Journal of Chromatography, 414 (1987) 365–379
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3454

VERSATILE ISOCRATIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR PROPRANOLOL AND ITS BASIC, NEUTRAL AND ACIDIC METABOLITES IN BIOLOGICAL FLUIDS

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(Received September 9th, 1986)

SUMMARY

A comprehensive high-performance liquid chromatographic method was developed for quantitating propranolol and its known metabolites in serum, bile and urine. Analysis was performed before and after incubation of the samples with β-glucuronidase–arylsulfatase to quantitate both free and conjugate forms of the oxidative metabolites. Fractionation of the basic, neutral and acidic metabolites was achieved by differential pH solvent extraction. 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 that were subjected to enzymatic hydrolysis. The original aqueous sample was titrated with acid to pH 1, followed by extraction of the remaining acidic metabolites into either *n*-butanol–dichloromethane (with unhydrolyzed serum) or carbon tetrachloride (with all other samples). Chromatographic separation of the metabolites in the different extracts was achieved on a reversed-phase C_{18} column, using a single isocratic mobile phase consisting of 0.044 *M* pH 2.7 phosphate buffer, tetrahydrofuran, methanol and acetonitrile, with the addition of *n*-butylamine as a competing base to control retention volume and peak shape. Detection and quantitation of propranolol and its metabolites in the low nanogram to sub-nanogram range was afforded by fluorescence at a low UV excitation wavelength. The coefficients of variation for replicate assay of spiked samples were uniformly less than 6% for all the analytes.

INTRODUCTION

Propranolol is a widely used β-adrenoceptor antagonist for the treatment of arrhythmias, thyrotoxicosis, angina pectoris and hypertension. It has also been used as a pharmacokinetic probe in elucidating the complex mechanisms involved in the uptake and metabolism of drugs by the liver. Metabolic studies in dogs, rats and humans [1,2] revealed three primary pathways of biotransformation, viz. N-dealkylation, aromatic hydroxylation and glucuronidation of the hydroxyl

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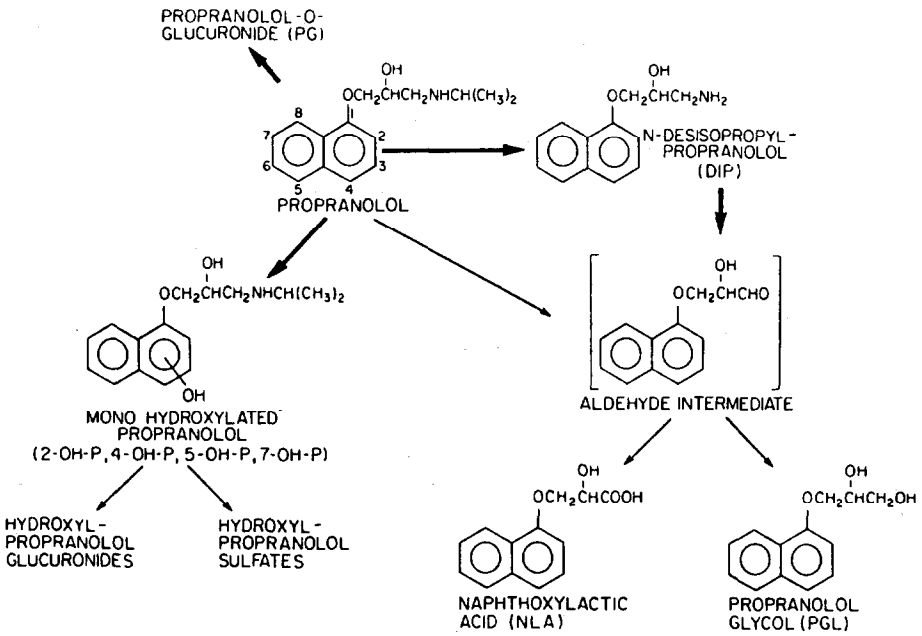

Fig. 1. Major pathways of propranolol metabolism.

group at the 2-position of the side-chain. More recently, ethereal sulfate of propranolol has been identified in dog serum [3]. A scheme illustrating the major products of propranolol metabolism is shown in Fig. 1. More than 30 metabolites, displaying a wide spectrum of physicochemical properties, have been identified to date. For example, polar metabolites with basic and acidic functional groups are present together with neutral non-polar compounds. Hence, a comprehensive and quantitative study of propranolol metabolism requires a flexible and efficient analytical procedure that can cope with the varied nature of the metabolic species.

Traditional methods for quantitating intact propranolol in biological samples include radioimmunoassay [4], direct fluorometric procedure [5–9], thin-layer chromatography [10–13] and gas–liquid chromatography [14–17]. Most of these methods are not easily adaptable for metabolite measurements. Analysis of propranolol metabolites has mostly been accomplished with high-performance liquid chromatography (HPLC) [3,18–31]. However, the existing methods are usually directed at a select group of propranolol metabolites, e.g. the basic species. None of the described procedures is capable of resolving the recently discovered regioisomeric monohydroxyl metabolites of propranolol (viz. 4-, 5- and 7-hydroxypropranolol), which have been shown to possess β-blocking activity. The most versatile methods reported to date involve the use of gas chromatography–mass spectrometry (GC–MS) [1,32–37]. Although mass-selective detection provides the necessary resolution capability, it requires access to an expensive mass spectrometer and it is not cost-effective for routine high-througput analytical usage.

