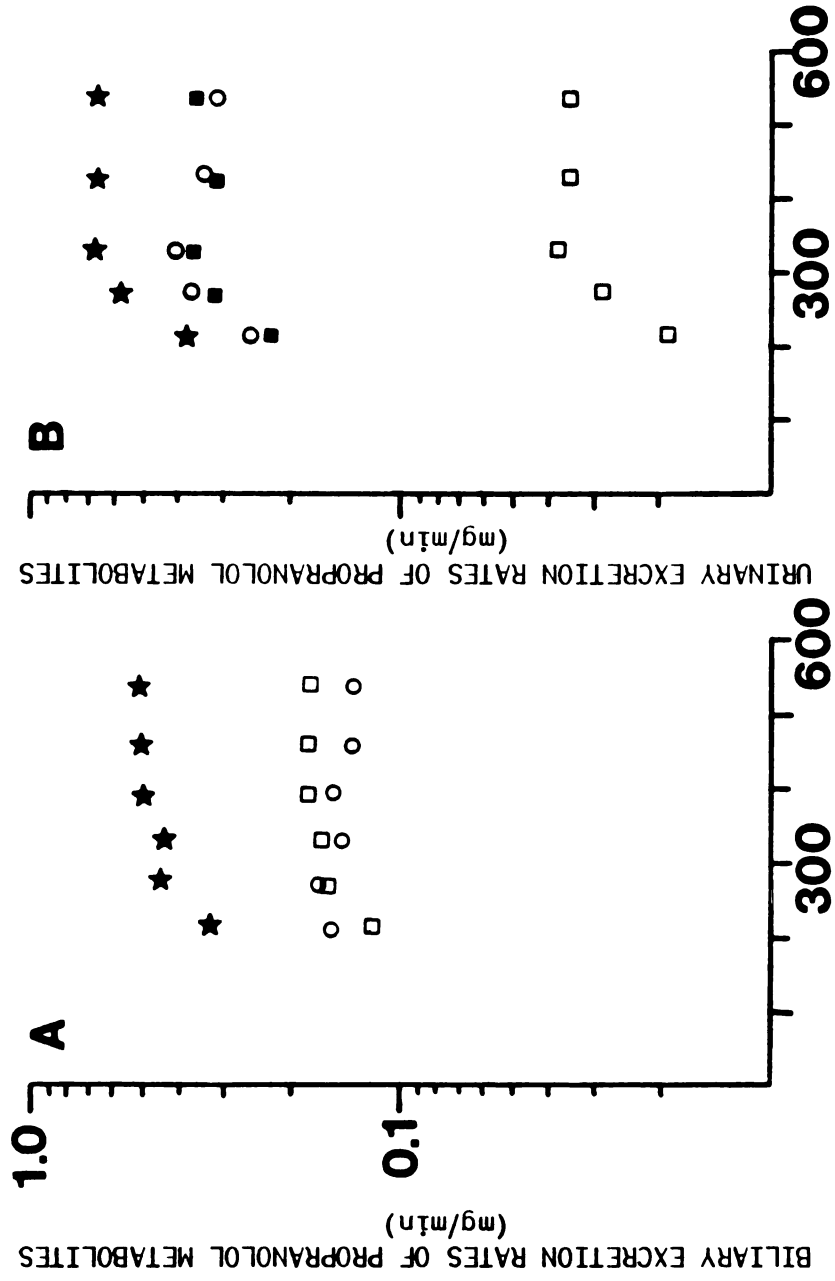


FIG VII-11. Biliary (A) and urinary (B) excretion rate-time plots of propranolol metabolites in dog C during a hind-limb propranolol infusion (3.149 mg/min). See key of FIG VII-6 for details.

TABLE VII-2. URINARY AND BILIARY STEADY-STATE EXCRETION RATES OF PROPRANOLOL METABOLITES AT VARIOUS INFUSION RATES OF PROPRANOLOL IN DOGS A, B, AND C.

Inf Rate (mg/min)	$C_{prop,ss}$ plasma (ng/ml)	Urinary NLA (µg/min)	Gly-G (µg/min) Urine	Gly-G (µg/min) Bile	Prop-G (µg/min) Urine	Prop-G (µg/min) Bile	4-OH-P-G (µg/min) Urine	4-OH-P-G (µg/min) Bile
				DOG A				
0.9235	170	136	-	46	27.3	52	107	80
1.4183	470	320	-	96	76	135	185	125
2.0879	780	490	-	160	115	230	290	165
3.0306	1200	520	-	260	155	420	310	225
4.0852	1800	760	-	320	300	740	420	300
6.272	3000	1050	30	350	620	1200	540	340
				DOG B				
1.012	400	200	9	71	115	89	92	63
1.89	1200	320	15	130	240	275	190	115
2.656	1700	460	28	175	430	400	250	130
3.4535	2500	480	27	195	700	650	310	135
				DOG C				
0.998	400	155	8.5	57	96	80	120	82
1.927	780	345	22	120	400	300	235	120
3.149	1250	360	37	180	680	510	350	140
4.28	1700	450	45	270	1050	860	440	175

prop= propranolol; Prop-G= propranolol glucuronide; 4-OH-P-G= 4-hydroxypropranolol glucuronide; NLA= α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide

This may be due to the larger molecular weight of the glucuronide of propranolol glycol. The glucuronides of propranolol and 4-hydroxypropranolol were excreted in both urine and bile. There was always more 4-hydroxypropranolol glucuronide present in urine than in bile. The percent of dose excreted as the various metabolites in urine and bile as the propranolol was increased for all three dogs is summarized in Table VII-3 and in Figure VII-12. It can be seen that the percent recovered as α-naphthoxylactic acid, propranolol glycol glucuronide, and 4-hydroxypropranolol glucuronide all show a decreasing trend as the dose of propranolol was increased. Propranolol glucuronide showed the reverse trend, namely the percent recovered kept on rising with increasing dose. This information suggests that the pathways of formation of α-naphthoxylactic acid, propranolol glycol, and 4-hydroxypropranolol are saturable whereas propranolol glucuronide formation is first-order.

Elimination clearances of the metabolites: Plots of the steady-state excretion rates of the various metabolites in bile and urine versus their plasma concentrations at different propranolol steady-state infusions were shown in Figure VII-13. The fact that linear relationships were observed indicates that the elimination of the metabolites obeys first-order kinetics. This is important to our analysis of the data because the assumption was made that all secondary metabolic processes after the formation of M_i from propranolol were linear, which of course includes the elimination of M_i'.

The α-naphthoxylactic acid metabolite exhibits some tubular reabsorption phenomena at plasma concentrations below 10 μg/ml. Fortunately, all the steady-state studies were in the linear elimination range of α-naphthoxylactic acid. Even though plasma metabolites were

TABLE VII-3. PERCENT OF DOSE EXCRETED AS THE VARIOUS METABOLITES IN URINE AND BILE IN DOGS.

Inf Rate (mg/min)	NLA	Gly-G		NLA+ Gly-G	Prop-G			4-OH-P-G			All Four Metabolites		
	Urine	Urine	Bile	Urine+Bile	Urine	Bile	Total	Urine	Bile	Total	Urine	Bile	Total
						DOG A							
0.9235	16.47	-	5.93	22.4	2.96	5.63	8.6	10.92	8.16	19.1	30.35	19.72	50.1
1.4183	25.22	-	8.05	33.3	5.36	9.52	14.9	12.29	8.30	20.6	42.87	25.87	68.7
2.0879	26.23	-	9.12	35.3	5.51	11.02	16.5	13.08	7.44	20.5	44.82	27.58	72.4
3.0306	19.17	-	10.2	29.4	5.11	13.86	19.0	9.63	6.99	16.6	33.91	31.05	65
4.0852	20.79	-	9.32	30.1	7.34	18.11	25.5	9.68	6.92	16.6	37.81	34.35	72.2
6.272	18.71	0.57	6.64	25.9	9.89	19.13	29.0	8.11	5.11	13.2	37.28	30.88	68.1
						DOG B							
1.012	22.09	1.06	8.34	31.5	11.36	8.79	20.2	8.56	5.86	14.4	43.07	22.99	66
1.89	18.92	0.94	8.18	28.0	12.7	14.55	27.2	9.47	5.73	15.2	42.03	28.46	70.5
2.656	19.36	1.25	7.84	28.5	16.19	15.06	31.3	8.87	4.61	13.5	45.67	27.51	73.2
3.4535	15.53	0.93	6.72	23.2	20.27	18.82	39.1	8.45	3.68	12.1	45.18	29.22	74.4
						DOG C							
0.998	17.36	1.01	6.79	25.1	9.62	8.02	17.6	11.32	7.74	19.1	39.31	22.55	62
1.927	20.01	1.36	7.41	28.8	20.76	15.57	36.3	11.49	5.87	17.4	53.62	28.85	82.5
3.149	12.78	1.4	6.8	21.0	21.59	16.20	37.8	10.47	4.19	14.7	46.24	27.19	73.4
4.28	11.75	1.25	7.5	20.5	24.53	20.09	44.6	9.68	3.85	13.5	47.21	31.44	79

Prop-G= propranolol glucuronide; 4-OH-P-G= 4-hydroxypropranolol glucuronide

NLA=α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide

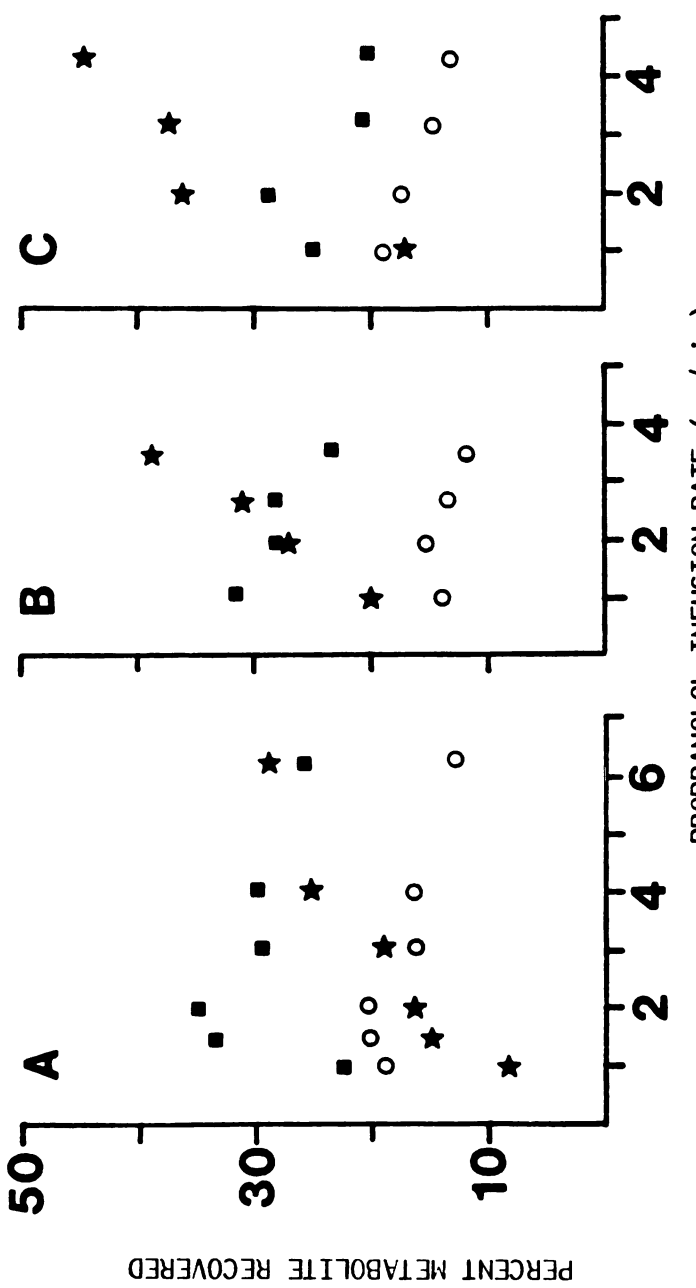


FIG VII-12. Percent metabolite recovered in dogs A, B, and C at various rates of infusion of propranolol.

