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Direct high-performance liquid chromatographic determination in urine of the enantiomers of propranolol and its major basic metabolite 4-hydroxypropranolol

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ABSTRACT

A method is described for quantitation of underivatized enantiomers of propranolol and its major basic metabolite, 4-hydroxypropranolol, in urine samples by high-performance liquid chromatography with fluorescence detection, using a cellulose tris(3,5-dimethylphenylcarbamate) chiral stationary phase. This method was found to be precise and accurate for the measurement of propranolol and 4-hydroxypropranolol concentrations in urine from pharmacokinetic investigations. This method represents the first assay for direct determination of 4-hydroxypropranolol enantiomers. The ability to easily measure 4-hydroxypropranolol enantiomers is valuable because the stereoselective disposition of propranolol is primarily due to stereoselective metabolism in the pathway responsible for generation of 4-hydroxypropranolol.

INTRODUCTION

Propranolol is a β-adrenergic receptor blocker which is widely used in the treatment of hypertension, angina pectoris, arrhythmias, post-myocardial infarction patients, migraine and other conditions [1]. It is administered as a racemic mixture, and the β-blocking potency of (−)-propranolol is approximately 100 times greater than that of (+)-propranolol. Metabolism and plasma protein binding of propranolol are stereoselective, and (−)-propranolol concentrations are typically higher than (+)-propranolol concentrations by 20–50% following oral administration [2,3].

Propranolol is eliminated almost exclusively by hepatic metabolism to produce at least fourteen metabolites, and the three primary metabolic pathways of propranolol are ring oxidation, side-chain oxidation and glucuronidation (see Fig. 1) [4]. Propranolol glucuronide (PG) is the only metabolite of initial glucuronidation, naphthoxylactic acid (NLA) is the primary metabolite of side-chain oxidation and 4-hydroxypropranolol (HOP) is the primary metabolite of ring oxidation. HOP is further conjugated to sulfate (HOP-S) and glucuronide (HOP-G) conjugates. Walle *et al.* [4] have shown that these three metabolites (HOP-S/HOP-G, PG, NLA) account for about 70% of the metabolites produced and that NLA and HOP are excellent markers for their respective pathways. Thus it is possible to assess clearance through the three primary metabolic pathways of propranolol without quantitating all of its metabolites [4]. This approach has been employed in numerous propranolol pharmacokinetic studies to assess the metabolic path-

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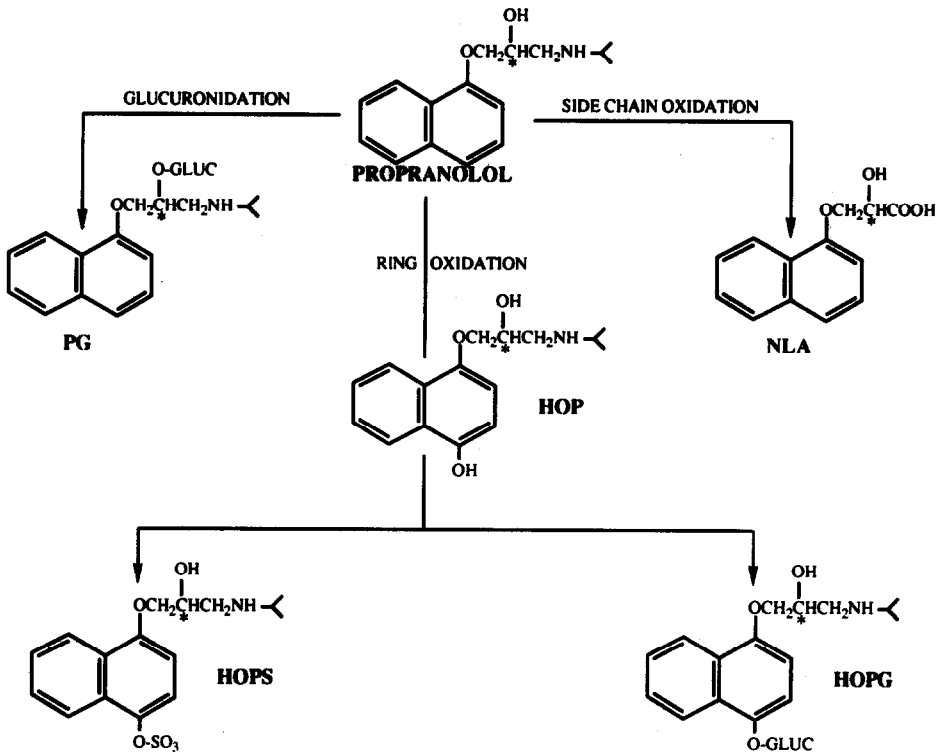