This paper decribes a comprehensive and highly sensitive HPLC fluorescence assay for propranolol and its six major metabolites (see Fig. 1). By combining an intensive clean-up procedure with an optimized enzymatic hydrolysis of the

conjugate substrates, our method is applicable to a variety of biological fluids including serum, bile and urine. Chromatographic separation of basic, neutral and acidic metabolites was accomplished with a single isocratic mobile phase. This procedure has been successfully applied to a series of quantitative metabolism studies with propranolol in the rat.

EXPERIMENTAL

Materials and reagents

Propranolol hydrochloride, N-desisopropylpropranolol, pronethalol, naphthoxylactic acid and 4-hydroxypropranolol hydrochloride were kindly supplied by Imperial Chemical Industries (Macclesfield, U.K.). 1-Naphthol, naphthalene ethanol and naphthoxyacetic acid were purchased from Aldrich (Milwaukee, WI, U.S.A.). Propranolol glycol, 2-, 5- and 7-hydroxypropranolol were gifts from Dr. Wendel Nelson (Department of Medicinal Chemistry, University of Washington, Seattle, WA, U.S.A.). β-Glucuronidase–arylsulfatase extracted from mollusks (Types H-1 and H-2), β-glucuronidase from beef liver (Glucurase®), purified arylsulfatase from *Aerobacter aerogenes* (Type III) and saccharo-1,4-lactone were obtained from Sigma (St. Louis, MO, U.S.A.). Additional supplies of β-glucuronidase–arylsulfatase were also purchased from Calbiochem-Behring (La Jolla, CA, U.S.A.) and Endo Labs. (Wilmington, DE, U.S.A.). High-purity solvents suitable for HPLC were supplied by Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). *n*-Butylamine (D-4 reagent) was obtained from Waters Assoc. (Milford, MA, U.S.A.). All other chemicals were of analytical grade.

Preparation of standards

Concentrated stock solutions of propranolol and each of the metabolites were prepared separately in methanol. Starting with the concentrated stock solutions, working solutions were prepared containing 15 μg/ml 4-hydroxypropranolol, 10.8 μg/ml 5-hydroxypropranolol, 10 μg/ml 7-hydroxypropranolol, 14.4 μg/ml N-desisopropylpropranolol, 16.6 μg/ml propranolol, 19.4 μg/ml naphthoxylactic acid and 6.8 μg/ml propranolol glycol. Each of the working solutions contained 5 mg/ml ascorbic acid. Standard solutions for spiking were prepared from each of the preceding list of stock solutions by 1:2, 1:5, 1:10 and 1:25 dilution, respectively. The isomeric hydroxypropranolols, N-desisopropylpropranolol and propranolol constituted the basic fraction. The neutral fraction contained propranolol glycol and the acidic fraction included naphthoxylactic acid. Naphthoxyacetic acid and 4-hydroxynaphthoxylactic acid are known to be minor metabolites derived from oxidative metabolism of naphthoxylactic acid. Since our focus is on the major metabolic species of propranolol, we did not include derivatives of naphthoxylactic acid in our measurements. However, preliminary work indicated that quantitation of naphthoxyacetic acid is feasible with the present assay.

Calibration standards for each type of biological fluid were prepared by spiking the blank biological medium with 20 μl of each standard solution. During the development of this assay, serum, bile and urine were obtained from rats. The

procedure has also been validated with human specimens. All calibration standards were prepared at the beginning of each assay run and were found to be stable at 4 °C for at least one week.

Sample preparation

Extraction procedures. Fig. 2 outlines the extraction procedure for the unhydrolyzed serum samples. Sequential extraction with *n*-butanol–dichloromethane (1:50, v/v) at basic and acidic pH yielded two fractions: the first extract contained the basic and neutral metabolites; the second extract contained the acidic metabolites. The final organic extracts were evaporated gently under dry nitrogen and reconstituted in the mobile phase, and aliquots were injected onto the HPLC column.