Key: Propranolol glucuronide (★); 4-hydroxypropranolol glucuronide (○); α-naphthoxylactic acid + propranolol glycol glucuronide (■).

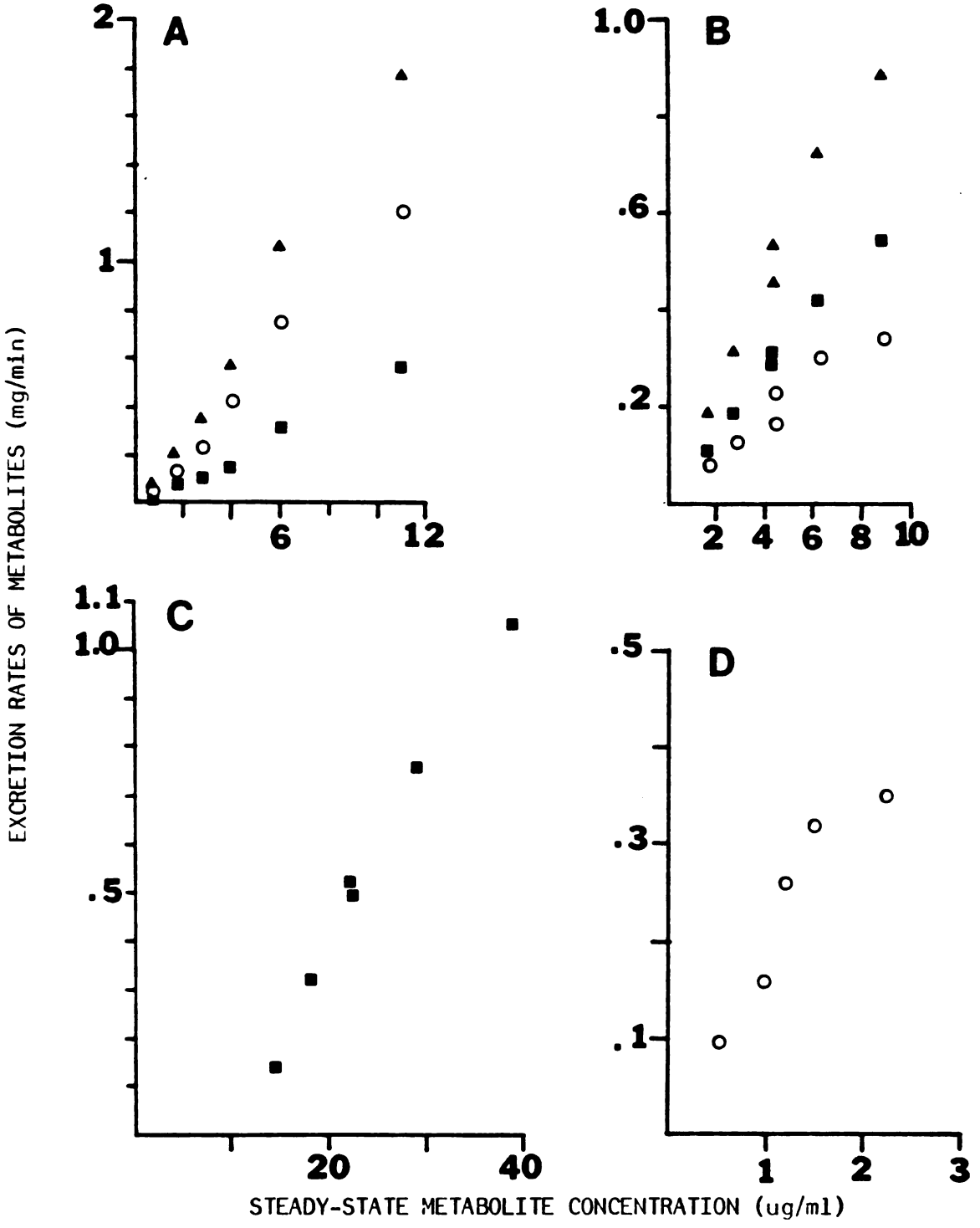


FIG VII-13. Biliary (○), urinary (■), and total (▲) excretion rates of metabolites versus steady-state plasma metabolite concentrations in dog A. (A)= propranolol glucuronide; (B)= 4-hydroxypropranolol glucuronide; (C)= α-naphthoxylactic acid; (D)= propranolol glycol glucuronide.

not measured in steady-state experiments in dogs B and C, a subsequent single dose study in dog B confirmed linear elimination of the metabolites.

Data treatments: In order to convert the data to the form where Equation VII-6 can be applied, two manipulations are necessary. First, the plasma concentration of propranolol needed to be converted to blood concentration. Due to the variable blood/plasma ratios, an INTERPOLATE* computer program was used to convert plasma concentration values to their equivalent blood concentrations using the blood and plasma concentration data listed in Table VII-1. The interpolated steady-state blood propranolol concentrations are shown in Table VII-4.

Second, both the excretion rates of the metabolites and the propranolol blood concentration had to be converted to molar units, namely, mole/unit time and mole/unit volume. Using the following molecular weights of propranolol and the metabolites (molecular weights: α-naphthoxylactic acid, 232; propranolol glycol, 218; propranolol, 259.3, and 4-hydroxypropranolol, 275.3), the excretion rates were converted to μmole/min and propranolol concentration to μmole/ml.

Table VII-5 shows the total steady-state excretion rates of the metabolites of propranolol versus the steady-state blood propranolol concentrations in the correct units. Plots of the data are shown in Figure VII-14. If the $K_{m,f_{M_i}}$ of a metabolic process is much less than $C_{b,prop,ss}$, the enzyme system involved will be operating under first order conditions and a linear relationship will be observed between

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* The program INTERPOLATE is a public procedure in the PROPHET system and is used to calculate function values for functions which are not expressed in closed form. Given a set of independent variable values and a corresponding set of function values, INTERPOLATE forces a cubic spline function through these data pairs and then uses this spline function to calculate estimates of the dependent variable for each new value of the independent variable (Perry and Kuhlmann, 1979).

TABLE VII-4. INTERPOLATED STEADY-STATE BLOOD PROPRANOLOL CONCENTRATIONS FROM PLASMA DATA USING THE INTERPOLATE COMPUTER PROGRAM.

	Steady State Plasma Conc (ng/ml)	Interpolated Steady State Blood Conc (ng/ml)
Dog A	170	126
	470	361
	780	616
	1200	961
	1800	1597
	3000	3398
Dog B	400	370
	1200	1149
	1700	1732
	2500	3320
Dog C	400	557
	780	1224
	1250	2129
	1700	3195

TABLE VII-5. TOTAL STEADY-STATE EXCRETION RATES OF PROPRANOLOL METABOLITES VERSUS STEADY-STATE PROPRANOLOL BLOOD CONCENTRATIONS.

$C_{b,prop,ss}$ (μmole/ml)	Total Steady-State Excretion Rates (μmole/min)		
	NLA + Gly-G	Prop-G	4-OH-P-G
	DOG A		
4.85×10^{-4}	0.80	0.31	0.68
13.9×10^{-4}	1.82	0.81	1.13
23.8×10^{-4}	2.85	1.33	1.65
37.1×10^{-4}	3.43	2.22	1.94
61.6×10^{-4}	4.74	4.01	2.62
131.1×10^{-4}	6.27	7.02	3.20
	DOG B		
14.3×10^{-4}	1.23	0.79	0.56
44.3×10^{-4}	2.04	1.99	1.11
66.8×10^{-4}	2.91	3.20	1.38
128.0×10^{-4}	3.09	5.21	1.62
	DOG C		
21.5×10^{-4}	0.97	0.68	0.73
47.2×10^{-4}	2.14	2.70	1.29
82.1×10^{-4}	2.55	4.59	1.78
123.2×10^{-4}	3.39	7.37	2.23

prop= propranolol; Prop-G= propranolol glucuronide;
4-OH-P-G= 4-hydroxypropranolol glucuronide;
NLA= α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide.