Fig. 1. Schematic representation of metabolism of propranolol to propranolol glucuronide (PG), naphthoxylactic acid (NLA) and 4-hydroxypropranolol (HOP). HOP is further conjugated to glucuronide (HOP/G) and sulfate (HOP/S) conjugates.

ways responsible for propranolol clearance in various populations [5,6].

Urinary concentrations of propranolol metabolites have typically been quantitated using achiral HPLC assays, GC–MS, or by derivatization of the metabolite [3,5,6]. To date, there are no assays available which allow direct determination of the enantiomers of any of the major metabolites of propranolol. In this paper, we describe an assay for the direct determination of the enantiomers of propranolol and its major basic metabolite, HOP, in urine. Because essentially no propranolol is excreted unchanged in urine [4], the propranolol measured in this urinary assay represents PG from which the glucuronide has been cleaved. The propranolol and HOP enantiomer data derived from urine samples using this assay can therefore be used to assess partial metabolic clearances via initial glucuronidation and ring oxidation, respectively. The ability to quantitate HOP enantiomers with a simple assay is particularly valuable as it has been shown that the stereoselective disposition of propranolol is primarily due to stereoselective metabolism in the ring oxidation pathway [6].

EXPERIMENTAL

Materials

Racemic propranolol hydrochloride, (+)-propranolol hydrochloride and (−)-propranolol hydrochloride were provided by Ayerst Labs. (New York, NY, USA). Racemic HOP was kindly provided by John Pieper (University of Colorado, Denver, CO, USA). The internal standard, racemic verapamil hydrochloride, was provided by Knoll Pharmaceuticals (Whippany, NJ, USA). Hexane in the mobile phase (Mallinckrodt, St. Louis, MO, USA) was HPLC grade. Ethanol in the mobile phase (Florida Distillers, Lake Alfred,

FL, USA) was U.S.P. grade. N,N-Diethylamine, sodium acetate and sodium carbonate from Fisher Scientific (Fairlawn, NJ, USA) were reagent grade. Ethyl acetate (Mallinckrodt) and *l*-ascorbic acid (Sigma, St. Louis, MO, USA) were also reagent grade. The β-glucuronidase–arylsulfatase mixture (G 0876) was purchased from Sigma.

Apparatus

The chromatographic system was composed of a constant-flow reciprocating pump (Constametric III, Laboratory Data Control, Riviera Beach, FL, USA) and fluorescence detector (Spectraflow 980, Kratos Analytical, Ramsey, NJ, USA). The guard and analytical (250 mm × 4.6 mm I.D.) columns were packed with cellulose adsorbed onto 10-μm macroporous silica (Chiralcel OD, Daicel Chemical Industries, Los Angeles, CA, USA). The analytical column was heated using a Bioanalytical Systems LC-22A column heater (West Lafayette, IN, USA). Samples were injected on column using a Rheodyne (Cotati, CA, USA) Model 7125 injection valve fitted with a 100-μl loop. The detector response was quantitated using a C-R5A Chromatopac integrator (Shimadzu Scientific Instruments, Columbia, MD, USA).

Chromatographic conditions

The mobile phase consisted of hexane–ethanol–diethylamine (91:9:0.1, v/v) pumped at 1.0 ml/min. The guard column was at room temperature, while the analytical column was kept at 37°C. The excitation wavelength was 240 nm, and a 320-nm high-pass filter was used as a fluorescence emission cut-off filter.

