HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC METHODS FOR THE STEREOISOMER SEPARATION OF 4-HYDROXYPROPRANOLOL AND THE GLUCURONIDE CONJUGATES OF PROPRANOLOL AND 4-HYDROXYPROPRANOLOL IN BIOLOGICAL SAMPLES

by

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THESIS

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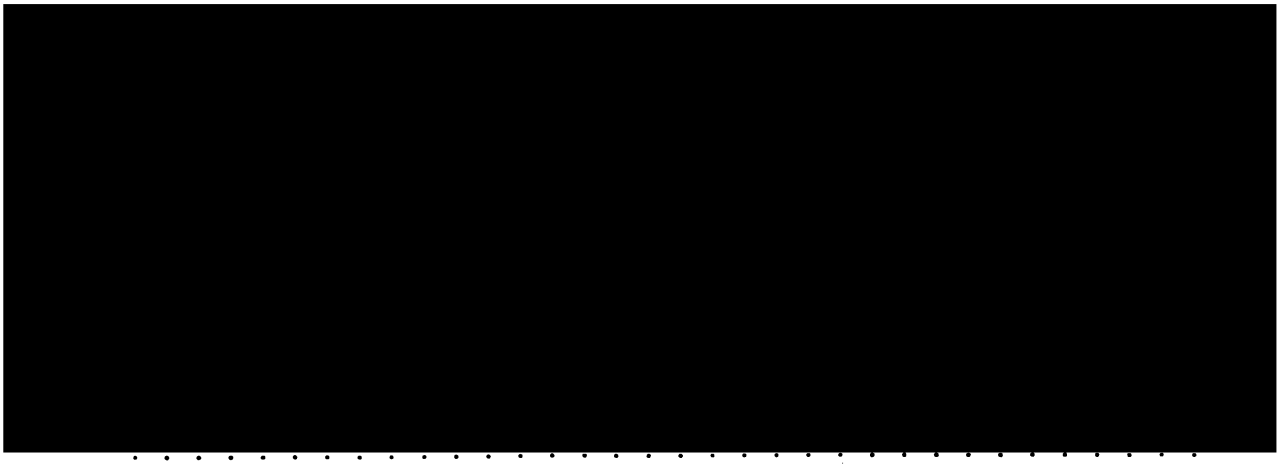

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Table of Contents

List of Tables

List of Figures

ABSTRACT

A high performance liquid chromatographic (HPLC) method has been developed for the determination of the R and S enantiomers of 4-hydroxypropranolol in human plasma. After extraction from plasma, 4-hydroxypropranolol is dried and derivatized with 2, 3, 4, 6-tetra-o-acetyl-ß-D-glucopyranosyl isothiocyanate. 4-Methyl-propranolol was used as an internal standard. The mobile phase consisted of a mixture of 50% acetonitrile, 0.15% phosphoric acid and 0.06% triethylamine. The separation was carried out on a 4.6 x 250 mm C18 column (5 μm). With a fluorescence detector (Ex : 325 nm and Em : 400 nm), the detection limit in plasma was 1 ng/ml for each enantiomer of 4-hydroxypropranolol when using 0.5 ml of plasma. The coefficients of variation for inter-day and intra-day precision were less than 10%, while the average extraction recovery from plasma was 78%.

In order to understand the stereospecific disposition and metabolism of propranolol, an HPLC method has also been developed to directly separate (R)-, (S)-glucuronide conjugates of propranolol and 4-hydroxypropranolol in urine. A reverse phase Spherisorb C8 column (4.6 x 250 mm, 5μ) was used. The (R)-, (S)-glucuronide diastereomers of propranolol and 4-hydroxypropranolol can be separated using a stepwise gradient solvent system which is programmed by flow rate. The mobile phase consisted of Solvent I (Pump A): CH_3OH/0.1 M $NH_4H_2PO_4$, 10/90; and Solvent II (Pump B): CH_3OH/0.1 M $NH_4H_2PO_4$, 60/40. The total flow rate (Pump A flow rate and Pump B flow rate) is 1 ml/min. The stepwise gradient program was expressed by the flow rate of Pump A: 1 ml/min, 29 min; 0.6 ml/min, 13 min; 0.5 ml/min, 16 min; 0.4 ml/min, 5 min; 0.36 ml/min, 3 min; 0.35 ml/min, 3 min ; 0.33 ml/min, 6 min. Two fluorescence detectors were connected in series. One was set at Ex 325 nm and Em 400 nm for (R)-, (S)-4-hydroxypropranolol and (R)-, (S)-4-hydroxypropranolol-glucuronide and the other was set at Ex 280 nm and Em 340 nm for (R)-, (S)-propranolol and (R)-, (S)-propranolol-o-glucuronide. Good linear relationships were obtained over a range of 50-12000 ng/ml of

propranolol and 4-hydroxypropranolol. The method achieves a detection sensitivity of 50 ng/ml of each glucuronide diastereomer when using 0.1 ml of urine, and has been used in the studies of 12 human subjects. There is a clear stereoselectivity in the metabolic clearance, favoring (S)-(-)-glucuronides formation of propranolol and 4-hydroxypropranolol in urine.

INTRODUCTION

I. General

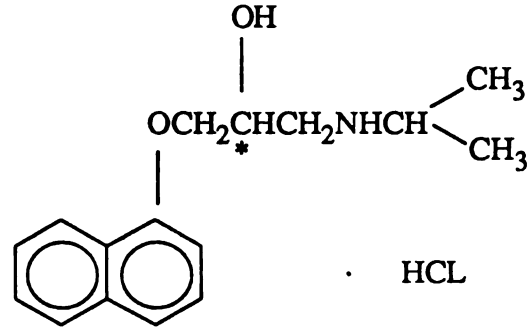
Propranolol (P), (R)-, (S)-1-isopropylamino-3-(1-nathoxy)-2-propanol {Fig. 1.(a)}, was discovered by Imperial Chemical Industries, Ltd. [1] and has been marketed in the United States as an anti-hypertensive drug. The levorotatory (S)-(-)-P is about 100 times more potent as a beta-blocker than the dextrorotatory (R)-(+)-enantiomer which has only a membrane stability activity, (S)-(-)-P is believed to be largely responsible for the clinical effects of the racemic drug [2].

4-Hydroxypropranolol (HOP) {Fig. 1.(a)}, a major metabolite formed in man only after oral administration of P [3, 4], has the same ß-blocker potency as does P. However, the (S)-(-)-HOP is responsible for the beta blocking effects of a racemic mixture of HOP [5].

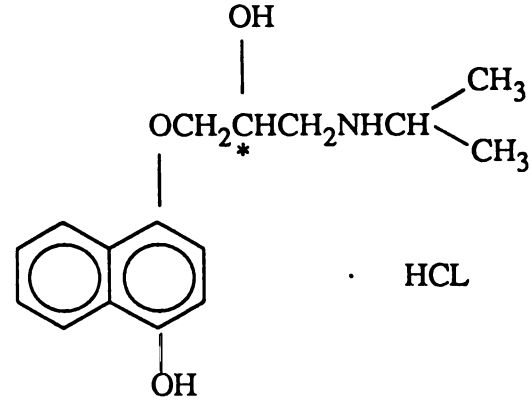
II. Mechanism of Action

P is a non-selective beta-adrenergic blocking agent indicated for the treatment of angina pectoris, hypertension, cardiac arrhythmias, thyrotoxicosis, as well as many other diseases. P antagonizes catecholamines at both $ß_1$- and $ß_2$-adrenergic receptors within the myocardium and within bronchial and vascular smooth muscle. When P is first administered to a hypertensive patient, blood pressure decreases primarily as a result of a decrease in cardiac output. With continued treatment, however, cardiac output returns toward normal while blood pressure remains low, owing to decreased peripheral vascular resistance.

Beta blockade in kidney has been proposed as contributing to the anti-hypertensive effect observed with ß-blockers. P inhibits the stimulation of renin production by

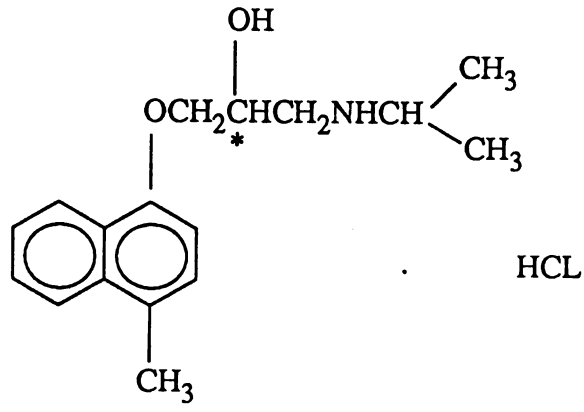


(R)-, (S)- Propranolol ($C_{16}H_{21}NO_2$ HCl, Mo wt.=295.84)

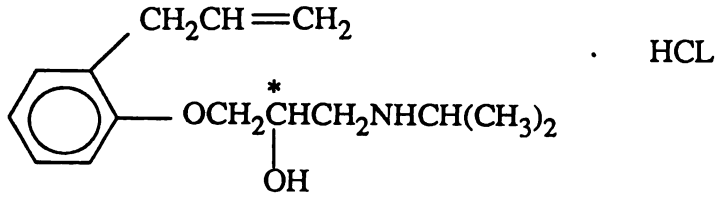


(R)-, (S)- 4-Hydroxypropranolol ($C_{16}H_{22}NO_3$ HCl , Mo.wt.=311.84)

Figure 1. (a) Chemical Structures of (R)-, (S)-Propranolol and (R)-, (S)-4-Hydroxypropranolol



(R)-, (S)- Methylpropranolol ($C_{17}H_{24}NO_2$ HCl, Mo. wt. =309.84)



(R)-, (S)- Alprenolol ($C_{15}H_{23}NO_2$ HCl, Mo. wt.=285.84)

Figure 1. (b) Chemical Structures of Internal Standards : (R)-, (S)-4-Methylpropranolol and(R)-, (S)-Alprenolol

catecholamines in animals and humans [6]. Following treatment with P, a reduction in plasma renin activity has been reported in both normal [7] and hypertensive subjects [8].

Some ß-blockers possess intrinsic sympathomimetic activity (ISA) or partial agonist activity. Clinically, drugs with ISA are thought to be as efficacious in the treatment of hypertension, angina, and arrhythmias as other ß-blockers [9]. P has no agonist activity [10]. HOP is a partial agonist in that it has ISA as well as non-selective ß-adrenoreceptor blocking potency. This ISA was shown by the increase in heart rate produced after its administration to rats of catecholamines [11].

III. Pharmacokinetic Studies

1. Absorption

P is almost completely absorbed from the GI tract, following its oral administration. After absorption from the GI tract, P is bound by the liver through nonspecific tissue binding. Following oral administration, the drug does not reach the systemic circulation until hepatic binding sites are saturated [12]. The amount of drug that reaches the circulation after oral administration depends on the amount of drug metabolized on the first pass through the liver. Because P has a high hepatic extraction ratio, clearance of this drug is blood-flow dependent [5].

2. Metabolism of P in Man and Animals

P is eliminated from the body in all animal species after undergoing metabolic transformations. In man, less than 1% of the oral dose is recovered unchanged in the urine [13]. All of the oral dose can be accounted for in urine as metabolites [4]. The four major metabolic pathways for P are o-dealkylation, side chain oxidation, ring oxidation, glucuronic acid conjugation, and sulfate conjugation [14]. A metabolic scheme for P in man is shown in Figure 2.

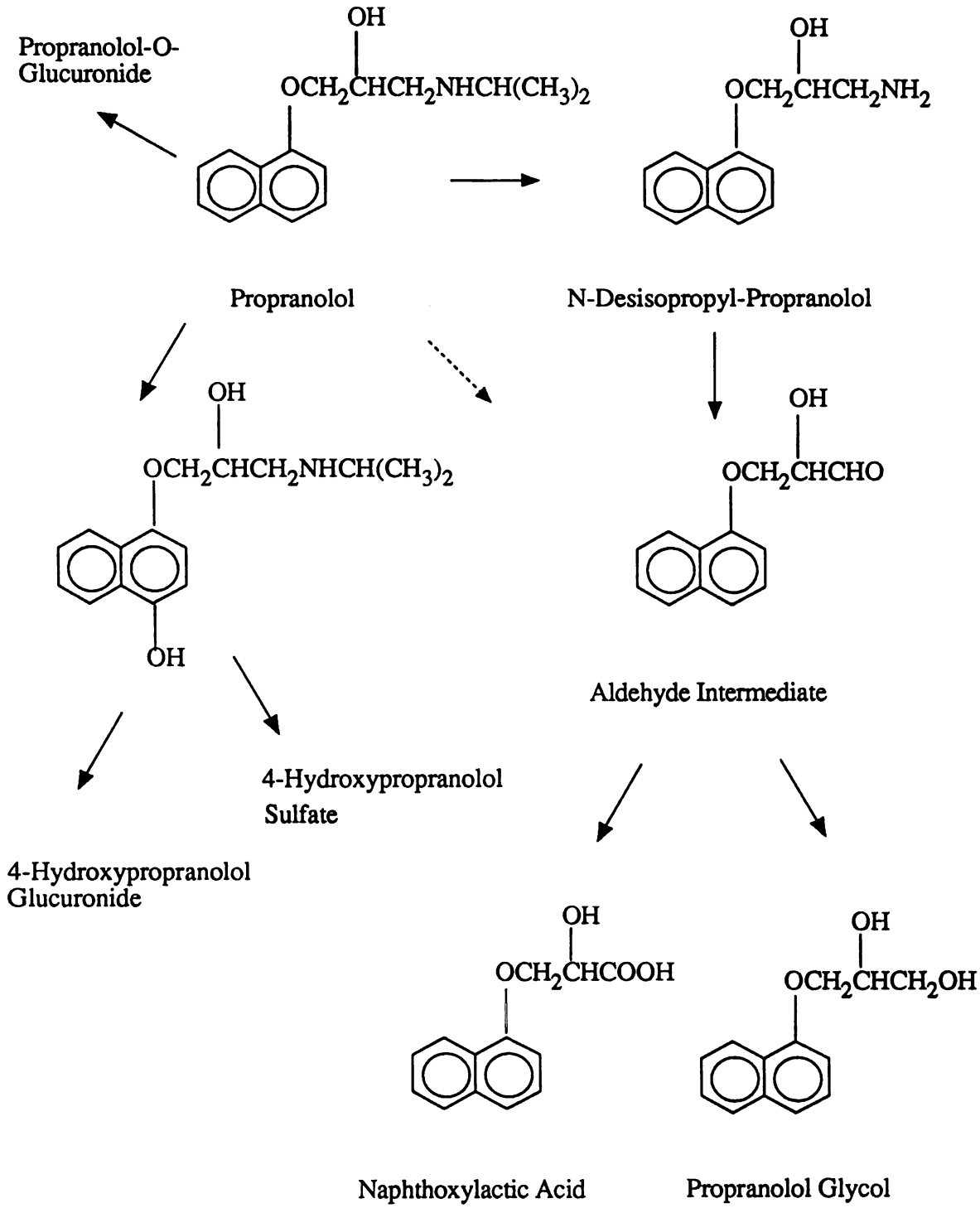
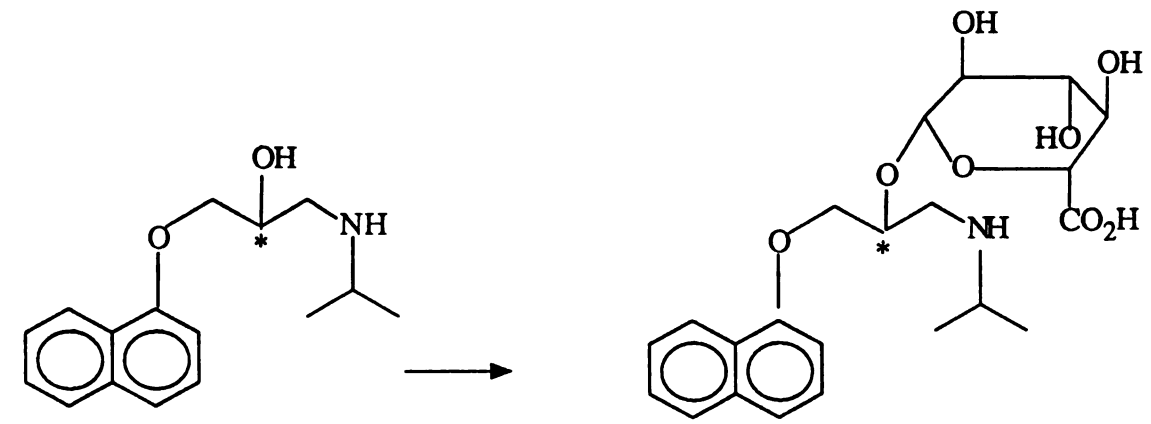


Figure 2. Major Pathways of Propranolol Metabolism in Man [59]

Propranolol-O-Glucuronide (P-O-G) and 4-Hydroxypropranolol Glucuronide (HOP-G) (Fig. 3) are found largely in both plasma and urine. Although the specific site of glucuronidation in man is not known, the liver is believed to be the major organ responsible for this metabolic process [15]. P-O-G plasma levels exceeded P levels by about 7 times after multiple oral doses, whereas P-O-G and P levels were of the same order after i. v. dose of P in normal human subjects [16]. This difference suggested P-O-G cumulation after repeated P administration. Although many metabolites of P have been identified, known metabolites account for only 60% of the oral dose [16]. The major metabolites are P-O-G, HOP-G or sulfate, and α-naphthoxylactic acid. Almost all P in plasma and urine appears as the glucuronide conjugate. P-O-G (as measured by excretion in urine) in man accounts for 3% to 18% of the dose when the P dose is increased from 40 to 320 mg daily [17]. Virtually all HOP in plasma and urine appear as glucuronide or sulfate conjugates. About 1% of the dose in urine was excreted as HOP. And 3% to 12% of the P dose was excreted as HOP-G when the oral dose is increased from 40 to 320 mg daily [18].

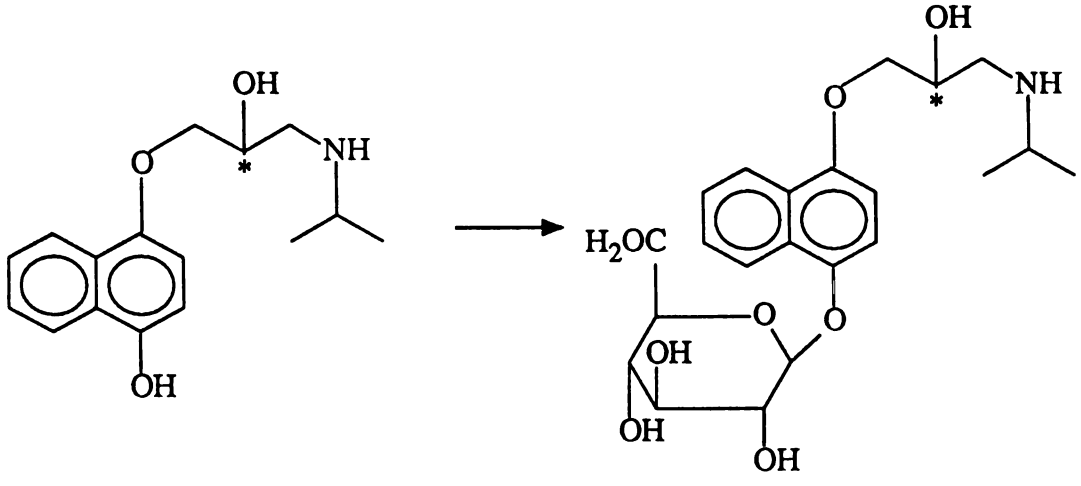
Ehrsson first observed that stereoselective glucuronidation was occurring in the dog [19]. After administration of the (R)- and (S)-P separately to two dogs (3x15 mg for 2 days), he observed that glucuronide concentrations of the (S)-(-)-enantiomer in the 24-hr urine were about 16-fold greater than for the glucuronide of (R)-(+)-P. A study in the dog reported that 4 times higher plasma concentrations of the (S)-(-)-isomer of P-O-G than of the (R)-(+)-isomer after single 160 mg oral doses of racemic P. Silber *et al.* found that (S)-(-)-P and its corresponding glucuronide conjugate were greater than that for (R)-(+)-P and its corresponding conjugate in human plasma following oral doses of 40-320 mg/day P to a steady state [5].

Ring hydroxylated metabolites have been identified in rats, hamsters, dogs, and humans [20, 21]. However, the precise mechanism of P ring hydroxylation is not yet clear. The oxidative pathways (side-chain oxidaton and ring oxidation) appear to be mediated by distinct cytochrome p-450 isoenzymes [22, 23, 24]. After initial 3, 4-epoxidation, the HOP



Propranolol

Propranolol-O-Glucuronide



4-Hydroxypropranolol

4-Hydroxypropranolol Glucuronide

Figure 3. Metabolic Scheme for the Formation of 4-Hydroxypropranolol and Glucuronide Conjugates [56]

formed is almost completely conjugated with glucuronic acid and sulfate [25]. Both conjugation pathways appear to be stereoselective in man with glucuronic acid conjugation favoring the (-)-enantiomer and sulfoconjugation favoring the (+)-enantiomer [26]. Walle *et al.* has identified a sulfate ester of HOP as a major metabolite of P in dogs and humans. Whereas HOP was excreted entirely as a glucuronic acid conjugate in both rats and hamsters. It was present very little or no sulfate conjugate of HOP in these latter species [27].

3. Distribution of P

P is widely distributed into body tissues including lungs, liver, kidneys, and heart. P readily crosses the blood-brain barrier and the placenta. In man, the apparent volume of distribution (Vd_{β}) of P was reported to be 236±51 liters [28].

P is about 95% bound to plasma proteins. In man, the percent free was reported to be 6.96±1.57 [29]. Both free and protein-bound P are metabolized. Increased plasma protein binding of the drug increases its metabolism and decreases its volume of distribution, resulting in a shorter terminal half-life [12].

4. Elimination of P

The whole blood clearance of P was reported as 1.08±0.22 l/min in subjects given 10 mg i. v. doses [28]. The disposition of P after oral administration is much more variable between individuals than after intravenous administration. These differences have been largely attributed to differences in first-pass extraction by liver. Therefore, oral doses will be much larger than i. v. doses in order to obtain the same clinical effects. P appears in the plasma within 30 min, and peak plasma concentrations occur about 2 hours after oral doses in fasted individuals [28, 30]. After i. v. doses of 0.5 mg of P, peak plasma concentrations of 40 ng/ml are produced in 1 min and the drug is undetectable in 5 min. The AUCs (area under the curve of plasma concentration-time profile) after 4 mg i.v. doses of P were

6.6±2.2 (ng·hr/ml) for HOP and 55±11 (ng·hr/ml) for P. After 20 and 80 mg oral doses, the AUCs, respectively, for HOP were 59±9 (ng·hr/ml) and 162±21 (ng·hr/ml) and for P were 72±9 (ng·hr/ml) and 306±46 (ng·hr/ml) [18]. Peak HOP concentrations were reached at 1 to 1.5 hours after the oral doses. There was a rapid decline in plasma HOP after 1.5 hours when HOP-G was still rising to levels above HOP levels about 5-fold. In most subjects, steady-state P concentrations were achieved two days after administration of either conventional P or long-acting P [31]. In contrast, HOP did not accumulate. The half-lives of (S)-(-)-P and its glucuronide conjugates were greater than those of the (R)-(+)-enantiomer and its corresponding conjugates in humans [5].The half-life of P in humans after oral administration of the (R)-(+)-enantiomer alone was reported to be shorter than after a dose of the racemate [32], this may have been caused by a reduction in liver blood flow [33] and thereby reduced metabolic clearance induced by the more potent ß-blocking (S)-(-)-isomer. In addition, the half-lives of both enantiomers and their corresponding glucuronide conjugates increased with increasing dosing rate [12]. When usual therapeutic doses of P are administered chronically, the half-life ranges from 3.4-6 hours. Single-dose studies have shown a shorter half-life of 2-3 hours. This difference may be the result of initial removal of the drug into a large extravascular space (especially hepatic binding sites) or of a saturation of systemic clearance [12].

IV. Administration and Dosage

P is usually administered orally in divided doses. When P extended-release capsules are administered, the entire daily dose is given once daily. For life-threatening arrhythmias, the drug is given intravenously at a rate not exceeding 1 mg/min. Careful monitoring of ECG and central venous pressure should be performed during i. v. administration.

Since there is no consistent interpatient correlation between the dosage of P and therapeutic response, especially after oral administration, dosage must be carefully

individualized according to the response of the patient. Resting bradycardia and a reduction in the heart rate during exercise are indicators of P's ß-blocking effect. For the treatment of hypertension, the initial oral dosage of P is 40 mg twice daily as conventional tablets or 80 mg once daily as extended-release capsules. The usual effective oral dosage is 160-480 mg daily as conventional tablets or 120-160 mg once daily as extended-release capsules[12].

V. Side Effects and Toxicity

P increases bronchial tone and airway resistance. This effect may be of crucial importance in patients with bronchial asthma. In patients who are predisposed to heart failure or bradycardia, caution should be used in initiating a trial of ß-adrenergic blockade. Diabetics are sensitive to the effects of P [34]. The rate of recovery of blood glucose levels after insulin-induced hypoglycemia is reduced and the increase of plasma glycerol is prevented by P. These effects depend in part on the ß-adrenergic effects of catecholamines reflexly released in response to hypoglycemia.

Other factors involved in P-mediated changes in carbohydrate metabolism are : increased secretion of growth hormone [35] and inhibition of glucagon secretion [36].

P , as well as other lipophilic ß-adrenergic blockers, has been associated with various psychiatric symptoms, such as nightmares, hallucinations, insomnia, and depression [12]. The more lipid soluble drugs, because they penetrate the central nervous system better than the hydrophilic agents, are more likely to produce these side effects [37].

When P is discontinued after prolonged regular use, some patients experience an abstinence syndrome which includes nervousness, tachycardia or elevated blood pressure. Therefore, P should not be discontinued abruptly. Adverse GI effects such as nausea, vomiting, diarrhea, constipation and flatulence may occur in patients using P and occasionally necessitate reduction of dosage or withdrawl of the drug.

VI. Significance of the Assay

Study of the pharmacokinetic and pharmacodynamic behavior of a drug is important not only for the development of a better understanding of the drug's mechanism,but also for the assessment of a rational dosing regimen. Frequently, enantiomers possess widely differing pharmocological activity, disposition, and metabolism. The possible stereoselective disposition of P and its major metabolites is of interest since they have chiral centers at the α -carbon positions.

HOP, a major active metabolite of P, has (R)- and (S)-isomer, and traditional methods can not separate them. The HPLC method presented here is sensitive and precise for separation of the (R)-, (S)-isomer of HOP in human plasma by using precolumn derivatization. In addition, glucuronidation is an important elimination pathway for P and HOP. A simple HPLC method to directly separate the (R)- and (S)-glucuronide of P and HOP from urine without derivatization was first carried out. Most of the assays developed in the past few years for P focused on the parent drug and the metabolite HOP only. Some HPLC methods measuring the glucuronide conjugates of P and HOP were using indirect techniques (expressed as the difference in P or HOP concentrations between enzyme-hydrolyzed and nonhydrolyzed samples) [16, 18]. The indirect quantitative methods are not reliable since the HOP released after ß-glucuronidase (containing sulfatase) hydrolysis is not just due to (R)-, (S)-HOP-G; there is another HOP sulfate conjugate hydrolyzed to the free state. The HPLC method presented here can analyze P, HOP, (R)-, (S)-P-O-G, and (R)-, (S)-HOP-G in biological samples simultaneously and is therefore more accurate.

This study has clarified the stereoselectivity in the metabolic clearance of (R)-, (S)-HOP-G and (R)-, (S)-P-O-G from urinary excretion data, favoring in (S)-(-)-glucuronides of P and HOP in urine.

REVIEW OF ANALYTICAL METHODS

I. Determination of Total (R)-, (S)-P and (R)-, (S)-HOP

1. Simultaneous Determination of P and HOP in Plasma by GC-MS - T. Walle *et al.* [38]

A quantitative method for the simultaneous determination of P and its active metabolite HOP by GC-MS was described. Plasma samples were extracted at pH 9.6 with ethyl acetate. The extracts were derivatized with trifluoroacetic anhydride before separation and detection with a 10% OV-1 column and a gas chromatograph-mass spectrometer. The minimum detectable concentration of P was 1 ng/ml and of HOP, 5 ng/ml, using 1 ml plasma samples.

2. HPLC for the Simultaneous Analysis of P and HOP in Plasma - R. L. Nation [39]

A reverse phase HPLC assay was developed for the simultaneous quantitative analysis of P and HOP in plasma. The plasma was extracted with ethyl acetate in the presence of a 1 M carbonate buffer (pH 9.5). The sample was separated by a 30-cm μBondapak alkylphenyl column with a mobile phase of CH_3CN/0.06% H_3PO_4 (27:73). The fluorescence detector was operated with a 205 nm excitation wavelength and a KV 340 emission filter. The detection limits were 1 ng/ml for P and 5 ng/ml for HOP.

3. Determination of ß-Adrenergic Blocking Drugs as Cyclic boronates by GC with Nitrogen -Selective Detection - T. Yamaguchi *et al.* [40]

A GC-NPD method was developed to measure the cyclic boronate of P which was derivatized with phenylboronic acid. P was extracted with n-hexane containing 1.5% of isoamyl alcohol from alkaline plasma. A 2% OV-17 on Gas-Chrom Q column was used. The minimum detectable concentration in plasma was 1 ng/ml when sampling 1 ml of plasma.

4. HPLC for Determination of P and HOP in Plasma and Urine without Solvent Extraction- M. Lo and S. Riegelman [41]

A HPLC method without solvent extraction was developed. Method 1: Measurement of unconjugated P in plasma. The 0.2 ml plasma sample was precipitated by 0.4 ml acetonitrile. N-ethylpropranolol was the internal standard. The sample was injected onto a Lichrosorb RP-8 column. The mobile phase was composed of acetonitrile, methanol and 0.0871 M phosphoric acid (360:180:70). The fluorometer was set at an excitation wavelength of 230 nm and an emission wavelength of 340 nm. Method 2: Measurement of P, HOP in plasma and urine after enzymatic hydrolysis. A 0.2 ml sample of urine or a 0.4 ml sample of plasma were mixed with 0.2 ml IS, 0.1 ml of ascorbic acid, 0.04 ml of acetate buffer (1.4 M, pH 5.5) and 25 mg of ß-glucuronidase/aryl sulfatase (400 units/mg, Sigma G 0751) were added. The mixture was incubated at 37°C for 90 min. After precipitating the protein with 0.8 ml of acetonitrile, a 0.6 ml of the clear supernatant was sucked into 0.3 ml of 0.05 M phosphoric acid to form the final injection mixture. The mobile phase was composed of CH_3CN/CH_3OH/0.0871 M H_3PO_4 (300:90:66). The excitation was fixed at 310 nm. The fluorometer's emission wavelength was first set to 430 nm to measure HOP. After the elution of HOP,the emission was then changed to 350 nm for the detection of P.The limits of detection of the two compounds are about 20 ng/ml using 0.4 ml plasma and 100 ng/ml using 0.2 ml urine.

5. HPLC for Measurement of P in Plasma or Blood- P. K. Richard and J. J. Wood [42]

A fluorometric high performance liquid chromatographic method for quantitating human plasma and blood levels of P by using 100 µl of plasma or blood was developed. The mixture of plasma or blood and 1 M NaOH (pH 10) was extracted with 1% 1-butanol in heptane. The sample was injected onto a µBondapak phenyl column. The mobile phase, pH 3.5, consisted of acetonitrile-water-acetic acid-triethylamine (450:600:10:0.15). The limiting concentration that could be detected was 1 ng/ml.

6. HPLC for P and Its Basic Metabolites in Biological Fluids - E. C. Kwong and D. D. Shen [43]

A HPLC method was developed for quantitating P and its known metabolites in serum, bile and urine. The basic and neutral metabolites were extracted from the biological samples at pH 10.5 with 2% n-butanol in dichloromethane. Additional clean-up of the basic fraction by back-extraction into dilute acid was needed for those samples which were subjected to enzymatic hydrolysis. Chromatographic separation was achieved on a reversed-phase C18 column, using a simple isocratic mobile phase consisting of 0.044 M pH 2.7 phosphate buffer-tetrahydrofuran-methanol-acetonitrile (67:12:20:1), with the addition of 0.5 µM of n-butylamine as a competing base.

II. Determination of (R)-, (S)-P and (R)-, (S)-HOP Enantiomers

1. Radioimmunoassay for P Isomers - K. Kawashima *et al.* [44]

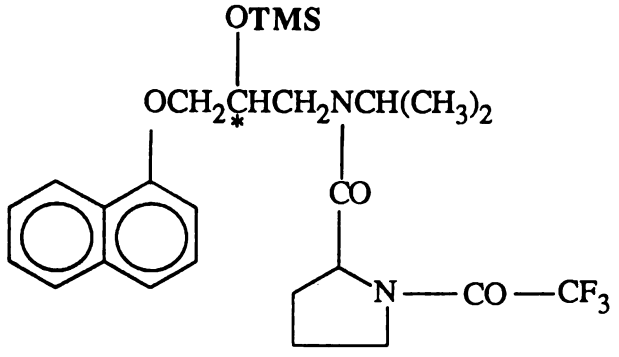
A stereospecific radioimmunoassay was developed to determine (R)-, (S)-P and (S)-P from biological samples. Antisera against (R)-, (S)-P was produced in rabbits immunized with (R)-, (S)-P conjugated to bovine serum albumin. The antiserum against (R)-, (S)-P recognized both (R)- and (S)-P to the same degree. The antibody in antiserum from rabbit immunized with (S)-P-bovine serum albumin immunogen showed stereospecificity and differentiated (S)-P from (R)-isomer. The process for preparation of immunogen and immunization of animals is tedious. Besides, it requires radioactive P. For a specific activity of 11 c/mmol of tritiated P, the minimum amount of the compound that could be detected was 10 pg.