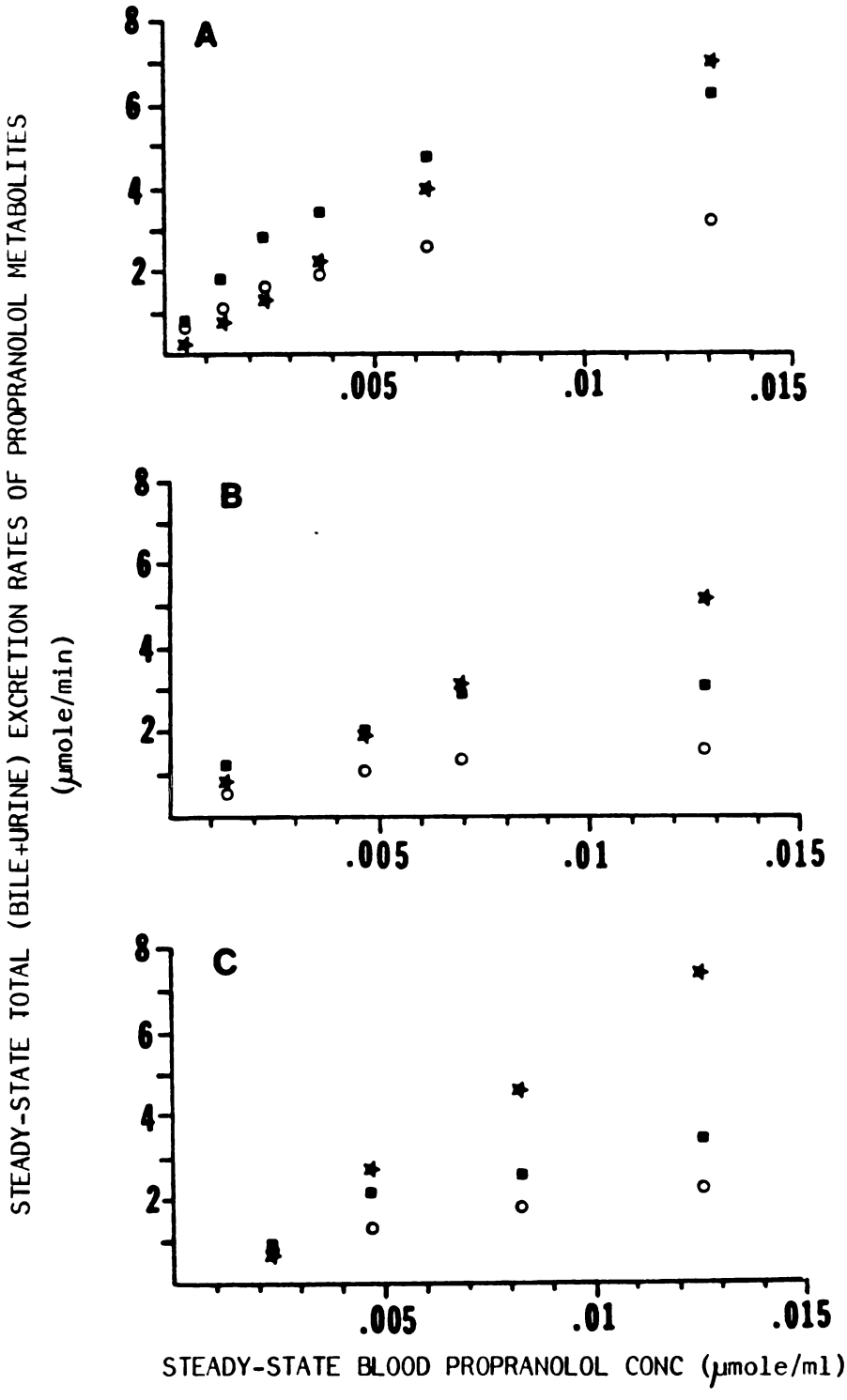

FIG VII-14. Steady-state excretion rates (bile+urine) of propranolol metabolites versus propranolol blood concentration in dogs A, B, and C.

Key: Propranolol glucuronide (★); 4-hydroxypropranolol glucuronide (○); α-naphthoxylactic acid + propranolol glycol glucuronide (■).

the (total excretion rate of $M_i')_{ss}$ and $C_{b,prop,ss}$. This is what is observed with propranolol glucuronide. If the metabolic pathway is capacity-limited (obeying Michaelis-Menten kinetics), then a hyperbolic relationship will be obtained as shown by the other three metabolites: α-naphthoxylactic acid, propranolol glycol, and 4-hydroxypropranolol.

The excretion rates and propranolol blood concentrations were then fitted to the Michaelis-Menten equation (see Equation VII-6) using the public procedure HYPERBOLIC* in the PROPHET system. Plots of the fit are shown in Figure VII-15 and 16 using the data from Table VII-5. The excretion rates of α-naphthoxylactic acid and propranolol glycol glucuronide were combined together since they arise from the same pathway, N-dealkylation. A summary of the fitted V_{max} and K_m for both the N-dealkylation and aromatic ring hydroxylation metabolic pathways giving rise to (α-naphthoxylactic acid + propranolol glycol) and 4-hydroxypropranolol are shown in Table VII-6. The propranolol glucuronidation pathway was simply fitted to a least-square linear regression equation. The K_m values ranged in magnitude from 0.00339 to 0.00983 μmole/ml. This corresponds to 0.88 to 2.5 μg/ml blood concentrations of propranolol. These K_m values are quite high compared to that in man which are in the range of 12 to 146 ng/ml (Silber *et al*, 1981). Thus, relatively high doses of propranolol are needed to partially saturate the enzymes responsible for propranolol's metabolism in this species. The excretion

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*The program HYPERBOLIC fits data to the enzyme equation v = Vmax · S/(Km + S). In this case, v is simply the steady-state excretion rate of the metabolites and (S) is the steady-state blood concentration of propranolol. The fit is carried out iteratively using a Taylor expansion of the nonlinear parameter Km (Baig and Reid-Miller, 1980a)

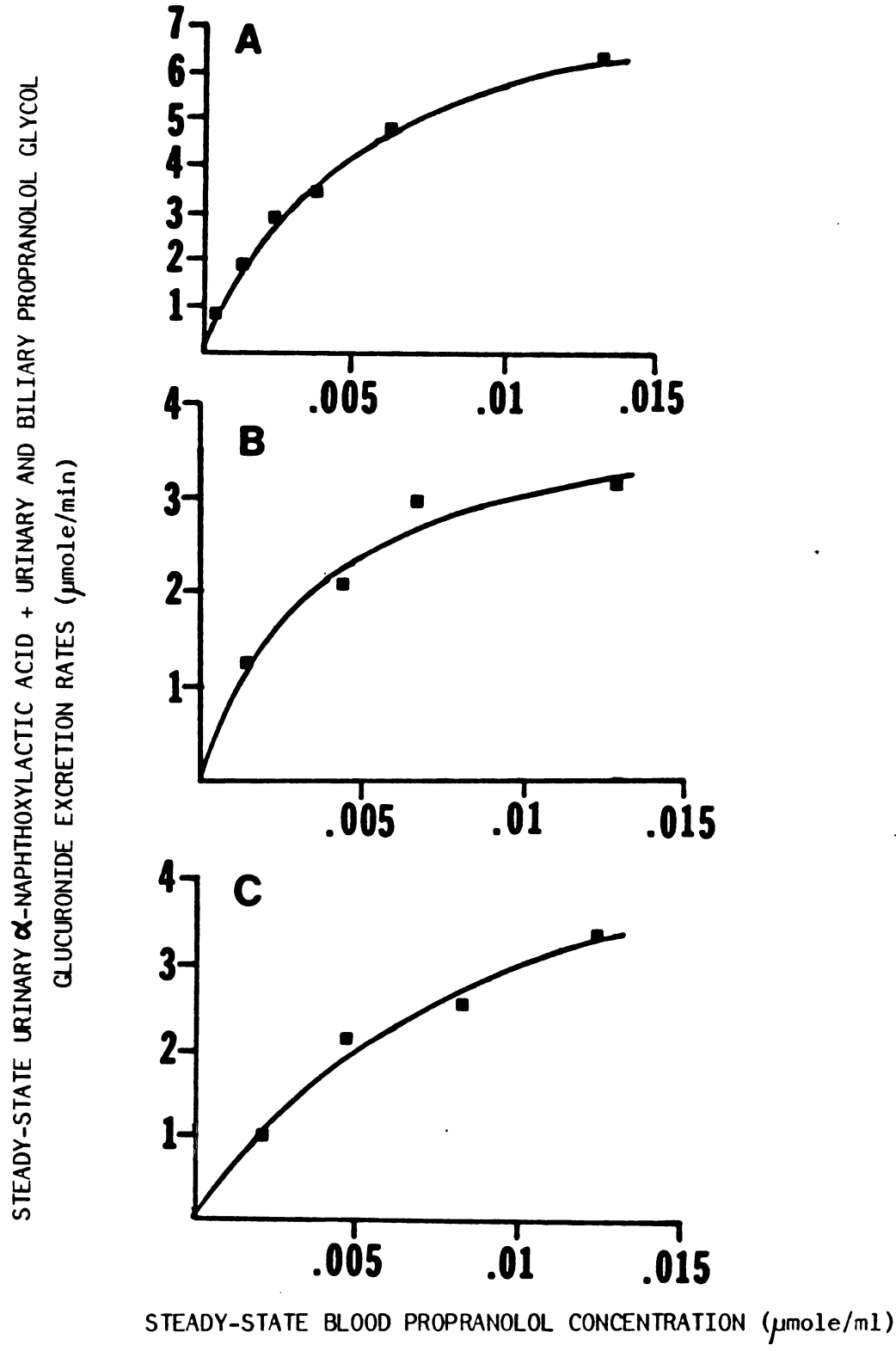

FIG VII-15. Total (bile+urine) excretion rates of α-naphthoxylactic acid and propranolol glycol glucuronide versus steady-state blood propranolol concentration in dogs A, B and C. The sum of α-naphthoxylactic acid and propranolol glycol glucuronide are presumed to be equivalent to the rate of formation of the precursor N-desisopropylpropranolol.

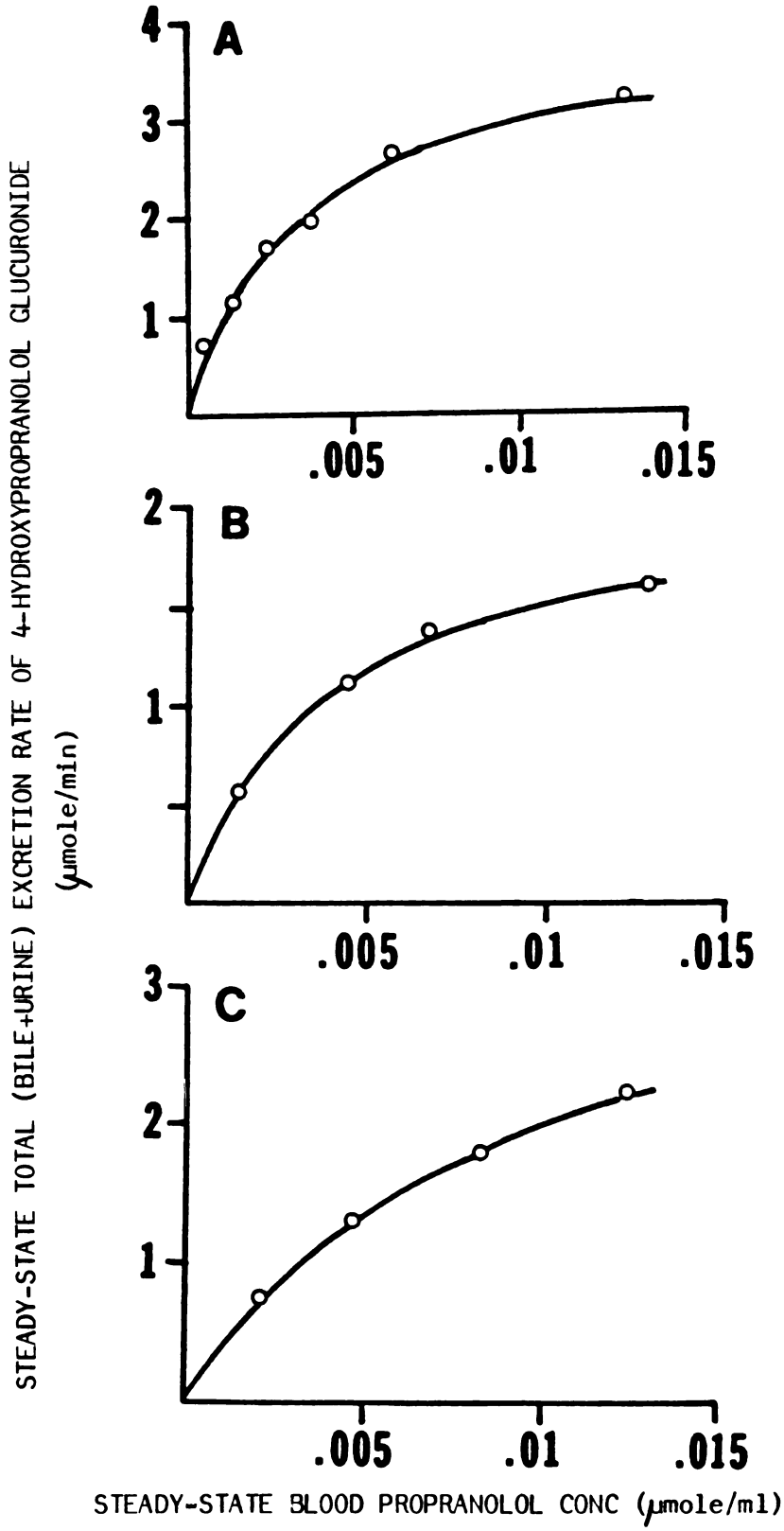

FIG VII-16. Steady-state total (bile+urine) excretion rates of 4-hydroxypropranolol glucuronide versus steady-state blood propranolol concentration.