Extraction procedure

Samples were extracted by placing 250 μl of urine in a screw-cap test tube with 15 μg of racemic verapamil (15 μl of a 1 μg/μl methanol solution). To this 100 μl of 1 *M* sodium acetate buffer (pH 4.8) containing 20 mg/ml *l*-ascorbic acid (to prevent oxidation of HOP) were added. Enzymes were also diluted in sodium acetate buffer (pH 4.8) at concentrations of 10 000 U of β-glucuronidase and 370 U of sulfatase per milliliter of enzyme solution. Samples were vortex-mixed after the addition of 100 μl of enzyme solution to the sample. As described by Harrison *et al.* [7], samples were incubated at 37°C for 1 h to allow cleavage of glucuronide and sulfate conjugates. The samples were then neutralized with 500 μl of 1 *M* sodium carbonate (pH 10) and vortex-mixed. Ethyl acetate (5 ml) was then added after which the samples were shaken for 10 min and centrifuged at 2000 *g* for 10 min. The organic layer was transferred to a clean culture tube and evaporated under nitrogen. Each sample was reconstituted with 250 μl of mobile phase and vortex-mixed for 15 s. A 50-μl aliquot of the sample was then injected on column. This extraction procedure was an adaptation of that published by Koshakji and Wood [8].

Extraction efficiency was determined at three concentrations (1, 6 and 12.5 μg base per ml of each enantiomer). For each concentration tested, five screw-cap test tubes and five culture tubes were spiked with the correct amounts of propranolol, HOP and verapamil solutions. The solutions were then evaporated to dryness under nitrogen. Blank urine (250 μl) was then added to the screw-cap test tubes, and these tubes were then subjected to the extraction procedure. All samples were reconstituted with 250 μl of mobile phase, and 50 μl were injected. Extraction efficiencies were calculated by comparing the mean peak areas of the unextracted samples with the mean peak areas of the extracted samples.

Calibration and reproducibility

Standards were constructed by spiking blank urine stocks to obtain concentrations of 2, 5, 12, 18 and 25 μg base per ml (7.71, 19.28, 46.27, 69.41 and 96.40 μmol base per l, respectively) for racemic propranolol and (7.51, 18.77, 45.05, 67.57 and 93.85 μmol base per l, respectively) for racemic HOP. Standard curves were constructed by analysis of extracted urine standards. The equation describing the standard curve was determined by weighted linear least-squares regression analysis. Each standard peak-area ratio was weighted to $1/\text{concentration}^2$. Goodness-of-fit was estimated by the coefficient of determination (r^2), calculated as the regression sum of squares/total sum of squares.

Within-day variability and precision were measured using control urine samples at three concentrations (4, 15 and 20 μg base per ml for racemic propranolol and HOP) with injection on the same day of five extracted samples of each control urine concentration. Between-day variability and precision were evaluated by injection of extracted samples of the three control concentrations for eight days.

Identity of each enantiomer of propranolol was verified by direct injection of single enantiomer standards. HOP enantiomer standards were not available. To determine the elution order of the HOP enantiomers, one investigator (J.A.J.) took a dose of pure (+)-propranolol and collected urine. An extracted urine sample produced only one HOP peak and it was assumed that this peak represented (+)-HOP.

Subject samples

As part of a propranolol enantiomer pharmacokinetic study, healthy male subjects took propranolol 80 mg every 8 h for a total of sixteen doses. Blood samples were collected at various times for 12 h after the final dose. Serum samples were analyzed for propranolol enantiomers by a chiral HPLC assay similar to the urine assay described here. The results of this study have been published [2]. During this study, some of the subjects also collected urine over the same 12-h period that blood samples were collected. The purpose of urine collection was to have subject urine samples for development of the current assay; urinary data were not utilized in the study. Urine was collected into containers to which 2 g of ascorbic acid had been added to prevent oxidation of the hydroxylated metabolites. Urine volume was measured and recorded and an aliquot of urine was stored at −20°C until analysis. Partial metabolic clearance values were calculated as the amount of drug excreted in urine in 12 h (Ae_{0-12}) divided by the area under the plasma concentration–time curve from 0 to 12 h (AUC_{0-12}).

RESULTS

The hexane–ethanol–diethylamine mobile phase allowed the enantiomers of propranolol to be well resolved from each other (mean R_s = 3.53), while also resolving the enantiomers of HOP (mean R_s = 3.76) within a run time of about 35 min. Mean capacity factors for verapamil, (+)-propranolol, (−)-propranolol, (+)-HOP, and (−)-HOP were 2.03, 2.64, 3.53, 9.74 and 7.36, respectively. The representative chromatograms shown in Fig. 2 demonstrate that