2. GC with a Flame Ionization Detector for Determination of Beta Adrenoceptor Antagonist Enantiomers - S. Caccia *et al.*[45]

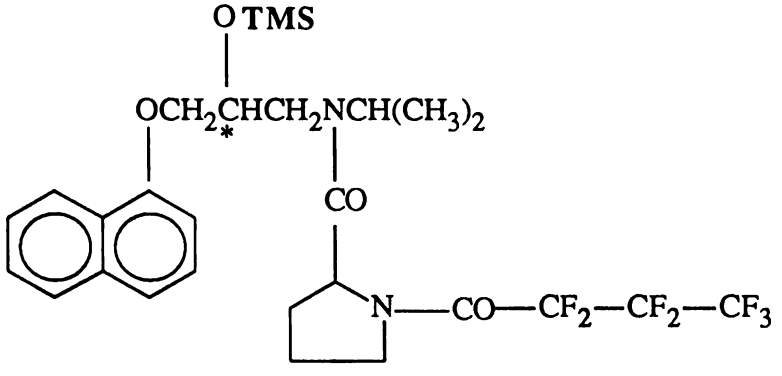
A FID-GC method was developed to separate the enantiomers of P and HOP. Enantiomers were reacted with N-trifluoroacetyl-1-prolyl-chloride (TPC) or N-heptafluorobutyryl-1-prolyl-chloride (HPC) and N, O-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) to form TPC-TMS or HPC-TMS derivatives (Fig. 4), which have acceptable thermal stability and suitable gas chromatographic properties. This method was used just for qualitative analysis.

3. GC with a Electron-Capture Detector for Simultaneous Determination of the P Enantiomers in Biological Samples - S. Caccia *et al.*[46]

An EC-GC method was developed to measure the HPC-TMS esters of P that were formed by derivatizing the secondary amino group of the drug with HPC. To make the derivatives more volatile, the hydroxy groups of the two diastereoisomers were esterified with BSTFA in the presence of benzene after heating at 50°C for 30 min. HPC derivatives



Propranolol TPC-TMS Derivative



Propranolol-HPC-TMC Derivative

Figure 4. Structures of Propranolol TPC-TMS and Propranolol HPC-TMS Derivatives

have the advantage over TPC analogs of shorter retention time and higher sensitivity with an ECD. A Carlo Erba Gas Chromatograph with a ^{63}Ni electron-capture detector was used. The overall recovery by this method was about 70-80% and the sensitivity was 25 ng per sample (blood or tissue). Nevertheless, the method lacks the specificity and sensitivity of the HPLC methods.

4. HPLC for Simultaneous Determination of (R)- and (S)-P in Human Plasma - J. Hermansson and C. V. Bahr [47]

A HPLC method based on a reversed-phase column with fluorimetric detection was developed. The method involved extraction of P from alkaline plasma with diethyl ether, and the formation of diastereomeric derivatives with the chiral reagent N-trifluoroacetyl-1-prolyl-chloride (TPC). The sample is injected onto a LiChrosorb RP-18 column (3.2 mm x 25 cm). The liquid chromatography system consisted of a high pressure pump, a loop injector, a fluorimeter set at 210 nm for excitation and at 340 nm for emission (cut-off filter), and a mobile phase of 45% acetonitrile in a phosphate buffer at pH 2.2. The minimum detectable concentration in plasma was 1 ng/ml for each isomer in 1 ml sample of plasma.

5. Ion-Pair Chromatography for Separation of P Isomers- C. Pettersson and G. Schill [48]

An ion-pair HPLC method was used to separate enantiomers of amines. The method was based on the diastereomeric ion pairs formed between the amines and optically active (+)-camphorsulphonic acid which show different distributions between an organic mobile phase and a stationary adsorbent. The separations were performed with LiChrosorb-DIOL

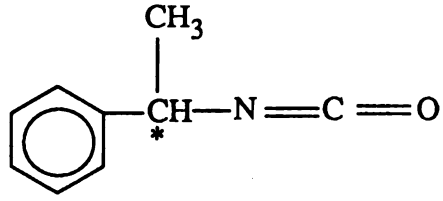
as the stationary phase and methylene chloride - 1-pentanol as the mobile phase. The detector was a Model 440 UV monitor, measuring at 254 nm.

6. Reversed-Phase Ion Pair Chromatography for Separation of (R)- and (S)-P as Their Diastereomeric Derivatives in Human Plasma - J. Hermansson [49]

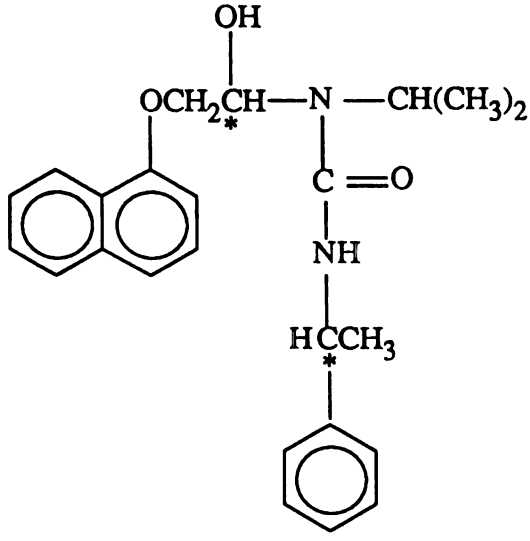
1 ml plasma was mixed with 1 ml 1 M carbonate buffer (pH 9.85) and extracted into 6 ml diethyl ether, then evaporated to dryness. Diastereomeric derivatives of P were prepared by reaction with symmetrical anhydrides of *tert*-butoxycarbonyl -L-alanine and *tert*-butoxycarbonyl -L-leucine followed by treatment with trifluoroacetic acid (TFA) at 0°C to remove the *tert* -butoxycarbonyl group. Separation of the diastereomeric derivatives was accomplished by reversed-phase ion-pair chromatography using a LiChrosorb RP-18 column and 25% acetonitrile in a 0.04 M phosphate buffer (pH 3.0) and N, N-dimethyl-octyl-amine (0.0194 M) as the mobile phase. The derivatization procedure was complicated and tedious.

7. Procedure for the Chiral Derivatization and Chromatographic Resolution of (R)-(+)- and (S)-(-)-P - J. A. Thompson *et al.*[50]

A reverse phase HPLC assay was developed for separation of (R)-(+)- and (S)-(-)-P. Racemic P was treated with (R)-(+)- or (S)-(-)-1-phenyl-ethyl-isocyanate (PEIC) to form diastereomeric derivatives which can be resolved chromatographically. The reaction with P occurred rapidly at 25°C to form a urea derivative with the secondary amino group of the drug (Fig. 5). Several investigators have noted that N-trifluoroacetyl-1-prolyl-chloride (TPC) can racemize during storage. The alternative chiral reagent, 1-phenyl-ethyl-isocyanate (PEIC) is chemically quite stable. The sample is injected onto a Spherisorb C18 5 µm column. The liquid chromatography system consisted of an Altex Model 110 pump, a



1-Phenylethyl Isocyanate (PEIC)



Urea derivative

Figure 5. Structures of PEIC and Urea Derivative

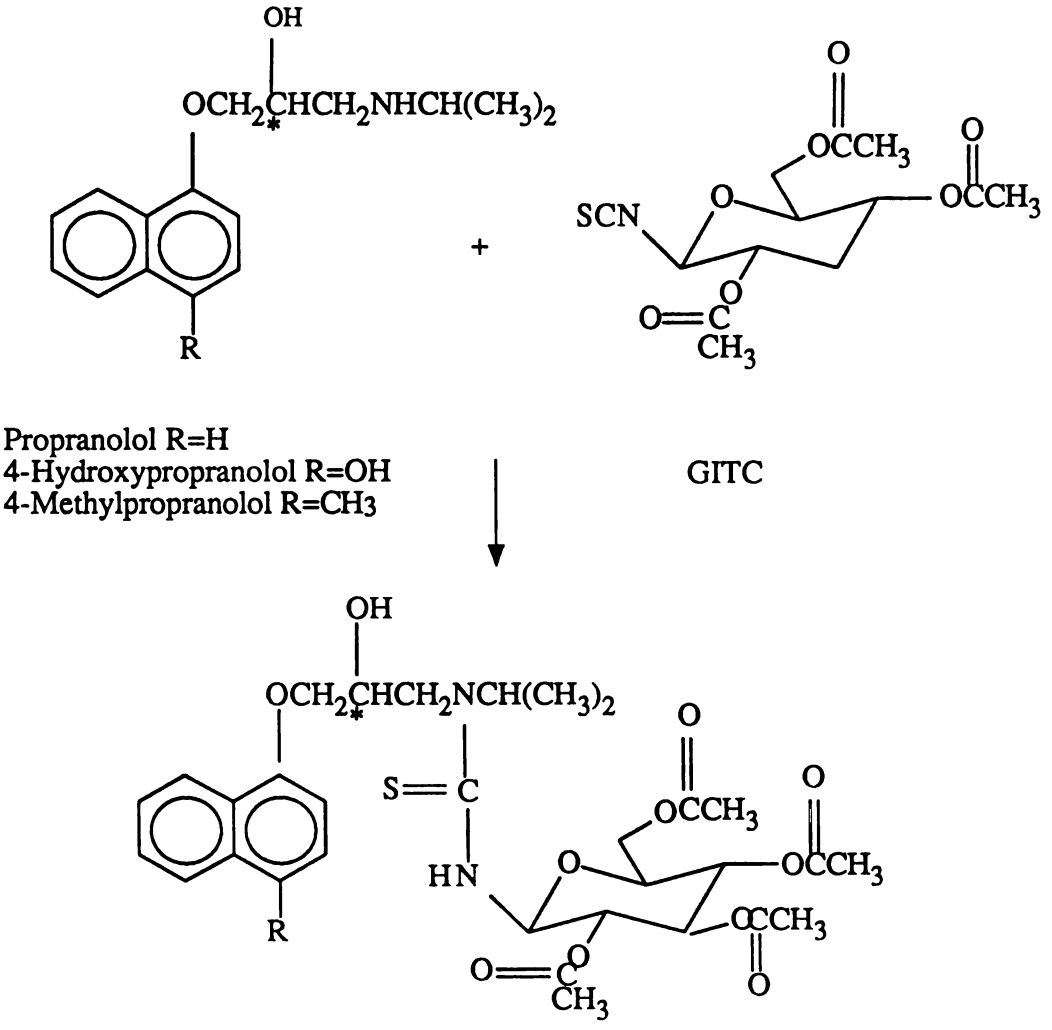
Kratos SF 970 fluorometer with excitation at 220 nm and emission at 340 nm, and a mobile phase of methanol/water (65:35). The method was not applied to the assay for (R)-, (S)-P in biological samples.

8. Resolution of the Enantiomers of P and Other Beta-Adrenergic Antagonists by HPLC- A. J. Sedman and J. Gal [51]

For separation of P and HOP enantiomers, 2, 3, 4, 6-tetra-o-acetyl-ß-D-glucopyranosyl isothiocyanate (GITC) was used as a derivatizing agent.The isothiocyanato group reacts rapidly and selectively with primary and secondary amines to form the corresponding thiourea derivative (Fig. 6). The derivatization is much simpler than the previously described procedure. GITC is commercially available and is chemically and stereochemically stable. A Waters HPLC series with a UV detector was used with the following conditions : Ultrasphere ODS reversed-phase column; two solvent systems of 50% of CH_3CN in 0.02 M aqueous $NH_4H_2PO_4$ and 58% of CH_3CN in 0.02 M aqueous $NH_4H_2PO_4$ - the former for separation of HOP enantiomers, the latter for separation of P enantiomers; and a UV detector at 254 nm. But the procedure for the resolution of the enantiomers was not applied to the assay for these compounds in biological fluids.

9. The Direct Enantiomeric Determination of (-)- and (+)-P in Human Serum by HPLC on a Chiral Stationary Phase - I. W. Wainer *et al.*[52]

A HPLC method was developed to measure P enantiomers in blood. Pronethalol was used as the reference standard. The heparinated whole blood was extracted with diethyl ether in the presence of 1 M carbonate buffer (pH 10). The mixture was shaken for 10 min and centrifuged, and the ether layer was collected and cooled to 0°C. Phosgene (10 µl of a 12.5% solution in toluene) was then added. The P enantiomeric 2-oxazolidones were then



Thiourea Derivative

Figure 6. Structures of GITC and Thiourea Derivative

resolved by chromatography on the chiral stationary phase (CSP). The P 2-oxazolidone derivatives were produced by facile condensation of the P with phosgene (Fig. 7). The column was a Regis-packed Pirkle Type 1-A (25 cm x 4.6 mm I. D.) with a α-aminopropyl packing of 5 μm spherical particles modified with (R)-N-(3, 5-dinitrobenzoyl) phenylglycine. The mobile phase was hexane-isopropanol-acetonitrile (97:3:1). A Perkin-Elmer Model LS-4 fluorescence detector was set at 290 nm for excitation and at 335 nm for emission. The detection limit was 0.5 ng/ml of blood.

10. HPLC for the Simultaneous Determination of P and HOP Enantiomers after Chiral Derivatization - M. J. Wilson and T. Walle [53]

A silica gel HPLC method was developed to determine the enantiomers of both P and HOP simultaneously after derivatization with (+)-1-phenyl-ethyl-isocyanate (PEI). The HPLC system consisted of a high pressure pump, a loop injector, and a Model 440 UV detector with a 313 nm filter. The column was either a 5 μm C18 or a 10 μm silica from Alltech Assoc. The mobile phases were methanol-water (70:30) and methanol-chloroform (1.2:100), respectively.

11. Chiral Separation of Aminoalcohols by Ion-Pair Chromatography - C. Pettersson and M. Josefesson [54]

A chiral counter ion, N-benzoxycarbonyl-glycyl-L-proline (ZGP), added to the organic mobile phase (dichloromethane), was used for separation of enantiomers of aminoalcohols with LiChrosorb DIOL as the solid phase. Retention was regulated by the concentration of the counter ion or by the addition of triethylamine to the mobile phase. The ion-pair chromatographic system with ZGP was used for separation of the (R)-, (S)-enantiomer of P in plasma. After adjustment of the pH in 1 ml plasma with 0.1 ml 4 M NaOH, the

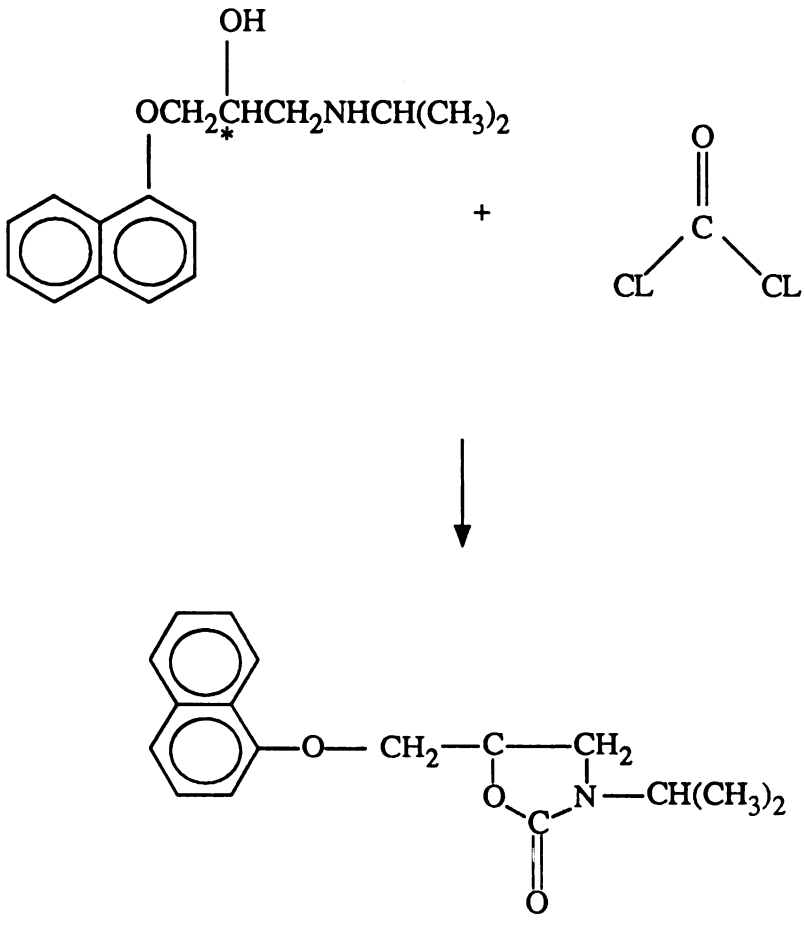


Figure 7. Synthesis of Propranolol 2-Oxazolidone Derivative

solution was extracted with 4 ml of an organic phase (hexane:dichloromethane = 4:1). A mobile phase containing 2.5 mM ZGP and 0.2 mM triethylamine in dichloromethane (500 ppm H_2O) was used. The fluorometric detection was monitored with excitation at 290 nm and an emission wavelength of 350 nm.

12. Stereoselective Analysis of P in Plasma by HPLC Using Precolumn Derivatization - Steven T. Wu *et al.* [55]

A sensitive HPLC method was developed to measure P enantiomers in plasma samples from 12 human subjects. After extraction from plasma with acetonitrile, P was dried and derivatized with GITC. The product was detected fluorometrically (Ex 280 nm, Em 340 nm). The separation was carried out on a 4.6 x 250 mm Altex C18 column. The mobile phase consisted of a mixture of 58% CH3CN, 0.1% phosphoric acid and 0.06% triethylamine. The method achieved a detection sensitivity of 1 ng/ml for each enantiomer when using 0.5 ml plasma samples.

III. Determination of Diastereomeric Propranolol-O-Glucuronide and Diastereomeric 4-Hydroxypropranolol Glucuronide

1. Identification of Diastereomeric Propranolol-O-Glucuronide by GC-MS - H. Ehrsson [17]

The 24 hr urine samples of patients who were given P orally (4 x 80 mg daily) were transferred to an Amberlite XAD-2 column. The column was eluted with distilled water and methanol; and P-O-G was collected. The presence of the glucuronide was determined by GC as the TPC derivative after hydrolysis with Glusulase. The P-O-G fraction was evaporated to dryness. The residue was resuspended in methanol, then treated with

trifluoroacetic anhydride (TFA), and injected onto a glass G. C. column (1% OV-1). The mass spectrometric analysis was carried out on a LKB 9000s instrument with an ionizing electron energy of 20 ev.

2. LC to assay the Formation of Diastereomeric Glucuronides of P and HOP by Rat Liver Microsomes - J. A. Thompson *et al.* [56]

Incubations were performed by shaking microsomal suspensions containing microsomal protein from rat liver, 10 mM UDPGA, and P or HOP at 37°C. The P-O-G samples were analyzed on a 4.6 x 250 mm Ultrasphere ODS column with a mobile phase consisting of CH_3OH/0.1M $NH_4H_2PO_4$ (56:44). The HOP-G diastereomer samples were analyzed on the same column with the same mobile phase solvents as above, but in the ratio 13:87. The UV detector was set at 225 nm. In addition, the identifications were performed by GC/MS techniques.

MATERIALS AND METHODS

I. Materials

Racemic propranolol hydrochloride (lot # 81F-0306), UDPGA, ß-glucuronidase (Type B-1), $MgCl_2$, and Tris, were obtained from Sigma Chemical Co. (St. Louis, MO). Racemic 4-hydroxypropranolol hydrochloride (ADM 441017181) was supplied by Imperial Chemical Inc., PLC Pharmaceutical Division (Macclesfield, Great Britain). 4-Methyl-propranolol hydrochloride {Fig. 1.(b)} was obtained from Ayerst Laboratories Inc. (New York, NY). (S)-alprenolol hydrochloride {Fig. 1.(b)} was donated by Dr. Hilde from West Germany. Methylene chloride was supplied by J. T. Baker Chemical Co. (Phillipsbury, NJ), while acetonitrile, methanol, phosphoric acid and monobasic ammonium phosphate were from Fisher Scientific Co. (Fair Lawn, NJ). All solvents mentioned above were certified HPLC grades. 2, 2-Dimethoxypropane and triethylamine were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). 2, 3, 4, 6-tetra-O-Acetyl-ß-D-Glucopyranosyl Isothiocyanate (GITC) was bought from Polysciences Inc. (Warrington, PA). Methylene chloride and acetonitrile were dried over molecular sieves.

II. Apparatus

A programmable solvent delivery pump (model 590, Waters Associates, Milford, MA), an automatic injector (WISP 710B, Waters Associates, Milford, MA), an Altex Ultrasphere ODS C18 or C8, 5 µm, 4.6 mm x 25 cm (Beckman Instruments Berkeley, CA), two Shimadzu Fluorescence detectors (model RF-530, Shimadzu ISI Instruspec Inc., Walnut Creek, CA), and two Hewlett-Packard Reporting Integrators (model # 3390A and # 3392A, Hewlett-Packard Co., Avondale, PA) were used.

III. Microsomes and Incubations

Rats were killed by cervical dislocation, and livers were immediately removed, weighed and homogenized in three volumes of ice cold KCL/Tris buffer (0.15 M:0.05 M, pH 7.4, at 37°C). Microsomes were prepared by differential centrifugation [57] and resuspended to give 2 g-equivalents of liver per ml of buffer. Microsomal protein was measured by the method of Lowry *et al.* [58]. The liver specimens were either used directly or after storage at -80°C. The incubations were performed in a system containing liver microsomes (10 mg of protein per ml), Tris·HCL buffer (50 mM, pH 7.4), $MgCl_2$ (10 mM), 10 mM UDPGA, and 0.2 mM HOP or P in stoppered tubes at 37°C for various times. Control incubation mixtures did not contain UDPGA. Total incubation volumes were 0.85 ml. Enzymatic reactions were terminated by adding equivalent volumes of acetonitrile and cooling to 0°C. The chromatograms are presented in Fig. 8.

IV. Drug Administration and Collection of Specimens

Twelve healthy volunteers began a standard diet without taking any medications for four weeks prior to the study. After having fasted for overnight, volunteers donated blank blood and urine samples just prior to the dosing. At approximately 8:00 AM, volunteers would begin taking two tablets of 40 mg of P (Inderal^R, manufactured by Ayerst) for a total dose of 80 mg of P. Relative to this dose, all urine voided during the following post-dose periods was collected, its volume measured, ascorbic acid (2 mg/ml) added, about a 20 ml aliquot saved and frozen for analysis : -1-0, 0-1, 1-2, 2-3, 3-4, 4-6, 6-8, 8-12, 12-24, 24-48, and 48-72 hours.

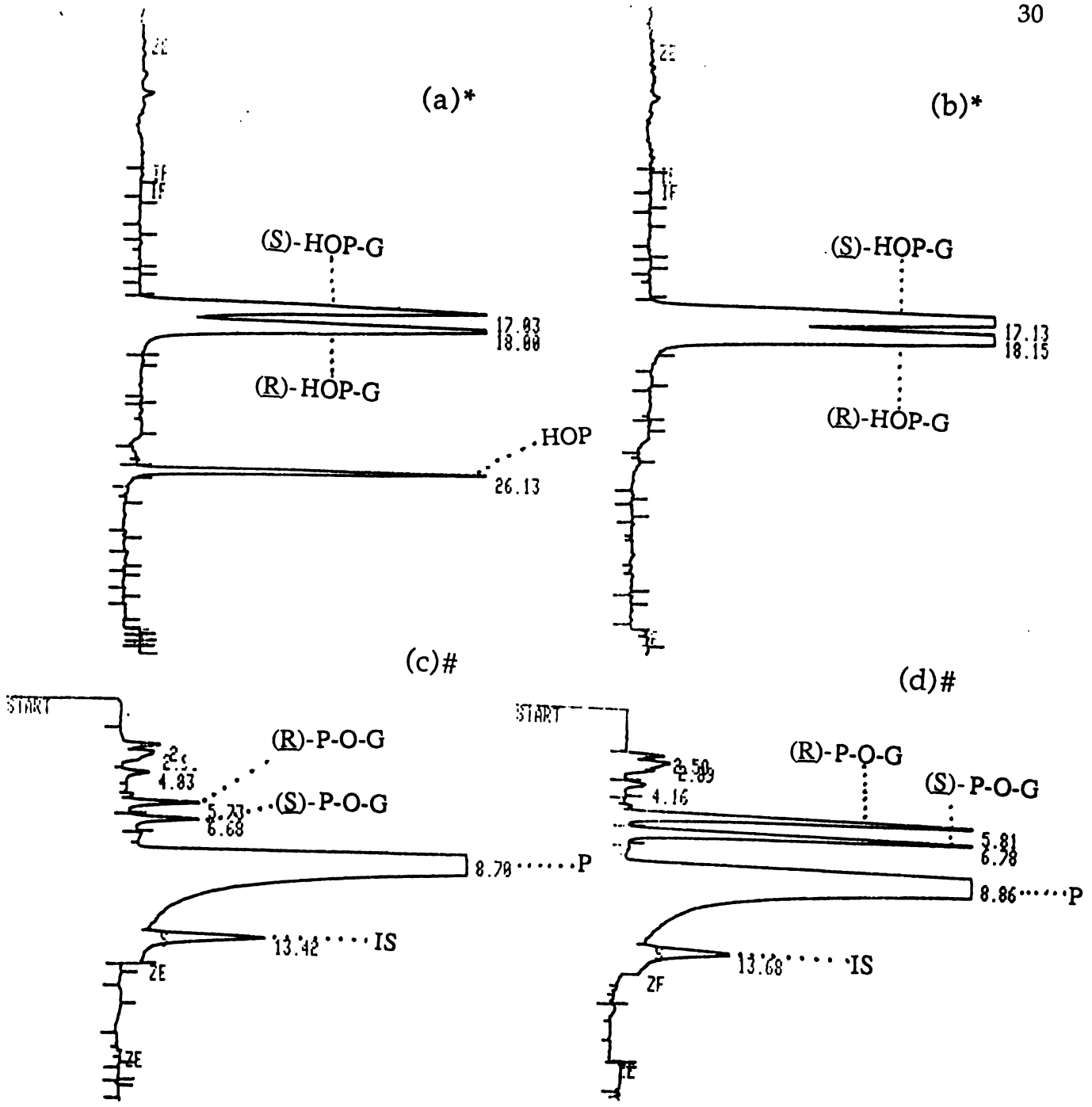


Fig. 8. HPLC chromatograms of (a): (R)-, (S)-HOPG from rat liver microsomal incubates consisting of 2 mM HOP substrate at 37° C for 20 min, (b): (R)-, (S)-HOPG, 2 mM HOP substrate at 37° C for 120 min, (c): (R)-, (S)-P-O-G from rat liver microsomal incubates consisting of 2 mM P substrate at 37° C for 20 min, internal standard is 4-Methyl-P, and (d): (R)-, (S)-P-O-G, 2 mM P substrate at 37° C for 120 min.

* Stepwise gradient mobile phase : 15% CH_3OH in 0.1M $NH_4H_2PO_4$, 18 min; 55% CH_3OH in $NH_4H_2PO_4$, 17 min.

\# Isocratic mobile phase : 55% CH_3OH in 0.1 M $NH_4H_2PO_4$.

V. Preparation of Standard Solutions

HOP-HCL standard stock solution is prepared by weighing 2.28 mg of HOP-HCL in 10 ml of 50% CH_3CN with 25% ascorbic acid. This stock solution has a concentration of 20.0 μg/ml HOP. A working solution of 2.00 μg/ml is made by diluting 5 ml of the stock solution with the same solvent to a volume of 50 ml in a volumetric flask. 4-Methyl-P is prepared as a stock solution of 100 μg/ml. A 1.00 μg/ml working solution of 4-methyl-P is prepared from this stock solution. (S)-alprenolol is prepared as a stock solution of 200 μg/ml. A 40.0 μg/ml working solution of (S)-alprenolol is prepared from this stock solution. GITC working solution is prepared at 100 μg/ml in dry acetonitrile.

VI. Extraction Procedures and Derivative Formation

To 0.5 ml of plasma, 50 μl of internal standard working solution (4-Methyl-P) is added. The mixture is vortexed for one min and 0.5 ml of 1 M K_2HPO_4 (pH=10.0) is added to make the mixture alkaline. Six milliliters of methylene chloride is added to extract the drug and internal standard into the methylene chloride. This organic layer is transferred to another tube and evaporated to dryness under nitrogen. Then, 1 ml of dry methylene chloride is added and, again, the mixture is evaporated to dryness under nitrogen. Next, 0.2 ml of 2, 2-dimethoxypropane is added and gently evaporated to dryness again to remove residual water. A 150 μl of GITC solution is added to derivatize the racemate into the (R)- and (S)-enantiomer and the mixture is left to stand overnight. Finally, the reaction mixture is evaporated to dryness. 200 μl of 50% acetonitrile is added to the products. 70 μl of the resulting solution is injected onto the column.

VII. Chromatographic Conditions for Separation of (R)- and (S)-HOP

The mobile phase was 50% acetonitrile, 0.15% of phosphoric acid, and 0.06% triethylamine. The solvent was degassed and filtered before use. The flow rate was 1.0 ml/min. A 4.6 x 250-mm ODS column (Altex) was used.

The fluorescence detector is set at 325 nm for excitation and at 400 nm for emission with a slit width of 20 nm. The retention times for (R)-, (S)-HOP, and (R)-, (S)-4-methyl-P were 16.5, 13.5, 32.5 and 24.5 minutes, respectively (Fig. 9).

VIII. Chromatographic Conditions for Separation of (R)- and (S)-P

The mobile phase was 58% acetonitrile, 0.3% of phosphoric acid, and 0.06% triethylamine. The solvent was degassed and filtered before use. The flow rate was 1.0 ml/min. A 4.6 x 250-mm ODS column (Altex) was used.

The fluorescence detector was set at 280 nm for excitation and at 340 nm for emission with a slit width of 20 nm. The retention times for (R)-, (S)-P, and (R)-, (S)-4-methyl-P were 10.5, 8.98, 14.2 and 11.6 minutes, respectively (Fig. 10).

IX. Chromatographic Conditions for Separation of (R)-, (S)-Glucuronide Conjugates of P and HOP

The (R)-, (S)-P-O-G and (R)-, (S)-HOP-G diastereomers were analyzed on a 4.6 x 250-mm Spherisorb C8 column (5 μ diameter). A stepwise gradient solvent system programmed by flow rate was used. It consisted of : Solvent I (Pump A), CH_3OH/0.1 M $NH_4H_2PO_4$, 10/90 and Solvent II (Pump B), CH_3OH/0.1 M $NH_4H_2PO_4$, 60/40. The total flow rate (Pump A flow rate and Pump B flow rate) was 1 ml/min. The stepwise

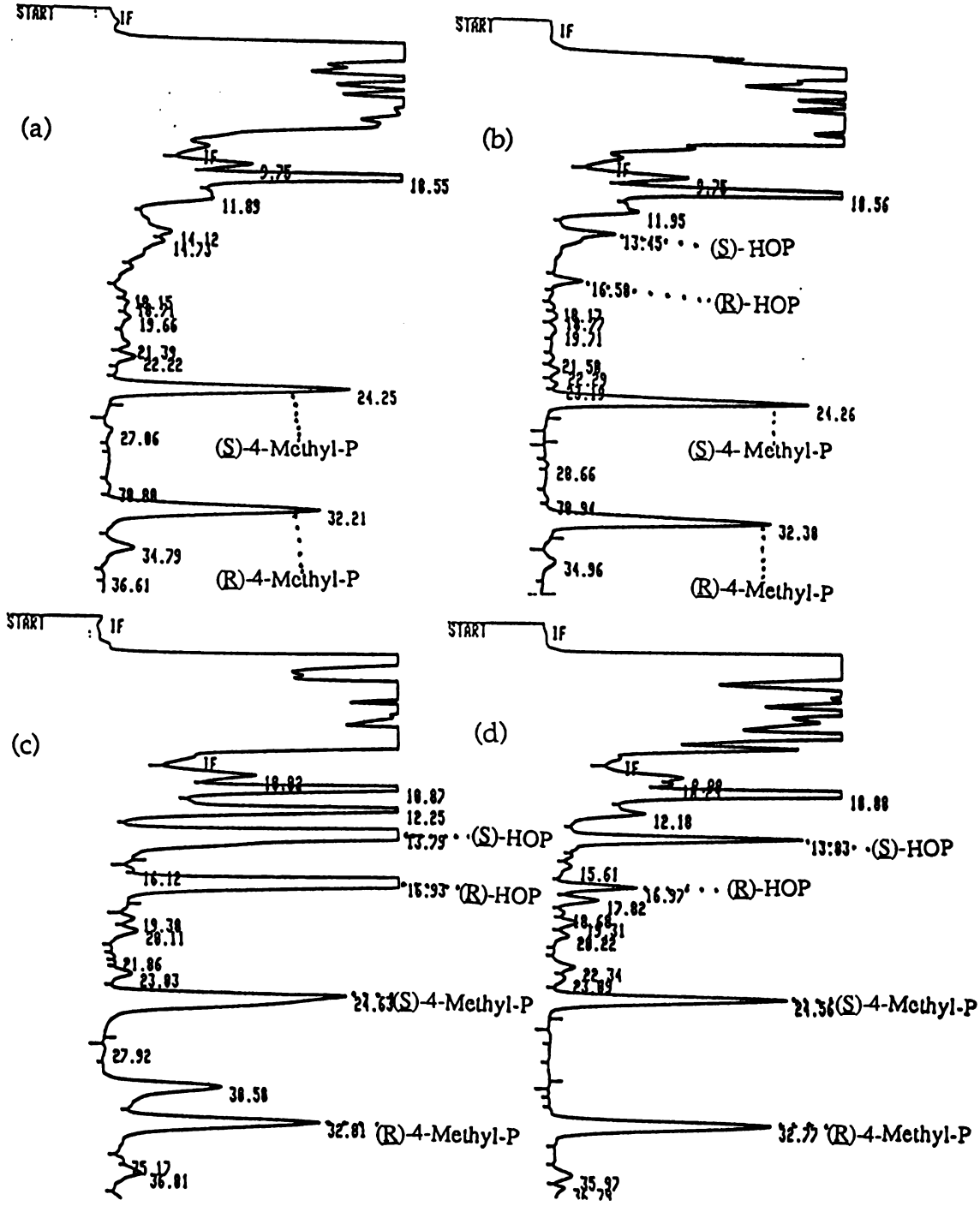


Fig. 9. HPLC chromatograms of (a): blank plasma spiked with internal standard (4-methyl-P), (b): plasma fortified with 1 ng/ml each of (R)-, (S)-HOP, (c): plasma fortified with 40 ng/ml each of (R)-, (S)-HOP, and (d): 1 hour plasma sample of one volunteer after 80 mg oral dose of (R)-, (S)-P, concentration : (R)-HOP 2.17 ng/ml, (S)-HOP 5.40 ng/ml.