TABLE VII-6. FITTED PHARMACOKINETIC PARAMETERS DESCRIBING THE HEPATIC ELIMINATION OF PROPRANOLOL IN DOGS.

	NLA + Gly-G		4-OH-P-G		Prop-G
	$Vmax_f$ (±S.E.) μmole/min	Km_f (±S.E.) μmole/ml	$Vmax_f$ (± S.E.) μmole/min	Km_f (± S.E.) μmole/ml	CL_f ml/min
Dog A	8.78 ± 0.31	0.00531 ± 0.00040	4.0 ± 0.22	0.00342 ± 0.00044	545
Dog B	4.0 ± 0.54	0.00339 ± 0.00127	2.13± 0.08	0.00394 ± 0.00040	406
Dog C	5.99 ± 1.18	0.00982 ± 0.00337	3.98± 0.18	0.00983 ± 0.00082	611

$Vmax_f$ and Km_f are the Michaelis-Menten enzyme parameters for the formation of the metabolites from propranolol; CL_f is the formation clearance of the metabolite from propranolol.
Prop-G= propranolol glucuronide; 4-OH-P-G= 4-hydroxypropranolol glucuronide;
NLA =α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide.

rates for α-naphthoxylactic acid and propranolol glycol glucuronide were also fitted separately to the Michaelis-Menten equation in dogs A and B. Similar K_m values were obtained compared to those from fitting the combined data. The V_{max} values also added up to the combined V_{max} (see Table VII-7). The individual values of V_{max} were used later in a single dose study to simulate data for α-naphthoxylactic acid and propranolol glycol glucuronide separately.

Fitting the unidentified metabolite(s) of propranolol: Approximately 70% of the metabolism of propranolol was accounted for by the metabolites measured in the present study (see Table VII-3). For modelling purposes, the rest of the metabolism was modelled using the data in Table VII-8 and the various fitted pharmacokinetic parameters of the known pathways by presuming that a single process occurred which obeyed either linear kinetics or as a saturable process according to Equation VII-7. A public procedure in the PROPHET system, FITFUN*, was used in the fitting of the data. The fitted data are shown in Figures VII-17 through 19 and the parameter estimates tabulated in Table VII-9. It can be seen that the data appear to be fitted better when the unknown pathway is described by a Michaelis-Menten process than by a linear process in dogs A and B. In dog C, it appears that either process can describe the data. The Akaike information criterion (Akaike, 1973; Akaike, 1976; Yamaoka *et al*, 1978) can be used to test models where the number of parameters differ. The smaller value indicates the better fitted model. The results, shown in columns 5 and 8 of Table VII-9, indicate that the Michaelis-Menten

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*FITFUN is an iterative method using the Marquardt-Levenberg approach to convergence. It combines the advantages of the Gauss-Newton and the steepest descent methods (Baig and Reid-Miller, 1980b).

TABLE VII-7. COMPARISON OF $Vmax_f$ and Km_f VALUES OF α-NAPHTHOXY-LACTIC ACID AND PROPRANOLOL GLYCOL WHEN FITTED TOGETHER OR FITTED SEPARATELY.

		$Vmax_f$ (μmole/min)	Km_f (μmole/ml)
DOG A	NLA + Gly-G	8.78	0.00531
	NLA	6.31	0.00550
	Gly-G	2.48	0.00486
DOG B	NLA + Gly-G	4.00	0.00339
	NLA	2.64	0.00314
	Gly-G	1.36	0.00395

$Vmax_f$ and Km_f are the Michaelis-Menten enzyme parameters for the formation of the metabolites from propranolol.
NLA= α-naphthoxylactic acid
Gly-G= propranolol glycol glucuronide

TABLE VII-8. PROPRANOLOL INFUSION RATES AND STEADY-STATE BLOOD CONCENTRATIONS USED IN MODELLING THE FOURTH UNKNOWN PATHWAY.

	Propranolol Infusion Rate (μmole/min)	Steady-State Blood Propranolol Conc (μmole/ml)
DOG A	3.56	0.000485
	5.47	0.00139
	8.052	0.00238
	11.69	0.00371
	15.755	0.00616
	24.19	0.01311
DOG B	3.903	0.00143
	7.29	0.00443
	10.24	0.00668
	13.32	0.0128
DOG C	3.849	0.00215
	7.432	0.00472
	12.14	0.00821
	16.51	0.01232

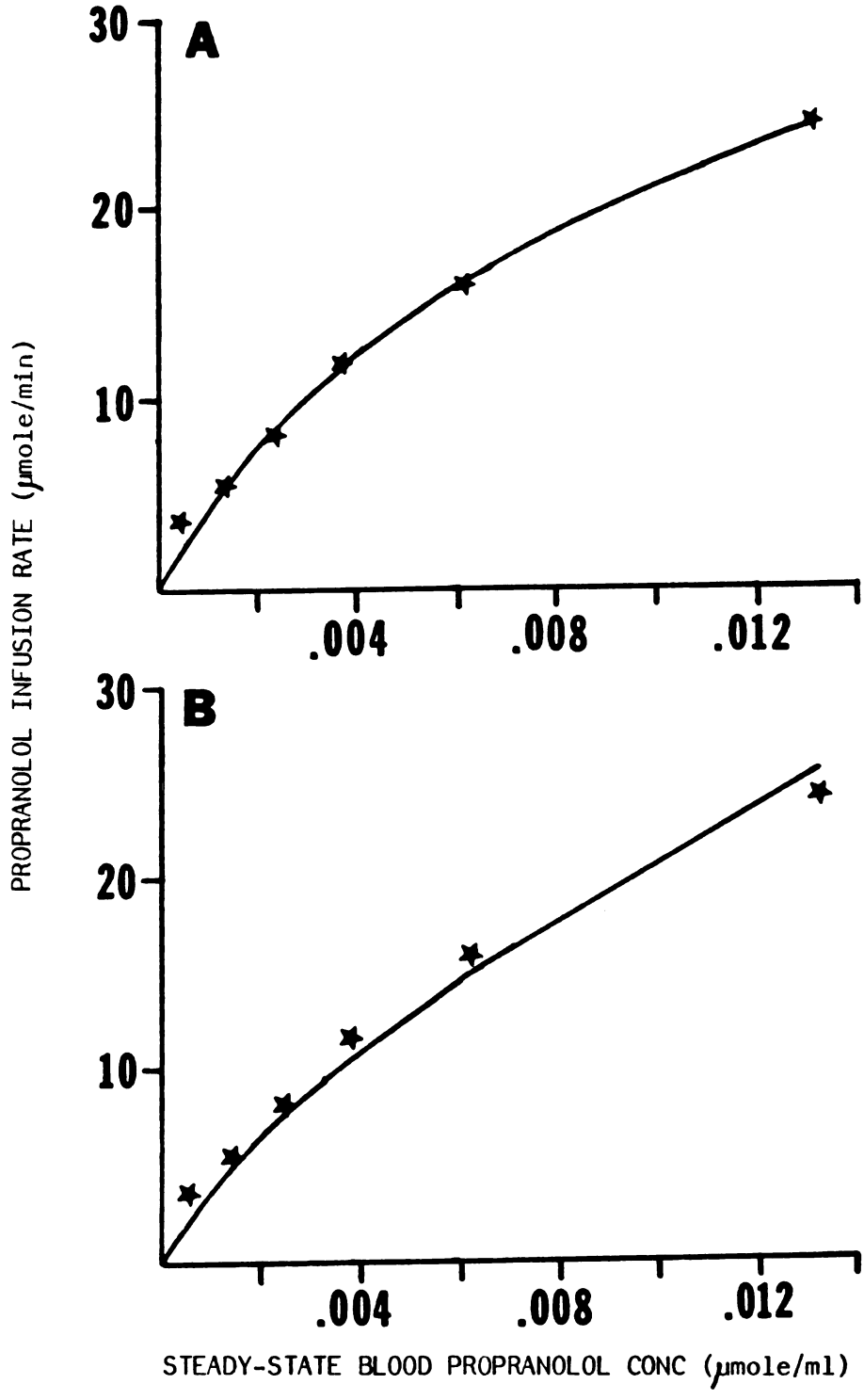

FIG VII-17. The solid lines represent computer fits where all the known clearance parameters of elimination are fixed while the unknown pathway is defined in FIG (A) as obeying Michaelis-Menten kinetics and FIG (B) linear kinetics for dog A.