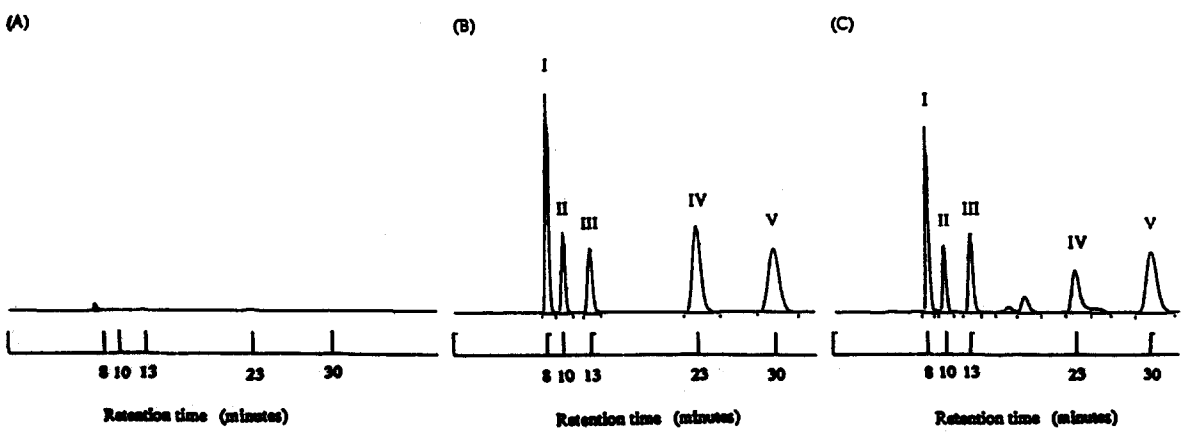

Fig. 2. Representative chromatograms of (A) extracted blank urine, (B) extracted urine spiked with 12 μg/ml base of racemic propranolol and 4-hydroxypropranolol (46.27 and 45.05 μmol base per l, respectively) and (C) extracted subject sample. Concentrations in the subject sample were: (+)-propranolol 5.83 μg/ml, (−)-propranolol 8.81 μg/ml, (+)-4-hydroxypropranolol 6.72 μg/ml and (−)-4-hydroxypropranolol 3.87 μg/ml (22.48, 33.97, 25.23 and 14.53 μmol/l, respectively). Chromatographic peaks: I = racemic verapamil (internal standard); II = (+)-propranolol; III = (−)-propranolol; IV = (−)-4-hydroxypropranolol; V = (+)-4-hydroxypropranolol.

TABLE I

PRECISION AND ACCURACY IN THE DETERMINATION OF PROPRANOLOL ENANTIOMERS FROM SPIKED URINE SAMPLES

Spiked concentration[a] (μmol/l)	(+)-Propranolol		(−)-Propranolol	
	Observed concentration[b] (μmol/l)	C.V. (%)	Observed concentration (μmol/l)	C.V. (%)
Within-day variation (n=5)				
38.56	37.75	1.74	37.06	1.78
28.92	28.88	4.10	28.69	4.40
7.71	7.10	4.55	7.13	2.79
Between-day variation (n=8)				
38.56	39.02	6.49	38.71	6.51
28.92	29.11	8.71	28.84	8.07
7.71	7.48	5.72	7.48	9.14

[a] All concentrations reported in terms of base.

[b] Reported observed concentrations represent the mean values from all experiments.

there were no endogenous interferences to any of the peaks of interest under the detection conditions chosen. It is interesting to note that the elution order of the enantiomers of HOP (− before +) was opposite that for propranolol enantiomers (+ before −).

Standard curves were linear over the concentration range used, with coefficients of determination greater than 0.99 for the propranolol enantiomers and greater than 0.98 for the HOP enantiomers. Tables I and II summarize the results of within- and between-day measurements of accuracy and precision for the enantiomers of both compounds. The extraction efficiency over the range 1–12.5 μg base per ml was 77.7% (C.V. = 6.6%) for both enantiomers of propranolol and 66.2 and 66.9% (C.V. = 8.5 and 7.2%) for (+)- and (−)-HOP, respectively. The extraction efficiency for the internal standard, verapamil, was 71.9% (C.V. = 7.5%). The limit of detection was selected as a signal-to-noise ratio of 4:1, and was determined to be 0.1 μg base per ml for each enantiomer of propranolol and 0.2 μg base per ml for each enantiomer of HOP.