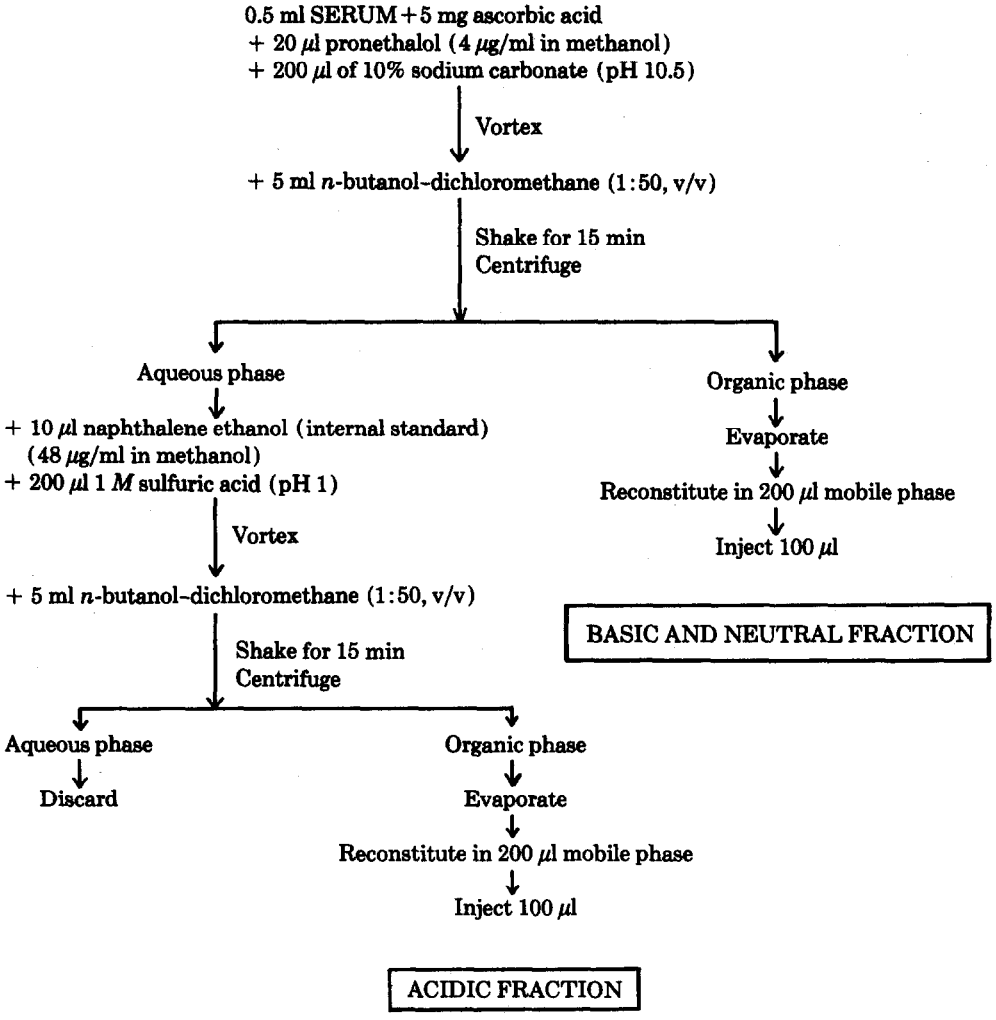

Fig. 2. Extraction scheme for unhydrolyzed serum samples.

The extraction and clean-up scheme for prehydrolyzed urine and posthydrolyzed serum, bile and urine samples is shown in Fig. 3. Additional clean-up steps are needed for the basic and neutral extract because of the appearance of background interference during chromatographic analysis. During back-extraction with 1.2 *M* hydrochloric acid, propranolol along with the neutral glycol metabolite are retained in the organic phase, while the hydroxyl metabolites are transferred into the mineral acid. The inability of dilute hydrochloric acid to back-extract propranolol from an organic solvent phase was observed also by Lefebvre et al. [26]. These investigators found that less than 0.1% of propranolol was removed from a chloroform–pentanol solution when it was extracted with 1 *M* hydrochloric acid.

Enzymatic hydrolysis of conjugate metabolites. The isomeric hydroxylated propranolols are present mainly in the form of glucuronide and sulfate conjugates in urine. Synthetic compounds of these conjugate metabolites are not available. At present, measurements of the conjugate metabolites must rely on enzymatic hydrolysis and indirect assay of the liberated aglycones. A preliminary study was carried out to explore the optimal conditions for hydrolysis. The final conditions

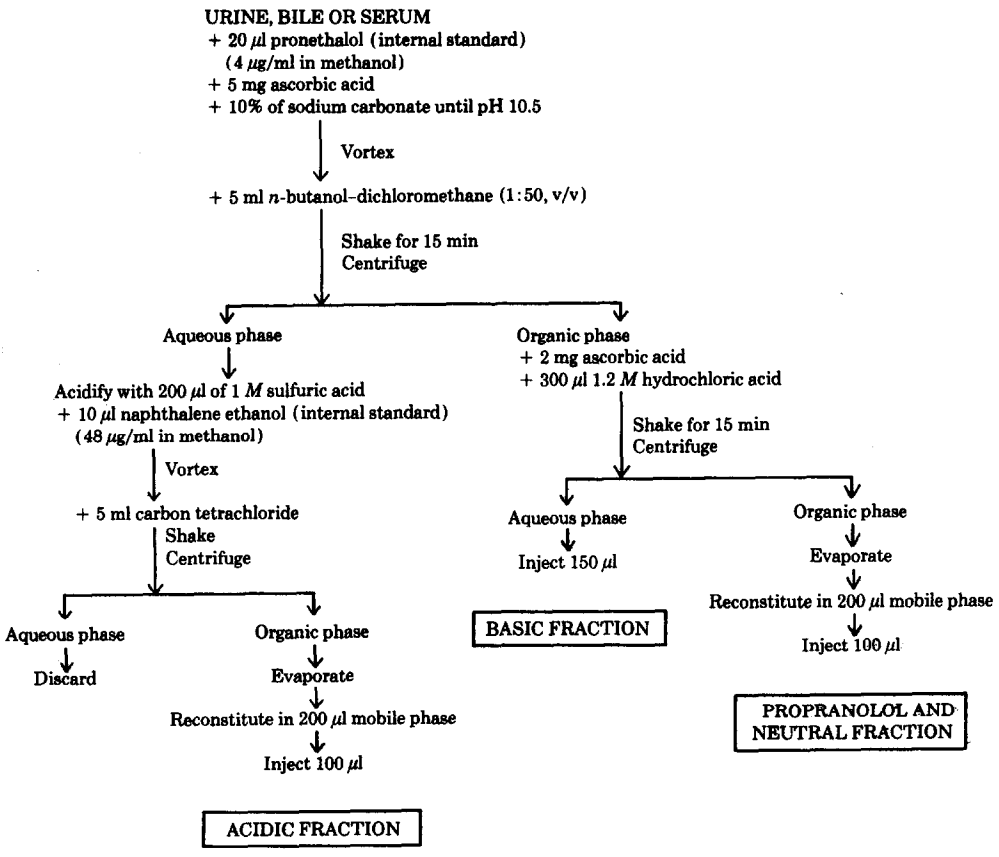

Fig. 3. Extraction scheme for hydrolyzed serum and all urine and bile samples.