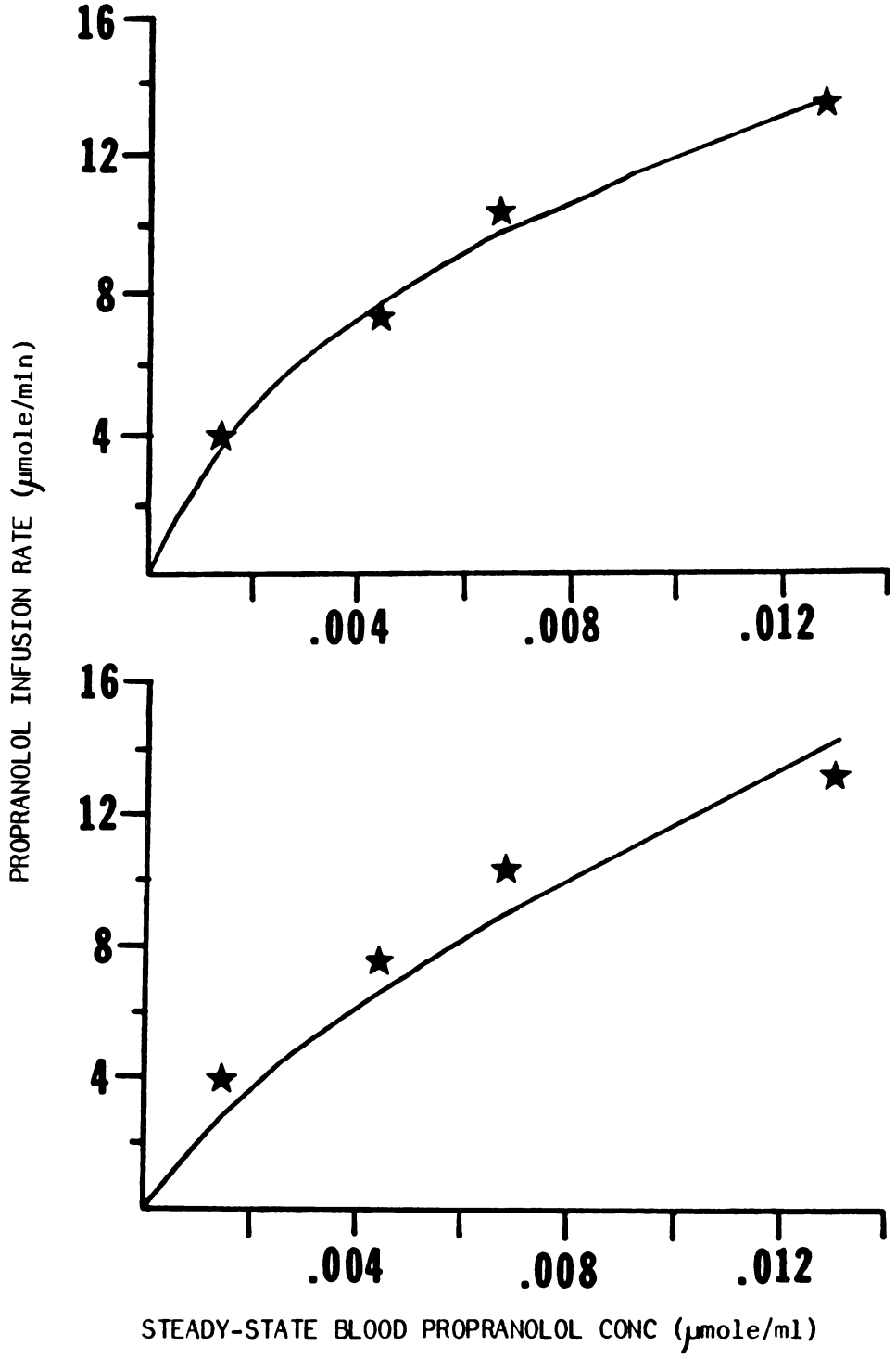

FIG VII-18. The solid lines represent computer fits where all the known clearance parameters of elimination are fixed while the unknown pathway is defined in FIG (A) as obeying Michaelis-Menten kinetics and FIG (B) linear kinetics for dog B.

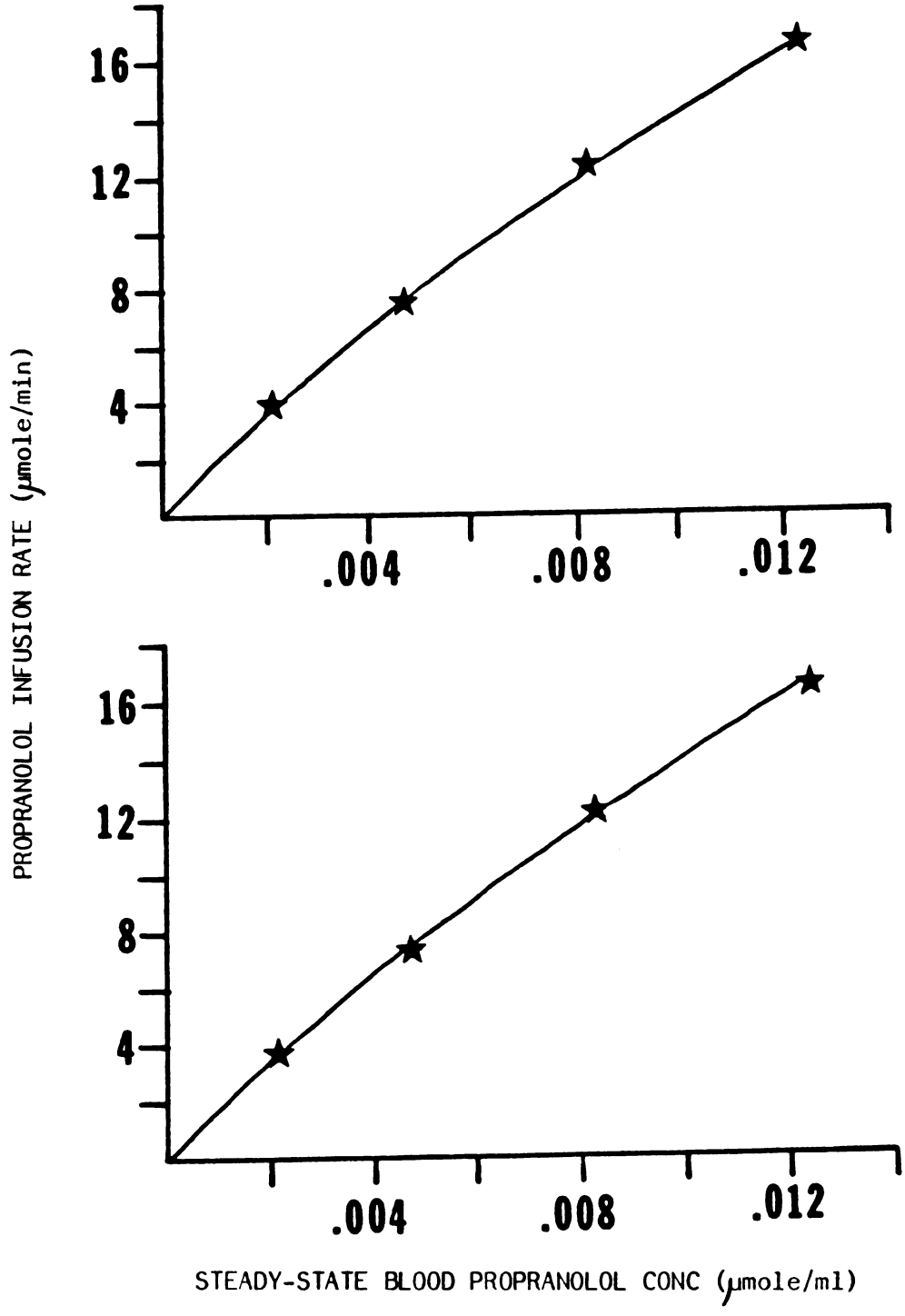

FIG VII-19. The solid lines represent computer fits where all the known clearance parameters of elimination are fixed while the unknown pathway is defined in FIG (A) as obeying Michaelis-Menten kinetics and FIG (B) linear kinetics for dog C.

TABLE VII-9. CHARACTERISTICS OF THE FOURTH UNKNOWN PATHWAY WHEN FITTED AS EITHER A MICHAELIS-MENTEN OR A LINEAR PROCESS.

	Michaelis-Menten Process				Linear Process		
	$Vmax_f$(±SD) μmole/min	Km_f(±SD) μmole/ml	RSS[a]	AIC[b]	CL_f ml/min	RSS[a]	AIC[b]
DOG A	11.98± 3.80	0.0078± 0.0047	2.083	9.137	671±36	8.844	17.26
DOG B	4.21± 1.56	0.00274±0.00316	0.5887	1.351	338±25	4.158	9.125
DOG C	23.16±18.05	0.0697±0.0620	0.04986	-10.99	290±3	0.07839	-10.73

a) RSS= Residual Sum of Squares.

b) AIC= Akaike Information Criterion = N x ln(RSS) + 2p, where N is the number of observations and p the number of parameters.

model is the better model for all three dogs although the observed difference in dog C is minimal.

Single dose studies: Using all the parameters describing the hepatic metabolism of propranolol to various metabolites estimated in the steady-state experiments, prediction of the metabolite concentration-time profiles following a short hind-limb infusion to the liver was attempted. Some parameters were still unknown including the volumes of distribution of the metabolites and the tissue distribution rate constants of propranolol following a dose to the liver. Thus some arbitrary assignment of parameters was necessary.

The experimentally-determined blood concentration-time profiles of propranolol and its metabolites following a short hind-limb infusion in dogs A and B are summarized in Tables VII-10 and 11. The fitting of the data was approached as follows:

1. Elimination clearances of the metabolites were obtained in both bile and urine and summed to give total elimination clearances. The excretion rates and the blood concentrations at the mid-point of the determination of the excretion rate of the metabolites were tabulated as shown in Table VII-12 and 13 for both dogs. The data are plotted in Figures VII-20 and 21. It can be seen that the elimination clearances are shown in Table VII-14; they vary from 46 to 341 ml/min.
2. Differential equations describing the rate of change of propranolol and its metabolites' blood concentrations were defined. The experimentally-determined parameter values and the fitted (unknown) pathway parameters listed in Table VII-14 are included as constants in these equations. The resultant set of differential equations are summarized in Table VII-15 and 16 for dogs A and B, respectively.

TABLE VII-10. BLOOD CONCENTRATION-TIME PROFILES OF PROPRANOLOL AND METABOLITES AFTER A SHORT 1.17 GM PROPRANOLOL HIND-LIMB INFUSION IN DOG A.

TIME[a] (min)	Propranolol and Metabolites Blood Conc in μmole/ml				
	$C_{b,Prop}$	$C_{b,Prop-G}$	$C_{b,NLA}$	$C_{b,Gly-G}$	$C_{b,4-OH-P-G}$
0	.0121	.01333	.0384	.00618	.0075
15.1	.00938	.01396	.0393	.00596	.0088
56	.00662	.01331	.0467	.0056	.0102
95.3	.00469	.0117	.0473	.00441	.00992
137	.00406	.00888	.0455	.00353	.00871
179	.00328	.00728	.0443	.00286	.0081
218	.00223	.00575	.0423	.002	.00721
256	.00147	.00472	.038	.0017	.00635
303	.0011	.0035	.0332	.00107	.00534
339	.000883	.00292	.0308	.000872	.00471
378	.000602	.00217	.0257	.000546	.00405
417	.000447	.00166	.0205	.000417	.00342
456	.000351	.00126	.0167	.000275	.0028

a) Time at the end of the infusion.

Prop= propranolol; Prop-G= propranolol glucuronide;
4-OH-P-G= 4-hydroxypropranolol glucuronide
NLA= α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide

TABLE VII-11. BLOOD CONCENTRATION-TIME PROFILES OF PROPRANOLOL AND METABOLITES AFTER A SHORT 0.73 GM PROPRANOLOL HIND-LIMB INFUSION IN DOG B.

TIME[a] (min)	Propranolol and Metabolites Blood Conc in μmole/ml				
	$C_{b,Prop}$	$C_{b,Prop-G}$	$C_{b,NLA}$	$C_{b,Gly-G}$	$C_{b,4-OH-P-G}$
0	.0108	.0121	.0174	.00356	.0042
16.9	.00848	.0151	.0179	.00372	.00501
57	.00557	.0146	.0176	.00367	.00545
97.3	.00433	.0112	.0154	.00306	.0052
139.7	.0036	.0088	.014	.00284	.00446
181.7	.00284	.00668	.0122	.00219	.00408
220.7	.00239	.00527	.0109	.00171	.00377
259.7	.00202	.00422	.0106	.00151	.0034
305.7	.00182	.00337	.00989	.00123	.00288
340.7	.00156	.0028	.00932	.00109	.00257
380.7	.00132	.00228	.00803	.000872	.0023
418.7	.00113	.00169	.00645	.000734	.00211
458.7	.000895	.00138	.00559	.000592	.00188

a) Time at the end of the infusion.