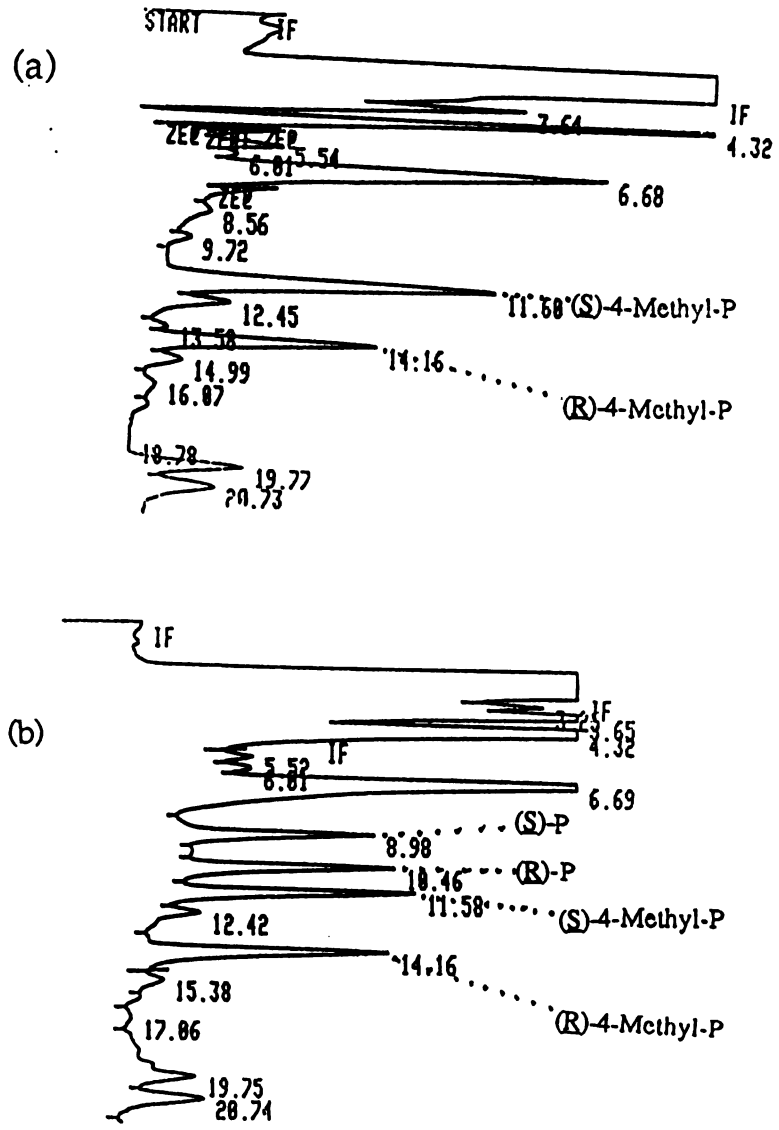


Fig. 10. HPLC chromatograms of (a): blank plasma spiked with internal standard (4-methyl-P), and (b): plasma fortified with 3 ng/ml each of (R)-, (S)-P

gradient program was expressed by the flow rate of Pump A: 1ml/min, 29 min; 0.6 ml/min, 13 min; 0.5 ml/min, 16 min; 0.4 ml/min, 5 min; 0.36 ml/min, 3 min; 0.35 ml/min, 3 min; 0.33 mi/min, 6 min. The two fluorescence detectors connected in series were set at Ex 325 nm and Em 400 nm for HOP-G and HOP, and at Ex 280 nm and Em 340 nm for P-O-G and P. The retention times for the (R)-, (S)-glucuronide diastereomers of P and HOP in urine are shown in Fig. 11, 12.

X. Sample Preparation for Determination of (R)-, (S)-Glucuronide Conjugates of P and HOP

To 100 µl of urine, 50 µl of internal standard working solution {40 ng/µl (S)-alprenolol} was added. The mixture was vortexed for 1 min and 25 µl of the resulting solution injected onto the column.

XI. Preparation and Quantitation of (R)-, (S)-Glucuronide Conjugates of HOP from the Microsomal Incubation Mixtures, and of (R)-, (S)-Glucuronide Conjugates of P from the Clinical Urine Samples

Because the synthetic glucuronides of P and HOP are not available at present, measurements of the conjugate metabolites must rely on enzymatic hydrolysis and indirect assay of the liberated aglycones. For pure preparation of the glucuronides of HOP from rat liver microsomal incubates, the incubated (R)- and (S)-HOP-G were isolated from HPLC column effluents. Since the concentration of (R)-, (S)-P-O-G in clinical urine samples is high, again, the (R)- and (S)-P-O-G were purified from HPLC column effluents by directly injecting the clinical urine samples. The mobile phase was the same as previously described. The injection volume was 150 µl (almost the maximum injection volume). The sensitivity of fluorescence detector was lowered down to 32-fold less than usual range 2.

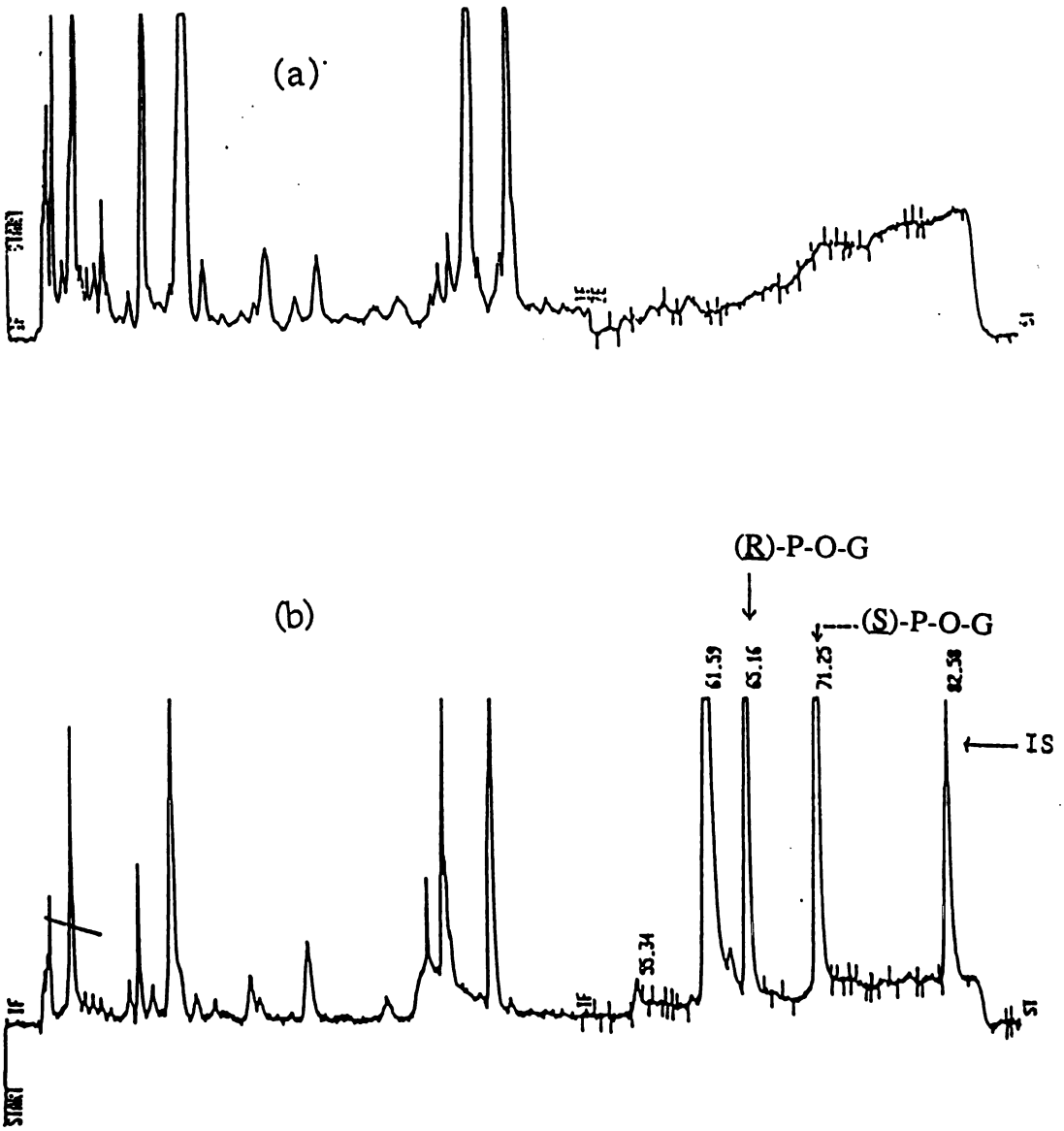


Fig. 11. HPLC chromatograms {with wavelength set for (R)-, (S)-P-O-G} of (a): blank urine, and (b): (R)-P-O-G (2.17 μg/ml) and (S)-P-O-G (2.23 μg/ml) in a 2-hr urine sample from one volunteer after a 80 mg oral dose of P; the internal standard was (S)-alprenolol.

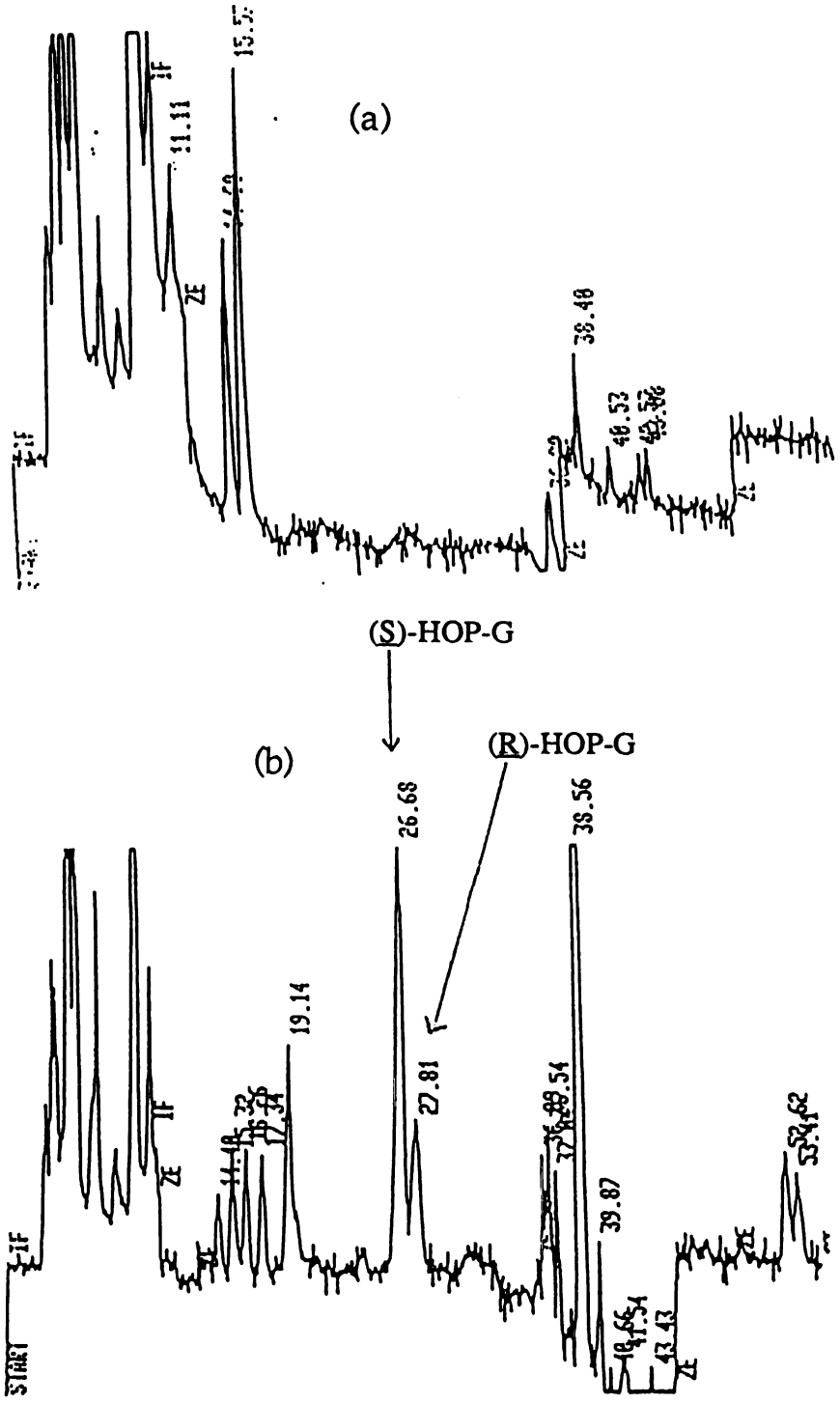


Fig. 12. HPLC chromatograms {with wavelength set for (R)-, (S)-HOP-G} of (a): blank urine, and (b): (R)-HOP-G (0.502 μg/ml) and (S)-HOP-G (1.12 μg/ml) in a 2-hr urine sample from one volunteer after a 80 mg oral dose of P.

Then, four peaks {Fig. 13 (a), (b) and Fig. 14 (a), (b)} were purified and collected individually.

Enzymatic hydrolysis of glucuronides involves prior removal of methanol from the collected solutions of glucuronides under nitrogen evaporation, addition of ascorbic acid, adjustment of the pH to 5.0 with 0.1 M NaOH, and incubation with 2000 units of ß-glucuronidase at 37°C for 15-20 hrs for hydrolysis of (R)-, (S)-P-O-G {Fig. 13 (c), (d)}, 3 hrs for hydrolysis of (R)-, (S)-HOP-G{Fig. 14 (c), (d)}. Controls were incubated without ß-glucuronidase. The P and HOP, liberated by hydrolysis of four peaks, could be quantitated. And, the four purified stock solutions {(R)- and (S)-P-O-G, and (R)-, and (S)-HOP-G} could be the calibrators to assay the biological samples. The quantitations of four stock solutions are shown in {Table 1 (a), (b)}. The working standard solutions were prepared by pooling the clinical urine samples (containing high concentration of glucuronides when tested in advance) together, then calibrated by stock solutions.

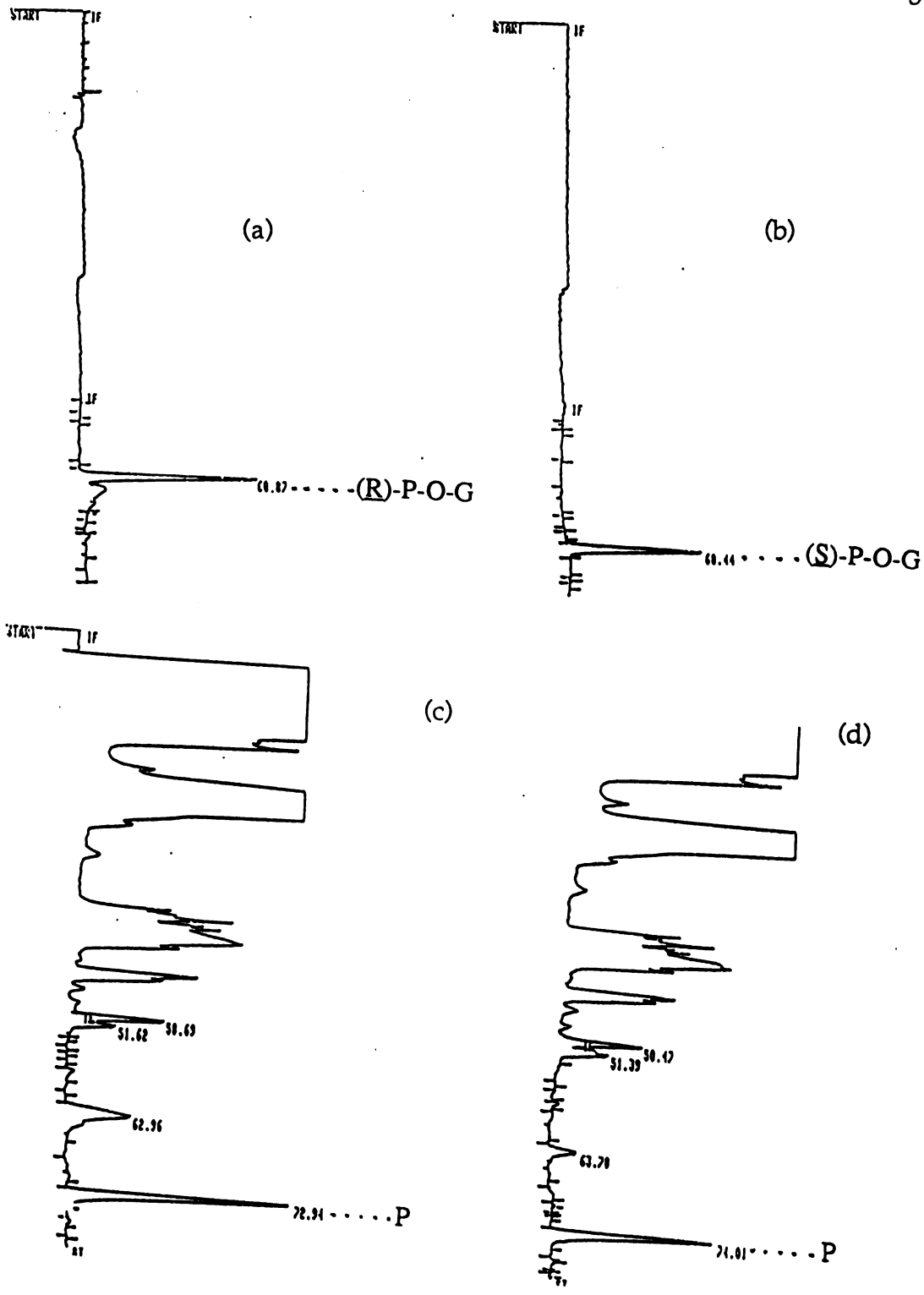


Fig. 13. HPLC chromatograms of (a): (R)-P-O-G purified from urine, (b): (S)-P-O-G purified from urine, (c): P released from enzyme hydrolysis of (R)-P-O-G, and (d): P released from enzyme hydrolysis of (S)-P-O-G.

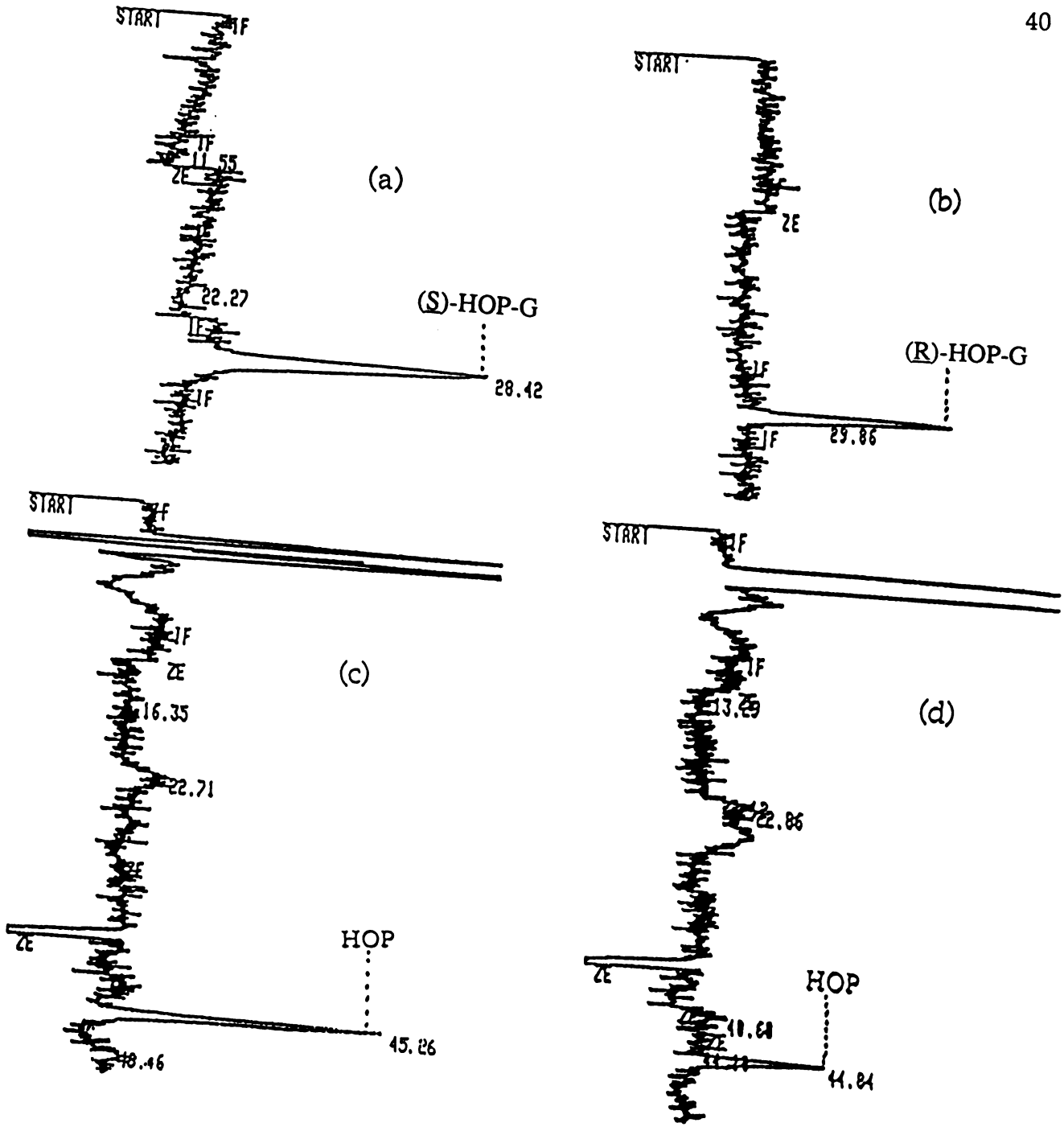


Fig. 14. HPLC chromatograms of (a): (S)-HOP-G purified from rat liver microsomal incubates, (b): (R)-HOP-G purified from rat liver microsomal incubates, (c): HOP released from enzyme hydrolysis of (S)-HOP-G, and (d): HOP released from enzyme hydrolysis of (R)-HOP-G.

Table 1 (a). Quantitation of R, S-Propranolol-O-Glucuronide (purified from urine)

Volume of Enzyme Hydrolysis[a] (µl)	R-P-O-G[b] Concentration (µg/ml)	S-P-O-G[b] Concentration (µg/ml)
100	0.305	0.197
100	0.317	0.183
100	0.279	0.188
200	0.317	0.200
200	0.301	0.191
200	0.342	*c*
Mean Concentration[b] (µg/ml)	**0.310**	**0.192**
SD	0.0209	0.0067
%CV	6.72	3.47

a. R and S-P-O-G were incubated with 2000 units of ß-glucuronidase at 37°C for 15 hrs.

b. Expressed as P.

c. Bad chromatogram.

Table 1 (b). Quantitation of R, S-4-Hydroxypropranolol-Glucuronide (purified from rat liver microsomal incubation)

Volume of Enzyme Hydrolysis* (µl)	R-HOP-G** Concentration (µg/ml)	S-HOP-G** Concentration (µg/ml)
100	0.268	0.726
100	0.277	0.789
150	0.267	0.755
150	0.280	0.664
200	0.289	0.752
200	0.271	0.704
Mean Concentration** (µg/ml)	**0.275**	**0.732**
SD	0.0086	0.044
%CV	3.11	6.01

*R and S-HOP-G were incubated with 2000 units of ß-glucuronidase at 37°C for 3 hrs.

** Expressed as HOP.

RESULTS

I. Development of Mobile Phases for Separations of (R)-, (S)-Glucuronide Diastereomers of P and HOP

Considerable experimentation was needed to determine the suitable mobile phases during the method development. Initially, by using a gradient solvent system consisting of Solvent I : CH_3OH/CH_3CN/0.3% H_3PO_4 (5:5:90), and of Solvent II : CH_3OH/CH_3CN/0.3% H_3PO_4 (45:45:10), running a continuous gradient program from Solvent I to Solvent II, some endogeneous peaks were found to interfere with the glucuronide peaks of P and HOP. With an ion-pairing reagent, SDS, another gradient solvent system consisting of solvent I, 20% CH_3CN in 0.2% SDS and 0.3% H_3PO_4, and of Solvent II, 80% CH_3CN in 0.2% SDS and 0.3% H_3PO_4, running from solvent I to Solvent II continuously, was tried. But the resolution of the (R)- and (S)-glucuronides was poor and analysis time (105 min) was too long. Finally, a stepwise gradient solvent system, which was programmed by flow rate to separate (R)- and (S)-glucuronides by using CH_3OH and 0.1 M $NH_4H_2PO_4$, was developed. The mobile phases consisted of Solvent I (Pump A): 10% CH_3OH in 0.1 M $NH_4H_2PO_4$; and Solvent II (Pump B): 60% CH_3OH in 0.1 M $NH_4H_2PO_4$. The total flow rate (Pump A flow rate and Pump B flow rate) was 1 ml/min. The stepwise gradient program was expressed by the flow rate of Pump A: 1 ml/min, 29 min; 0.6 ml/min, 13 min; 0.5 ml/min, 16 min; 0.4 ml/min, 5 min; 0.36 ml/min, 3 min; 0.35 ml/min, 3 min; 0.33 ml/min, 6 min.

II. Chromatographic Analysis of (R)-, (S)-Glucuronide Diastereomers of P and HOP from Microsomal Incubates and Urine

Typical chromatograms are presented in Fig. 11, 12. Because (R)- and (S)-P-O-G are much more lipophilic than (R)- and (S)-HOP-G, (R)- and (S)-HOP-G were eluted out by Solvent I (10% CH_3OH). But (R)- and (S)-P-O-G can not be eluted out until 35.0% CH_3OH is used. The diastereomers of HOP-G are more difficult to separate than those of P-O-G, requiring a more efficient column and conditions that produce greater retention volumes. In Fig. 10, there is one pair of interference peaks close to (R)-P-O-G. The interference peaks exist in chromatograms of all 12 subjects. After treatment with ß-glucuronidase, the interference peaks still exist, and they may represent other non-glucuronide metabolites of P in urine. The chromatograms of the treatment with or without ß-glucuronidase in urine are shown in Fig. 15, 16.

Evidence that the chromatographic peaks in Fig. 8 are indeed due to glucuronides was obtained by two methods : 1) no peaks are detected if either UDPGA or the substrate drug is omitted from microsomal incubates; 2) these peaks are not affected when microsomal supernatant fractions are incubated for an additional 20 hr at pH 5.0, but they disappear when ß-glucuronidase is included. Fig. 17 shows that the conversion of P and HOP to glucuronides by rat liver microsomes is linear over incubation times up to 2 hr and 1 hr, respectively. The diastereomer ratio is near 1 for both substrates of P and HOP at 2 mM. No significant stereoselectivity was observed in incubations containing 2 mM substrates (Fig. 17) [58]. HOP appears more easily glucuronidated than P does for the same incubation time and at 2 mM substrate concentration (Fig. 8).

III. Calibration Curves and Detection Sensitivities

Standard curves for plasma were obtained by calculating the ratios of peak heights of (R)-, (S)-HOP to that of the internal standard {(S)-4-methyl-P} and plotting that ratio versus the spiked concentration as shown in Fig. 18 and Table 2. Good accuracy and reproducibility are obtained over a range of 1-50 ng/ml of each enantiomer. The sensitivity

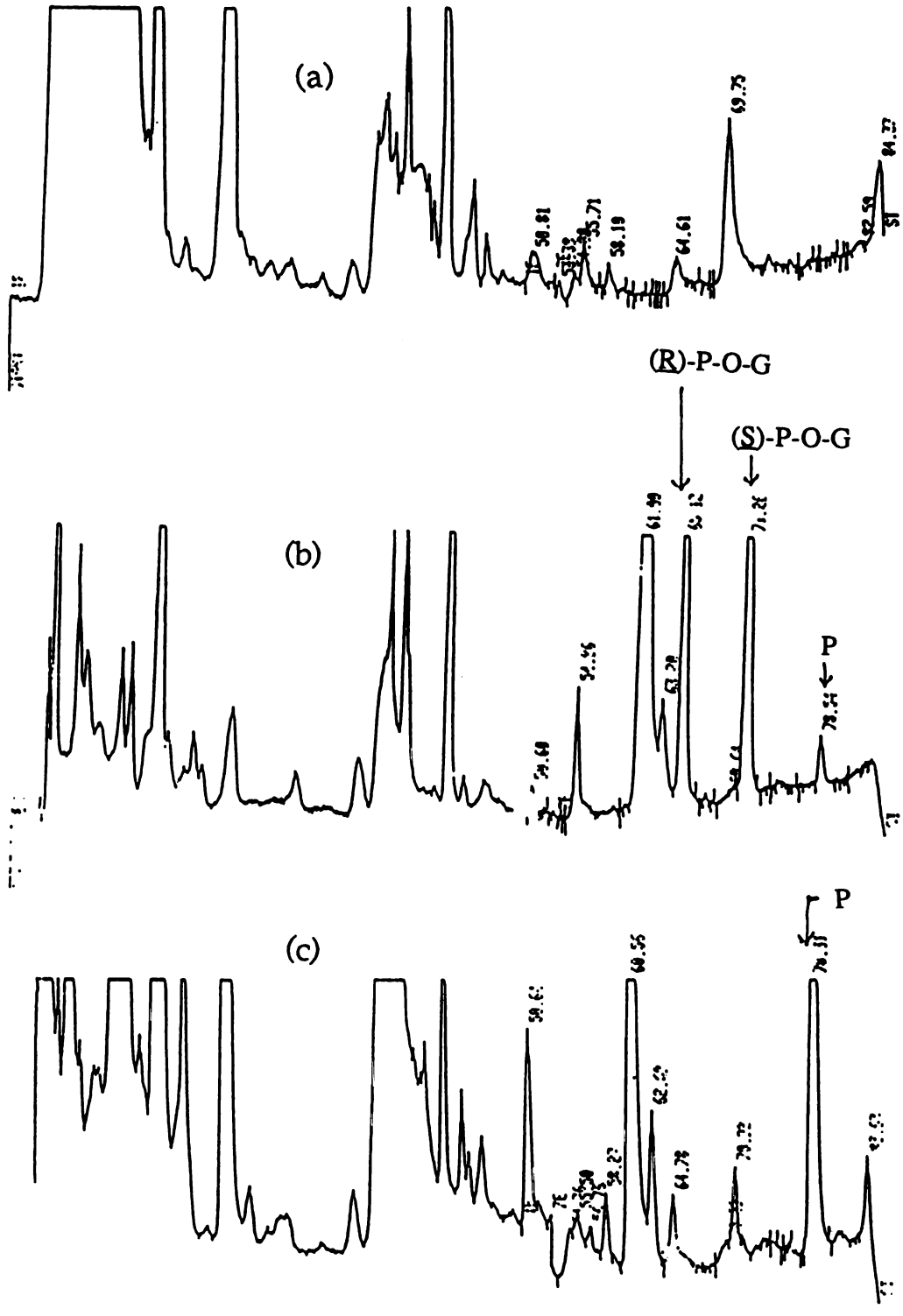


Fig. 15. HPLC chromatograms of (a): blank urine (with enzyme hydrolysis) for (R)-, (S)-P-O-G, (b): 2-hr urine sample of one volunteer (**without** enzyme hydrolysis), (c): 2-hr urine sample of the same volunteer as (b) (**with** enzyme hydrolysis).

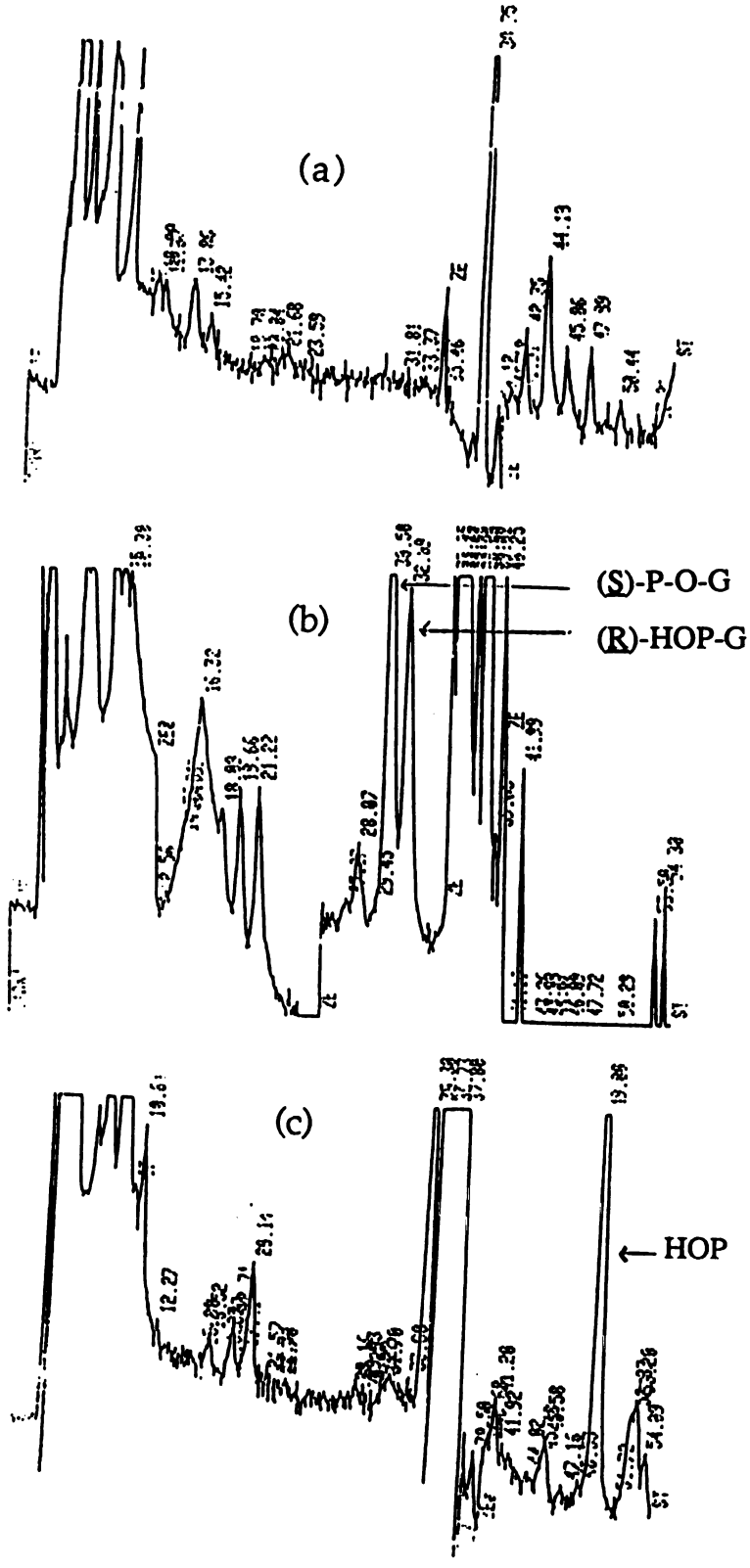


Fig. 16. HPLC chromatograms of (a): blank urine (with enzyme hydrolysis) for (R)-, (S)-HOP-G, (b): 3-hr urine sample of one volunteer (without enzyme hydrolysis), (c): 3-hr urine sample of the same volunteer as (b) (with enzyme hydrolysis).