adopted for the assay are as follows. An aliquot of bile (10 μl), urine (50 μl) or serum (0.5 ml) were placed in screw-capped tubes and to each were added 5 mg of ascorbic acid (as an anti-oxidant), 100 μl of 1 *M* sodium acetate buffer (pH 5.5) and 200 μl of water (except for serum samples). Then 10 mg of Sigma Type H-1 enzyme in lyophilyzed form, containing 350 U/mg β-glucuronidase (i.e., 3500 U) and 15 U/mg arylsulfatase (i.e., 150 U) activity, were added. After vortexing, the tubes were incubated for 4 h at 37°C in a water-bath. Following hydrolysis, the samples were subjected to extraction as described in Fig. 3.

Optimization of conditions for enzyme hydrolysis. The optimization study was carried out with a pooled 24-h urine collection from a male Sprague–Dawley rat (body weight 300 g) dosed orally with 6 mg/kg of *S*(−)-propranolol hydrochloride. The principal conjugate metabolites of propranolol in rat urine are the glucuronides of the regioisomeric monohydroxypropranolols. The hydrolysis procedure was optimized to achieve maximal yield of free 4-, 5- and 7-hydroxypropranolol. The source and amount of enzyme, sample volume, incubation temperature and incubation time were among the parameters examined for an efficient enzymatic hydrolysis of the conjugate species.

Four different preparations of β-glucuronidase–sulfatase from three commercial sources were evaluated: Sigma Type H-1 (partially purified powder) and Type H-2 (crude solution) mixed enzymes, Glusulase® (crude solution) and Calbiochem β-glucuronidase–arylsulfatase solution.

To determine the amount of enzymes required, the extent of release of the aglycone metabolites from a 50-μl aliquot of rat urine was measured after incubation with 5, 10, 20, 50 and 100 mg of enzyme, which corresponded to 1750–35 000 U of β-glucuronidase activity and 75–1500 U of arylsulfatase activity. During the early part of the investigation, incubations were carried out at 37°C for 90 min at pH 5.0, in accordance with the conditions recommended by Lo et al. [28].

The size of the urine aliquot, together with the substrate concentration (which depends on the administered dose of propranolol and the collection interval), also proved to be important considerations. Aliquots of urine, varying from 5 to 100 μl, were incubated with a fixed amount (10 mg) of Sigma Type H-1 β-glucuronidase–arylsulfatase. The extent of hydrolysis was compared between the different aliquot volumes of urine.

Lastly, the rate of hydrolysis was assessed at incubation temperatures of 37, 40 and 45°C. Incubation times of 2, 4, 6, 8 and 24 h were evaluated at 37°C.

Selective enzyme hydrolysis

The relative proportions of glucuronide and sulfate conjugates of the hydroxyl metabolites in rat urine were assessed by hydrolysis studies with purified enzymes and by the use of selective enzyme inhibitors.

Aliquots of rat urine (50 μl) were incubated separately with β-glucuronidase isolated from beef liver (4000 U) or purified bacterial arylsulfatase (3 U) at 37°C for 4 h. Similar incubation was carried out with the mixed Type H-1 enzymes in the presence of saccharo-1,4-lactone (2 mg), a selective inhibitor of β-glucuronidase.

Chromatographic conditions

The HPLC system consisted of a Waters Model 6000A pump for solvent delivery, a Model 710B Waters automatic injector and a 25-cm Beckman/Altex Ultrasphere reversed-phase C_{18} column (5 μm particle size) (Rainin Instrument, Woburn, MA, U.S.A.). A Schoeffel Model FS 970 fluorometer (Schoeffel Instruments, Westwood, NJ, U.S.A.) was used for detection. The excitation monochromator was set at 216 nm. A 280-nm cut-off filter was placed in the emission beam. The time constant was set at 6 s. The signal from the fluorometric detector was processed by a Waters Model 730 data module.

Optimum separation of propranolol and its metabolites was achieved with an isocratic mobile phase consisting of 0.044 *M* phosphoric acid (pH 2.7)–methanol–acetonitrile–tetrahydrofuran (67:20:1:12) to which 0.5 μM of the amine modifier, D-4 reagent, was added. The flow-rate was set at 1 ml/min and the column was heated to 40°C.

Assay performance

Extraction recovery of propranolol and its metabolites from serum and urine was assessed with spiked samples at four different levels over the relevant concentration ranges listed in Tables I–III. Recovery estimates were based on a comparison of the heights of the metabolite peaks obtained by an injection of extracts from spiked sample to the peak-height response of a known amount of the analytes injected directly on column (after taking volume and concentration variation into account). Minimum assay limit was defined as the sample concentration of an analyte that provides, upon injection of the extract, a detector response with a signal-to-noise ratio of at least 3:1. Reproducibility of the assay was assessed by replicate measurement ($n=4$) of samples spiked with the drug and its metabolites at mid-range concentrations of the standards. Linear calibration plots of propranolol and its metabolites were generated by least-squares regression of the

TABLE I

PERFORMANCE MEASURES FOR THE ASSAY OF PROPRANOLOL AND ITS FREE OXIDATIVE METABOLITES IN RAT SERUM ACCORDING TO EXTRACTION SCHEME IN FIG. 2

Analyte*	Minimum detection limit (ng/ml)	Concentration range (ng/ml)	Recovery (mean ± S.D., $n=4$) (%)	Coefficient of variation ($n=4$) (%)
4-OH-P	5.0	12.0–150	91.4 ± 6.3	3.6
5-OH-P	2.0	8.6–100	83.2 ± 4.2	2.0
7-OH-P	2.5	8.0–100	80.2 ± 3.4	2.6
DIP	2.5	11.5–144	91.1 ± 5.2	2.0
P	3.0	13.3–166	73.2 ± 6.2	3.5
NLA	4.5	15.5–194	91.8 ± 5.8	3.6
PGL	3.0	5.4– 68	60.2 ± 6.4	2.4

*5-OH-P = 5-hydroxypropranolol; 4-OH-P = 4-hydroxypropranolol; 7-OH-P = 7-hydroxypropranolol; DIP = N-desisopropylpropranolol; P = propranolol; NLA = naphthoxylactic acid; PGL = propranolol glycol.