Prop= propranolol; Prop-G= propranolol glucuronide
4-OH-P-G= 4-hydroxypropranolol glucuronide
NLA= α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide

TABLE VII-12. EXCRETION RATES OF THE METABOLITES VERSUS BLOOD CONCENTRATIONS OF THE METABOLITES AT T_{mid} IN DOG A GIVEN A SHORT HIND-LIMB INFUSION OF 1.17 GM PROPRANOLOL.

B I L E							U R I N E								
	Prop-G		4-OH-P-G		Gly-G			Prop-G		4-OH-P-G		Gly-G		NLA	
T_{mid} a)	dM/dt b)	$C_{b,M}$ c)	dM/dt	$C_{b,M}$	dM/dt	$C_{b,M}$	T_{mid}	dM/dt	$C_{b,M}$	dM/dt	$C_{b,M}$	dM/dt	$C_{b,M}$	dM/dt	$C_{b,M}$
100.2	478.5	3.023	270.1	2.73	272.6	0.962	122.1	288.2	2.65	304.1	2.6	17.8	0.86	492.7	10.8
139.5	345.1	2.34	214.6	2.4	213.1	0.79	184	219.5	1.89	280.6	2.23	13.5	.624	450.9	10.28
181.3	299.3	1.9	202.9	2.25	188.9	0.63	224	172.7	1.49	259	1.99	10.3	.437	408.8	9.8
221.3	251.8	1.5	196.2	2.0	161.5	0.44	303	91.1	0.94	167.5	1.49	4.57	.24	288.3	7.9
260	187.2	1.22	158.5	1.75	115.6	0.37	382	59.6	0.562	136.8	1.11	2.99	.119	274.8	5.9
300	141.5	0.96	127.9	1.5	86.8	0.25	422	39.9	0.43	112.8	0.94	1.89	.091	206.4	4.75
340	117.9	0.76	118.7	1.32	70.3	0.195	463	30.99	0.325	90.56	0.76	1.08	.059	166.1	3.8
380	95.8	0.58	107.7	1.13	55.1	0.121									
417	72.5	0.45	87.9	0.96	41.9	0.094									

a) Time in min after the end of the infusion

b) dM/dt = excretion rate in ug/min

c) $C_{b,M}$ = blood concentration of the metabolites in ug/ml

Prop-G= propranolol glucuronide; 4-OH-P-G= 4-hydroxypropranolol glucuronide; NLA=α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide

TABLE VII-13. EXCRETION RATES OF THE METABOLITES VERSUS BLOOD CONCENTRATIONS OF THE METABOLITES AT T_{mid} IN DOG B GIVEN A SHORT HIND-LIMB INFUSION OF 0.73 GM PROPRANOLOL.

Prop-G				4-OH-P-G			Gly-G			NLA	
T_{mid} a)	$C_{b,M}$ b)	Bile c) dM/dt	Urine dM/dt	$C_{b,M}$	Bile dM/dt	Urine dM/dt	$C_{b,M}$	Bile dM/dt	Urine dM/dt	$C_{b,M}$	Urine dM/dt
101.2	2.91	238.1	329.8	1.43	122.7	192.2	.666/	133.6	19.4	3.58	355.6
142.	2.28	159	205.5	1.23	92.2	136	.62	108.2	14.8	3.25	243.3
184.5	1.73	148.1	183.2	1.12	97.8	141.7	.478	110.6	15.3	2.83	244.6
224.7	1.37	97.1	116.6	1.04	70.6	102.9	.373	77	9.3	2.52	158.7
263.3	1.09	82.8	123.5	.935	67.1	118.6	.33	69.7	9.8	2.47	168.7
303.1	.873	67.6	86.2	.794	60.8	91	.269	59.6	6.9	2.29	140.3
347.5	.725	49.6	69.1	.707	49.4	82.8	.238	45.3	5.8	2.16	121.9
386.4	.591	43.2	52.3	.632	44.9	69.5	.19	39.6	4.3	1.86	106.2
424.4	.438	30.9	41.2	.582	35.8	61.6	.16	28.8	3.56	1.5	94.4
465.8	.359	28.3	31.3	.517	33.9	52.1	.129	27.7	2.59	1.3	76.3

a) Time in min after the end of the infusion; b) $C_{b,M}$= blood concentration of the metabolite in ug/ml
c) dM/dt = excretion rate in ug/min
Prop-G= propranolol glucuronide; 4-OH-P-G= 4-hydroxypropranolol glucuronide; NLA=α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide.

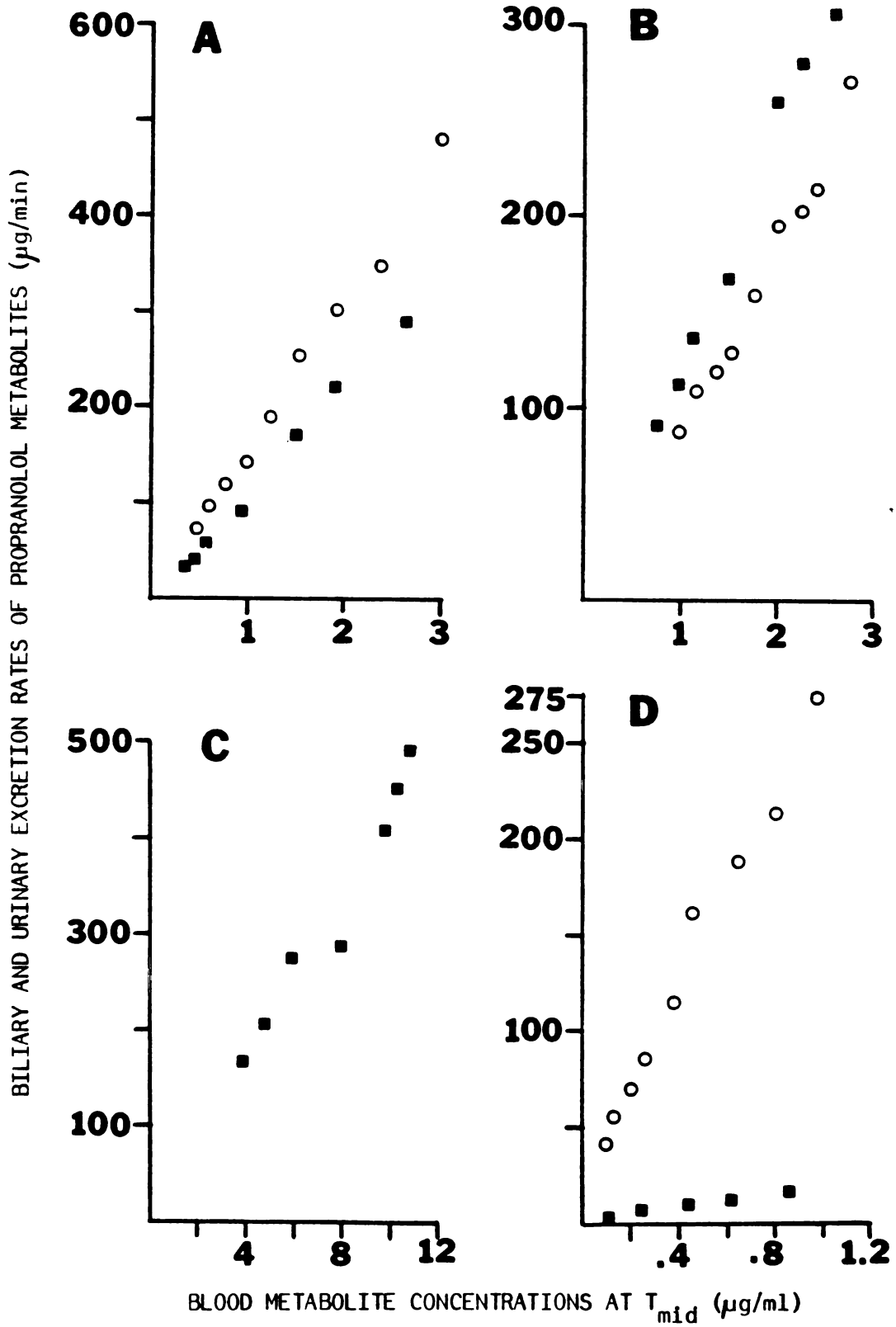

FIG VII-20. Biliary (O) and urinary (■) rates of excretion of propranolol metabolites versus their blood concentrations at mid-point of collection period in dog A after a short propranolol infusion. (A)= Propranolol glucuronide; (B)= 4-hydroxypropranolol glucuronide; (C)= α-naphthoxylactic acid; (D)= propranolol glycol glucuronide.