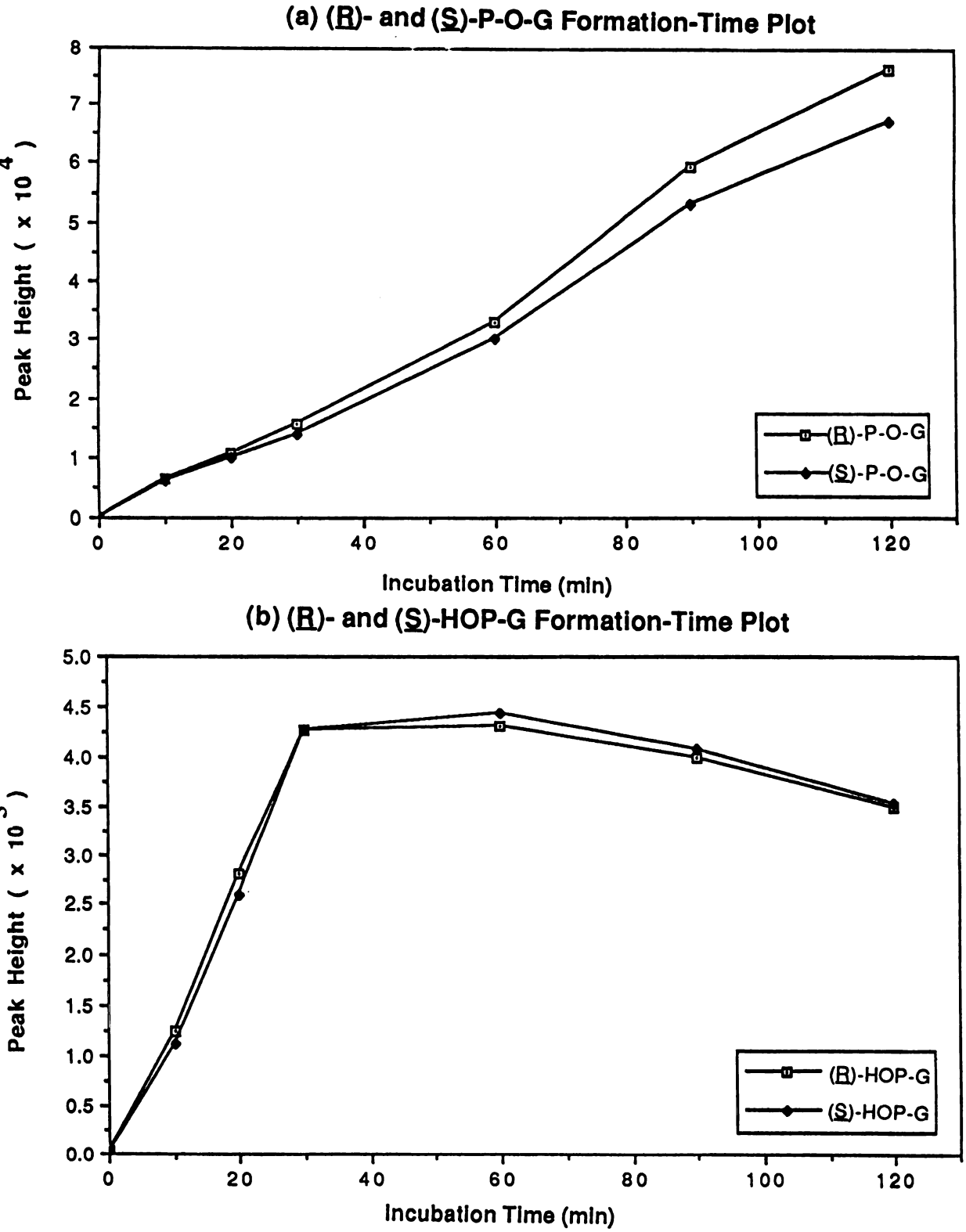


Fig. 17. (a): (R)- and (S)-P-O-G formation vs. time plot, 2 mM substrate of P at 37°C for 120 min, and (b): (R)- and (S)-HOP-G formation vs. time plot, 2 mM substrate of HOP at 37°C for 120 min.

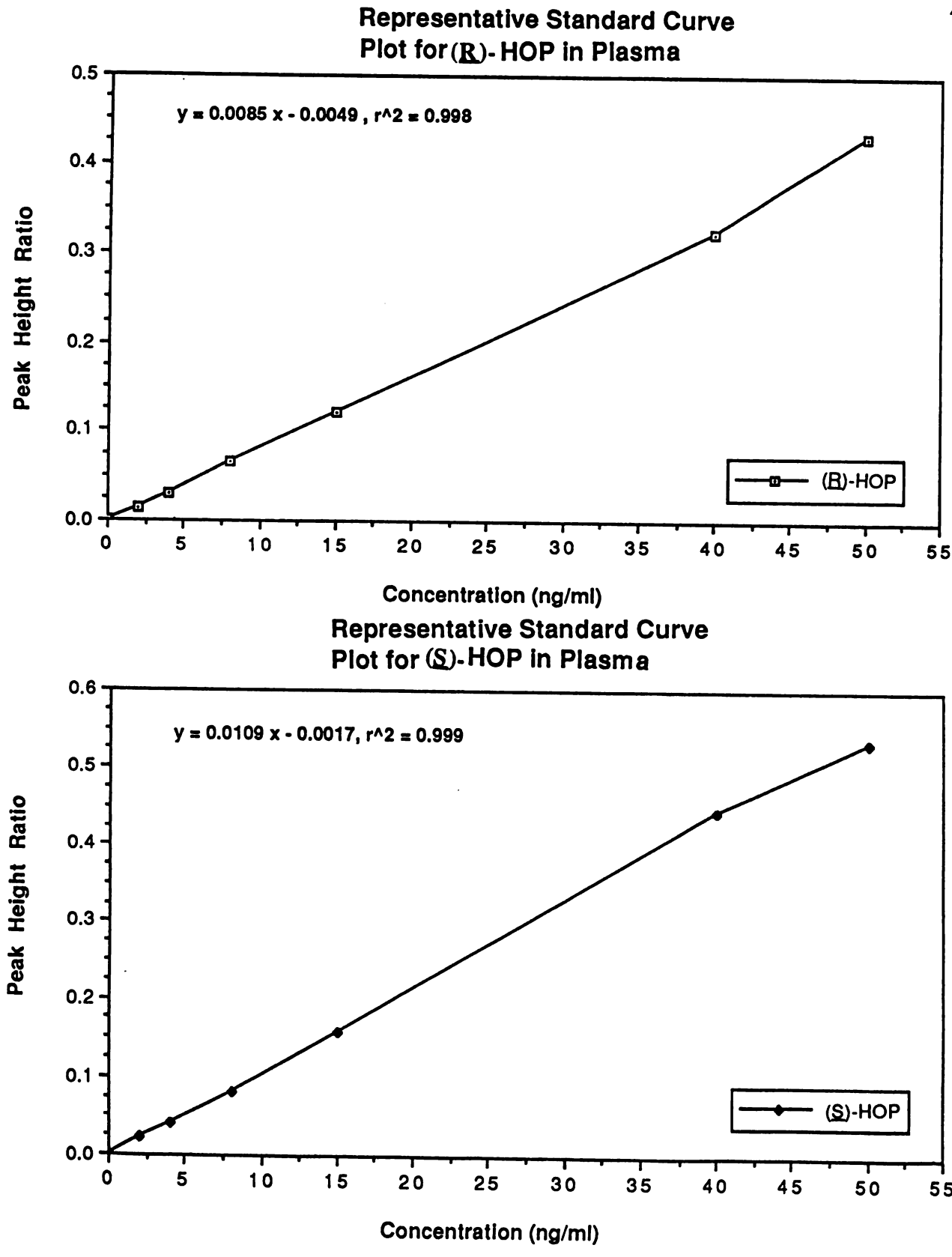


Fig. 18 Linearities of plasma calibration curves for (R)- and (S)-HOP

Table 2. (a) Representative Standard Curve for (R)-HOP in Plasma Assay

Spiked Concentration (ng/ml)	Peak Height Ratio	Calculated Concentration (ng/ml)
0.00	0.000	0.00
1.00	0.011	1.65
2.00	0.014	2.01
4.00	0.029	3.78
8.00	0.065	8.03
15.0	0.122	14.8
40.0	0.323	38.5
50.0	0.431	51.3

Regression equation :

$y = 0.0085 x - 0.003$, $r^2 = 0.998$

Table 2. (b) Representative Standard Curve for (S)-HOP in Plasma Assay

Spiked Concentration (ng/ml)	Peak Height Ratio	Calculated Concentration (ng/ml)
0.00	0.000	0.00
1.00	0.015	1.40
2.00	0.023	2.14
4.00	0.041	3.81
8.00	0.082	7.60
15.0	0.159	14.7
40.0	0.443	40.9
50.0	0.534	49.4

Regression equation :

$y = 0.0108 x - 0.0002$, $r^2 = 0.999$

of this method allows quantitation of 1 ng/ml of (R)-HOP and 1 ng/ml of (S)-HOP when using 0.5 ml plasma.

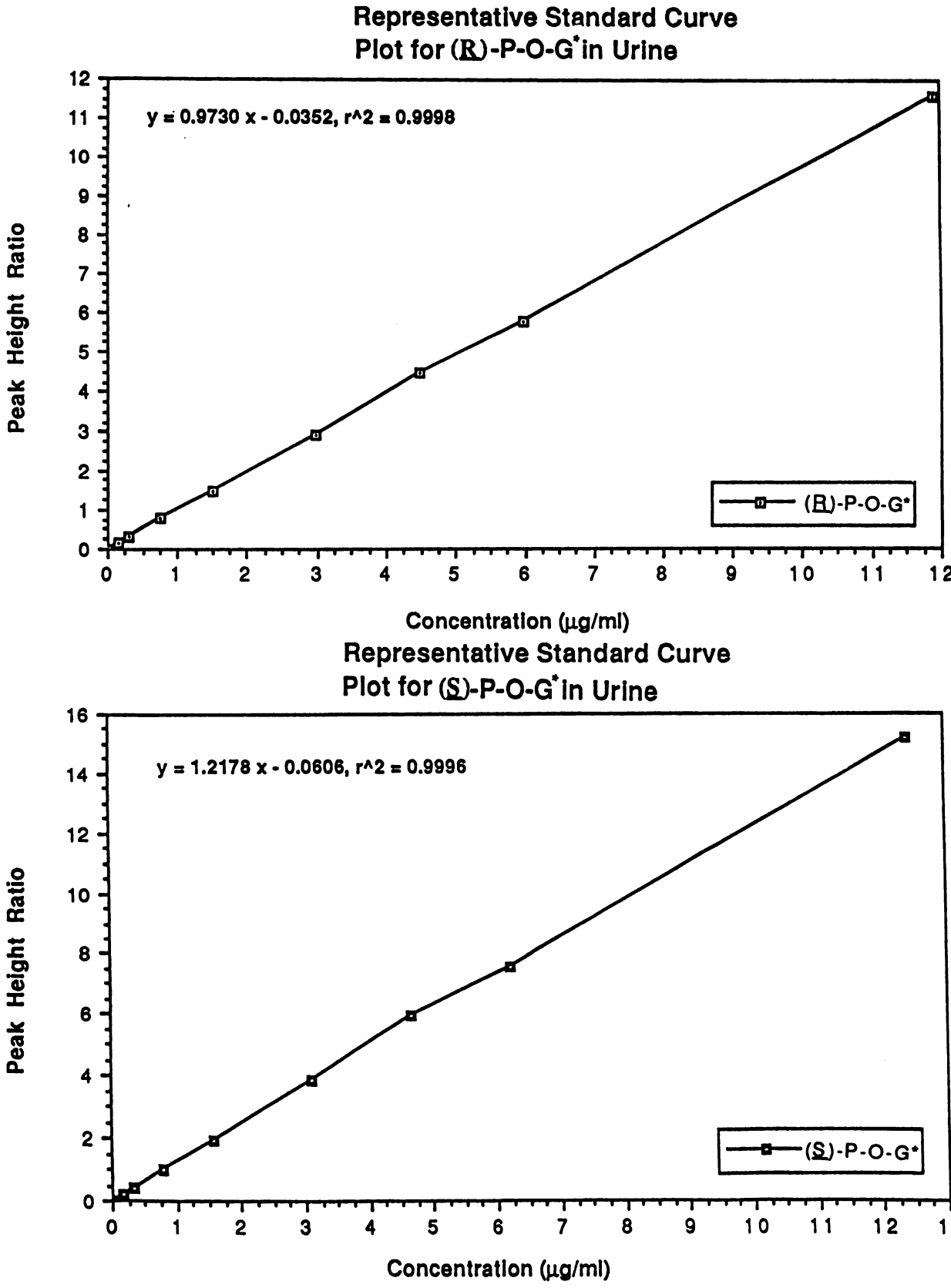
Standard curves for urine were obtained by calculating the ratios of the peak heights of (R)-, (S)-P-O-G, (R)-, and (S)-HOP-G to that of the internal standard {(S)-alprenolol} and plotting that ratio versus the spiked concentration (expressed as P and HOP individually) as shown in Fig. 19, 20 and Tables 3, 4.

IV. Precision

The precision of the methods over the working range was determined by the following procedure. Repeated assays were performed on plasma samples for (R)-, (S)-HOP or urine samples for (R)-, (S)-P-O-G and (R)-, (S)-HOP-G containing four different concentrations of the drug or its metabolites. These analyses performed on the same day were used to validate intra-day precision, while these analyses done on six consecutive days were used to validate inter-day precision. The results of the two studies appear in Tables 5, 6, 7, 8, 9, and 10. With the exception of the medium concentrations for the inter-day precision of (R)- and (S)-HOP in plasma, all the inter-day and intra-day precision data were within 10% CV.

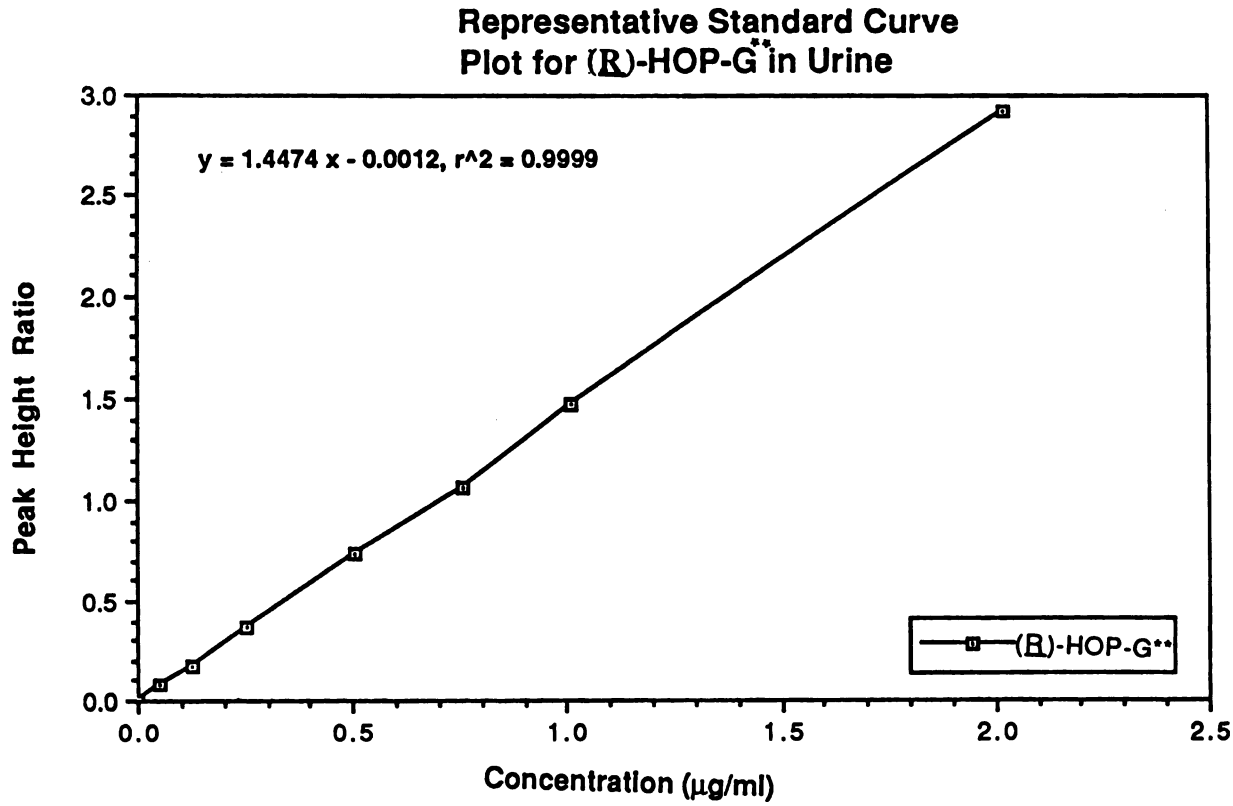
V. Extraction Recovery

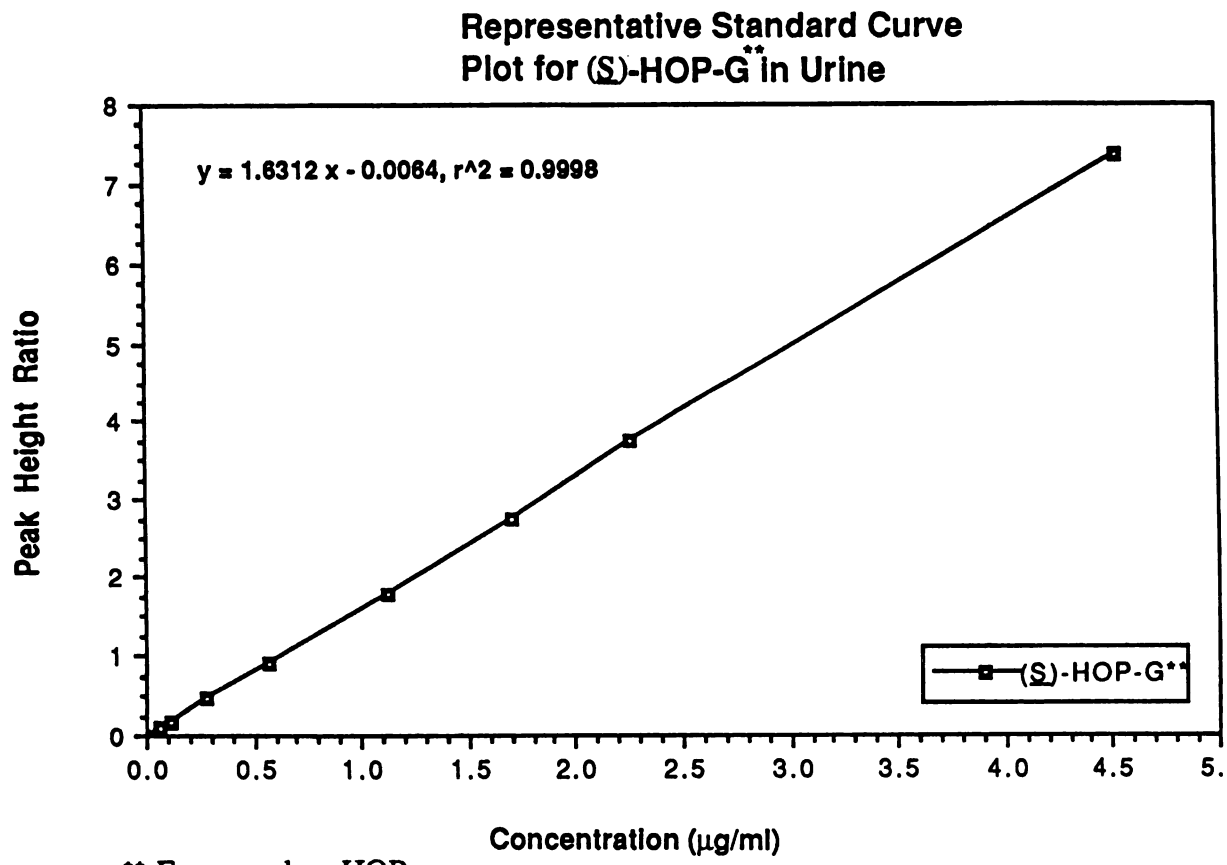
Recovery of HOP in plasma was determined by comparing the drug to internal standard peak height ratios of plasma samples extracted with acetonitrile to the peak height ratios of water sample not extracted with acetonitrile. Then, the internal standard was added to both two-thirds of the acetonitrile extract and the water samples. Finally, the mixture was evaporated to dryness. Thus, the resultant peak height ratio of plasma samples was multiplied by the factor 3/2 (4 ml of acetonitrile extract out of 6 ml of acetonitrile solution).



* Expressed as P.

Fig. 19 Linearities of urine calibration curves for (R)- and (S)-P-O-G





** Expressed as HOP.

Fig. 20 Linearities of urine calibration curves for (R)- and (S)-HOP-G

Table 3. (a) Representative Standard Curve for (R)-P-O-G in Urine Assay

Spiked Concentration* (μg/ml)	Peak Height Ratio	Calculated Concentration* (μg/ml)
0.000	0.000	0.000
0.149	0.172	0.140
0.299	0.320	0.293
0.746	0.778	0.763
1.49	1.48	1.49
2.98	2.93	2.98
4.47	4.50	4.59
5.97	5.78	5.90
11.9	11.6	11.9

Regression equation :
$y = 0.9730 x - 0.0352$, $r^2 = 0.9998$

Table 3. (b) Representative Standard Curve for (S)-P-O-G in Urine Assay

Spiked Concentration* (μg/ml)	Peak Height Ratio	Calculated Concentration* (μg/ml)
0.000	0.000	0.000
0.156	0.220	0.131
0.311	0.417	0.292
0.778	1.02	0.791
1.56	1.96	1.56
3.11	3.85	3.11
4.67	5.98	4.86
6.22	7.55	6.15
12.4	15.2	12.4

Regression equation :
$y = 1.2178 x - 0.0606$, $r^2 = 0.9996$

* Expressed as P.

Table 4. (a) Representative Standard Curve for (R)-HOP-G in Urine Assay

Spiked Concentration* (µg/ml)	Peak Height Ratio	Calculated Concentration* (µg/ml)
0.000	0.000	0.000
0.050	0.074	0.052
0.126	0.175	0.121
0.252	0.366	0.254
0.504	0.738	0.511
0.756	1.07	0.741
1.01	1.47	1.02
2.02	2.92	2.02

Regression equation :
$y = 1.4474 x - 0.0012$, $r^2 = 0.9999$

Table 4. (b) Representative Standard Curve for (S)-HOP-G in Urine Assay

Spiked Concentration* (µg/ml)	Peak Height Ratio	Calculated Concentration* (µg/ml)
0.000	0.000	0.000
0.057	0.104	0.067
0.113	0.170	0.108
0.283	0.467	0.290
0.566	0.911	0.562
1.13	1.78	1.10
1.70	2.74	1.68
2.26	3.76	2.31
4.53	7.37	4.52

Regression equation :
$y = 1.6312 x - 0.0064$, $r^2 = 0.9998$

* Expressed as HOP.

Table 5. (a) Intraday Precision for the (R)-HOP in Plasma Assay

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (40.0 ng/ml)								
35.2	37.0	41.7	42.6	38.8	41.2	39.4	2.94	7.46
Spiked concentration (20.0 ng/ml)								
21.6	21.5	21.8	19.8	18.6	21.3	20.8	1.26	6.09
Spiked concentration (8.00 ng/ml)								
6.60	7.10	7.47	6.23	8.34	6.85	7.10	0.741	10.4
Spiked concentration (4.00 ng/ml)								
3.99	3.62	4.49	4.12	4.12	4.62	4.16	0.357	8.58

Table 5. (b) Intraday Precision for the (S)-HOP in Plasma Assay

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (40.0 ng/ml)								
41.3	41.6	42.6	38.3	43.0	39.4	41.0	1.83	4.45
Spiked concentration (20.0 ng/ml)								
20.9	20.8	19.3	19.0	19.9	20.5	20.1	0.808	4.03
Spiked concentration (8.00 ng/ml)								
7.88	7.54	8.05	7.79	7.45	8.29	7.83	0.314	4.01
Spiked concentration (4.00 ng/ml)								
4.71	4.03	4.88	3.86	4.54	4.03	4.34	0.430	9.79

Table 6. (a) Interday Precision for the (R)-HOP in Plasma Assay

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (40.0 ng/ml)								
37.9	40.2	41.1	41.4	41.1	38.8	40.1	1.42	3.55
Spiked concentration (20.0 ng/ml)								
19.7	22.4	16.2	18.0	16.8	21.3	19.0	2.48	13.0
Spiked concentration (8.00 ng/ml)								
7.64	7.95	8.09	8.23	7.67	8.34	7.99	0.289	3.62
Spiked concentration (4.00 ng/ml)								
3.58	3.65	4.01	4.10	3.57	4.12	3.84	0.264	6.87

Table 6. (b) Interday Precision for the (S)-HOP in Plasma Assay

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (40.0 ng/ml)								
41.5	38.4	40.5	38.7	39.8	39.4	39.7	1.16	2.91
Spiked concentration (20.0 ng/ml)								
21.9	21.2	15.8	20.0	17.4	20.5	19.5	2.35	12.1
Spiked concentration (8.00 ng/ml)								
8.52	8.07	7.61	8.21	7.22	8.29	7.99	0.482	6.03
Spiked concentration (4.00 ng/ml)								
3.45	3.92	3.85	3.79	3.57	4.55	3.85	0.385	9.99

Table 7. (a) Intraday Precision for the (R)-P-O-G in Urine Assay*

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (2.98 µg/ml)								
2.97	3.04	3.04	3.03	3.03	2.93	3.01	0.045	1.50
Spiked concentration (2.24 µg/ml)								
2.28	2.20	2.22	2.22	2.22	2.14	2.21	0.047	2.11
Spiked concentration (1.49 µg/ml)								
1.48	1.46	1.51	1.49	1.45	1.49	1.48	0.019	1.29
Spiked concentration (0.597 µg/ml)								
0.603	0.602	0.631	0.603	0.609	0.592	0.607	0.013	2.14

Table 7. (b) Intraday Precision for the (S)-P-O-G in Urine Assay*

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (3.11 µg/ml)								
3.00	3.07	3.10	3.19	3.16	3.08	3.10	0.070	2.26
Spiked concentration (2.33 µg/ml)								
2.27	2.29	2.31	2.30	2.14	2.31	2.27	0.067	2.95
Spiked concentration (1.56 µg/ml)								
1.56	1.53	1.61	1.59	1.56	1.54	1.57	0.029	1.83
Spiked concentration (0.622 µg/ml)								
0.610	0.622	0.656	0.642	0.645	0.624	0.633	0.017	2.69

* Concentration is expressed as P.

Table 8. (a) Interday Precision for the (R)-P-O-G in Urine Assay*

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (2.98 µg/ml)								
2.91	2.91	2.97	2.97	2.86	2.92	2.93	0.043	1.47
Spiked concentration (2.24 µg/ml)								
2.20	2.20	2.31	2.16	2.37	2.28	2.25	0.089	3.97
Spiked concentration (1.49 µg/ml)								
1.47	1.43	1.52	1.47	1.46	1.57	1.47	0.033	2.22
Spiked concentration (0.597 µg/ml)								
0.607	0.576	0.596	0.573	0.597	0.582	0.588	0.013	2.21

Table 8. (b) Interday Precision for the (S)-P-O-G in Urine Assay*

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (3.11 µg/ml)								
3.13	3.06	2.94	3.25	3.12	3.20	3.12	0.110	3.52
Spiked concentration (2.33 µg/ml)								
2.23	2.27	2.23	2.50	2.48	2.34	2.34	0.138	5.90
Spiked concentration (1.56 µg/ml)								
1.58	1.49	1.50	1.55	1.54	1.67	1.53	0.036	2.35
Spiked concentration (0.622 µg/ml)								
0.602	0.597	0.624	0.605	0.611	0.625	0.611	0.012	1.92

* Concentration is expressed as P.

Table 9. (a) Intraday Precision for the (R)-HOP-G in Urine Assay*

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (0.504 μg/ml)								
0.477	0.505	0.476	0.502	0.504	0.485	0.491	0.013	2.65
Spiked concentration (0.378 μg/ml)								
0.358	0.377	0.420	0.378	0.362	0.338	0.372	0.028	7.44
Spiked concentration (0.252 μg/ml)								
0.243	0.249	0.259	0.257	0.242	0.258	0.251	0.008	3.06
Spiked concentration (0.101 μg/ml)								
0.100	0.097	0.095	0.103	0.102	0.099	0.099	0.003	2.92

Table 9. (b) Intraday Precision for the (S)-HOP-G in Urine Assay*

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (1.13 μg/ml)								
1.08	1.12	1.10	1.13	1.10	1.09	1.10	0.020	1.81
Spiked concentration (0.849 μg/ml)								
0.819	0.848	0.902	0.843	0.840	0.766	0.836	0.044	5.26
Spiked concentration (0.566 μg/ml)								
0.543	0.567	0.562	0.531	0.548	0.558	0.552	0.013	2.36
Spiked concentration (0.226 μg/ml)								
0.206	0.212	0.209	0.208	0.220	0.211	0.211	0.005	2.32

* Concentration is expressed as HOP.

Table 10. (a) Interday Precision for the (R)-HOP-G in Urine Assay*

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (0.504 μg/ml)								
0.513	0.533	0.485	0.539	0.506	0.503	0.513	0.022	4.29
Spiked concentration (0.378 μg/ml)								
0.384	0.390	0.385	0.390	0.391	0.387	0.388	0.004	0.90
Spiked concentration (0.252 μg/ml)								
0.241	0.238	0.252	0.264	0.243	0.242	0.248	0.011	4.33
Spiked concentration (0.101 μg/ml)								
0.110	0.109	0.092	0.101	0.104	0.091	0.101	0.008	8.09

Table 10. (b) Interday Precision for the (S)-HOP-G in Urine Assay*

1	2	3	4	5	6	Mean	SD	%CV
Spiked concentration (1.13 μg/ml)								
1.20	1.13	1.15	1.09	1.12	1.13	1.14	0.040	3.50
Spiked concentration (0.849 μg/ml)								
0.841	0.857	0.842	0.839	0.882	0.853	0.852	0.018	2.11
Spiked concentration (0.566 μg/ml)								
0.548	0.568	0.550	0.577	0.552	0.556	0.559	0.013	2.33
Spiked concentration (0.226 μg/ml)								
0.239	0.225	0.243	0.222	0.236	0.219	0.231	0.010	4.28

* Concentration is expressed as HOP.

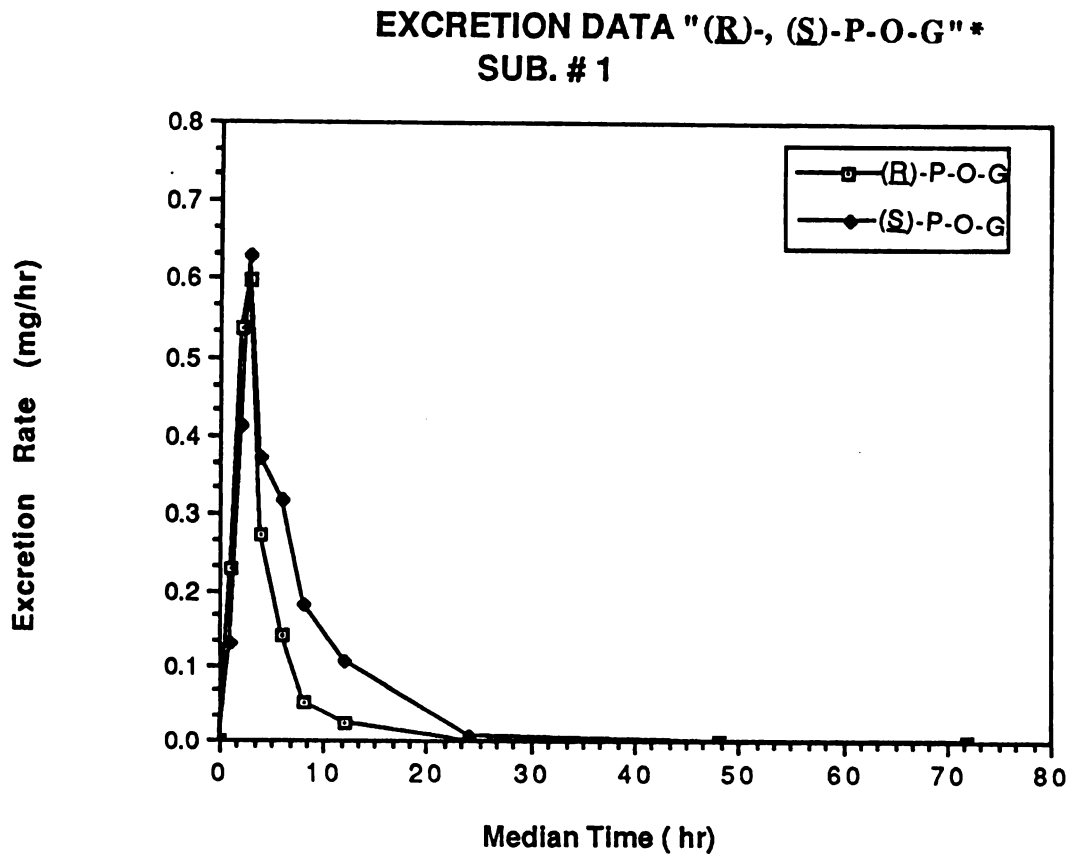
The recoveries at different concentrations for plasma appear in Table 11. The overall average recovery was 78.4%.