TABLE II

PERFORMANCE MEASURES FOR THE ASSAY OF PROPRANOLOL AND ITS FREE OXIDATIVE METABOLITES IN UNHYDROLYZED RAT URINE ACCORDING TO EXTRACTION SCHEME IN FIG. 3

Analyte*	Minimum detection limit (ng/ml)	Concentration range (ng/ml)	Recovery (mean ± S.D., $n=4$) (%)	Coefficient of variation ($n=4$) (%)
4-OH-P	5.0	12.0–150	84.8 ± 5.1	3.3
5-OH-P	2.0	8.6–108	80.3 ± 3.4	3.4
7-OH-P	3.0	8.0–100	77.9 ± 2.9	2.4
DIP	3.0	11.5–144	88.3 ± 3.7	4.6
P	3.0	13.3–166	70.5 ± 5.0	6.0
NLA	4.5	15.5–194	90.2 ± 5.1	3.0
PGL	3.0	5.4- 68	60.4 ± 4.8	3.0

*For full chemical names to the abbreviations see footnote Table I.

TABLE III

PERFORMANCE MEASURES FOR THE ASSAY OF TOTAL (FREE AND CONJUGATE) PROPRANOLOL METABOLITES IN HYDROLYZED RAT URINE ACCORDING TO EXTRACTION SCHEME IN FIG. 3

Analyte*	Minimum detection limit (ng/ml)	Concentration range (ng/ml)	Recovery (mean ± S.D., $n=4$) (%)	Coefficient of variation ($n=4$) (%)
4-OH-P	7.0	12.0–150	80.3 ± 8.4	5.6
5-OH-P	3.0	8.6–100	74.9 ± 4.2	4.0
7-OH-P	3.0	8.0–100	79.8 ± 4.0	3.8
DIP	3.0	11.5–144	84.6 ± 3.8	1.8
P	4.5	13.3–166	70.2 ± 6.3	3.1
NLA	4.5	15.5–194	85.2 ± 6.5	5.3
PGL	3.0	5.4- 68	60.1 ± 5.1	2.1

*For full chemical names to the abbreviations, see footnote Table I.

peak-height ratios of the analyte to internal standard against the standard concentrations. The regression parameters were used to calculate the analyte concentration corresponding to the peak-height ratios obtained from the test samples.

RESULTS AND DISCUSSION

Chromatographic separation

Resolution of the propranolol metabolites on the C_{18} reversed-phase column was accomplished with a single isocratic mobile phase using a judicious combination of organic modifiers and a critical buffer composition and pH. The first attempt at optimization was to establish an effective mobile phase pH range, within which the stability of the bonded stationary phase is not sacrificed. Mobile phases with moderately acidic pH values of 5.5–7, such as those reported by Simon

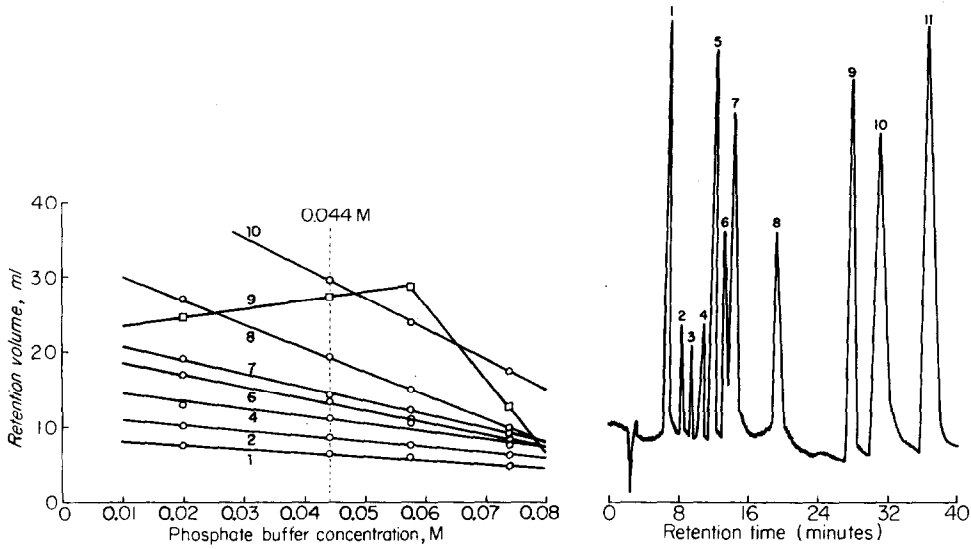

Fig. 4. Relationship between retention volume and mobile phase concentration of phosphate buffer for propranolol and eight of its metabolites. Key: 1=5-OH-P; 2=4-OH-P; 4=7-OH-P; 6=2-OH-P; 7=DIP; 8=propranolol; 9=PGL; and 10=NLA. See Fig. 1 for the full chemical names to the abbreviations.