Partial metabolic clearances were determined in a single subject (chromatogram shown in Fig. 2). Partial metabolic clearance via initial glucuronidation to form (+)- and (−)-PG (as assessed by urinary propranolol concentrations) in this subject were 398 and 434 ml/min, respectively.

TABLE II

PRECISION AND ACCURACY IN THE DETERMINATION OF 4-HYDROXYPROPRANOLOL ENANTIOMERS FROM SPIKED URINE SAMPLES

Spiked concentration[a] (μmol/l)	(+)-4-Hydroxypropranolol		(−)-4-Hydroxypropranolol	
	Observed concentration[b] (μmol/l)	C.V. (%)	Observed concentration (μmol/l)	C.V. (%)
Within-day variation (n=5)				
37.54	35.17	5.29	36.04	6.00
28.16	27.48	3.10	27.49	4.34
7.51	6.46	5.56	6.53	7.54
Between-day variation (n=8)				
37.54	36.56	11.23	36.83	10.81
28.16	27.03	7.84	27.33	7.68
7.51	6.98	6.25	7.17	5.09

[a] All concentrations in terms of base.

[b] Reported observed concentrations represent the mean values from all experiments.

Partial metabolic clearance values via ring oxidation to form (+)- and (−)-HOP were 434 and 179 ml/min, respectively.

DISCUSSION

The enantiomeric (+/−) ratios for clearances via two of the metabolic pathways of propranolol (glucuronidation and ring oxidation) were essentially identical to those reported by Ward *et al.* [6], validating that our method is useful for analyzing subject samples. The clearance values derived from PG were slightly higher in this subject than the average values reported by Ward *et al.* [6], while values derived from HOP data were substantially lower. Inter-subject variability is likely responsible for the small differences in PG data. The more substantial differences in HOP are likely the result of oxidation of this metabolite. This subject's urine sample had been stored for over eight months before analysis. HOP is known to be relatively unstable in physiologic fluids, and most studies have evaluated stability of HOP in the presence of ascorbic acid for no more than four weeks [8]. Our data and those of others suggest that for absolute values to be accurate, urine samples containing HOP should be analyzed as quickly as possible after sample collection.

The method we have described to quantitate propranolol enantiomers significantly speeds the analysis of propranolol enantiomer concentrations over previous methods which employ this column [9]. Methods in which mobile phases containing isopropanol are used elute the enantiomers as wide peaks with large separation factors [9] and run times of about 25 min for propranolol enantiomers alone. Ethanol has been found to produce narrower peaks and smaller separation factors than isopropanol on columns of this type [10]. Using the chromatographic conditions described, propranolol enantiomers are eluted in only 15 min. Use of these conditions for plasma samples would therefore yield significantly shorter run times than in currently published assays. The described method also allows the quantitation of the enantiomers of the major basic metabolite of propranolol, HOP. The faster elution of propranolol enantiomeric pairs made possible the elution of HOP enantiomers within a reasonable run time of about 35 min. Further shortening of retention times was limited by the need to resolve an endogenous interfering material in subject samples from the tailing edge of the (−)-HOP peak.

The greatest sensitivity for propranolol with the chosen detector was achieved at an excitation wavelength of 220 nm, however, HOP produced greater fluorescence when excited at 240 nm. At 220 nm the lowest standard HOP peaks were difficult to detect, while at 240 nm these peaks were easily detected. The peak area of the previously mentioned endogenous interfering material was also minimized with detection at 240 nm.

The standard curve range used was chosen to cover the wide range of propranolol and HOP concentrations expected when conducting pharmacokinetic studies. Due mainly to fluorescence detector noise characteristics, peak-area ratio variability increased as the concentration of the standard increased. This trend affected the results of the unweighted linear least-squares regression when used to evaluate the curve, resulting in high relative errors for low concentration samples. A weighted linear regression model (weighting = $1/\text{concentration}^2$) was found to reduce these errors and produce the best fit to the data.

In conclusion, the assay described produces acceptable results for the determination of urinary concentrations of propranolol and its major basic metabolite, HOP. The assay employs a simple liquid–liquid extraction and does not require derivatization to resolve the enantiomers of the two compounds. The ability to easily measure HOP enantiomers is valuable because the stereoselective disposition of propranolol is primarily due to stereoselective metabolism in the pathway responsible for generation of HOP.

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