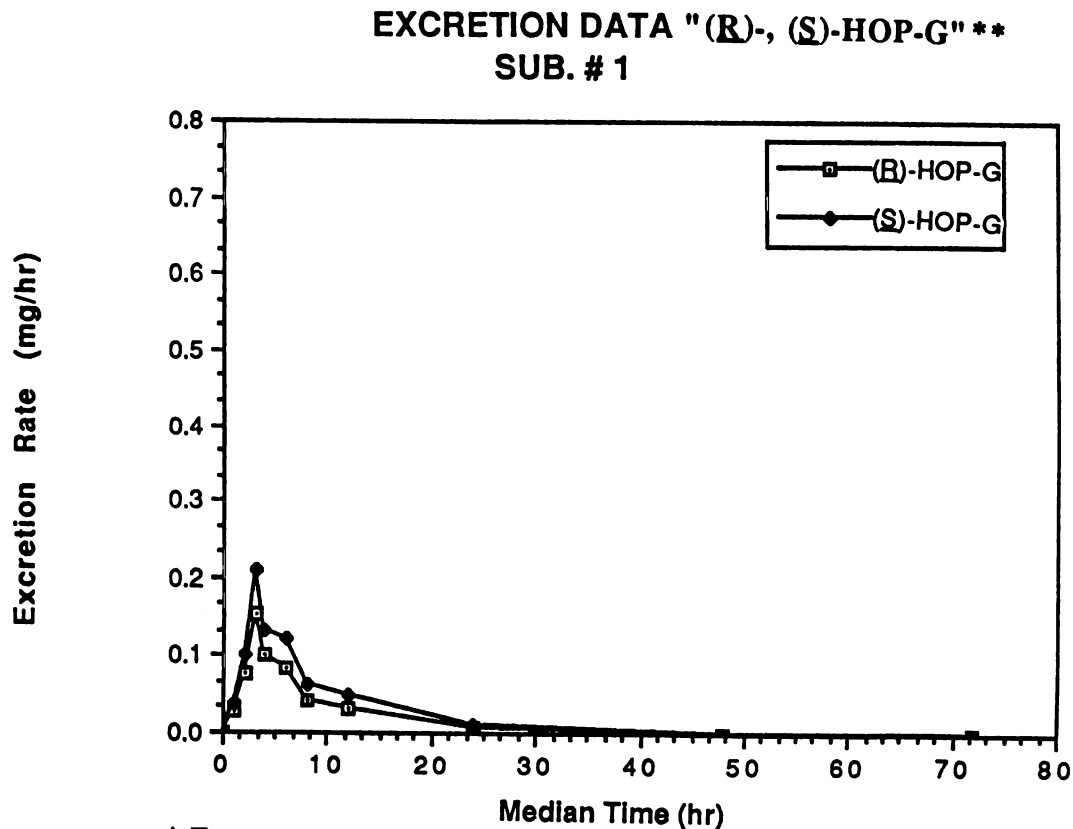
VI. Human Study

The (R)-, (S)-P-O-G and (R)-, (S)-HOP-G levels in twelve normal volunteers' urine were measured by the direct method. The urinary excretion rate-time curves are shown in Figures 21-32 and Tables 12-59. The mean excretion rate-time curve is shown in Fig. 33. From excretion data (Table 60), (R)-P-O-G levels are greater than (S)-P-O-G levels in 0-3 hours samples of urine, but (S)-P-O-G levels are higher than (R)-P-O-G levels in 3-72 hours samples. Fig. 34 and Fig. 35 depict that (S)-glucuronides accumulation levels are higher than (R)-glucuronides accumulation levels. The accumulation data (Table 61 and Fig. 36) indicate that (R)- and (S)-P-O-G accumulation levels are two times greater than (R)- and (S)-HOP-G accumulation levels. In Fig. 37, it is clear that the metabolic clearance of (R)-P-O-G is apparently faster than that of (S)-P-O-G because of progressively increasing ratios of (S)-P-O-G/(R)-P-O-G (from 0.5 to 6) with the excretion time (0-24 hours), whereas the clearance rates of (R)-HOP-G and (S)-HOP-G are no significantly different according to the consistent diastereomer ratio (from 2 to 3). A plasma concentration-time plot of (R)- and (S)-HOP is shown in Fig. 38. Tables 62 and 63 show that the excretion amounts of (R)-, (S)-P-O-G and (R)-, (S)-HOP-G are respectively 10.3 ± 2.56% and 4.60 ± 2.18% (mean ± SD) of the 80 mg oral dose of P. 14.9 ± 2.77% (mean ± SD) of the 80 mg P dose are excreted as glucuronides (Table 64). The urinary excretion ratio (S)-P-O-G/(R)-P-O-G is 1.35 ± 0.109 (mean ± SD) (Table 62), while the excretion ratio (S)-HOP-G/(R)-HOP-G is 2.17 ± 0.500 (mean ± SD) (Table 63). Those data demonstrate that the stereoselective glucuronidation in man is favoring in (S)-isomers.

Table 11. Extraction Recovery of HOP from Plasma

	Peak Height Ratio	
	Water	Plasma
High concentration (40.0 ng/ml)		
Sample 1	0.827	0.681
Sample 2	0.939	0.630
Sample 3	0.920	0.771
Mean ± SD	0.896 ± 0.060	0.674 ± 0.041
Recovery (0.674/0.896)100 = 75.2		
Medium concentration (20.0 ng/ml)		
Sample 1	0.440	0.300
Sample 2	0.436	0.319
Sample 3	0.443	0.334
Mean ± SD	0.440 ± 0.004	0.318 ± 0.017
Recovery (0.318/0.440)100 = 80.8		
Low concentration (10.0 ng/ml)		
Sample 1	0.243	0.193
Sample 2	0.253	0.195
Sample 3	0.214	0.185
Mean ± SD	0.236 ± 0.020	0.191 ± 0.005
Recovery (0.185/0.214)100 = 72.4		
X-Low concentration (4.00 ng/ml)		
Sample 1	0.118	0.085
Sample 2	0.109	0.110
Sample 3	0.122	0.102
Mean ± SD	0.116 ± 0.007	0.099 ± 0.013
Recovery (0.099/0.116)100 = 85.3		
Overall Average Recovery = 78.4%		



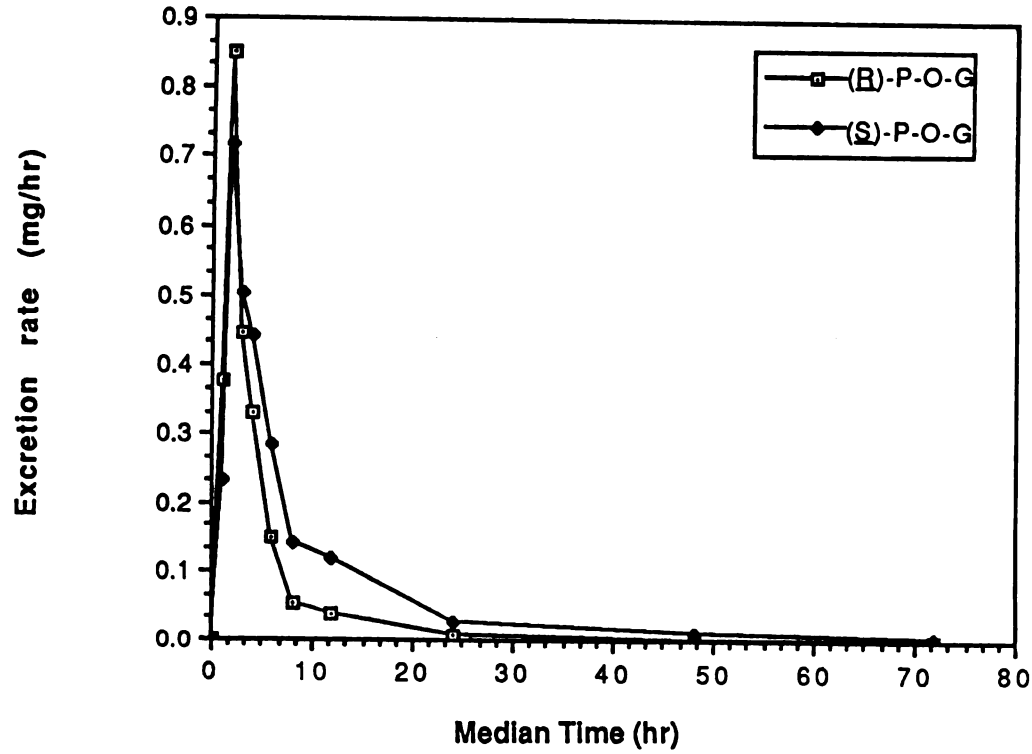


* Expressed as P.

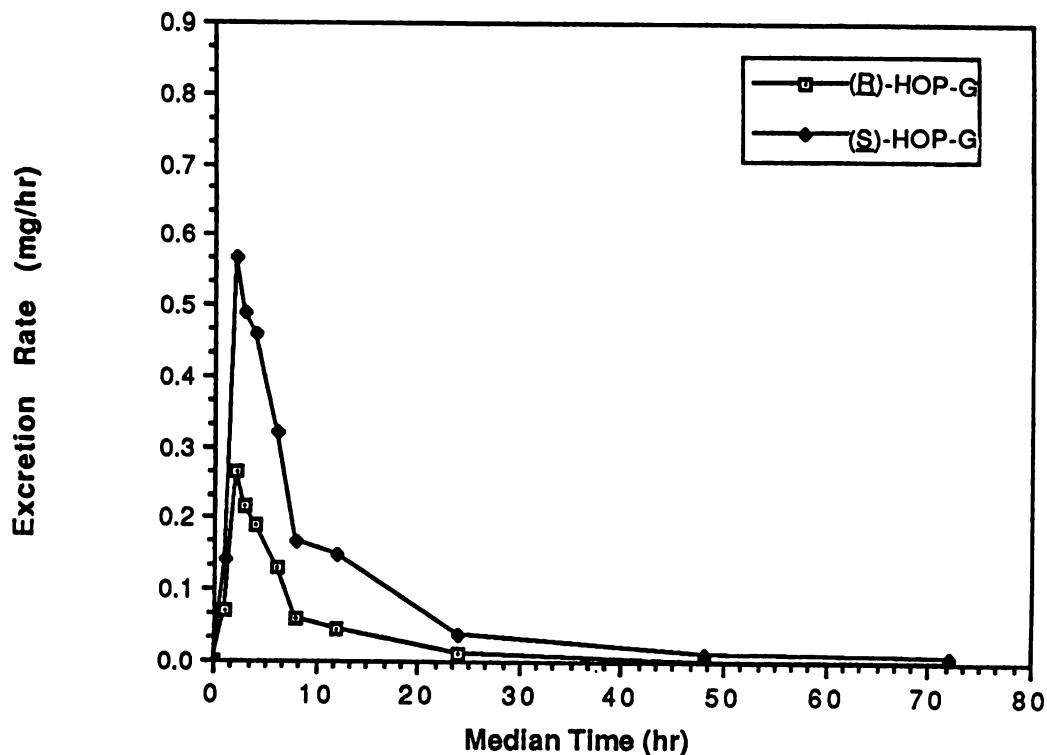
** Expressed as HOP.

Fig. 21

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 2



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 2

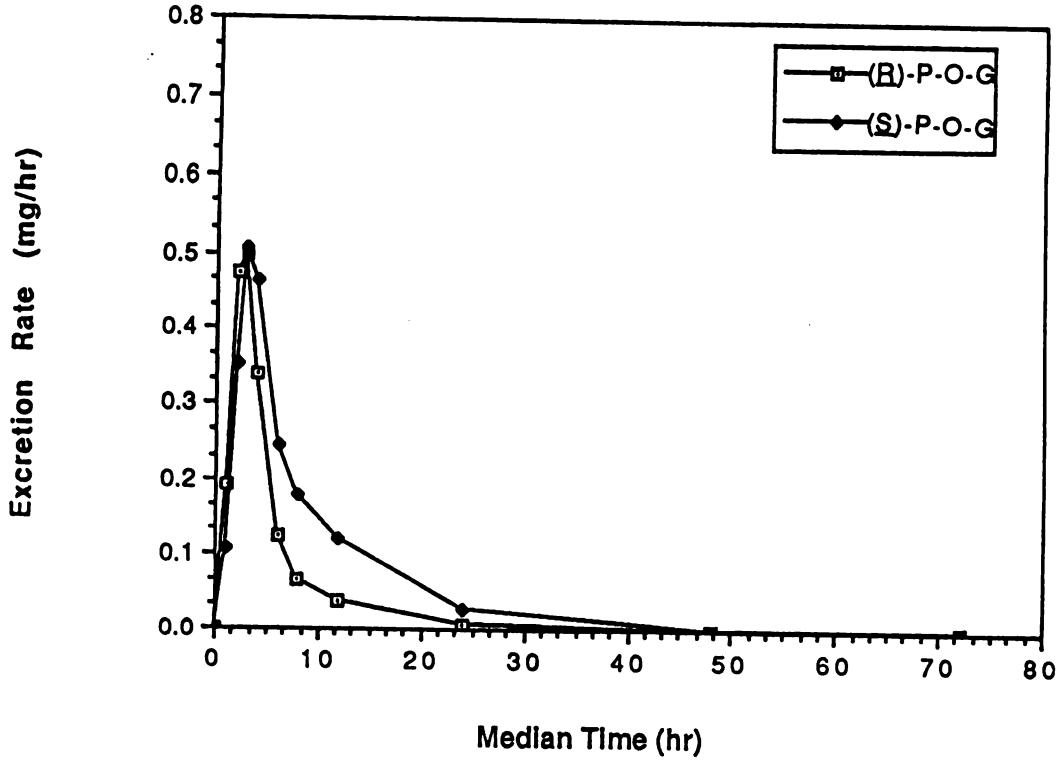


* Expressed as P.

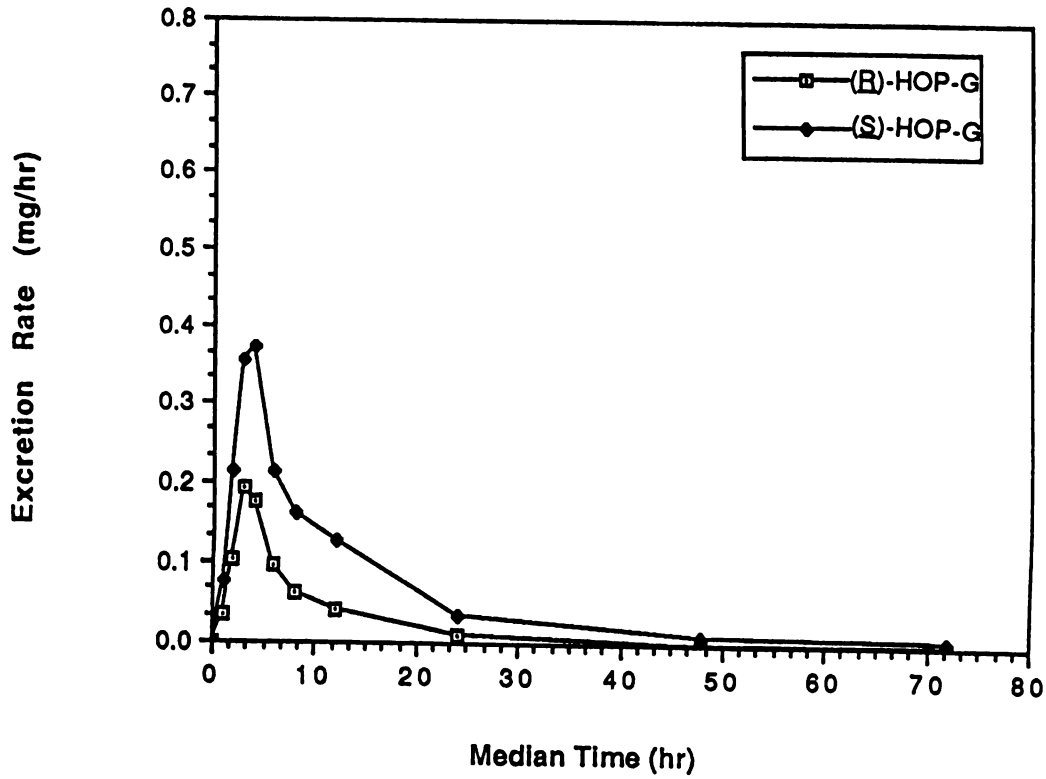
** Expressed as HOP.

Fig. 22

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 3



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 3

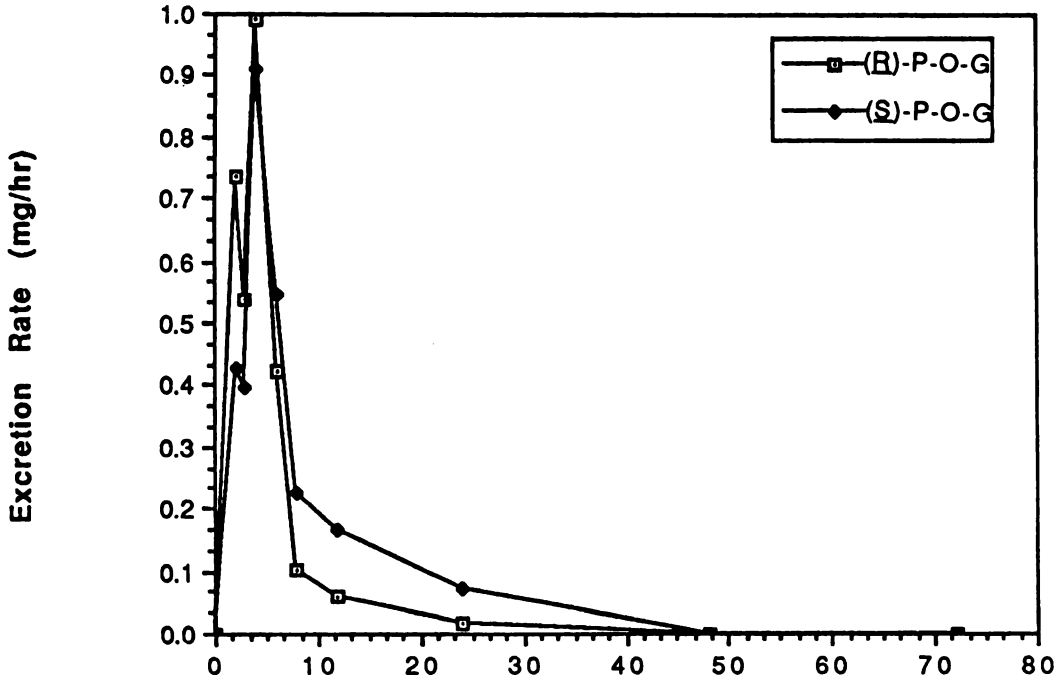


* Expressed as P.

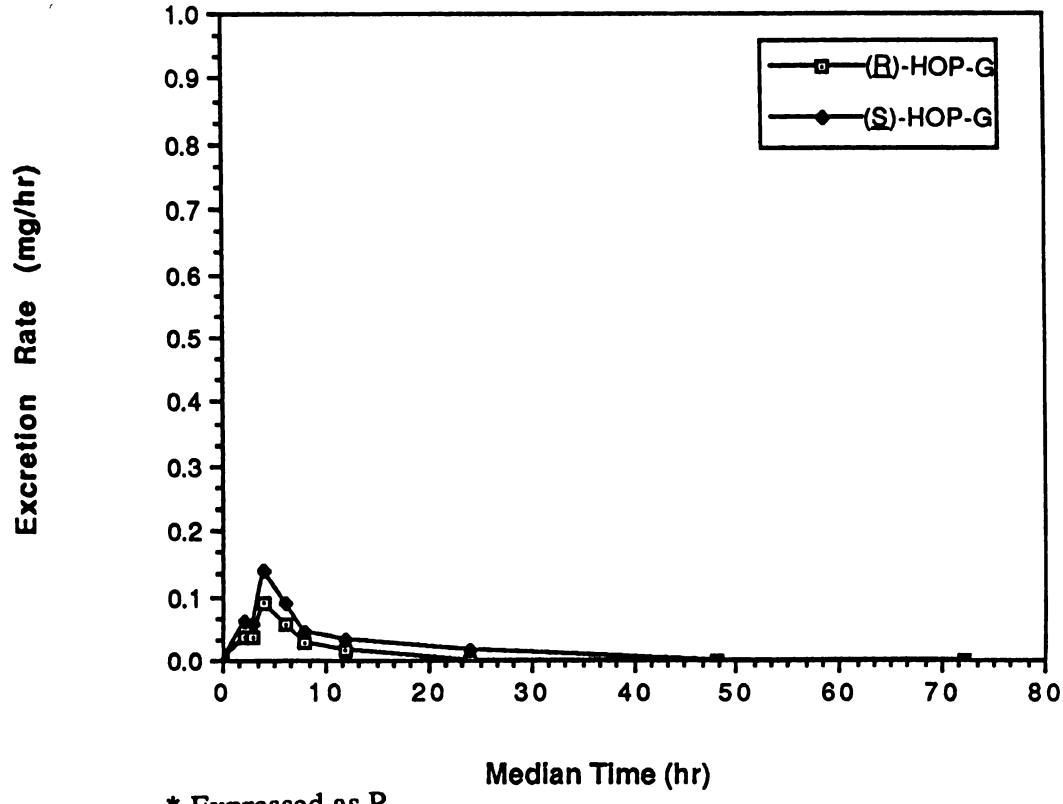
** Expressed as HOP.

Fig. 23

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. #4



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 4

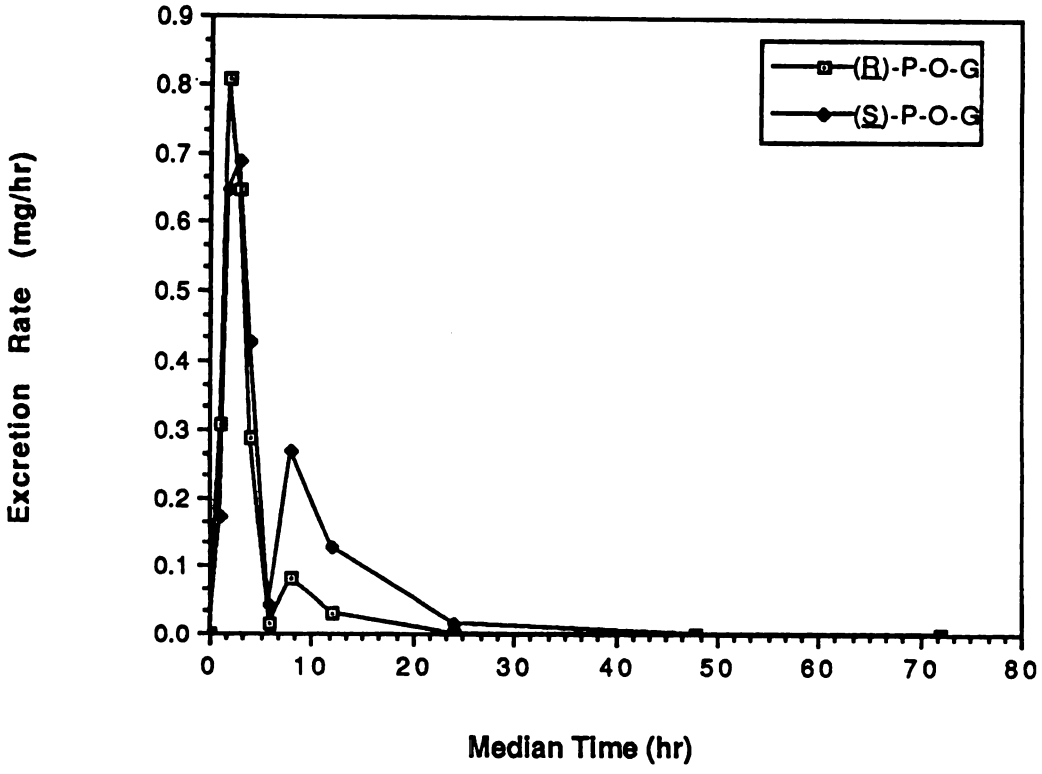


* Expressed as P.

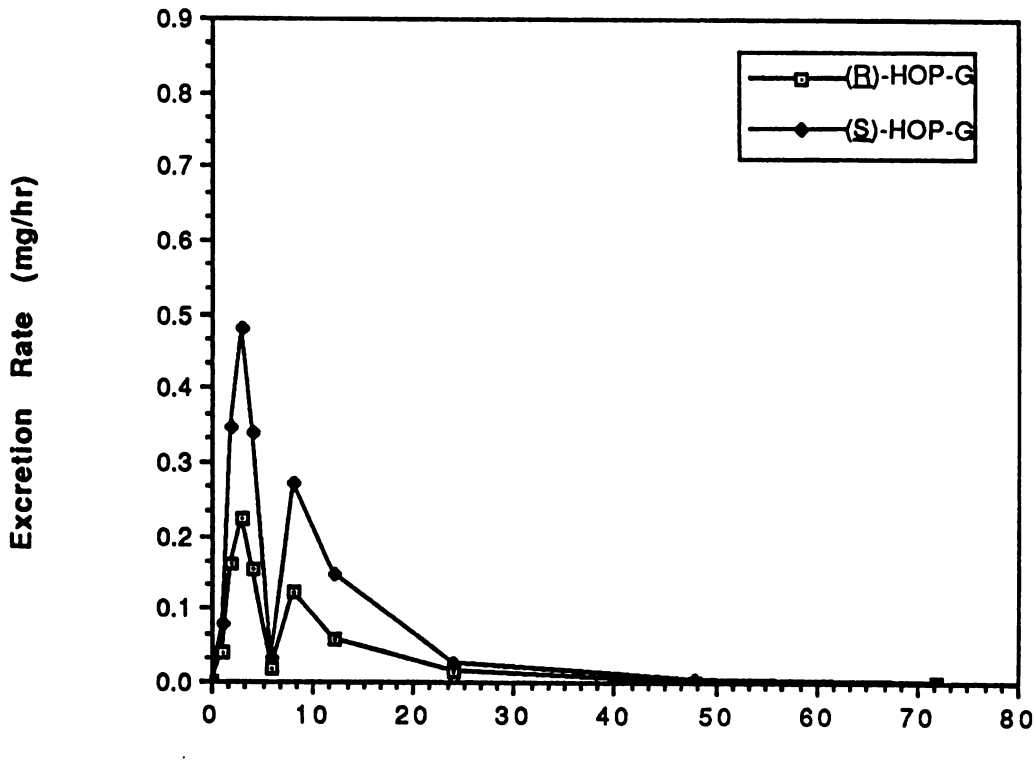
** Expressed as HOP.

Fig. 24

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 5



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 5

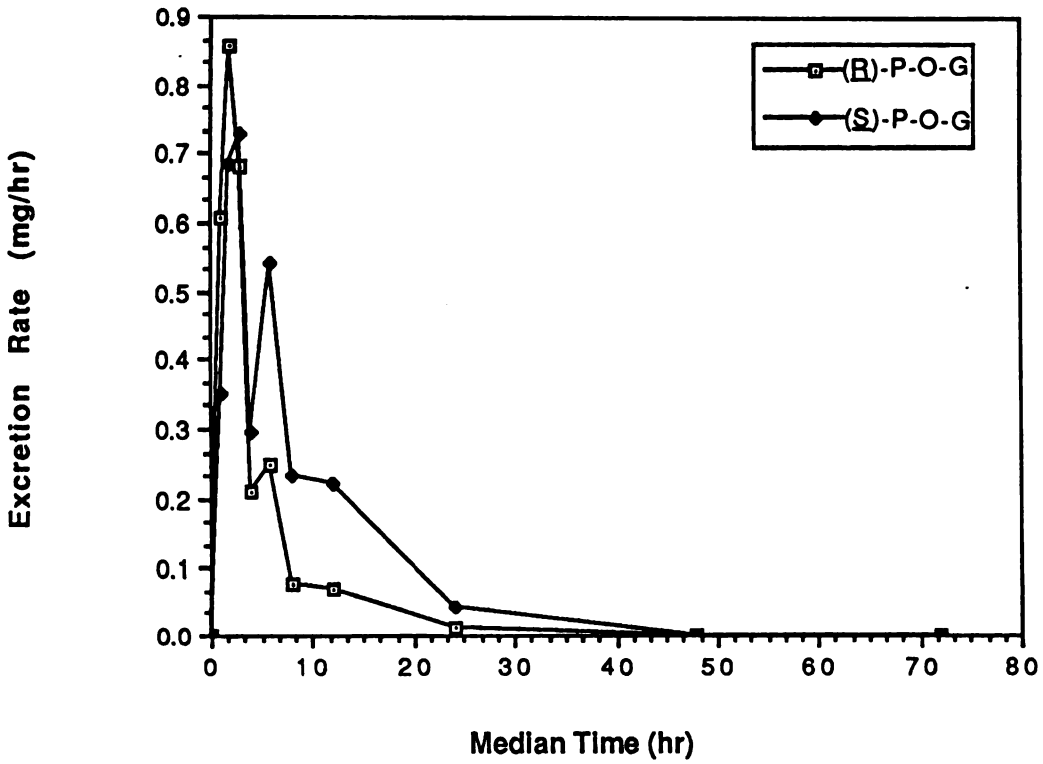


* Expressed as P.

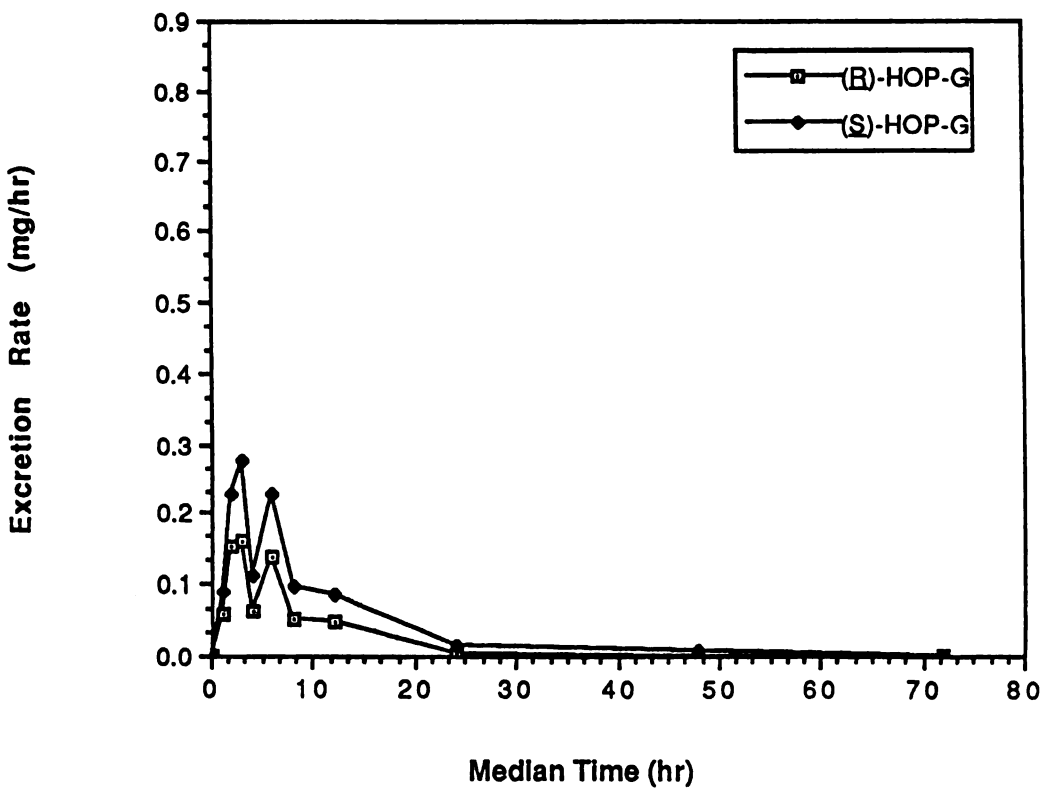
** Expressed as HOP.

Fig. 25

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 6



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 6

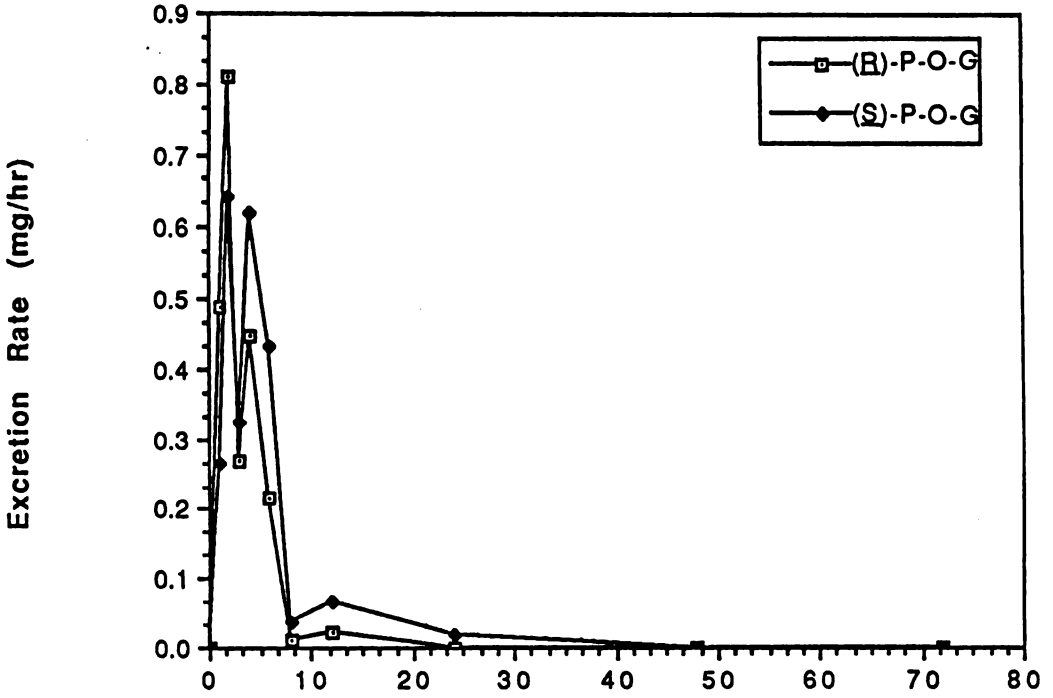


* Expressed as P.