Fig. 5. Representative chromatogram obtained with a standard mixture of propranolol and its metabolites. Peaks: 1=5-OH-P; 2=4-OH-P; 3=4-OH-NLA; 4=7-OH-P; 5=pronethalol (internal standard); 6=2-OH-P; 7=DIP; 8=propranolol; 9=PGL; 10=NLA; 11=naphthalene ethanol (internal standard). See Fig. 1 for the full chemical names to the abbreviations.

and Terry [38] and Nygard et al. [39], were evaluated. Unfortunately, these mobile phases resulted in excessive peak tailing for the basic metabolites. Lowering of pH led to an improvement in peak shape and increased sensitivity. A final pH of 2.7 was chosen, which provided an acceptable column lifetime (ca. six to eight months of constant use).

The use of a ternary solvent system was also evaluated. A proportion of 20:1:12 methanol–acetonitrile–tetrahydrofuran afforded the best resolution. Using this particular combination of organic co-solvents, the influence of phosphate buffer concentration on the retention volumes of the analytes was also evaluated. Except for the neutral metabolite, propranolol glycol, a decrease in retention volume with an increase in phosphate concentration was observed (Fig. 4). A concentration of 0.044 *M* was chosen, which provided adequate resolution and complete elution of the metabolites within an hour.

The basic metabolites presented peak-tailing problems. Peak symmetry was improved by the addition of amine modifiers such as triethylamine or *n*-butylamine. The alkylamines are known to reduce the peak tailing of basic drugs by suppressing ionic or adsorptive interaction of amine solutes with the uncapped silanol groups on the silica support [40]. *n*-Butylamine was selected because of its relatively low volatility and its stability in aqueous solution.

In general, elevating the temperature of the mobile phase above ambient pro-

motes solubilization of hydrophobic solutes in the mobile phase, leading to a shortened chromatographic retention [41]. A throughput time of ca. 30 min was attained at a column temperature of 40 ± 1 °C. At higher column temperatures, variation in 4-hydroxypropranolol peak height was observed, perhaps reflecting on-column degradation of this labile metabolite. A typical example of the separation of propranolol and its metabolites accomplished with the optimized separation conditions is illustrated in Fig. 5.

Sample preparation

Non-conjugate metabolites. A variety of extraction solvents had been used in previously reported assays for propranolol, viz. benzene, hexane, ethyl acetate, dichloromethane, ethyl ether and dichloromethane–alcohol combination. A mixture of 2% *n*-butanol in dichloromethane gave the best overall extraction yield for propranolol and its metabolites from serum. In the case of urine and other hydrolyzed specimens (see Fig. 3), carbon tetrachloride was chosen for the extraction step with the acidic fraction, as it yielded a cleaner extract than the *n*-butanol–dichloromethane co-solvent.

In most published methods the extraction of basic metabolites is performed at a pH close to the isoelectric point of 4-hydroxypropranolol (i.e. 9.6), possibly to avoid the increased risk of degradation of the 4-hydroxy metabolite at high pH. Our experience indicated that better recovery was attained at a higher pH of 10.5 without compromising the stability of the monohydroxylated metabolites. A similar finding has been reported by Simon and Babich-Armstrong [24].

As was mentioned, 4-hydroxypropranolol is known to undergo rapid oxidative degradation at alkaline pH [3,19,20,22,26,28,34]. The use of anti-oxidants to reduce degradative loss has been recommended. Amongst the anti-oxidants examined, ascorbic acid has proven to be the most accepted choice [28]. The advantages include the fact that ascorbic acid does not alter the initial pH of the biological fluid, thereby requiring less base to adjust the pH in the initial extraction step. Also, unlike the reducing sulfites, ascorbic acid does not interfere with arylsulfatase activity [3,42] in the hydrolysis of sulfo-conjugated metabolites. It should be noted that other positional isomers of monohydroxypropranolol do not exhibit the instability problems of the 4-hydroxyl compound.

All solutions of 4-hydroxypropranolol were spiked with ascorbic acid and stored at 4 °C between use. Biological samples were fortified with ascorbate at 200 mg/ml immediately upon collection, stored at −70 °C and analyzed within one month. Additional amounts of ascorbic acid were added to the samples at the start of the extraction procedure. Extracts were kept cool during handling and were processed for chromatography within 24 h.

Amongst the array of internal standards mentioned in the literature, pronethalol emerged as the most suitable candidate for the basic metabolite fraction. It had the desirable retention characteristics and was quantitatively extracted under alkaline conditions. However, because of the instability of pronethalol, working solutions of the internal standard need to be prepared daily. Naphthalene ethanol was chosen as the internal standard for the acidic extract mainly because it is eluted after naphthoxylactic acid, thus avoiding the numerous inter-