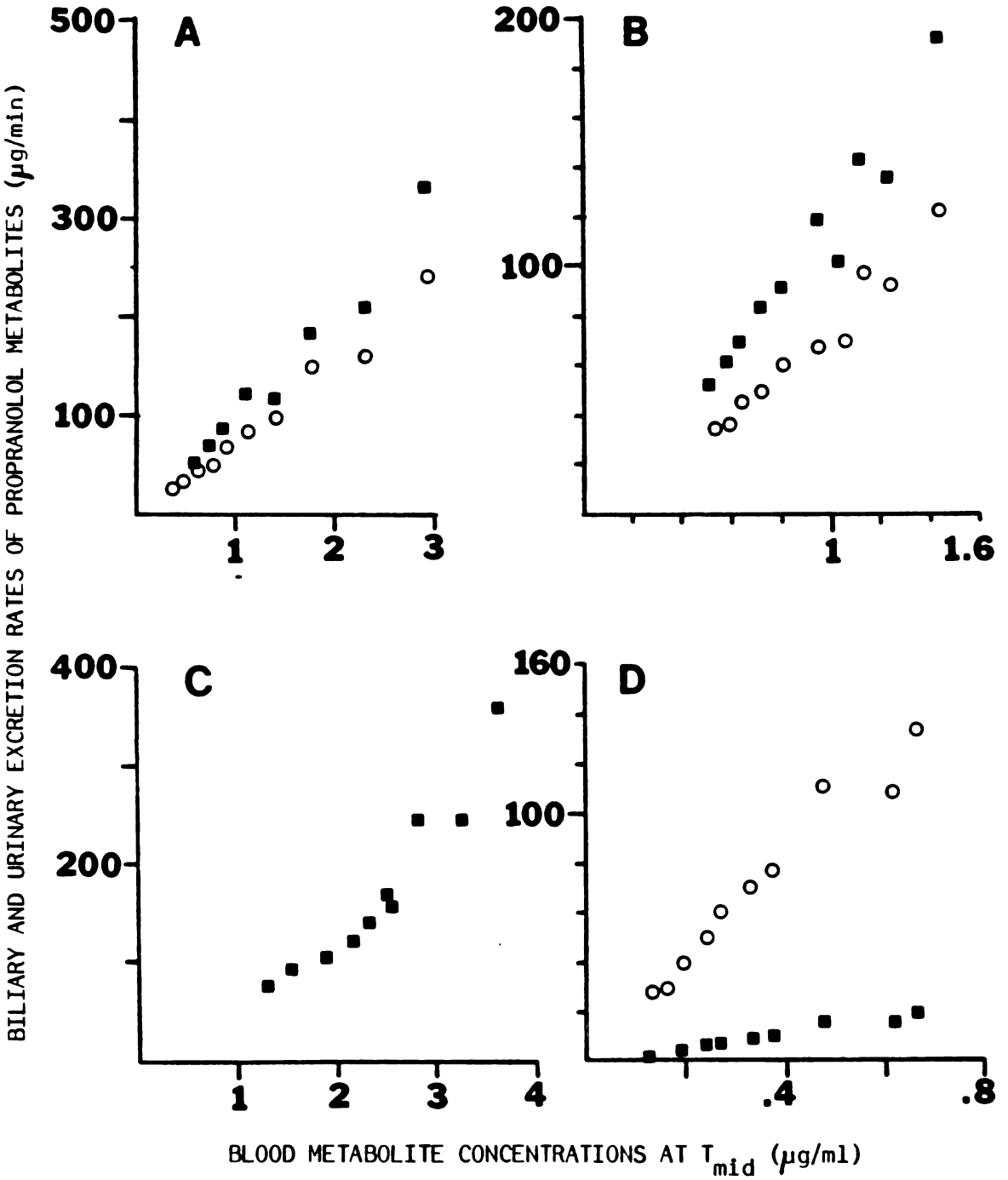

FIG VII-21. Biliary (○) and urinary (■) rates of excretion of propranolol metabolites versus their blood concentrations at mid-point of collection period in dog B after a short propranolol infusion. (A)= propranolol glucuronide; (B)= 4-hydroxypropranolol glucuronide; (C)=α-naphthoxylactic acid; (D)= propranolol glycol glucuronide.

TABLE VII-14. EXPERIMENTALLY-DETERMINED KINETIC PARAMETERS [a] WHICH WERE USED TO SIMULATE PROPRANOLOL AND METABOLITE BLOOD CONCENTRATION-TIME PROFILES IN DOGS A AND B.

	Metabolite Formation and Elimination Clearances												
	NLA			Gly-G			4-OH-P-G			Prop-G		Unknown	
	$Vmax_f$ [b]	Km_f [b]	CL_e [c]	$Vmax_f$	Km_f	CL_e	$Vmax_f$	Km_f	CL_e	CL_f [c]	CL_e	$Vmax_f$	Km_f
DOG A	6.31	.00531	46	2.47	.00531	341	4	.00342	215	545	265	11.98	.0078
DOG B	2.64	.00339	93	1.36	.00339	250	2.13	.00394	212	406	190	4.21	.00274

a) The values for the unknown metabolite are fitted values as described previously.
b) $Vmax_f$ and Km_f are the Michaelis-Menten enzyme parameters for the formation of the metabolites from propranolol in μmole/min and μmole/ml, respectively.
c) CL_f and CL_e are the formation and elimination clearances of the metabolite in ml/min.

Prop-G= propranolol glucuronide; 4-OH-P-G= 4-hydroxypropranolol glucuronide
NLA= α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide

TABLE VII-15. DIFFERENTIAL EQUATIONS DESCRIBING THE BLOOD CONCENTRATION-TIME PROFILES OF PROPRANOLOL AND ITS METABOLITES AFTER A SHORT HIND-LIMB INFUSION OF PROPRANOLOL IN DOG A.

$$\frac{dC_{b,prop}}{dt} = -C_{b,prop}/V_{c,prop} \cdot \left(\frac{6.31}{.00531 + C_{b,prop}} + \frac{2.47}{.00531 + C_{b,prop}} + \frac{4}{.00342 + C_{b,prop}} + \frac{11.98}{.0078 + C_{b,prop}} + 545 \right)$$

$$+ CL_T/V_{c,prop} \cdot (C_{T,prop} - C_{b,prop}) \quad (1)$$

$$\frac{dC_{b,NLA}}{dt} = 1/V_{c,NLA} \cdot \left(\frac{6.31 \times C_{b,prop}}{.00531 + C_{b,prop}} - 46 \times C_{b,NLA} \right) \quad (2)$$

$$\frac{dC_{b,Gly\text{-}G}}{dt} = 1/V_{c,Gly\text{-}G} \cdot \left(\frac{2.47 \times C_{b,prop}}{.00531 + C_{b,prop}} - 341 \times C_{b,Gly\text{-}G} \right) \quad (3)$$

$$\frac{dC_{b,4\text{-}OH\text{-}P\text{-}G}}{dt} = 1/V_{c,4\text{-}OH\text{-}P\text{-}G} \cdot \left(\frac{4 \times C_{b,prop}}{.00342 + C_{b,prop}} - 215 \times C_{b,4\text{-}OH\text{-}P\text{-}G} \right) \quad (4)$$

$$\frac{dC_{b,Prop\text{-}G}}{dt} = \left(545 \times C_{b,prop} - 265 \times C_{b,Prop\text{-}G} \right) / V_{c,\ Prop\text{-}G} \quad (5)$$

$$\frac{dC_{T,prop}}{dt} = CL_T / V_{T,prop} \cdot (C_{b,prop} - C_{T,prop}) \quad (6)$$

C_b and C_T are concentrations in blood and tissue, respectively; V_c and V_T are volumes of central and tissue compartments, respectively; CL_T is the clearance of propranolol from central into tissue compartment, prop= propranolol; Prop-G= propranolol glucuronide; 4-OH-P-G= 4-hydroxypropranolol glucuronide; NLA= α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide.

TABLE VII-16. DIFFERENTIAL EQUATIONS DESCRIBING THE BLOOD CONCENTRATION-TIME PROFILES OF PROPRANOLOL AND ITS METABOLITES AFTER A SHORT HIND-LIMB INFUSION OF PROPRANOLOL IN DOG 5.

$$\frac{dC_{b,prop}}{dt} = -C_{b,prop}/V_{c,prop} \cdot \left(\frac{2.64}{.00339 + C_{b,prop}} + \frac{1.36}{.00339 + C_{b,prop}} + \frac{2.13}{.00394 + C_{b,prop}} + \frac{4.21}{.00274 + C_{b,prop}} + 406\right) + CL_T/V_{c,prop} \cdot (C_{T,prop} - C_{b,prop}) \quad (1)$$

$$\frac{dC_{b,NLA}}{dt} = 1/V_{c,NLA} \cdot \left(\frac{2.64 \times C_{b,prop}}{.00339 + C_{b,prop}} - 93 \times C_{b,NLA}\right) \quad (2)$$

$$\frac{dC_{b,Gly\text{-}G}}{dt} = 1/V_{c,Gly\text{-}G} \cdot \left(\frac{1.36 \times C_{b,prop}}{.00339 + C_{b,prop}} - 250 \times C_{b,Gly\text{-}G}\right) \quad (3)$$

$$\frac{dC_{b,4\text{-}OH\text{-}P\text{-}G}}{dt} = 1/V_{c,4\text{-}OH\text{-}P\text{-}G} \cdot \left(\frac{2.13 \times C_{b,prop}}{.00394 + C_{b,prop}} - 212 \times C_{b,4\text{-}OH\text{-}P\text{-}G}\right) \quad (4)$$

$$\frac{dC_{b,Prop\text{-}G}}{dt} = \left(406 \times C_{b,Prop\text{-}G} - 190 \times C_{b,Prop\text{-}G}\right) / V_{c,Prop\text{-}G} \quad (5)$$

$$\frac{dC_{T,prop}}{dt} = CL_T / V_{T,prop} \cdot (C_{b,prop} - C_{T,prop}) \quad (6)$$

C_b and C_T are concentrations in blood and tissue, respectively; V_c and V_T are volumes of central and tissue compartments, respectively; CL_T is the clearance of propranolol from central into tissue compartment. prop= propranolol; Prop-G= propranolol glucuronide; 4-OH-P-G= 4-hydroxypropranolol glucuronide; NLA= α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide.

3. Using Equations 1 and 6 from Table VII-15, the volumes of distribution of propranolol in the central and tissue compartments and its distribution clearance into the tissue compartment were fitted using the FITDIFF program of PROPHET (Perry, 1979) with the propranolol blood concentration-time points from Table VII-10 of dog A. The volume of distribution of propranolol glucuronide was fitted using Equation 5 of Table VII-15 on the propranolol glucuronide blood data in the same way. The data of dog B were treated accordingly. The fitted values are tabulated in Table VII-17 and the fitted curves describing propranolol and its glucuronide were shown in Figures VII-22A and 23A. Very good fits were obtained with some reasonable volumes estimated. The volumes for propranolol and the glucuronide were 342.5 and 9.45 liters for dog A and 301.2 and 4.23 liters for dog B, respectively. The high volume of distribution of propranolol is to be expected due to its high lipophilicity. As for the glucuronide, its distribution is expected to be small because of its high polarity.

4. Finally, using all the above parameters which are either determined experimentally or fitted, and arbitrarily assigning volumes of distribution to α-naphthoxylactic acid, propranolol glycol glucuronide, and 4-hydroxypropranolol glucuronide (ranging from 2 to 7 liters; see Table VII-18), all the differential equations in Tables VII-15 and 16 were used to simulate data for α-naphthoxylactic acid, propranolol glycol glucuronide, and 4-hydroxypropranolol glucuronide with the DIFFEQ program (Perry, 1979). As shown in Figures VII-22B and 23B, the resultant curves are in reasonable agreement with the experimentally observed data. The poorest fit in each case is the propranolol glycol glucuronide. However, considering the number of parameters involved the result appears to confirm the validity of the model.

TABLE VII-17. FITTED VOLUMES OF DISTRIBUTION OF PROPRANOLOL AND PROPRANOLOL GLUCURONIDE AND FITTED DISTRIBUTION CLEARANCE OF PROPRANOLOL.

	DOG A	DOG B
$V_{c,Prop}$ [a]	342.5	301.2
$V_{T,Prop}$ [a]	129.0	239.4
$V_{c,Prop-G}$ [a]	9.45	4.23
$CL_{T,Prop}$ [b]	4936	3431

a) V_c and V_T are the volumes of the central and tissue compartments, respectively in liters.

b) CL_T is the distribution clearance of propranolol into tissue compartment in ml/min.