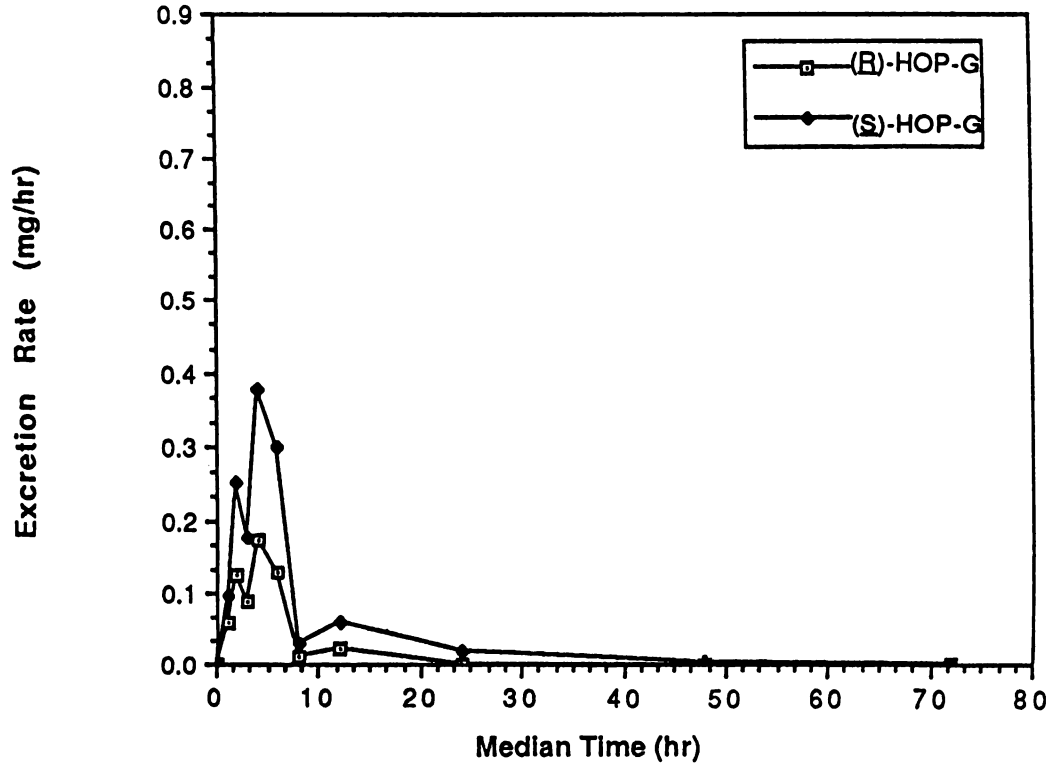
** Expressed as HOP.

Fig. 26

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 7



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 7

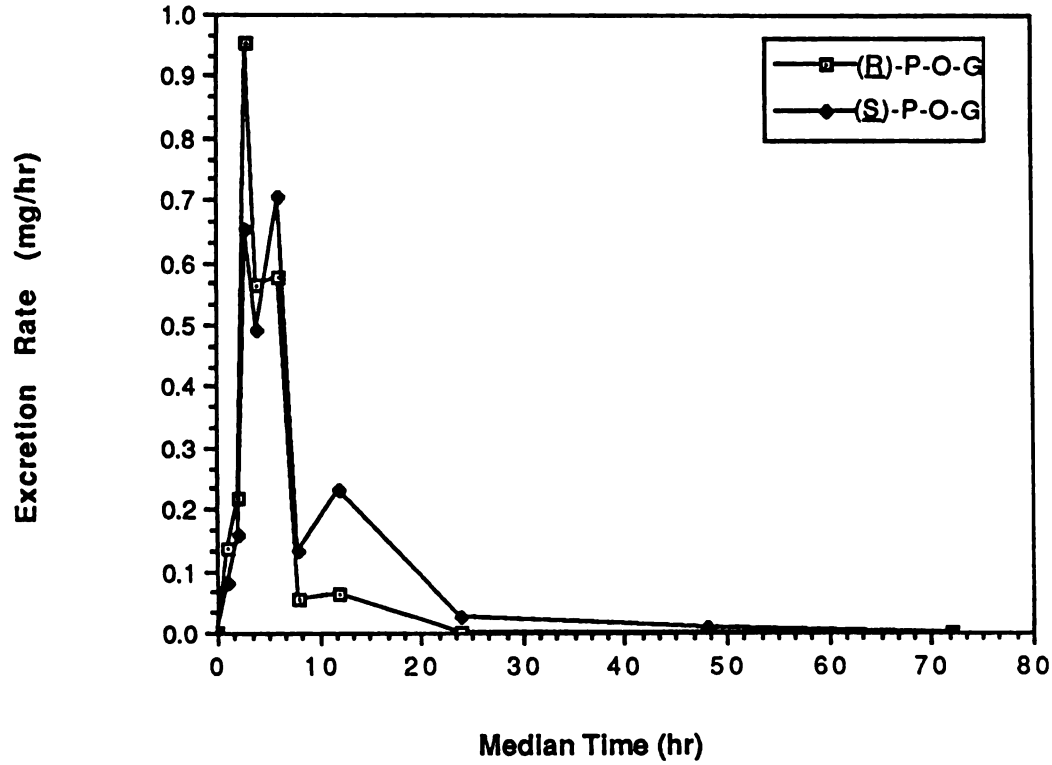


* Expressed as P.

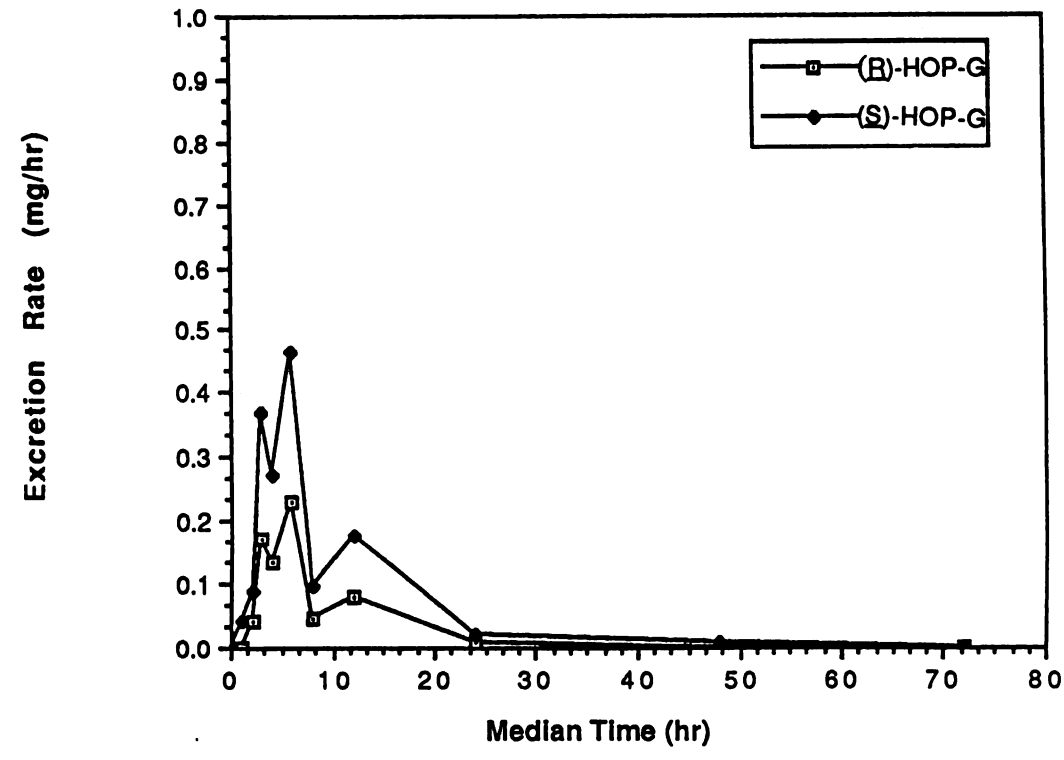
** Expressed as HOP.

Fig. 27

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 8



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 8

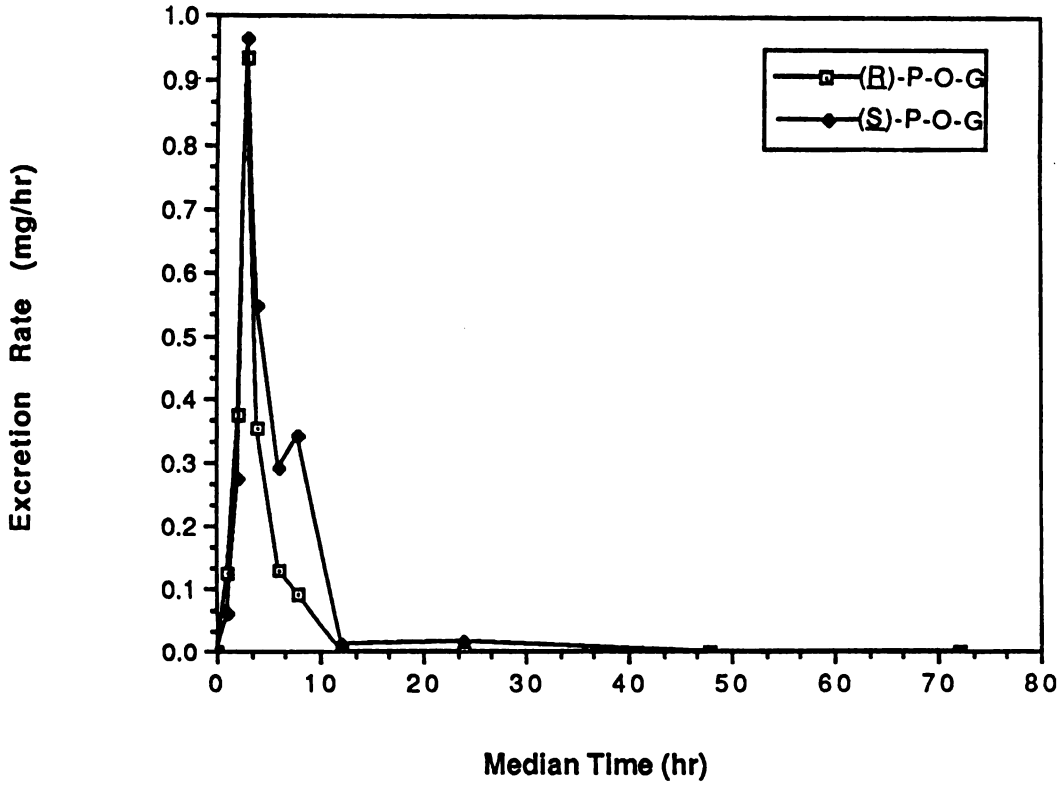


* Expressed as P.

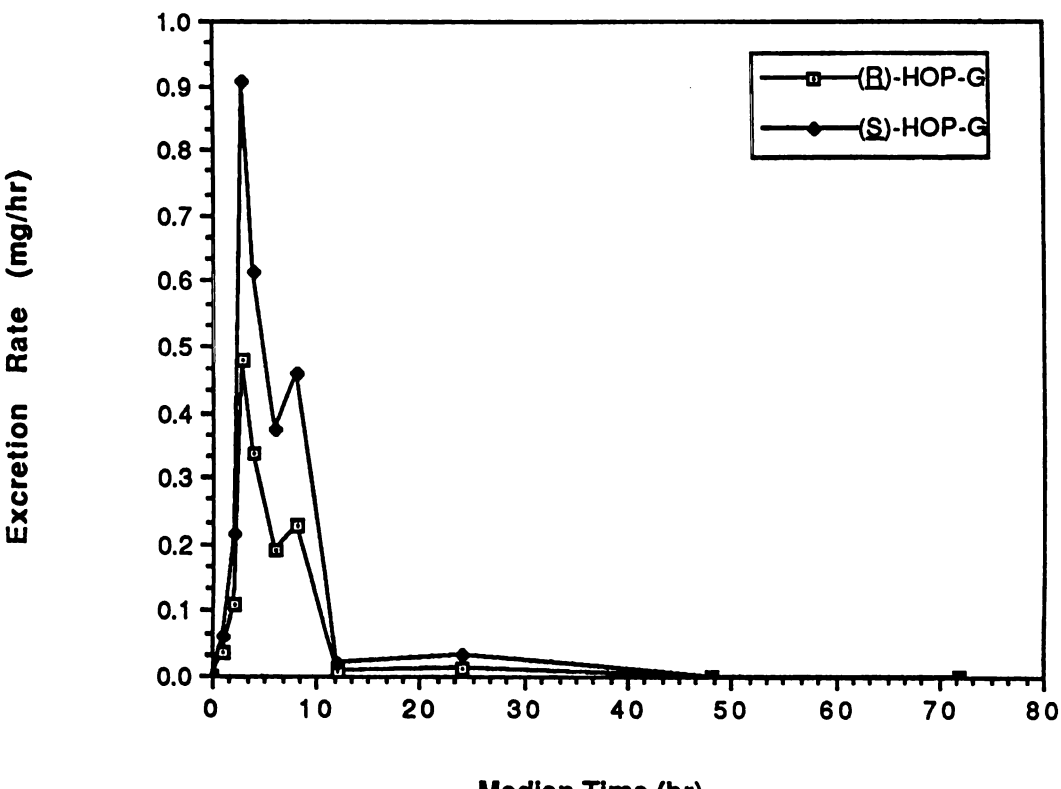
** Expressed as HOP.

Fig. 28

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 9



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 9

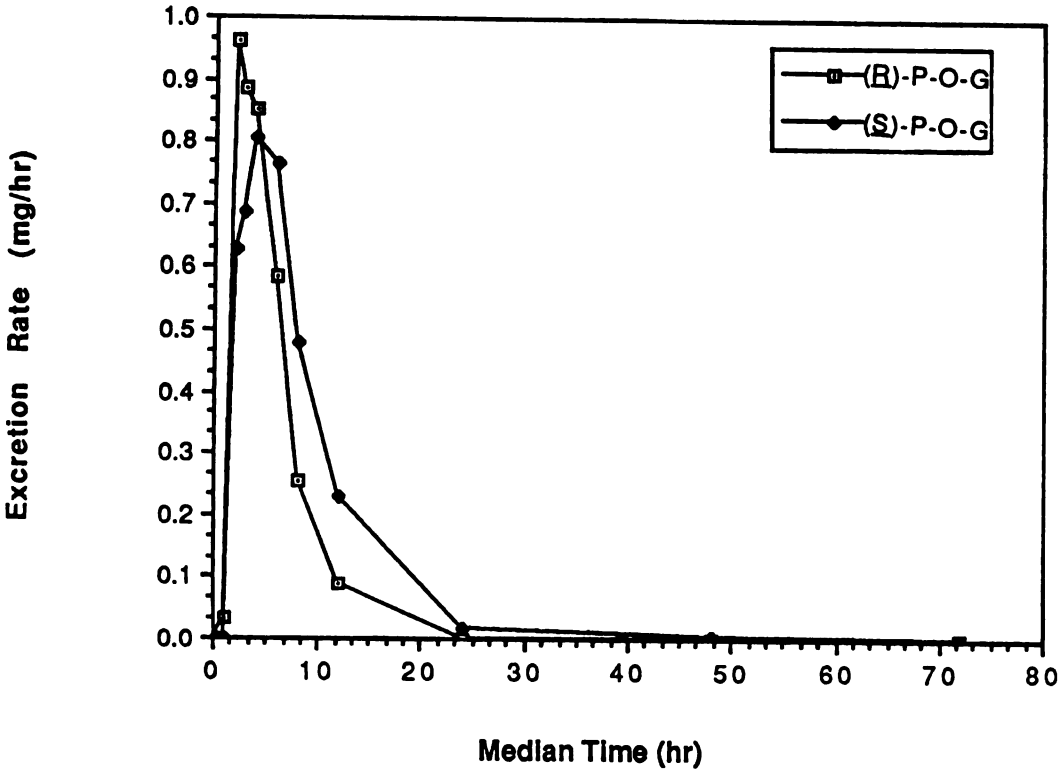


* Expressed as P.

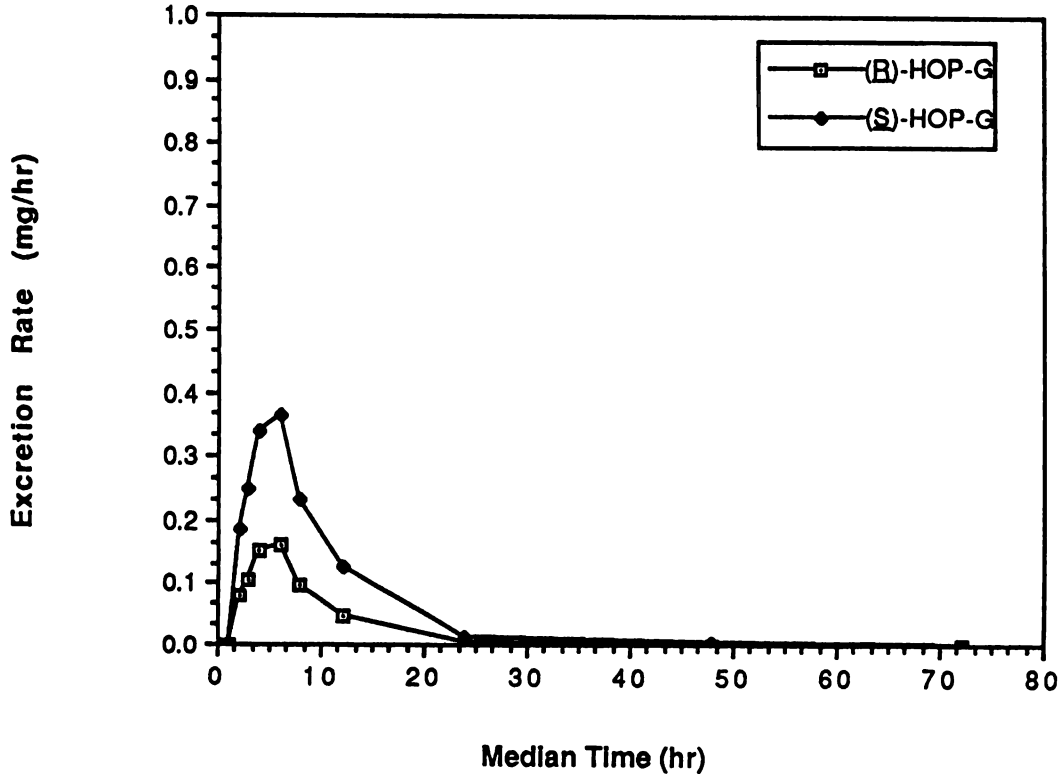
** Expressed as HOP.

Fig. 29

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 10



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 10

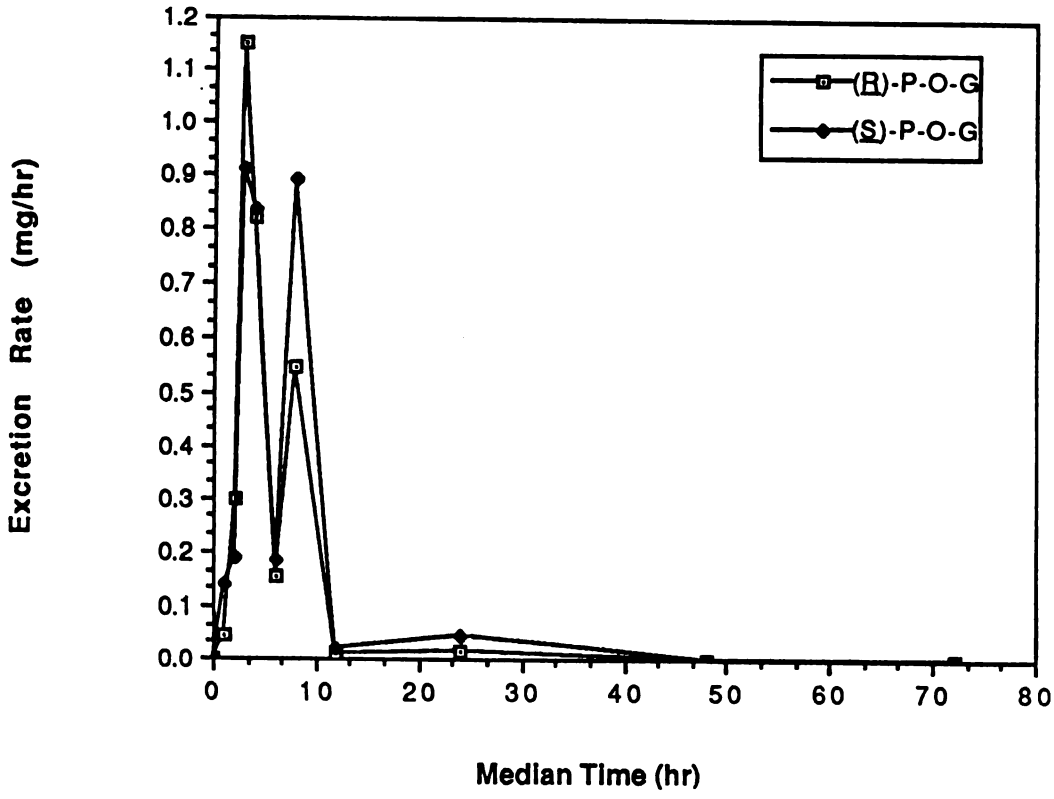


* Expressed as P.

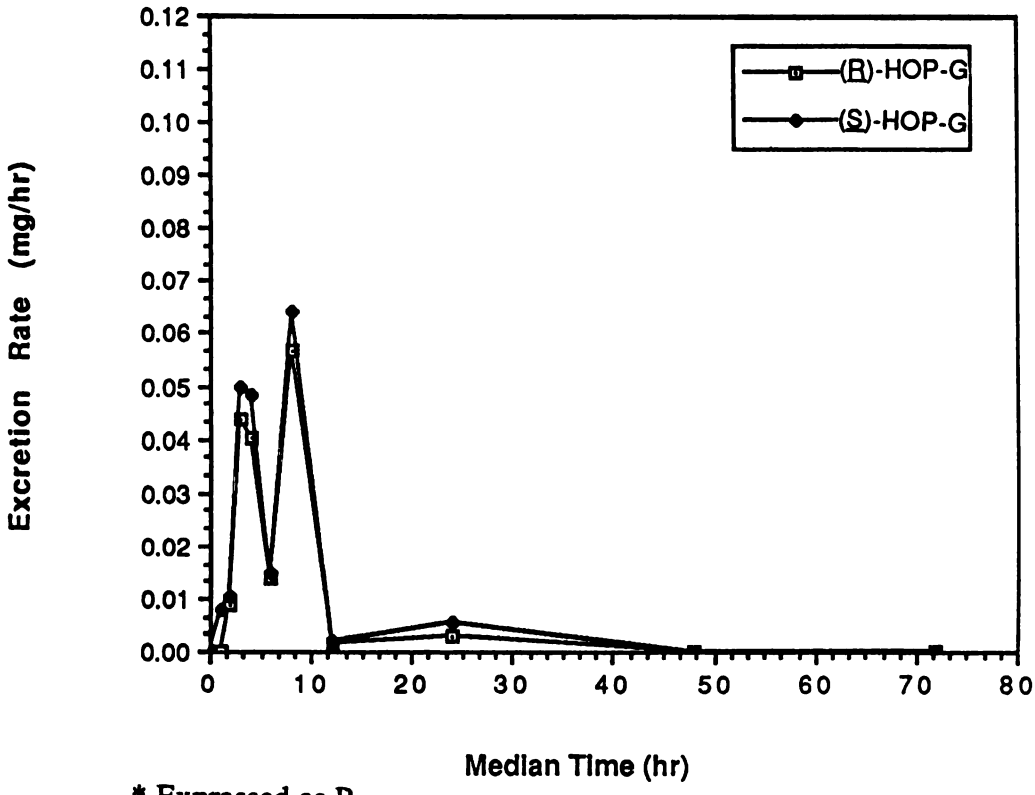
** Expressed as HOP.

Fig.30

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 11



EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 11

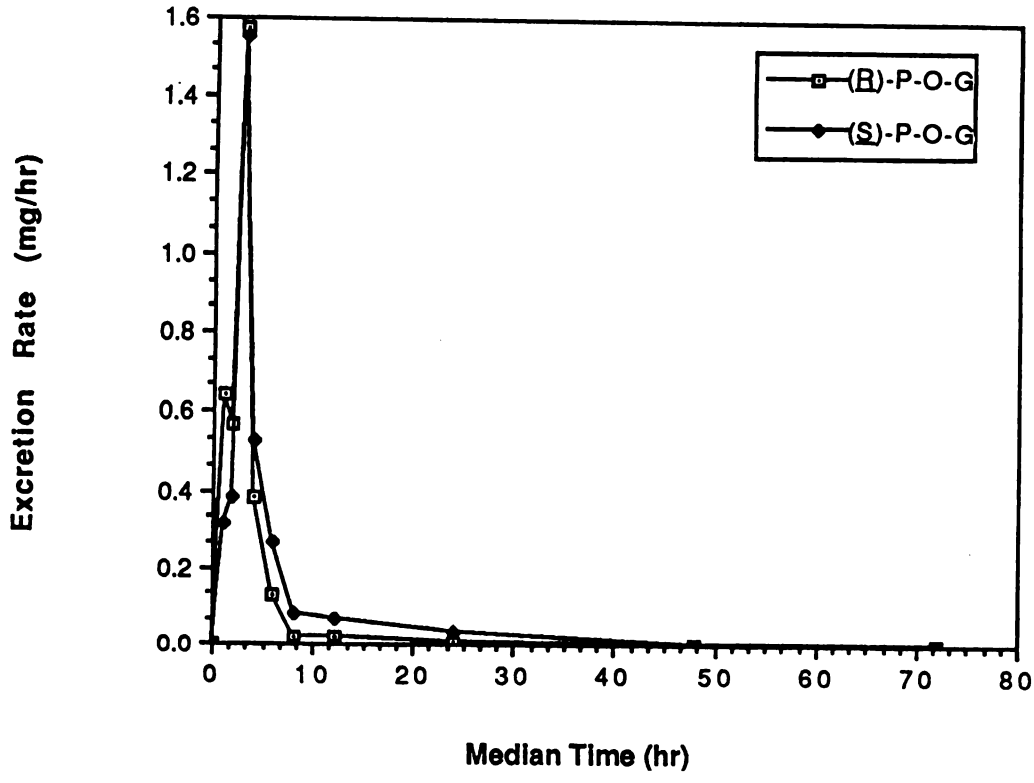


* Expressed as P.

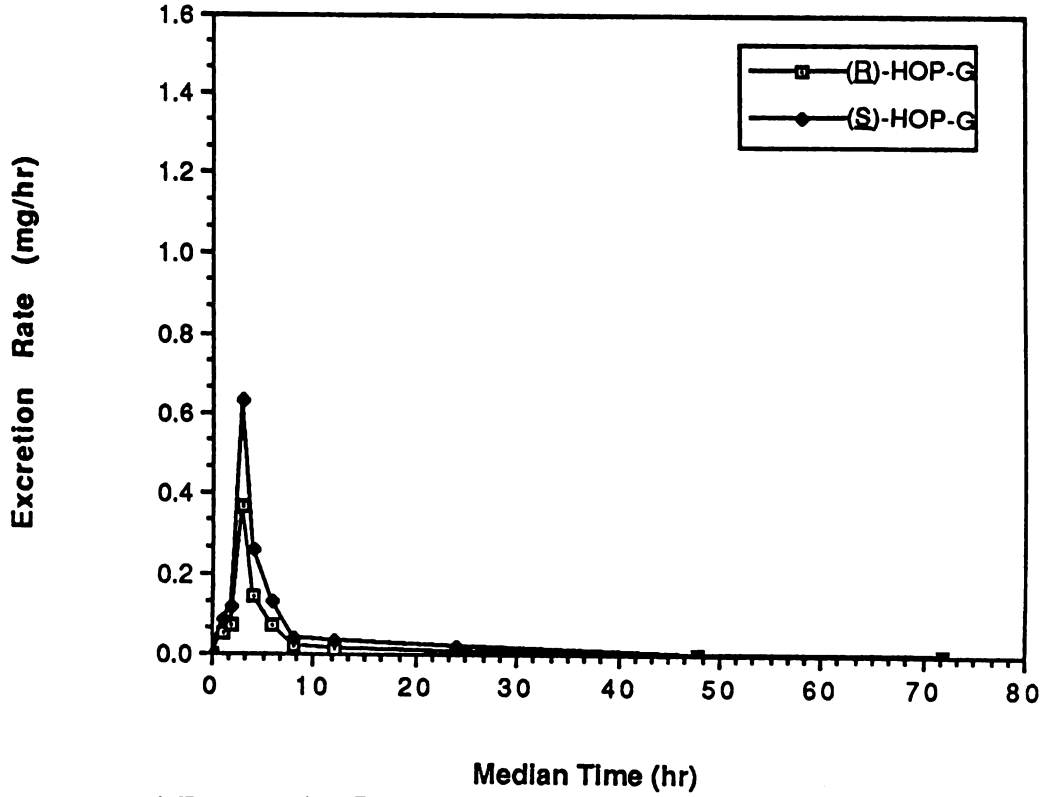
** Expressed as HOP.

Fig. 31

EXCRETION DATA "(R)-, (S)-P-O-G"*
SUB. # 12



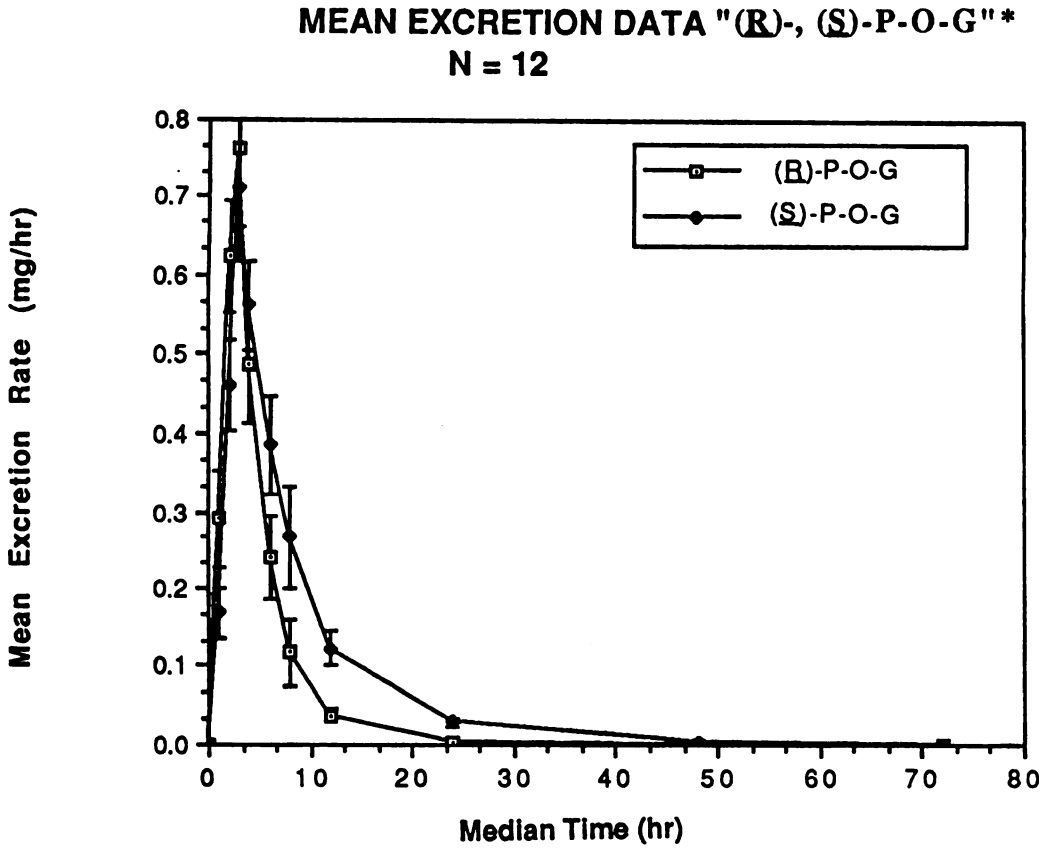
EXCRETION DATA "(R)-, (S)-HOP-G"**
SUB. # 12

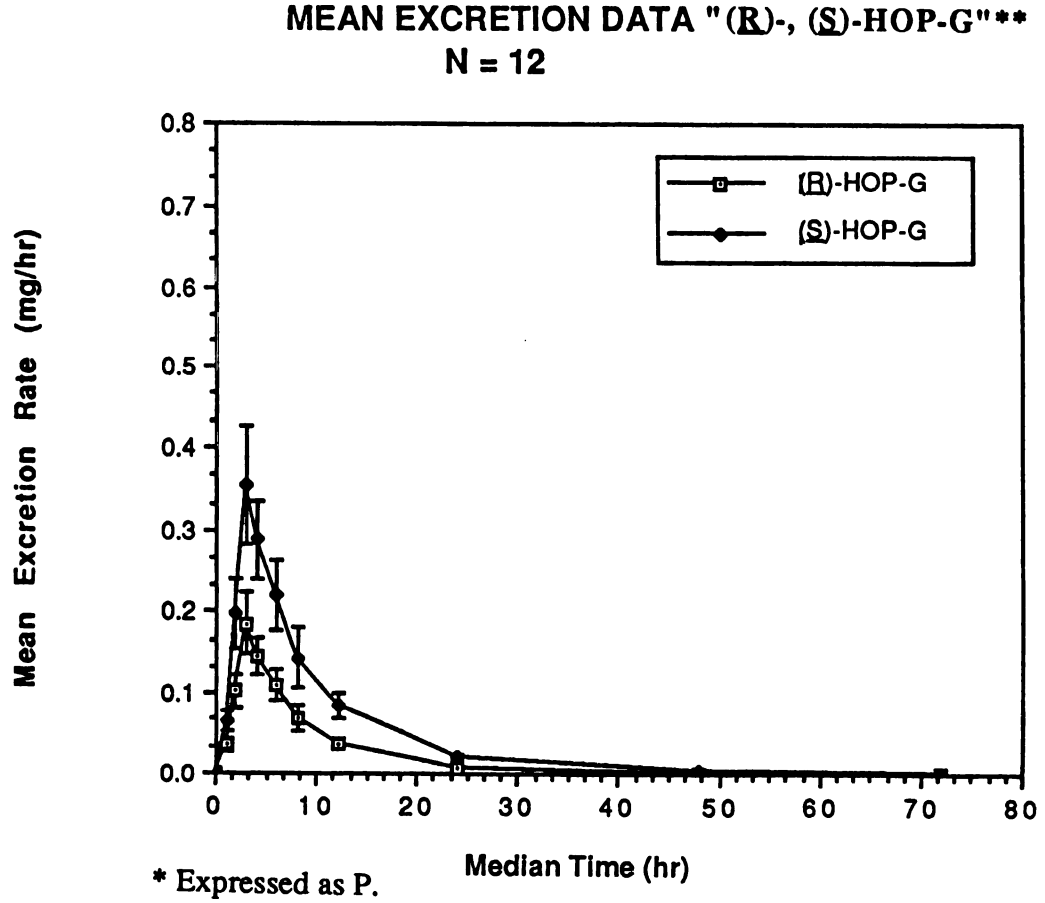


* Expressed as P.