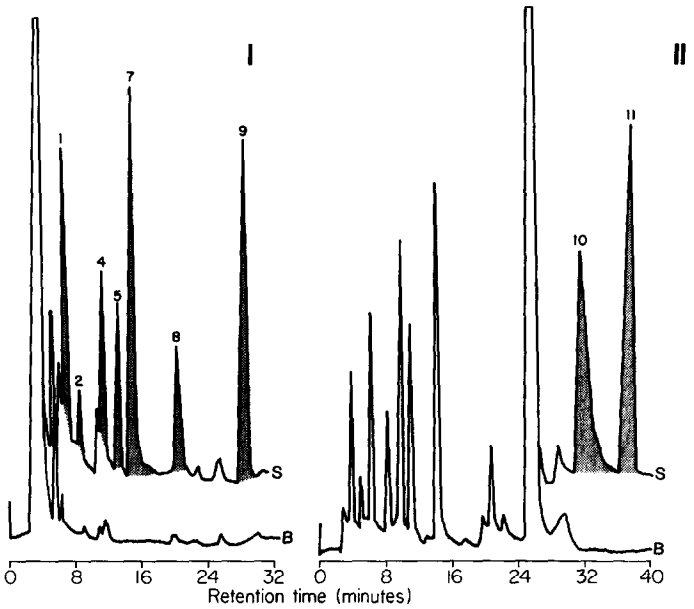

Fig. 6. Representative chromatograms of the basic and neutral (panel I) and acidic (panel II) extracts from blank (B) and spiked (S) rat serum prior to enzymatic hydrolysis. Peaks: 1=5-OH-P; 2=4-OH-P; 4=7-OH-P; 5=pronethalol (internal standard); 7=DIP; 8=propranolol; 9=PGL; 10=NLA; 11=naphthalene ethanol (internal standard). See Fig. 1 for the full chemical names to the abbreviations.

fering peaks that are present in the early part of the chromatogram.

Representative chromatograms of extracts obtained from serum and urine according to the procedures outlined in Figs. 2 and 3 are presented in Figs. 6 and 7. In all instances, the chromatograms were clear of interfering peaks in the areas of interest. Recovery data for the extraction procedures with unhydrolyzed serum and urine samples are summarized in Tables I–III. Except for propranolol glycol, the extraction yields were in the range 70–90% for all the analytes. More importantly, upon replicate extraction the absolute recovery is reproducible with a coefficient of variation between 4 and 10%.

Conjugate metabolites. Enzymatic hydrolysis of the conjugate metabolites of propranolol was evaluated under a wide variety of conditions. Incubation with the Sigma Type H-1 partially purified powder of β-glucuronidase–arylsulfatase provided the cleanest extract and was chosen for use. The other enzyme preparations contained large amounts of fluorescent contaminants, which interfered with the HPLC assay.

Fig. 8 shows that for a 50-μl aliquot of urine, the maximal yield of the aglycone monohydroxyl metabolites was observed when the added amount of enzyme was 10 mg or more (i.e., 3500 U of β-glucuronidase and 150 U of arylsulfatase activity). With larger aliquots of urine, more enzymes were required to effect complete hydrolysis, possibly owing to an increase in the amount of substrate or to the presence of endogenous constituents in urine that are capable of inhibiting the

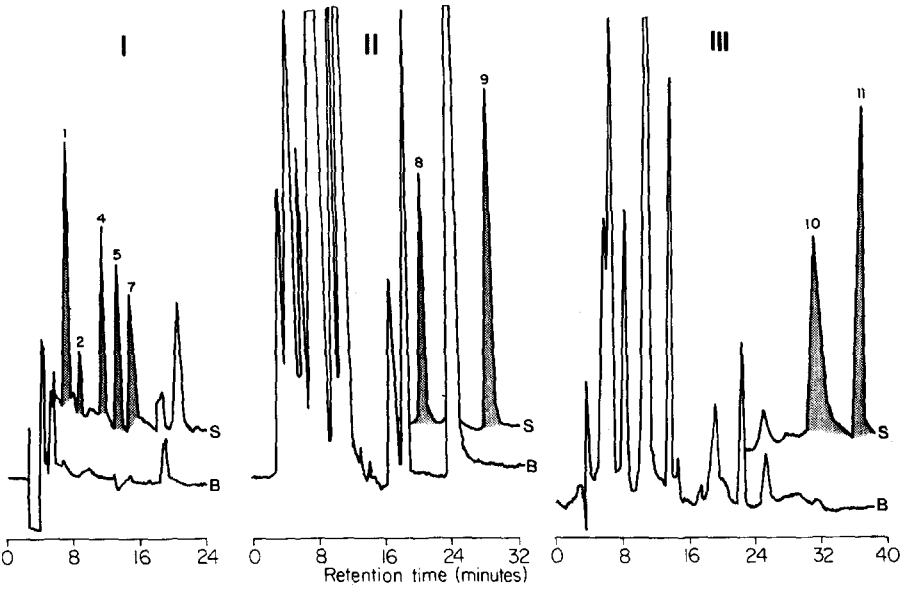

Fig. 7. Representative chromatograms of the basic (panel I), neutral (panel II) and acidic (panel III) extracts from blank (B) and spiked (S) rat urine prior to enzymatic hydrolysis. See legend to Fig. 6 for the key to the numbering of peaks. Note that pronethalol serves as internal standard for both the neutral and basic fractions, since its partition between dichloromethane and 1.2 *M* hydrochloric acid is reproducible.

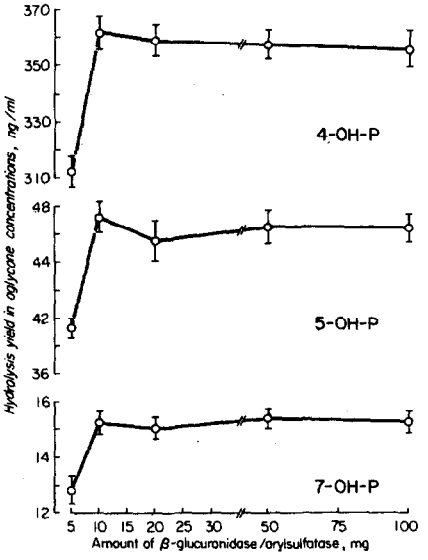

Fig. 8. Extent of hydrolysis of conjugated monohydroxypropranolols (4-, 5- and 7-hydroxypropranolol) in a 50-μl aliquot of rat urine as a function of the amount of β-glucuronidase-arylsulfatase enzymes added to the incubation mixture. The Sigma Type H-1 enzyme powder contains 350 U/mg β-glucuronidase and 15 U/mg arylsulfatase.