Prop= propranolol; Prop-G= propranolol glucuronide

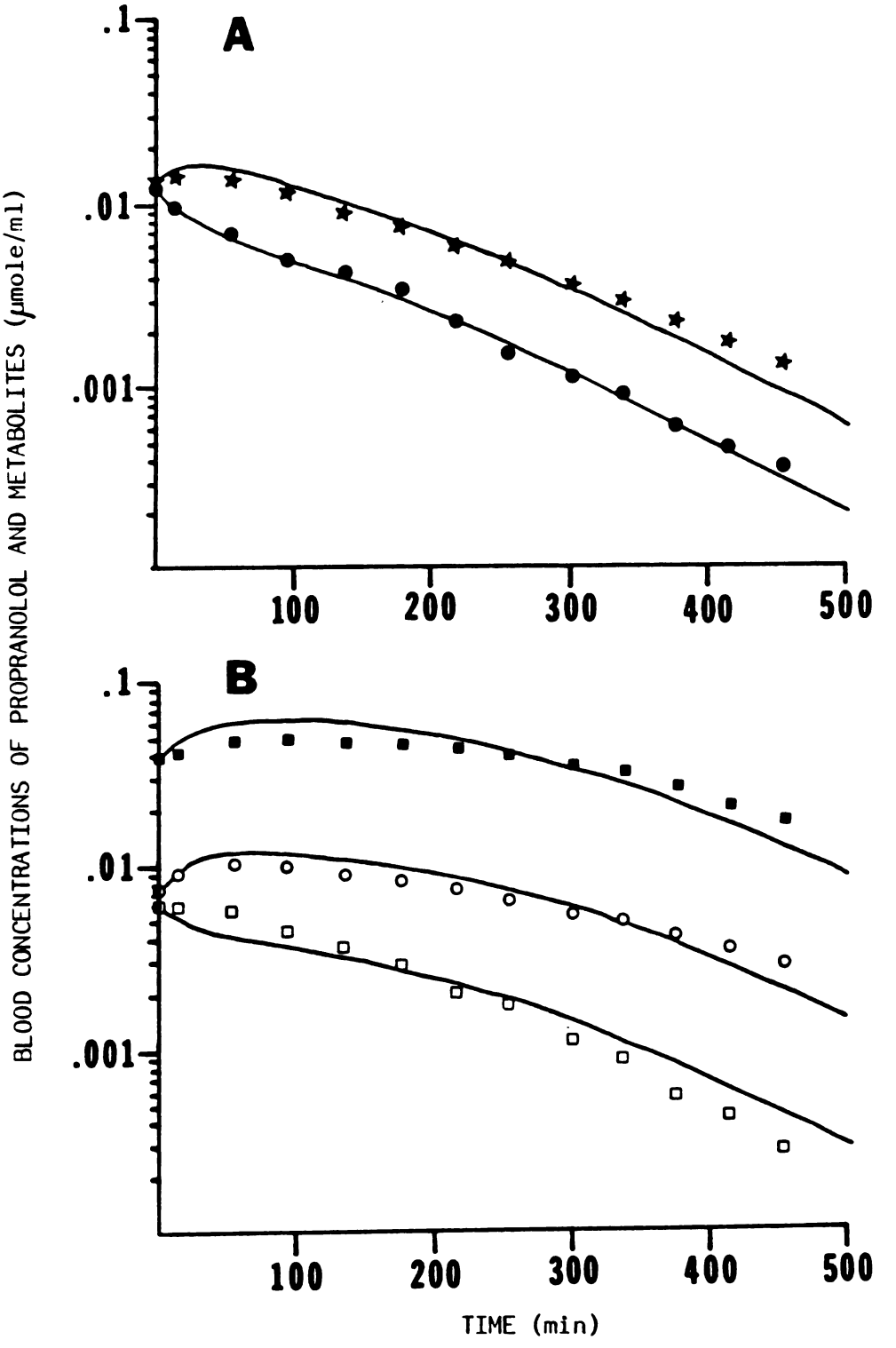

FIG VII-22. Simulation of blood concentration-time profiles of propranolol and metabolites after a short 1.17 gm propranolol hind-limb infusion using kinetic parameters obtained from steady-state studies in dog A.
Key: (A) Propranolol (●); propranolol glucuronide (★)
(B) 4-hydroxypropranolol glucuronide (○); ∝-naphthoxylactic acid (■); propranolol glycol glucuronide (□).

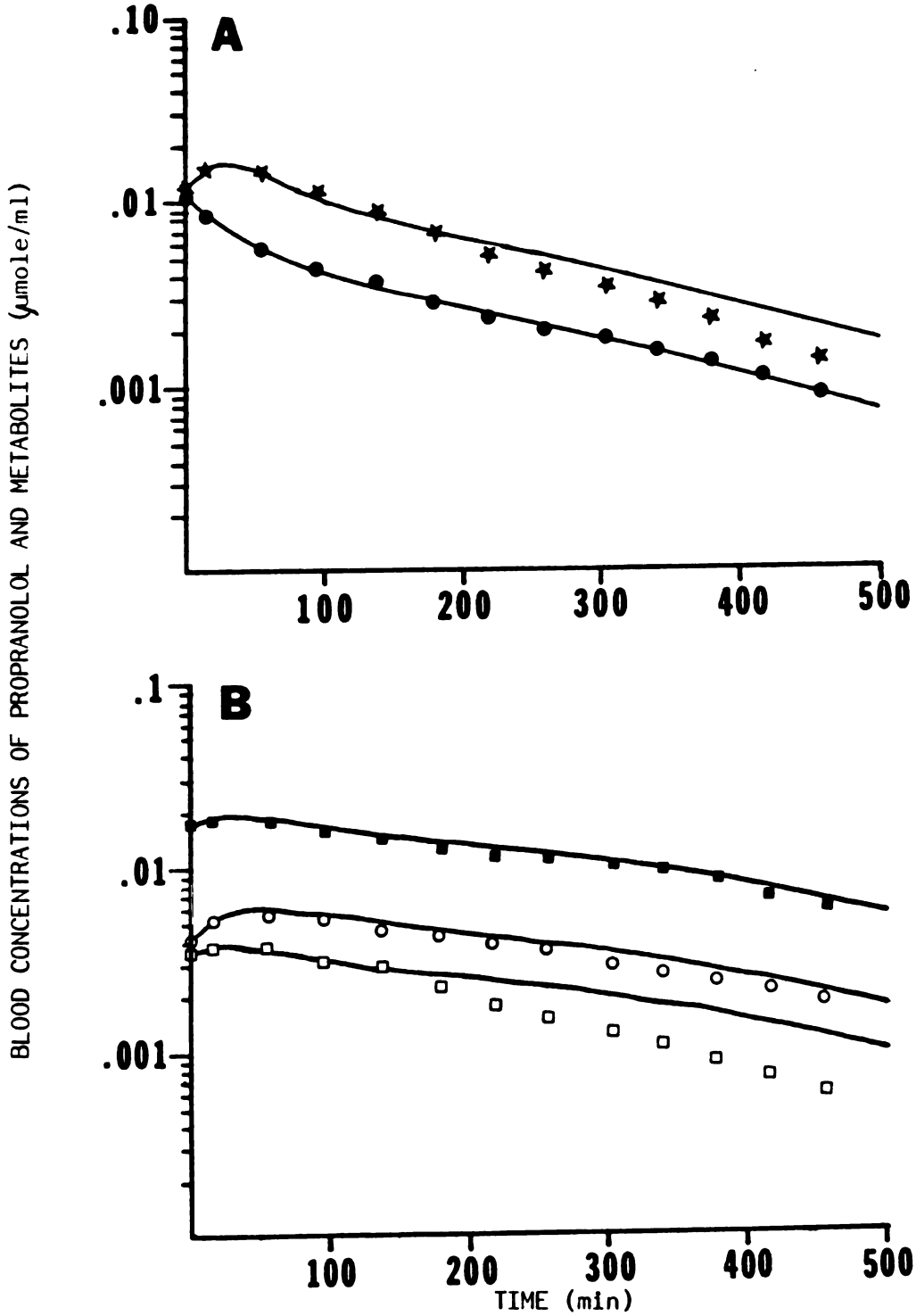

FIG VII-23. Simulation of blood concentration-time profiles of propranolol and metabolites after a short 0.73 gm propranolol hind-limb infusion using kinetic parameters obtained from steady-state studies in dog B.
Key: (A) Propranolol (●); propranolol glucuronide (★)
(B) 4-Hydroxypropranolol glucuronide (○); α-naphthoxy-lactic acid (■); propranolol glycol glucuronide (□).

TABLE VII-18. ARBITRARY VOLUMES OF DISTRIBUTION OF PROPRANOLOL METABOLITES.

	DOG A	DOG B
$V_{c,NLA}$	3	2
$V_{c,Gly-G}$	4	3
$V_{c,4-OH-P-G}$	7	6

Volume in liters

4-OH-P-G= 4-hydroxypropranolol glucuronide;

NLA= α-naphthoxylactic acid; Gly-G= propranolol glycol glucuronide

CHAPTER VIII. SUMMARY, CONCLUSION AND RECOMMENDED ADDITIONAL STUDIES

1. SUMMARY

Portacaval transposition surgery was performed in a total of 7 dogs. Only one died as a direct result of the surgery. The health of the other animals remained excellent throughout the period of the study. Liver blood flow is elevated in these animals as shown by the increased systemic clearance (37%) of propranolol in 4 of the 5 dogs after the operation.

Bile was collected using a Thomas cannula chronically emplaced in the duodenum. During each experiment urine was collected from the bladder using a Swan-Ganz flow-directed catheter. No irritation or inflammation of the urinary tract was observed.

Propranolol and its major metabolites in plasma, blood, urine, and bile were quantitated using high-pressure liquid chromatography with fluorescence detection. Two methods, a direct injection technique after a simple protein precipitation step and a double extraction technique, were used.

Propranolol did not undergo gastrointestinal metabolism to any significant extent in the 5 dogs that were portacavally transposed. Nonlinear hepatic metabolism was observed by infusing propranolol directly to the liver to achieve 4 to 6 separate steady-states. The data indicated that 2 of its major metabolic pathways, side chain oxidation and aromatic ring hydroxylation, are saturable. The third major pathway, glucuronidation, was found to be linear within the dosage range studied. In addition, evidence was obtained that an unidentified metabolite(s) is also formed by a saturable process.

2. CONCLUSION

The portacavally transposed dog appears to be a valuable model in pharmacokinetic studies because it allows the investigator to quantitate independently gastrointestinal and hepatic contributions to first-pass metabolism. To date, this is the only chronic dog model available for such purposes. Previous methods are either acute preparations or nonphysiological.

The well-stirred model for the hepatic extraction of propranolol appears to be appropriate if capacity-limited metabolism is incorporated.

Recent observations in our laboratory of normal volunteers who are given oral doses of propranolol chronically indicate that the same pathways of metabolism are saturable in both man and dog, except that the O-glucuronidation pathway is also saturable in man. However, the values of K_m obtained for the three pathways showing nonlinear behavior are higher in dogs, 0.9 to 2.6 μg/ml propranolol, than in man, 0.012 to 0.146 μg/ml. Thus, relatively high doses of propranolol were needed to partially saturate the hepatic enzymes in this animal species. This observation may not hold for propranolol in other species.

3. ADDITIONAL STUDIES

The portacaval transposition dog model is ideally suited for studies of other drugs that undergo gastrointestinal metabolism, for example, salicylamide, propoxyphene, and erythromycin.

Further work is required to identify the other 30% of the metabolites of propranolol and the nature of their disposition.

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