** Expressed as HOP.

Fig. 32





* Expressed as P.

** Expressed as HOP.

Fig. 33 Mean excretion rate vs time plot of twelve subjects for (R)-, (S)-P-O-G and (R)-, (S)-HOP-G. Error bars represent standard errors.

Clinical Samples for Urine Study Number 88-1-a

Table 12. Subject 1 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	62	NA	NA
0.00 - 1.00	1.28	180	0.23	0.230
1.00 - 2.00	3.06	175	0.54	0.536
2.00 - 3.00	2.55	235	0.60	0.598
3.00 - 4.00	0.739	370	0.27	0.273
4.00 - 6.00	0.438	640	0.28	0.140
6.00 - 8.00	0.552	186	0.10	5.14E-2
8.00 - 12.00	0.281	320	0.09	2.24E-2
12.00 - 24.00	*	460	NA	NA
24.00 - 48.00	*	910	NA	NA
48.00 - 72.00	*	822	NA	NA
Total Amount Excreted:			2.11	mg

Table 13. Subject 2 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	37	NA	NA
0.00 - 1.00	2.10	180	0.38	0.379
1.00 - 2.00	4.25	200	0.85	0.850
2.00 - 3.00	2.07	215	0.44	0.445
3.00 - 4.00	1.85	180	0.33	0.332
4.00 - 6.00	0.990	300	0.30	0.149
6.00 - 8.00	0.666	155	0.10	5.16E-2
8.00 - 12.00	0.516	285	0.15	3.67E-2
12.00 - 24.00	*	630	NA	NA
24.00 - 48.00	*	812	NA	NA
48.00 - 72.00	*	680	NA	NA
Total Amount Excreted:			2.55	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 14. Subject 3 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	35	NA	NA
0.00 - 1.00	0.557	350	0.20	0.195
1.00 - 2.00	1.56	304	0.47	0.474
2.00 - 3.00	1.41	352	0.50	0.497
3.00 - 4.00	0.966	350	0.34	0.338
4.00 - 6.00	0.557	440	0.25	0.123
6.00 - 8.00	0.755	172	0.13	6.49E-2
8.00 - 12.00	0.608	255	0.16	3.88E-2
12.00 - 24.00	0.160	555	0.09	7.39E-3
24.00 - 48.00	*	915	NA	NA
48.00 - 72.00	*	832	NA	NA
Total Amount Excreted:			2.12	mg

Table 15. Subject 4 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	36	NA	NA
0.00 - 1.00	NS	NS	NA	NA
1.00 - 2.00	10.2	72	0.73	0.733
2.00 - 3.00	8.99	60	0.54	0.539
3.00 - 4.00	3.82	260	0.99	0.992
4.00 - 6.00	3.51	240	0.84	0.421
6.00 - 8.00	2.98	68	0.20	0.101
8.00 - 12.00	1.95	125	0.24	6.10E-2
12.00 - 24.00	0.229	920	0.21	1.76E-2
24.00 - 48.00	*	1880	NA	NA
48.00 - 72.00	*	910	NA	NA
Total Amount Excreted:			3.76	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 16. Subject 5 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	48	NA	NA
0.00 - 1.00	2.93	105	0.31	0.307
1.00 - 2.00	9.51	85	0.81	0.808
2.00 - 3.00	5.37	120	0.64	0.645
3.00 - 4.00	3.41	85	0.29	0.290
4.00 - 6.00	0.213	160	0.03	1.70E-2
6.00 - 8.00	0.954	170	0.16	8.11E-2
8.00 - 12.00	0.380	320	0.12	3.04E-2
12.00 - 24.00	*	440	NA	NA
24.00 - 48.00	*	506	NA	NA
48.00 - 72.00	*	1020	NA	NA
Total Amount Excreted:			2.37	mg

Table 17. Subject 6 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	42	NA	NA
0.00 - 1.00	1.78	340	0.61	0.606
1.00 - 2.00	2.52	340	0.86	0.856
2.00 - 3.00	1.29	530	0.68	0.683
3.00 - 4.00	1.20	175	0.21	0.210
4.00 - 6.00	1.24	400	0.50	0.249
6.00 - 8.00	0.851	182	0.15	7.75E-2
8.00 - 12.00	0.471	600	0.28	7.07E-2
12.00 - 24.00	0.169	800	0.14	1.13E-2
24.00 - 48.00	*	1508	NA	NA
48.00 - 72.00	*	1020	NA	NA
Total Amount Excreted:			3.43	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 18. Subject 7 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	70	NA	NA
0.00 - 1.00	1.51	325	0.49	0.490
1.00 - 2.00	2.85	284	0.81	0.809
2.00 - 3.00	0.926	290	0.27	0.269
3.00 - 4.00	1.36	332	0.45	0.450
4.00 - 6.00	1.96	220	0.43	0.216
6.00 - 8.00	0.897	28	0.03	1.26E-2
8.00 - 12.00	0.259	325	0.08	2.10E-2
12.00 - 24.00	*	1300	NA	NA
24.00 - 48.00	*	1040	NA	NA
48.00 - 72.00	*	450	NA	NA
Total Amount Excreted:			2.56	mg

Table 19. Subject 8 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	60	NA	NA
0.00 - 1.00	0.595	230	0.14	0.137
1.00 - 2.00	1.38	160	0.22	0.222
2.00 - 3.00	2.32	410	0.95	0.952
3.00 - 4.00	3.65	155	0.57	0.566
4.00 - 6.00	3.29	350	1.15	0.576
6.00 - 8.00	1.20	92	0.11	5.52E-2
8.00 - 12.00	0.491	540	0.26	6.62E-2
12.00 - 24.00	*	500	NA	NA
24.00 - 48.00	*	1002	NA	NA
48.00 - 72.00	*	900	NA	NA
Total Amount Excreted:			3.40	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 20. **Subject 9 Treatment A (R)-P-O-G#**

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	60	NA	NA
0.00 - 1.00	0.615	200	0.12	0.123
1.00 - 2.00	2.50	150	0.37	0.374
2.00 - 3.00	2.17	430	0.93	0.935
3.00 - 4.00	1.10	320	0.35	0.353
4.00 - 6.00	0.723	352	0.25	0.127
6.00 - 8.00	0.746	240	0.18	8.95E-2
8.00 - 12.00	*	50	NA	NA
12.00 - 24.00	*	368	NA	NA
24.00 - 48.00	*	620	NA	NA
48.00 - 72.00	*	920	NA	NA
Total Amount Excreted:			2.22	mg

Table 21. **Subject 10 Treatment A (R)-P-O-G#**

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	31	NA	NA
0.00 - 1.00	0.226	150	0.03	3.39E-2
1.00 - 2.00	6.40	150	0.96	0.961
2.00 - 3.00	4.66	190	0.89	0.885
3.00 - 4.00	5.50	155	0.85	0.853
4.00 - 6.00	4.04	290	1.17	0.585
6.00 - 8.00	2.55	200	0.51	0.255
8.00 - 12.00	1.32	270	0.36	8.92E-2
12.00 - 24.00	*	658	NA	NA
24.00 - 48.00	*	500	NA	NA
48.00 - 72.00	*	550	NA	NA
Total Amount Excreted:			4.77	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 22. Subject 11 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	74	NA	NA
0.00 - 1.00	0.464	102	0.05	4.73E-2
1.00 - 2.00	2.34	128	0.30	0.299
2.00 - 3.00	5.83	197	1.15	1.15
3.00 - 4.00	4.57	180	0.82	0.822
4.00 - 6.00	1.40	218	0.30	0.152
6.00 - 8.00	6.09	180	1.10	0.548
8.00 - 12.00	1.45	27	0.04	9.77E-3
12.00 - 24.00	0.428	470	0.20	1.68E-2
24.00 - 48.00	*	500	NA	NA
48.00 - 72.00	*	1010	NA	NA
Total Amount Excreted:			3.96	mg

Table 23. Subject 12 Treatment A (R)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	92	NA	NA
0.00 - 1.00	4.89	132	0.65	0.646
1.00 - 2.00	5.36	106	0.57	0.568
2.00 - 3.00	4.89	322	1.57	1.57
3.00 - 4.00	2.38	160	0.38	0.380
4.00 - 6.00	1.10	236	0.26	0.129
6.00 - 8.00	0.598	80	0.05	2.39E-2
8.00 - 12.00	0.285	240	0.07	1.71E-2
12.00 - 24.00	0.168	400	0.07	5.59E-3
24.00 - 48.00	*	1600	NA	NA
48.00 - 72.00	*	810	NA	NA
Total Amount Excreted:			3.61	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 24. Subject 1 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	102	NA	NA
0.00 - 1.00	0.729	180	0.13	0.131
1.00 - 2.00	2.36	175	0.41	0.413
2.00 - 3.00	2.68	235	0.63	0.630
3.00 - 4.00	1.01	370	0.37	0.372
4.00 - 6.00	0.998	640	0.64	0.319
6.00 - 8.00	1.96	186	0.36	0.182
8.00 - 12.00	1.33	320	0.42	0.106
12.00 - 24.00	0.205	460	0.09	7.86E-3
24.00 - 48.00	*	910	NA	NA
48.00 - 72.00	*	822	NA	NA
Total Amount Excreted:			3.07	mg

Table 25. Subject 2 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	37	NA	NA
0.00 - 1.00	1.28	180	0.23	0.231
1.00 - 2.00	3.58	200	0.72	0.716
2.00 - 3.00	2.34	215	0.50	0.503
3.00 - 4.00	2.45	180	0.44	0.441
4.00 - 6.00	1.90	300	0.57	0.285
6.00 - 8.00	1.83	155	0.28	0.142
8.00 - 12.00	1.67	285	0.48	0.119
12.00 - 24.00	0.472	630	0.30	2.48E-2
24.00 - 48.00	0.305	812	0.25	1.03E-2
48.00 - 72.00	0.196	680	0.13	5.55E-3
Total Amount Excreted:			3.90	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 26. Subject 3 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	35	NA	NA
0.00 - 1.00	0.310	350	0.11	0.108
1.00 - 2.00	1.16	304	0.35	0.354
2.00 - 3.00	1.44	352	0.51	0.505
3.00 - 4.00	1.33	350	0.47	0.465
4.00 - 6.00	1.12	440	0.49	0.247
6.00 - 8.00	2.10	172	0.36	0.180
8.00 - 12.00	1.89	255	0.48	0.120
12.00 - 24.00	0.595	555	0.33	2.75E-2
24.00 - 48.00	*	915	NA	NA
48.00 - 72.00	*	832	NA	NA
Total Amount Excreted:			3.10	mg

Table 27. Subject 4 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	36	NA	NA
0.00 - 1.00	NS	NS	NA	NA
1.00 - 2.00	5.96	72	0.43	0.429
2.00 - 3.00	6.61	60	0.40	0.397
3.00 - 4.00	3.51	260	0.91	0.912
4.00 - 6.00	4.56	240	1.09	0.547
6.00 - 8.00	6.64	68	0.45	0.226
8.00 - 12.00	5.36	125	0.67	0.167
12.00 - 24.00	0.942	920	0.87	7.22E-2
24.00 - 48.00	*	1880	NA	NA
48.00 - 72.00	*	910	NA	NA
Total Amount Excreted:			4.82	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 28. Subject 5 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	48	NA	NA
0.00 - 1.00	1.66	105	0.17	0.174
1.00 - 2.00	7.59	85	0.64	0.645
2.00 - 3.00	5.73	120	0.69	0.687
3.00 - 4.00	5.02	85	0.43	0.427
4.00 - 6.00	0.518	160	0.08	4.15E-2
6.00 - 8.00	3.17	170	0.54	0.269
8.00 - 12.00	1.60	320	0.51	0.128
12.00 - 24.00	0.412	440	0.18	1.51E-2
24.00 - 48.00	*	506	NA	NA
48.00 - 72.00	*	1020	NA	NA
Total Amount Excreted:			3.25	mg

Table 29. Subject 6 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	42	NA	NA
0.00 - 1.00	1.03	340	0.35	0.351
1.00 - 2.00	2.01	340	0.68	0.684
2.00 - 3.00	1.37	530	0.73	0.727
3.00 - 4.00	1.70	175	0.30	0.297
4.00 - 6.00	2.70	400	1.08	0.540
6.00 - 8.00	2.57	182	0.47	0.234
8.00 - 12.00	1.48	600	0.89	0.222
12.00 - 24.00	0.636	800	0.51	4.24E-2
24.00 - 48.00	*	1508	NA	NA
48.00 - 72.00	*	1020	NA	NA
Total Amount Excreted:			5.00	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 30. Subject 7 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	70	NA	NA
0.00 - 1.00	0.816	325	0.27	0.265
1.00 - 2.00	2.26	284	0.64	0.643
2.00 - 3.00	1.12	290	0.32	0.324
3.00 - 4.00	1.87	332	0.62	0.621
4.00 - 6.00	3.95	220	0.87	0.434
6.00 - 8.00	2.67	28	0.07	3.73E-2
8.00 - 12.00	0.830	325	0.27	6.75E-2
12.00 - 24.00	0.169	1300	0.22	1.83E-2
24.00 - 48.00	*	1040	NA	NA
48.00 - 72.00	*	450	NA	NA
Total Amount Excreted:			3.28	mg

Table 31. Subject 8 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	60	NA	NA
0.00 - 1.00	0.346	230	0.08	7.97E-2
1.00 - 2.00	0.993	160	0.16	0.159
2.00 - 3.00	1.60	410	0.66	0.655
3.00 - 4.00	3.17	155	0.49	0.491
4.00 - 6.00	4.03	350	1.41	0.705
6.00 - 8.00	2.90	92	0.27	0.133
8.00 - 12.00	1.70	540	0.92	0.229
12.00 - 24.00	0.603	500	0.30	2.51E-2
24.00 - 48.00	0.203	1002	0.20	8.46E-3
48.00 - 72.00	*	900	NA	NA
Total Amount Excreted:			4.48	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 32. Subject 9 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	60	NA	NA
0.00 - 1.00	0.319	200	0.06	6.37E-2
1.00 - 2.00	1.84	150	0.28	0.276
2.00 - 3.00	2.24	430	0.96	0.963
3.00 - 4.00	1.71	320	0.55	0.546
4.00 - 6.00	1.65	352	0.58	0.290
6.00 - 8.00	2.85	240	0.68	0.341
8.00 - 12.00	0.969	50	0.05	1.21E-2
12.00 - 24.00	0.560	368	0.21	1.72E-2
24.00 - 48.00	*	620	NA	NA
48.00 - 72.00	*	920	NA	NA
Total Amount Excreted:			3.37	mg

Table 33. Subject 10 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	31	NA	NA
0.00 - 1.00	*	150	NA	NA
1.00 - 2.00	4.18	150	0.63	0.627
2.00 - 3.00	3.61	190	0.69	0.686
3.00 - 4.00	5.20	155	0.81	0.807
4.00 - 6.00	5.27	290	1.53	0.764
6.00 - 8.00	4.79	200	0.96	0.479
8.00 - 12.00	3.36	270	0.91	0.227
12.00 - 24.00	0.310	658	0.20	1.70E-2
24.00 - 48.00	0.225	500	0.11	4.68E-3
48.00 - 72.00	*	550	NA	NA
Total Amount Excreted:			5.83	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P. .

Clinical Samples for Urine Study Number 88-1-a

Table 34. Subject 11 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	74	NA	NA
0.00 - 1.00	1.35	102	0.14	0.137
1.00 - 2.00	1.48	128	0.19	0.190
2.00 - 3.00	4.62	197	0.91	0.911
3.00 - 4.00	4.64	180	0.84	0.835
4.00 - 6.00	1.68	218	0.37	0.183
6.00 - 8.00	9.92	180	1.79	0.893
8.00 - 12.00	3.01	27	0.08	2.03E-2
12.00 - 24.00	1.14	470	0.53	4.46E-2
24.00 - 48.00	*	500	NA	NA
48.00 - 72.00	*	1010	NA	NA
Total Amount Excreted:			4.84	mg

Table 35. Subject 12 Treatment A (S)-P-O-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	92	NA	NA
0.00 - 1.00	2.39	132	0.32	0.316
1.00 - 2.00	3.63	106	0.38	0.385
2.00 - 3.00	4.82	322	1.55	1.55
3.00 - 4.00	3.30	160	0.53	0.528
4.00 - 6.00	2.23	236	0.53	0.264
6.00 - 8.00	1.97	80	0.16	7.89E-2
8.00 - 12.00	1.11	240	0.27	6.66E-2
12.00 - 24.00	1.04	400	0.41	3.45E-2
24.00 - 48.00	*	1600	NA	NA
48.00 - 72.00	*	810	NA	NA
Total Amount Excreted:			4.14	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as P.

Clinical Samples for Urine Study Number 88-1-a

Table 36. Subject 1 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	102	NA	NA
0.00 - 1.00	0.150	180	0.03	2.70E-2
1.00 - 2.00	0.430	175	0.08	7.53E-2
2.00 - 3.00	0.655	235	0.15	0.154
3.00 - 4.00	0.272	370	0.10	0.100
4.00 - 6.00	0.267	640	0.17	8.53E-2
6.00 - 8.00	0.454	186	0.08	4.22E-2
8.00 - 12.00	0.373	320	0.12	2.99E-2
12.00 - 24.00	0.153	460	0.07	5.85E-3
24.00 - 48.00	*	910	NA	NA
48.00 - 72.00	*	822	NA	NA
Total Amount Excreted:			0.80	mg

Table 37. Subject 2 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	37	NA	NA
0.00 - 1.00	0.394	180	0.07	7.09E-2
1.00 - 2.00	1.32	200	0.26	0.264
2.00 - 3.00	1.02	215	0.22	0.218
3.00 - 4.00	1.05	180	0.19	0.189
4.00 - 6.00	0.870	300	0.26	0.130
6.00 - 8.00	0.751	155	0.12	5.82E-2
8.00 - 12.00	0.646	285	0.18	4.61E-2
12.00 - 24.00	0.204	630	0.13	1.07E-2
24.00 - 48.00	*	812	NA	NA
48.00 - 72.00	*	680	NA	NA
Total Amount Excreted:			1.43	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Clinical Samples for Urine Study Number 88-1-a

Table 38. Subject 3 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	35	NA	NA
0.00 - 1.00	9.99E-2	350	0.03	3.50E-2
1.00 - 2.00	0.331	304	0.10	0.101
2.00 - 3.00	0.541	352	0.19	0.190
3.00 - 4.00	0.499	350	0.17	0.175
4.00 - 6.00	0.428	440	0.19	9.43E-2
6.00 - 8.00	0.706	172	0.12	6.07E-2
8.00 - 12.00	0.667	255	0.17	4.25E-2
12.00 - 24.00	0.248	555	0.14	1.15E-2
24.00 - 48.00	*	915	NA	NA
48.00 - 72.00	*	832	NA	NA
Total Amount Excreted:			1.12	mg

Table 39. Subject 4 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	36	NA	NA
0.00 - 1.00	NS	NS	NA	NA
1.00 - 2.00	0.534	72	0.04	3.85E-2
2.00 - 3.00	0.625	60	0.04	3.75E-2
3.00 - 4.00	0.346	260	0.09	8.99E-2
4.00 - 6.00	0.473	240	0.11	5.68E-2
6.00 - 8.00	0.790	68	0.05	2.69E-2
8.00 - 12.00	0.565	125	0.07	1.76E-2
12.00 - 24.00	*	920	NA	NA
24.00 - 48.00	*	1880	NA	NA
48.00 - 72.00	*	910	NA	NA
Total Amount Excreted:			0.40	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Clinical Samples for Urine Study Number 88-1-a

Table 40. Subject 5 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	48	NA	NA
0.00 - 1.00	0.373	105	0.04	3.92E-2
1.00 - 2.00	1.90	85	0.16	0.161
2.00 - 3.00	1.86	120	0.22	0.224
3.00 - 4.00	1.84	85	0.16	0.157
4.00 - 6.00	0.209	160	0.03	1.67E-2
6.00 - 8.00	1.46	170	0.25	0.124
8.00 - 12.00	0.734	320	0.23	5.87E-2
12.00 - 24.00	0.375	440	0.17	1.38E-2
24.00 - 48.00	*	506	NA	NA
48.00 - 72.00	*	1020	NA	NA
Total Amount Excreted:			1.26	mg

Table 41. Subject 6 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	42	NA	NA
0.00 - 1.00	0.171	340	0.06	5.80E-2
1.00 - 2.00	0.452	340	0.15	0.154
2.00 - 3.00	0.304	530	0.16	0.161
3.00 - 4.00	0.363	175	0.06	6.35E-2
4.00 - 6.00	0.697	400	0.28	0.139
6.00 - 8.00	0.580	182	0.11	5.28E-2
8.00 - 12.00	0.317	600	0.19	4.75E-2
12.00 - 24.00	6.99E-2	800	0.06	4.66E-3
24.00 - 48.00	*	1508	NA	NA
48.00 - 72.00	*	1020	NA	NA
Total Amount Excreted:			1.07	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Clinical Samples for Urine Study Number 88-1-a

Table 42. Subject 7 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	70	NA	NA
0.00 - 1.00	0.185	325	0.06	6.00E-2
1.00 - 2.00	0.449	284	0.13	0.127
2.00 - 3.00	0.301	290	0.09	8.73E-2
3.00 - 4.00	0.524	332	0.17	0.174
4.00 - 6.00	1.17	220	0.26	0.129
6.00 - 8.00	0.715	28	0.02	1.00E-2
8.00 - 12.00	0.260	325	0.08	2.11E-2
12.00 - 24.00	*	1300	NA	NA
24.00 - 48.00	*	1040	NA	NA
48.00 - 72.00	*	450	NA	NA
Total Amount Excreted:			0.81	mg

Table 43. Subject 8 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	60	NA	NA
0.00 - 1.00	*	230	NA	NA
1.00 - 2.00	0.256	160	0.04	4.10E-2
2.00 - 3.00	0.420	410	0.17	0.172
3.00 - 4.00	0.869	155	0.13	0.135
4.00 - 6.00	1.32	350	0.46	0.232
6.00 - 8.00	0.958	92	0.09	4.40E-2
8.00 - 12.00	0.585	540	0.32	7.89E-2
12.00 - 24.00	0.187	500	0.09	7.81E-3
24.00 - 48.00	*	1002	NA	NA
48.00 - 72.00	*	900	NA	NA
Total Amount Excreted:			1.31	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Table 44. Subject 9 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	60	NA	NA
0.00 - 1.00	0.180	200	0.04	3.61E-2
1.00 - 2.00	0.743	150	0.11	0.111
2.00 - 3.00	1.11	430	0.48	0.478
3.00 - 4.00	1.05	320	0.34	0.337
4.00 - 6.00	1.10	352	0.39	0.193
6.00 - 8.00	1.89	240	0.45	0.226
8.00 - 12.00	0.659	50	0.03	8.24E-3
12.00 - 24.00	0.407	368	0.15	1.25E-2
24.00 - 48.00	*	620	NA	NA
48.00 - 72.00	*	920	NA	NA
Total Amount Excreted:			1.98	mg

Table 45. Subject 10 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	31	NA	NA
0.00 - 1.00	*	150	NA	NA
1.00 - 2.00	0.535	150	0.08	8.03E-2
2.00 - 3.00	0.551	190	0.10	0.105
3.00 - 4.00	0.992	155	0.15	0.154
4.00 - 6.00	1.12	290	0.33	0.163
6.00 - 8.00	0.977	200	0.20	9.77E-2
8.00 - 12.00	0.722	270	0.19	4.87E-2
12.00 - 24.00	9.63E-2	658	0.06	5.28E-3
24.00 - 48.00	9.64E-2	500	0.05	2.01E-3
48.00 - 72.00	*	550	NA	NA
Total Amount Excreted:			1.17	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Clinical Samples for Urine Study Number 88-1-a

Table 46. Subject 11 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	74	NA	NA
0.00 - 1.00	*	102	NA	NA
1.00 - 2.00	6.96E-2	128	0.01	8.91E-3
2.00 - 3.00	0.223	197	0.04	4.39E-2
3.00 - 4.00	0.224	180	0.04	4.03E-2
4.00 - 6.00	0.128	218	0.03	1.40E-2
6.00 - 8.00	0.634	180	0.11	5.70E-2
8.00 - 12.00	0.220	27	0.01	1.48E-3
12.00 - 24.00	7.93E-2	470	0.04	3.11E-3
24.00 - 48.00	*	500	NA	NA
48.00 - 72.00	*	1010	NA	NA
Total Amount Excreted:			0.28	mg

Table 47. Subject 12 Treatment A (R)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	92	NA	NA
0.00 - 1.00	0.382	132	0.05	5.04E-2
1.00 - 2.00	0.661	106	0.07	7.01E-2
2.00 - 3.00	1.14	322	0.37	0.366
3.00 - 4.00	0.897	160	0.14	0.143
4.00 - 6.00	0.607	236	0.14	7.16E-2
6.00 - 8.00	0.419	80	0.03	1.68E-2
8.00 - 12.00	0.241	240	0.06	1.44E-2
12.00 - 24.00	0.186	400	0.07	6.19E-3
24.00 - 48.00	*	1600	NA	NA
48.00 - 72.00	*	810	NA	NA
Total Amount Excreted:			0.94	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Clinical Samples for Urine Study Number 88-1-a

Table 48. Subject 1 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	102	NA	NA
0.00 - 1.00	0.204	180	0.04	3.68E-2
1.00 - 2.00	0.582	175	0.10	0.102
2.00 - 3.00	0.896	235	0.21	0.211
3.00 - 4.00	0.357	370	0.13	0.132
4.00 - 6.00	0.384	640	0.25	0.123
6.00 - 8.00	0.689	186	0.13	6.41E-2
8.00 - 12.00	0.607	320	0.19	4.86E-2
12.00 - 24.00	0.236	460	0.11	9.04E-3
24.00 - 48.00	*	910	NA	NA
48.00 - 72.00	*	822	NA	NA
Total Amount Excreted:			1.16	mg

Table 49. Subject 2 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	37	NA	NA
0.00 - 1.00	0.786	180	0.14	0.141
1.00 - 2.00	2.83	200	0.57	0.566
2.00 - 3.00	2.28	215	0.49	0.491
3.00 - 4.00	2.55	180	0.46	0.459
4.00 - 6.00	2.14	300	0.64	0.322
6.00 - 8.00	2.16	155	0.34	0.168
8.00 - 12.00	2.08	285	0.59	0.148
12.00 - 24.00	0.747	630	0.47	3.92E-2
24.00 - 48.00	0.346	812	0.28	1.17E-2
48.00 - 72.00	0.233	680	0.16	6.59E-3
Total Amount Excreted:			4.14	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Table 50. Subject 3 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	35	NA	NA
0.00 - 1.00	0.216	350	0.08	7.56E-2
1.00 - 2.00	0.701	304	0.21	0.213
2.00 - 3.00	1.01	352	0.36	0.356
3.00 - 4.00	1.06	350	0.37	0.372
4.00 - 6.00	0.966	440	0.42	0.212
6.00 - 8.00	1.88	172	0.32	0.162
8.00 - 12.00	1.97	255	0.50	0.126
12.00 - 24.00	0.732	555	0.41	3.38E-2
24.00 - 48.00	0.252	915	0.23	9.61E-3
48.00 - 72.00	0.239	832	0.20	8.28E-3
Total Amount Excreted:			3.10	mg

Table 51. Subject 4 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	36	NA	NA
0.00 - 1.00	NS	NS	NA	NA
1.00 - 2.00	0.867	72	0.06	6.24E-2
2.00 - 3.00	0.947	60	0.06	5.68E-2
3.00 - 4.00	0.544	260	0.14	0.141
4.00 - 6.00	0.753	240	0.18	9.04E-2
6.00 - 8.00	1.28	68	0.09	4.35E-2
8.00 - 12.00	1.03	125	0.13	3.23E-2
12.00 - 24.00	0.221	920	0.20	1.70E-2
24.00 - 48.00	*	1880	NA	NA
48.00 - 72.00	*	910	NA	NA
Total Amount Excreted:			0.86	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Clinical Samples for Urine Study Number 88-1-a

Table 52. Subject 5 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	48	NA	NA
0.00 - 1.00	0.751	105	0.08	7.88E-2
1.00 - 2.00	4.10	85	0.35	0.348
2.00 - 3.00	4.00	120	0.48	0.480
3.00 - 4.00	4.00	85	0.34	0.340
4.00 - 6.00	0.417	160	0.07	3.34E-2
6.00 - 8.00	3.20	170	0.54	0.272
8.00 - 12.00	1.85	320	0.59	0.148
12.00 - 24.00	0.643	440	0.28	2.36E-2
24.00 - 48.00	0.164	506	0.08	3.46E-3
48.00 - 72.00	*	1020	NA	NA
Total Amount Excreted:			2.82	mg

Table 53. Subject 6 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	42	NA	NA
0.00 - 1.00	0.258	340	0.09	8.79E-2
1.00 - 2.00	0.665	340	0.23	0.226
2.00 - 3.00	0.524	530	0.28	0.278
3.00 - 4.00	0.645	175	0.11	0.113
4.00 - 6.00	1.14	400	0.46	0.229
6.00 - 8.00	1.08	182	0.20	9.81E-2
8.00 - 12.00	0.579	600	0.35	8.68E-2
12.00 - 24.00	0.242	800	0.19	1.61E-2
24.00 - 48.00	0.114	1508	0.17	7.15E-3
48.00 - 72.00	*	1020	NA	NA
Total Amount Excreted:			2.07	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Clinical Samples for Urine Study Number 88-1-a

Table 54. Subject 7 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	70	NA	NA
0.00 - 1.00	0.295	325	0.10	9.58E-2
1.00 - 2.00	0.886	284	0.25	0.251
2.00 - 3.00	0.615	290	0.18	0.178
3.00 - 4.00	1.13	332	0.38	0.376
4.00 - 6.00	2.72	220	0.60	0.299
6.00 - 8.00	2.09	28	0.06	2.93E-2
8.00 - 12.00	0.722	325	0.23	5.87E-2
12.00 - 24.00	0.166	1300	0.22	1.80E-2
24.00 - 48.00	8.33E-2	1040	0.09	3.61E-3
48.00 - 72.00	*	450	NA	NA
Total Amount Excreted:			2.10	mg

Table 55. Subject 8 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	60	NA	NA
0.00 - 1.00	0.181	230	0.04	4.17E-2
1.00 - 2.00	0.556	160	0.09	8.90E-2
2.00 - 3.00	0.897	410	0.37	0.368
3.00 - 4.00	1.74	155	0.27	0.270
4.00 - 6.00	2.65	350	0.93	0.464
6.00 - 8.00	2.06	92	0.19	9.50E-2
8.00 - 12.00	1.30	540	0.70	0.175
12.00 - 24.00	0.497	500	0.25	2.07E-2
24.00 - 48.00	0.225	1002	0.23	9.39E-3
48.00 - 72.00	*	900	NA	NA
Total Amount Excreted:			3.06	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Clinical Samples for Urine Study Number 88-1-a

Table 56. Subject 9 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	60	NA	NA
0.00 - 1.00	0.303	200	0.06	6.07E-2
1.00 - 2.00	1.44	150	0.22	0.216
2.00 - 3.00	2.11	430	0.91	0.907
3.00 - 4.00	1.92	320	0.61	0.615
4.00 - 6.00	2.12	352	0.75	0.374
6.00 - 8.00	3.83	240	0.92	0.459
8.00 - 12.00	1.67	50	0.08	2.09E-2
12.00 - 24.00	1.12	368	0.41	3.44E-2
24.00 - 48.00	*	620	NA	NA
48.00 - 72.00	*	920	NA	NA
Total Amount Excreted:			3.96	mg

Table 57. Subject 10 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	31	NA	NA
0.00 - 1.00	*	150	NA	NA
1.00 - 2.00	1.23	150	0.18	0.185
2.00 - 3.00	1.33	190	0.25	0.252
3.00 - 4.00	2.19	155	0.34	0.340
4.00 - 6.00	2.52	290	0.73	0.365
6.00 - 8.00	2.35	200	0.47	0.235
8.00 - 12.00	1.85	270	0.50	0.125
12.00 - 24.00	0.222	658	0.15	1.22E-2
24.00 - 48.00	0.229	500	0.11	4.77E-3
48.00 - 72.00	*	550	NA	NA
Total Amount Excreted:			2.73	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

#Expressed as HOP.