TABLE IV

EXTENT OF ENZYMATIC HYDROLYSIS OF THE CONJUGATED MONOHYDROXYLATED METABOLITES OF PROPRANOLOL IN RAT URINE AS A FUNCTION OF INCUBATION TIME

Aglycone metabolite	Pre-hydrolysis concentration (ng/ml)	Post-hydrolysis concentration* (ng/ml)				
		2 h	4 h	6 h	8 h	24 h
4-Hydroxypropranolol	26.0±0.3	362 ±2	384 ±2	339 ±2	329 ±2	271 ±2
5-Hydroxypropranolol	10.3±0.6	39.2±0.9	46.1±0.8	45.2±1.1	44.9±0.8	42.6±0.8
7-Hydroxypropranolol	2.5±0.2	12.9±0.5	16.3±0.8	16.4±0.9	15.9±0.8	16.1±0.8

*Free and conjugated metabolite.

β-glucuronidase–arylsulfatase enzymes. Hence the amount of enzyme used for a given aliquot of sample may differ, depending on the source and nature of the biological fluids. The recommended amount of enzyme in the present study is more than sufficient for complete hydrolysis of conjugate metabolites in samples collected from animal studies involving low and moderately high dosages of propranolol (less than 10 mg/kg).

In order to shorten the hydrolysis time, incubation was carried out at elevated temperatures. In the presence of excess ascorbic acid, 4-hydroxypropranolol appears to be stable at temperatures below 40°C. Above 40°C, a significant decrease in the yield of free 4-hydroxypropranolol was noted, probably as a result of accelerated degradation. Hence the incubation temperature was set at 37°C. When the incubation time was varied, maximal yield of the aglycone metabolite was attained within 4 h (see Table IV). A notable decrease in the recovery of 4-hydroxypropranolol was observed at incubation times longer than 8 h.

Both sulfate and glucuronide conjugates of propranolol and its hydroxylated

TABLE V

COMPARISON OF AGLYCONE YIELDS FROM VARIOUS HYDROLYTIC TREATMENTS OF RAT URINE CONTAINING CONJUGATES OF THE MONOHYDROXYLATED METABOLITES OF PROPRANOLOL

Treatment	Total concentration (ng/ml)		
	4-Hydroxy-propranolol	5-Hydroxy-propranolol	7-Hydroxy-propranolol
Pre-hydrolysis	26.0±0.6	10.3±0.6	2.5±0.2
Type H-1 β-glucuronidase–arylsulfatase	380 ±4	44.8±0.8	16.5±0.5
Type H-1 enzymes plus saccharo-1,4-lactone	23.4±0.2	13.1±0.1	2.8±0.5
Beef liver glucuronidase	385 ±2	44.1±0.3	15.5±0.4
Purified bacterial arylsulfatase	24.3±0.8	12.6±0.4	2.5±0.7

metabolites have been reported to be formed in humans and in dogs [1,43]. The exact nature of the conjugate metabolites of propranolol has not been investigated in the rat. Table V compares the amounts of aglycone metabolites released after incubation with mixed enzyme preparation in the presence of a selective β-glucuronidase inhibitor and after hydrolysis with either purified β-glucuronidase or arylsulfatase enzyme. Hydrolysis was negligible in the presence of a specific β-glucuronidase inhibitor, saccharo-1,4-lactone, or upon incubation with purified arylsulfatase alone, indicating the absence of sulfate conjugates in rat urine. Furthermore, incubation with pure beef liver β-glucuronidase preparation (Glucurase) yielded the same amount of aglycone as the Type H-1 mixed enzyme preparation.

For all post-hydrolysis samples, a back-extraction step was included in the treatment of the basic and neutral fraction (Fig. 3). This is necessary to eliminate chromatographic interference from endogenous constituents. The mean extraction recovery for each of the spiked propranolol metabolites (in free form) from urine subjected to the hydrolysis procedure are presented in Table III.

Assay performance

Data on the sensitivity limit and precision of the assay are included in Tables I–III. By using a low UV excitation wavelength in the fluorescence detection, as recommended by Krol et al. [43], quantitation of low levels of propranolol metabolites in small volumes of blood/serum samples (ca. 1–5 ng/ml in 0.5–1.0 ml) became feasible. This assay should be applicable to studies of propranolol metabolite kinetics in blood. The only existing assays with comparable sensitivity are those involving GC–MS.

The within-assay coefficients of variation are consistently less than 6% for each of the metabolites. Our experience to date indicates that the same degree of reproducibility can be achieved between assay runs, when repeated measurements on actual samples are made.

CONCLUSION

The present assay procedure offers several distinct advantages. It allows simultaneous determination of all six of the major propranolol metabolites in a single sample, thereby avoiding the problems of multiple assays. The use of an isocratic mobile phase allows easy automation of the chromatographic procedure. Finally, it is a cost-effective alternative to the existing GC–MS assays.

ACKNOWLEDGEMENT

This work was supported by a United States Public Health Service Grant HL-33389 from the National Heart, Lung and Blood Institute of the National Institutes of Health.

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