Clinical Samples for Urine Study Number 88-1-a

Table 58. Subject 11 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	74	NA	NA
0.00 - 1.00	8.04E-2	102	0.01	8.20E-3
1.00 - 2.00	8.31E-2	128	0.01	1.06E-2
2.00 - 3.00	0.252	197	0.05	4.97E-2
3.00 - 4.00	0.269	180	0.05	4.85E-2
4.00 - 6.00	0.135	218	0.03	1.47E-2
6.00 - 8.00	0.715	180	0.13	6.43E-2
8.00 - 12.00	0.259	27	0.01	1.75E-3
12.00 - 24.00	0.145	470	0.07	5.67E-3
24.00 - 48.00	*	500	NA	NA
48.00 - 72.00	*	1010	NA	NA
Total Amount Excreted:			0.35	mg

Table 59. Subject 12 Treatment A (S)-HOP-G#

Time Interval (hours)	Conc. (mcg/ml)	Volume (ml)	Amount (mg)	Ex Rate (mg/hr)
-1.00 - 0.00	*	92	NA	NA
0.00 - 1.00	0.665	132	0.09	8.78E-2
1.00 - 2.00	1.14	106	0.12	0.120
2.00 - 3.00	1.96	322	0.63	0.630
3.00 - 4.00	1.63	160	0.26	0.261
4.00 - 6.00	1.15	236	0.27	0.136
6.00 - 8.00	0.944	80	0.08	3.78E-2
8.00 - 12.00	0.552	240	0.13	3.31E-2
12.00 - 24.00	0.555	400	0.22	1.85E-2
24.00 - 48.00	*	1600	NA	NA
48.00 - 72.00	*	810	NA	NA
Total Amount Excreted:			1.80	mg

* = Below Assay Sensitivity C = Combined Sample BC = Bad Chromatogram
NS = No Sample NR = Not Run NA = Not Applicable

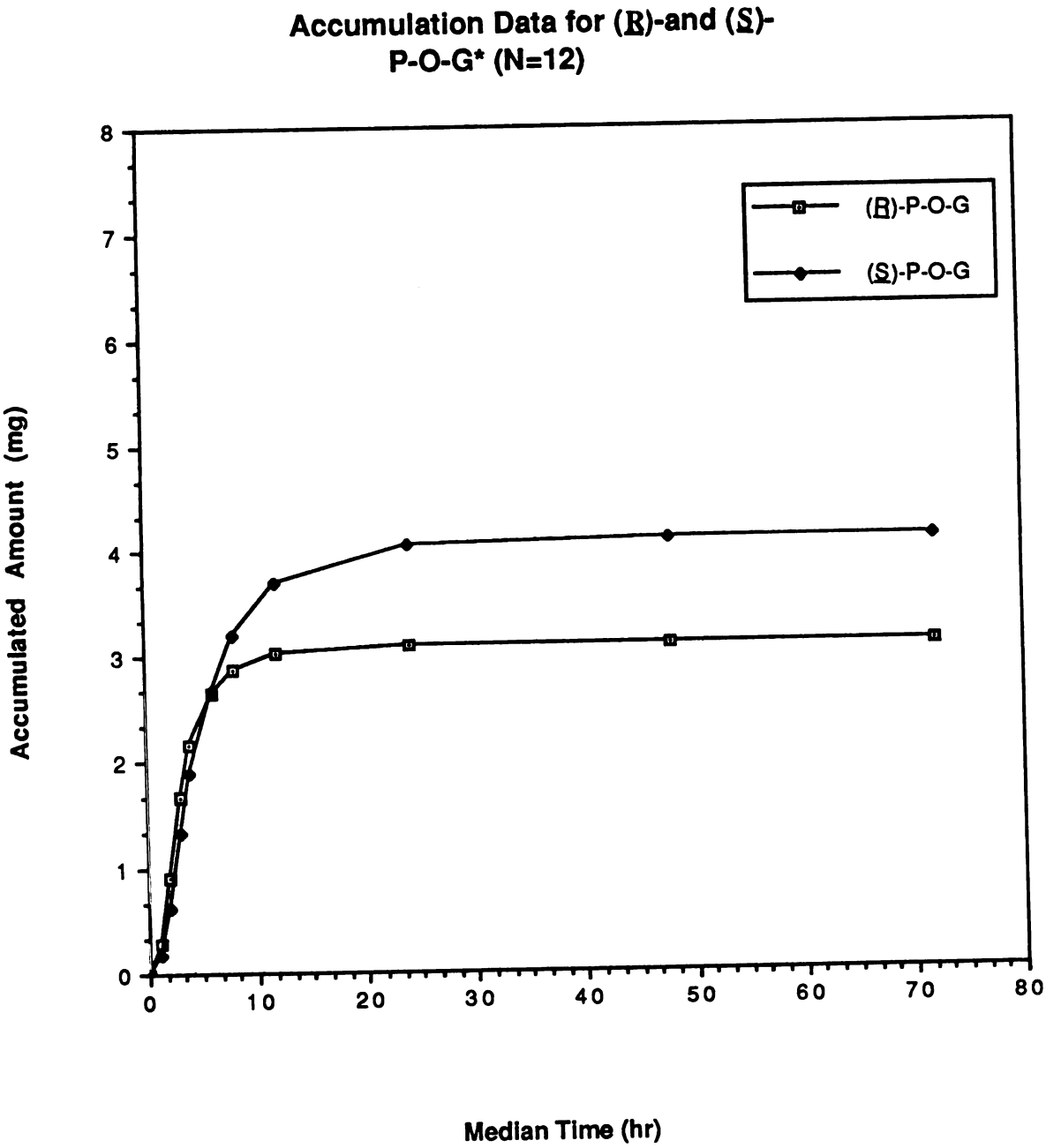
#Expressed as HOP.

Table 60. Mean Excretion Rate vs. Time (N=12)

Time Interval (hrs)	(R)-P-O-G Ex. Rate (mg/hr)	(S)-P-O-G Ex. Rate (mg/hr)	(R)-HOP-G Ex. Rate (mg/hr)	(S)-HOP-G Ex. Rate (mg/hr)
-1.00-0.00	0.000	0.000	0.000	0.000
0.00-1.00	0.290	0.169	0.034	0.065
1.00-2.00	0.624	0.460	0.103	0.199
2.00-3.00	0.764	0.712	0.186	0.355
3.00-4.00	0.488	0.562	0.147	0.289
4.00-6.00	0.240	0.385	0.110	0.222
6.00-8.00	0.118	0.266	0.068	0.144
8.00-12.00	0.039	0.124	0.035	0.084
12.00-24.00	0.005	0.029	0.007	0.021
24.00-48.00	0.000	0.002	0.000	0.004
48.00-72.00	0.000	0.001	0.000	0.001

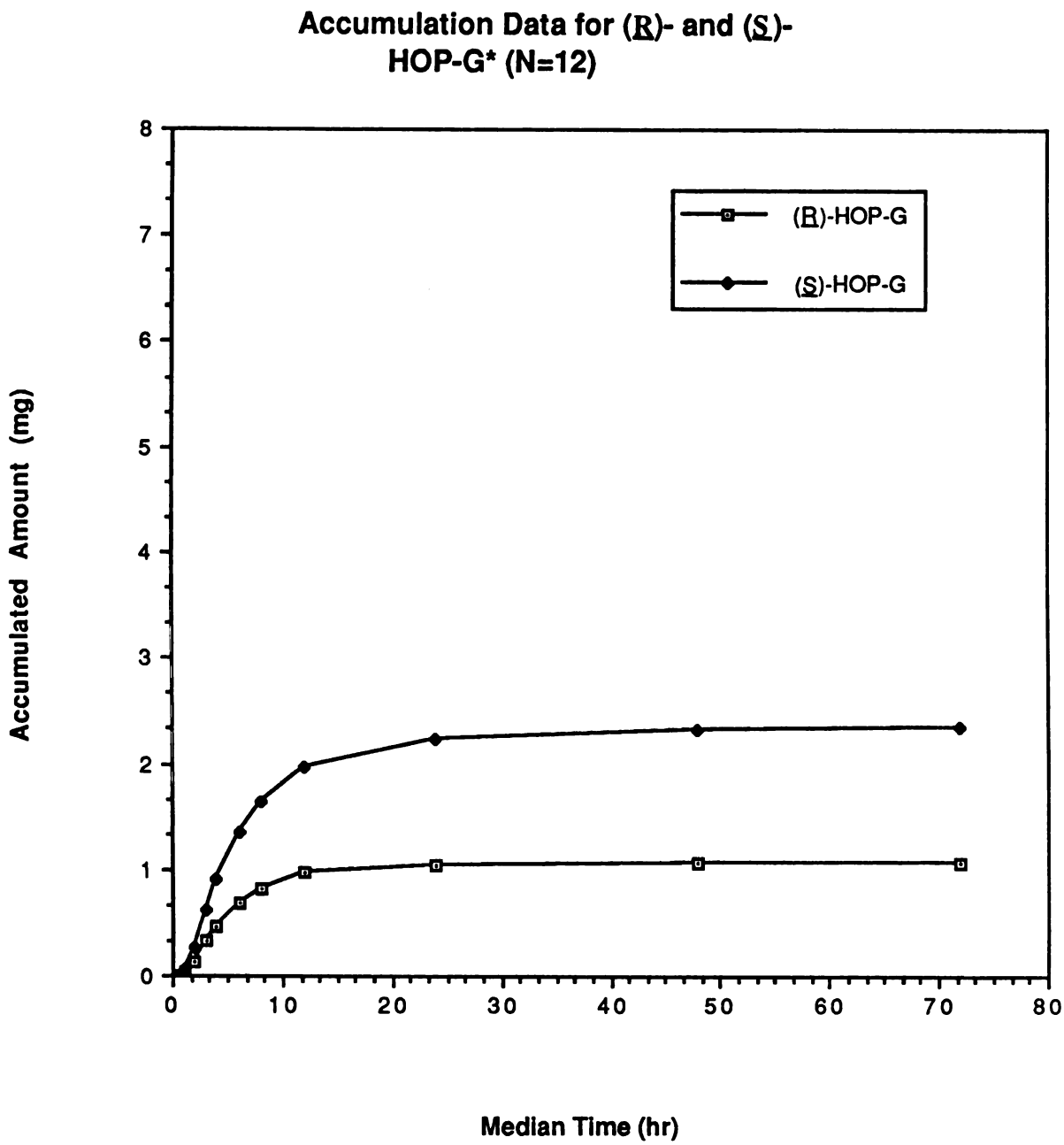
Table 61. Accumulated Amount (mg) of (R)- and (S)-Glucuronide of P and HOP

Time Interval (hrs)	(R)-P-O-G (mg)	(S)-P-O-G (mg)	(R)-HOP-G (mg)	(S)-HOP-G (mg)	(R)-P-O-G and (S)-P-O-G (mg)	(R)-HOP-G and (S)-HOP-G (mg)
-1.00-0.00	0.000	0.000	0.000	0.000	0.000	0.000
0.00-1.00	0.290	0.169	0.034	0.065	0.459	0.099
1.00-2.00	0.915	0.629	0.137	0.264	1.543	0.401
2.00-3.00	1.679	1.340	0.323	0.619	3.019	0.942
3.00-4.00	2.167	1.902	0.470	0.908	4.069	1.378
4.00-6.00	2.647	2.672	0.691	1.352	5.320	2.042
6.00-8.00	2.883	3.205	0.827	1.640	6.088	2.466
8.00-12.00	3.037	3.700	0.965	1.974	6.737	2.939
12.00-24.00	3.096	4.046	1.047	2.223	7.142	3.269
24.00-48.00	3.096	4.094	1.051	2.321	7.147	3.373
48.00-72.00	3.096	4.106	1.051	2.350	7.148	3.373



* Expressed as P.

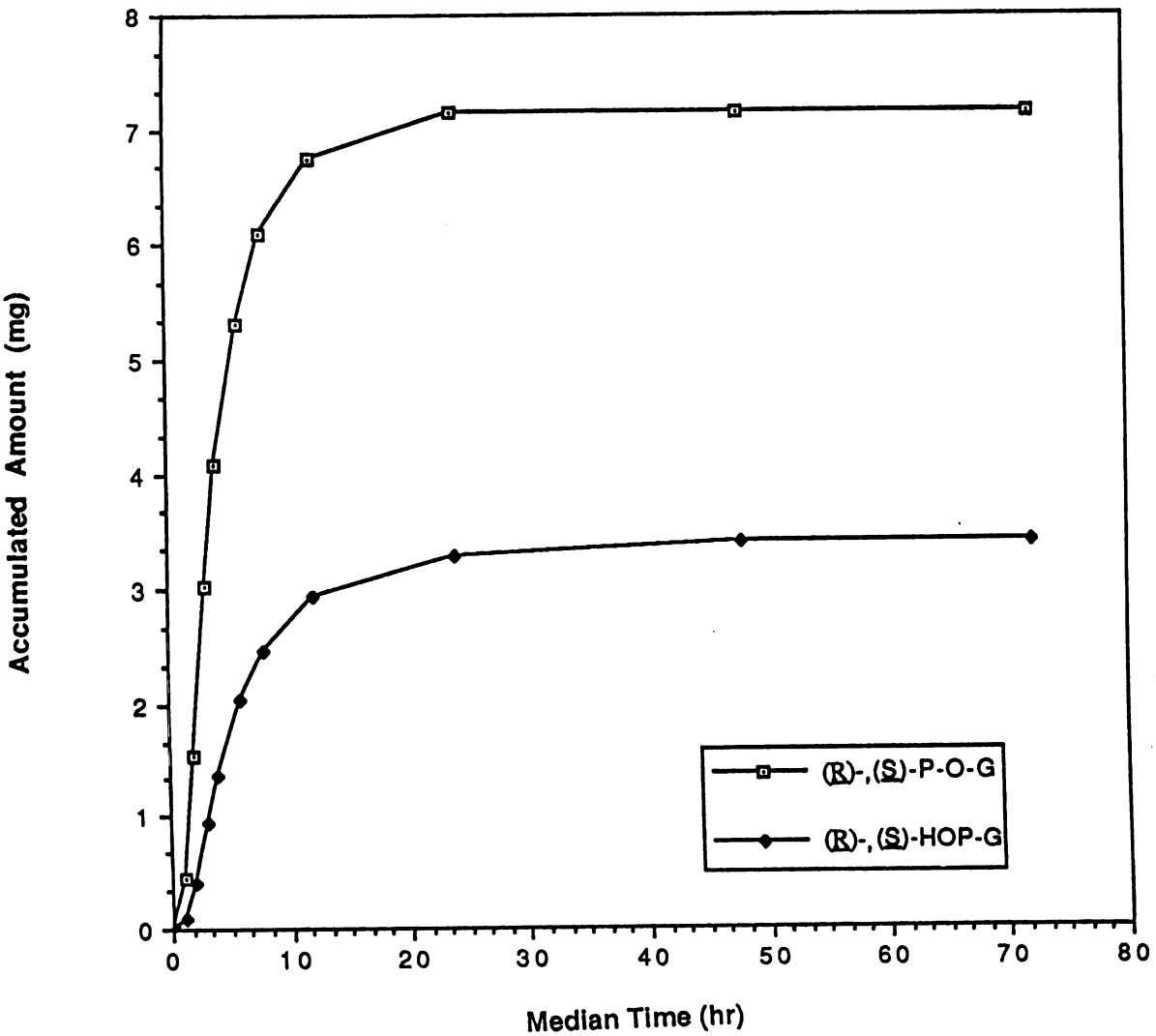
Fig. 34. Amount accumulated for (R)- and (S)-P-O-G respectively



* Expressed as HOP.

Fig. 35. Amount accumulated for (R)- and (S)-HOP-G respectively

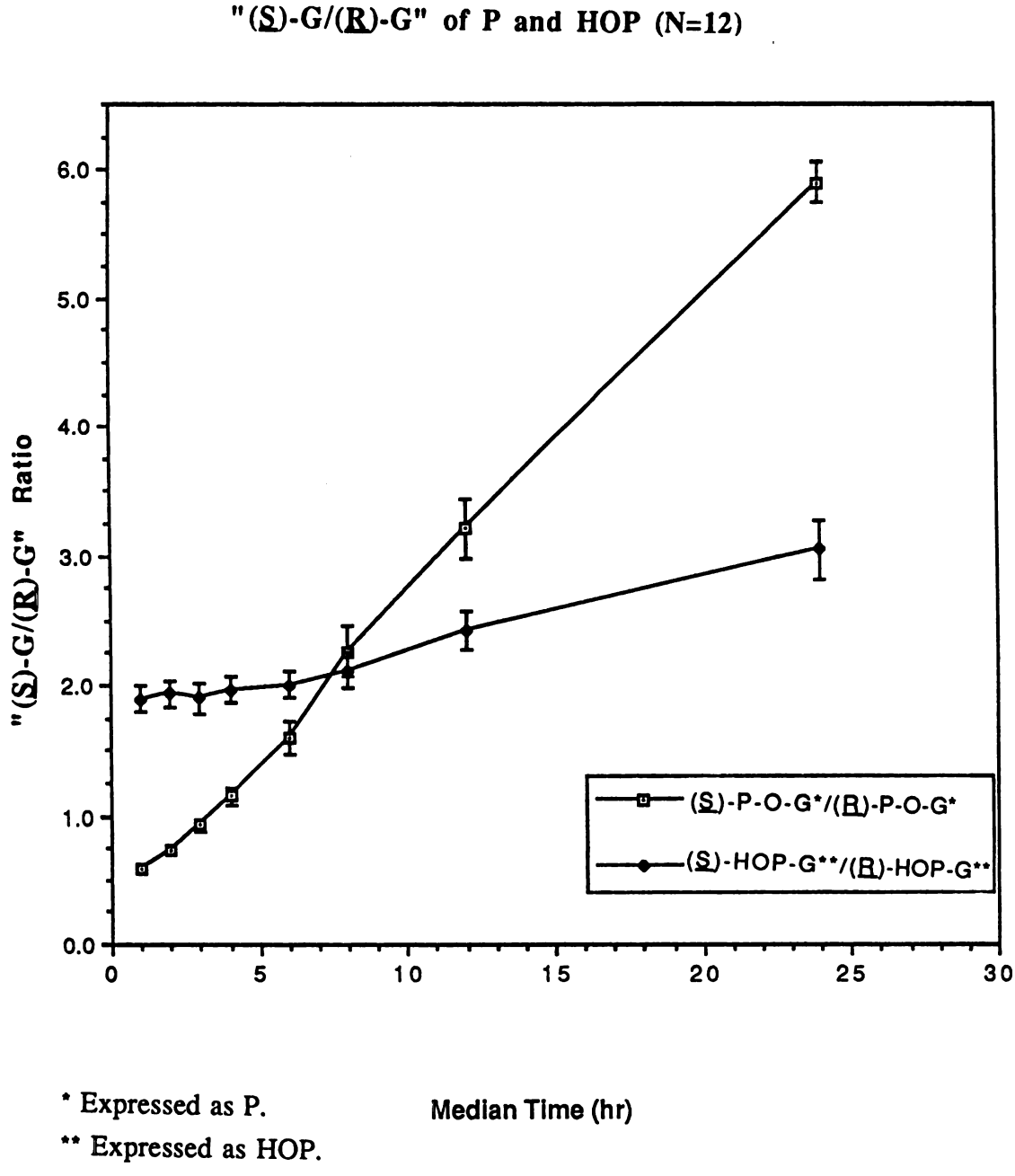
Mean Accumulation Data (N = 12)
"(R)-, (S)-P-O-G"* and "(R)-, (S)-HOP-G"**



* = The Sum of Accumulated Amount for Both (R)- and (S)-P-O-G, expressed as P.

** = The Sum of Accumulated Amount for Both (R)- and (S)-HOP-G, expressed as HOP.

Fig. 36 Amount accumulated for (R)- and (S)-P-O-G, (R)- and (S)-HOP-G, respectively.



* Expressed as P.

** Expressed as HOP.

Fig. 37 "(S)-glucuronide/(R)-glucuronide" ratio vs time plot (N=12). Error bars represent standard errors.

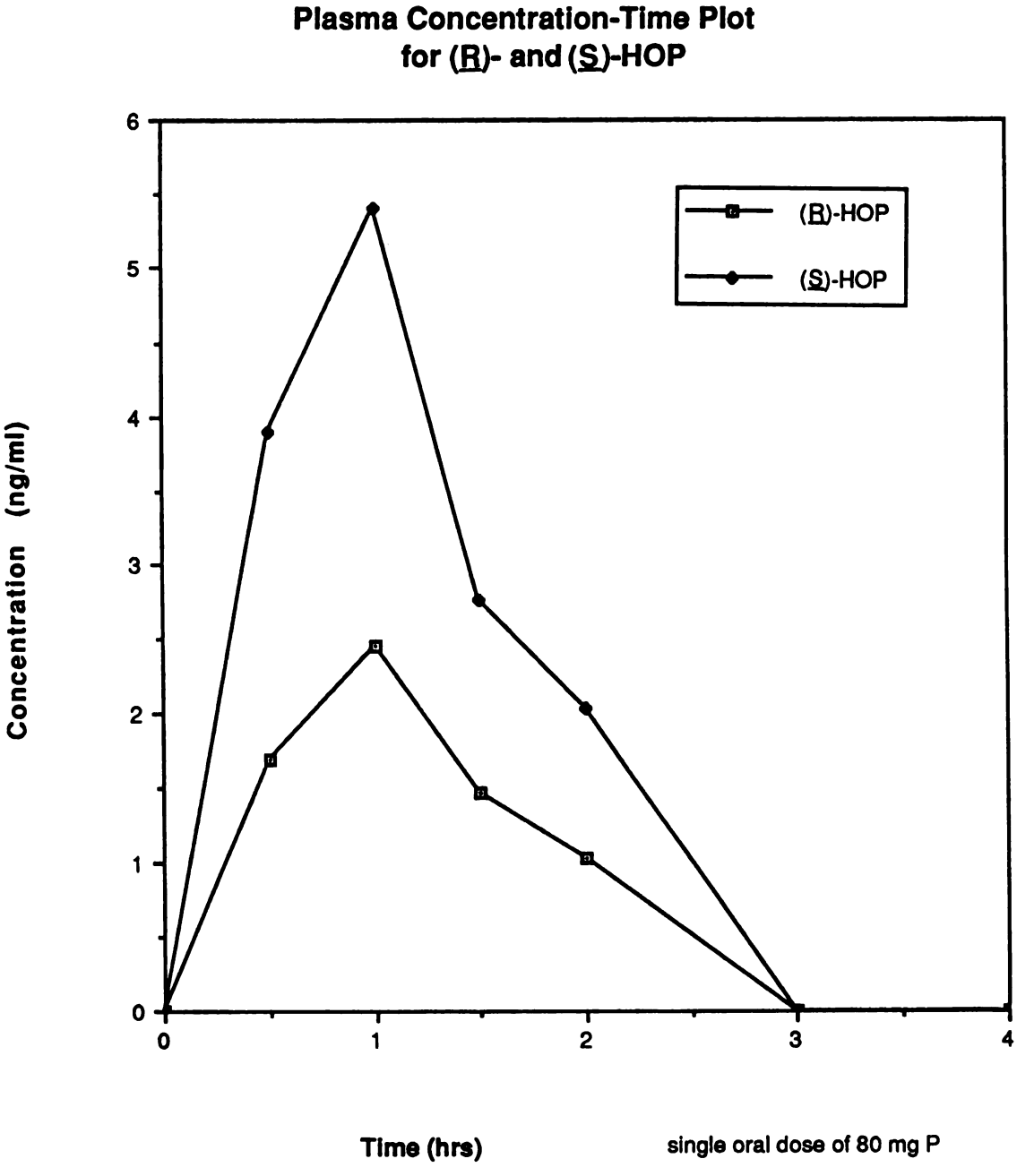


Fig. 38. Plasma concentration-time plot for (R)- and (S)-HOP in one human subject

Table 62. Excretion Amount (mg) of (R)- and (S)-P-O-G

Subj. No.	(R)-P-O-G* (mg)	(S)-P-O-G* (mg)	Ext. Amount of (R)- and (S)-P-O-G*	(R)- and (S)-P-O-G*%	(S)-P-O-G* /(R)-P-O-G*
1	2.110	3.070	5.180	7.39%	1.455
2	2.763	3.900	6.663	9.50%	1.412
3	2.120	3.010	5.130	7.32%	1.420
4	3.768	4.834	8.602	12.27%	1.283
5	2.365	3.250	5.615	8.01%	1.374
6	3.607	5.309	8.916	12.71%	1.472
7	2.564	3.357	5.921	8.44%	1.309
8	3.476	4.491	7.966	11.36%	1.292
9	2.238	3.370	5.608	8.00%	1.506
10	4.797	5.826	10.623	15.15%	1.214
11	3.960	4.861	8.821	12.58%	1.228
12	3.610	4.262	7.872	11.22%	1.181
MEAN	3.115	4.128	7.243	10.33%	1.345
SD	0.871	0.945	1.799	2.56%	0.109
%CV	27.97%	22.89%	24.84%	24.78%	8.10%

* Expressed as P.

Table 63. Excretion Amount (mg) of (R)- and (S)-HOP-G

Subj. No.	(R)-HOPG* (mg)	(S)-HOPG* (mg)	Ext. Amount of (R)- and (S)-HOPG*	Ext. Amount of (R)- and (S)-HOPG**	(R)- and (S)-HOPG**%	(S)-HOPG* /(R)-HOPG*
1	0.802	1.158	1.961	1.847	2.63%	1.444
2	1.436	4.142	5.577	5.254	7.49%	2.885
3	1.129	3.263	4.392	4.137	5.90%	2.889
4	0.404	0.862	1.266	1.193	1.70%	2.133
5	1.272	2.829	4.101	3.863	5.51%	2.225
6	1.080	2.175	3.255	3.066	4.37%	2.014
7	0.811	2.102	2.913	2.744	3.91%	2.590
8	1.309	3.066	4.375	4.121	5.88%	2.343
9	1.987	3.964	5.951	5.606	7.99%	1.995
10	1.172	2.736	3.908	3.681	5.25%	2.335
11	0.278	0.348	0.626	0.590	0.84%	1.253
12	0.936	1.801	2.736	2.577	3.68%	1.924
MEAN	1.051	2.370	3.422	3.223	4.60%	2.169
SD	.458	1.190	1.622	1.528	2.18%	0.500
%CV	43.59%	50.19%	47.40%	47.41%	47.39%	23.05%

* Expressed as HOP.
** Expressed as P.

Table 64. Total (mg) and % OF P-O-G and HOP-G Excreted in Urine after an 80 mg Oral Dose OF P

Subj. No.	Excretion Amount of Total Glucuronides*	Total* %
1	7.027	10.02%
2	11.917	16.99%
3	9.267	13.21%
4	9.795	13.97%
5	9.478	13.51%
6	11.982	17.09%
7	8.665	12.36%
8	12.087	17.24%
9	11.214	15.99%
10	14.304	20.40%
11	9.411	13.42%
12	10.449	14.90%
MEAN	10.460	14.92%
SD	1.939	2.77%
%CV	18.53%	18.57%

* Expressed as P.

DISCUSSION

Many drugs are optically active and are commonly used as racemic mixtures of the enantiomeric forms. However, there may be large differences between enantiomers in their pharmacological activity, metabolism, and elimination. To study the pharmacokinetic and pharmacodynamic behaviors of the two enantiomeric conformations requires a precise and sensitive analytical assay that separates and quantitates the individual (R)- and (S)-isomer in biological samples. The precolumn derivatization HPLC procedure presented here is suitable for this purpose. Also, for a better understanding of the stereoselective disposition and metabolism of P, another HPLC method was developed to directly separate (R)- and (S)-glucuronide conjugates of P and HOP in urine.

I. Optimization of the Precolumn Derivatization Method

During the development of the precolumn derivatization assay, various parameters were evaluated to assess the optimum conditions.

Among the commercially available derivatization agents, N-trifluoroacetyl-prolyl-chloride (TPC) was tried first [44, 46], but was found to rapidly undergo racemization during storage. Therefore, it is unsuitable for examining stereoselective metabolism of drugs. Another reagent, 1-phenyl-ethyl-isocyanate (PEIC), is chemically more stable, but the retention time is too long for better separation in either normal or reverse phases [52] and the sensitivity is not adequate for this assay. Hermansson [48], using *t*-butoxycarbonyl-*l*-alanine or *t*-butoxycarbonyl-*l*-leucine as a derivatization agent, did achieve a detection sensitivity down to 1 ng/ml when using 1 ml of plasma from one human subject. However, this assay is not a convenient routine method. 2, 3, 4, 6-tetra-O-acetyl-ß-D-glucopyranosyl isothiocyanate (GITC), was initially used in the resolution of amino

acids and epinephrine [60, 61, 62] and also was found to be applicable to the resolution of ß-blockers in pure chemical reaction [51]. Steven T. Wu *et al.*[55] used GITC to resolve P enantiomers in human plasma for the first time. The method presented here details the application of GITC resolve HOP enantiomers in human plasma.

The reaction between HOP (or P) and GITC, in principle, gives two pairs of monosubstituted diastereomers. Namely, GITC can attach to the amino group to give urea derivatives or it can react with the alcohol moiety to form carbamate derivatives. However, the urea derivatives are the major products, as indicated in the literature [49]. Isothiocyanate groups react rapidly and selectively with primary and secondary amines to form the corresponding thiourea derivatives [49]. Here, P and HOP are secondary amines that react with GITC. The reaction should be carried out in an alcohol and water free medium to get a consistent result. Therefore, all reagents (such as methylene chloride, and acetonitrile) should be free of water to allow complete derivatization. Traces of water were further removed chemically by using 2, 2-dimethoxypropane as a scavenger. The activated molecular sieve was also used to remove moisture from methylene chloride and acetonitrile.

Because the pK_a of HOP (and P) is about 9.5, the base 1 M K_2HPO_4 (pH 10) was used to make the plasma solution alkaline to facilitate the extraction. 4-Methyl-P, an analog of P, is a very stable and ideal internal standard, since the methyl group on the naphthalene ring is far away from the reaction site, and would have little effect on reactivity.

HOP in plasma is less stable than P, due to oxidation of phenolic hydroxyl group in the moiety, antioxidant such as ascorbic acid must be added to clinical plasma samples as soon as the plasma is separated from blood. With two fluorescence detectors connected in series, this precolumn derivatization method has been tried to separate each enantiomer of (R)-, (S)-P and (R)-, (S)-HOP in plasma simultaneously by using a reverse-phase system similar to the one used for separating (R)-, (S)-HOP. This simple and precise method has a sensitivity of 1 ng/ml for each (R)-, (S)-HOP enantiomer when 0.5 ml plasma samples

were used, and will be used in a clinical pharmacokinetic study of hypertension patients in the future.

II. Improvement of the Indirect Method for Determination of (R)- and (S)-Glucuronide Diastereomers of P and HOP in Human Urine

Because (R)- and (S)-glucuronide conjugates of P and HOP are diastereomers, the simple HPLC method we developed is the first to directly separate each glucuronide diastereomer of (R)-, (S)-P and (R)-, (S)-HOP without derivatization or use of a chiral column.

Measurement of the glucuronide conjugate must depend upon enzymatic hydrolysis and indirect assay of the liberated aglycones because no authentic samples are available. Some HPLC methods mentioned previously were based upon indirect methods (measurement of the difference in P or HOP concentrations between enzyme-hydrolyzed and nonhydrolyzed samples) to determine the glucuronide concentrations , and a further step has to be done to measure (R)- and (S)-isomer [16, 18]. Those indirect methods are unreliable because the HOP released after ß-glucuronidase (containing significant sulfatase) hydrolysis also may include HOP released from (R)-, (S)-HOP sulfate conjugates. Therefore, the simple and accurate method described here precludes the over estimation of the (R)-, (S)-HOP-G concentration caused by sulfate hydrolysis. The (R)-, (S)-P-O-G and (R)-, (S)-HOP-G stock solutions, respectively, were isolated from human urine and rat liver microsomal incubates and were measured by an indirect method (Table 1). The working standard solutions were produced by pooling the clinical urine samples (containing high concentration of glucuronides when tested in advance), then calibrated by comparison to the stock solutions. Ascorbic acid was added to all standard solutions and to urine samples from twelve subjects to prevent degradation of the glucuronide conjugates (especially HOP-G) [18].

The glucuronidation of HOP principally could occur at either the aliphatic or the phenolic hydroxyl group, and evidence for the latter has been found only in humans, *i. e.* containing an unsubstituted ß-blocking side chain [63] (Fig. 3).

The activity of glucuronyltransferases in rat liver microsomes is about 10-fold higher for the conjugation of HOP than for the conjugation of P (Fig. 17) [56].The *in vitro* results (Fig. 8 and Fig. 17) suggest that the glucuronidation of HOP occurs nonstereoselectively in rat liver when 2 mM HOP was used as the substrate. Neither stereoselectivity was observed in the formation of (R)-, (S)-P-O-G when 2 mM P was used as the substrate [56]. But in human urine, the *in vivo* data : (S)-P-O-G/(R)-P-O-G ratio, 1.35±0.109 (mean±SD); (S)-HOP-G/(R)-HOP-G ratio, 2.17±0.500 (mean±SD) demonstrate that the stereoselectivities of both P-O-G and HOP-G appear to favor formation of the (S)-(-)-isomers. In addition, Fig. 37 suggests that (S)-P-O-G has a slower elimination rate compared to (R)-P-O-G. These findings may lend support to the theory proposed by Walle *et al.*[16] and Silber *et al.*[5].

Unconjugated HOP and P represent, respectively, about 1% and 0.2-0.8% of the oral dose in urine [18] [64]. HOP sulfate has been identified as a major metabolite and accounts for 17 to 18% of a 960 mg oral dose of P in the urine of a human subject [27]. From our urinary excretion data, 4.60 ± 2.18% (mean ± SD) of an 80 mg oral dose of P is excreted as HOP-G, and 10.3 ± 2.56% (mean ± SD) of the dose is excreted as P-O-G. Therefore, total glucuronide excretion is 14.9 ± 2.77% (mean ± SD). These data of urinary excretion may reflect the changes that occur in plasma and correlate well with the literature [17, 18]. This quantitative assay for urine has also been successfully applied to analysis of plasma samples (after precipitation with acetonitrile). Hence, it will be used in a future clinical pharmacokinetic study involving plasma analysis.

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