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Simultaneous determination of propranolol and 4-hydroxypropranolol enantiomers after chiral derivatization using reversed-phase high-performance liquid chromatography

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SUMMARY

A reversed-phase high-performance liquid chromatographic method is described, which allows the simultaneous quantification of propranolol and 4-hydroxypropranolol enantiomers in human plasma. After extraction from plasma (pH 10.5) using ethyl acetate, the enantiomers are derivatized with *R*-(+)-phenylethylisocyanate as chiral derivatization reagent and triethylamine as basic catalyst in chloroform. Ascorbic acid is used to prevent 4-hydroxypropranolol from oxidation during the extraction. Chromatographic separation on ODS columns and fluorescence detection (228 nm/>340 nm) allows sensitive quantitation of all derivatives. Incubation of the plasma samples with β-glucuronidase/arylsulfatase and the use of the specific β-glucuronidase inhibitor saccharo-1,4-lactone allows the quantitation of both the sulfate and glucuronide conjugates of the enantiomers. The method was applied to human plasma samples from a subject after administration of 60 mg racemic propranolol three times daily.

INTRODUCTION

Propranolol is a non-selective β-adrenergic blocking agent used in the treatment of hypertension, cardiac arrhythmias, angina pectoris and prophylaxis of secondary acute myocardial infarction [1]. Commercial propranolol (Inderal®) is a racemic mixture, with the (−)-enantiomer being about 100 times more potent than its optical antipode. Pharmacokinetics and metabo-

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lism of propranolol are associated with large differences between the enantiomers in both animals [2,3] and man [4,5]. Previously described methods for separation of propranolol enantiomers including radioimmunoassay [6], radioreceptor assays [7] and gas chromatography–mass spectrometry using deuterium-labeled pseudoracemates [3] have some disadvantages. These methods either are very time-consuming and expensive, or lack specificity.

High-performance liquid chromatographic (HPLC) assays using chiral stationary phases have recently been introduced [8,9], but to date HPLC assays using optically active derivatization reagents are the most common procedure in clinical studies [10–14]. *R*-(+)-Phenylethylisocyanate (PEIC) is frequently used as derivatization reagent. It is relatively stable and shows no racemisation during storage [13]. Separation of the derivatized propranolol enantiomers can be achieved with reversed-phase chromatography [12,13]. Furthermore, *R*-(+)-PEIC can be applied to the separation of the enantiomers of 4-hydroxypropranolol [14], a propranolol metabolite (Fig. 1) with qualitatively similar biological activity to propranolol [15].

There are two possible ways the derivatization reagent can react: (1) only the secondary amino group will be derivatized forming the mono-PEIC derivative (urea derivative) or (2) both the secondary amino group and the phenolic hydroxy group are derivatized forming di-PEIC derivatives (urea and carbamate derivatives). Reaction of the chiral secondary hydroxy group has not been observed for either propranolol, 4-hydroxypropranolol [13] or other β-blockers like pindolol [16]. Wilson and Walle [14] reported that without any basic catalyst 4-hydroxypropranolol forms a mixture of both derivatives. After derivatization the carbamate end of the di-PEIC derivative can be selectively hydrolyzed using 0.1 *M* hydrochloric acid [14]. The resulting mono-PEIC derivatives were separated using silica gel HPLC, whereas it was not possible to separate the derivatized enantiomers with reversed-phase chromatography [14].

In our investigations we are using triethylamine as basic modifier during the derivatization reaction. This results in the exclusive formation of the di-PEIC derivative. Both the enantiomers of the di-PEIC derivative of 4-hydroxypropranolol and the enantiomers of the propranolol derivatives were determined simultaneously using reversed-phase HPLC.

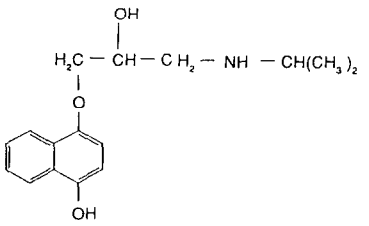

Fig. 1. Structure of 4-hydroxypropranolol.

The method was applied to determine the stereochemical composition of propranolol and 4-hydroxypropranolol and their glucuronide and sulfate conjugates in human plasma and urine after oral administration of racemic propranolol.

EXPERIMENTAL

Reagents

R-(+)-PEIC (purity > 98%) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and (+)-and (−)-propranolol hydrochloride, triethylamine, β-glucuronidase (with sulfatase activity, partial purified, G-0751), saccharo-1,4-lactone from Sigma (St. Louis, MO, U.S.A.). Chloroform and methanol (HPLC grade) were obtained from Fischer Scientific (Fair Lawn, NJ, U.S.A.) and used without further purification.

Instrumentation

The HPLC system consisted of a Constametric III G high-pressure pump (LDC/Milton Roy, Riviera Beach, FL, U.S.A.), an ISS 100 automatic injector (Perkin Elmer, Norwalk, CT, U.S.A.), a Kratos FS 970 fluorescence detector (Kratos Analytical Instruments, Ramsey, NJ, U.S.A.) and a Whatman Partisil 5 ODS-3 column (12.5 cm × 4.6 mm, 5 μm particle size, Fisher Scientific). The excitation wavelength was set at 228 nm and a 340-nm cut-off filter was used for emission. The mobile phase consisted of methanol–water (60.5:39.5, v/v) and the flow-rate was 1.2 ml/min.

Plasma extraction

To 0.5 ml of plasma 1.0 ml of phosphate buffer (0.2 *M*; pH 10.5), 5 mg of ascorbic acid and 4.0 ml of ethyl acetate were added. The mixture was then shaken vigorously for 10 min and centrifuged at 1500 *g* for 15 min. The organic phase (3 ml) was removed and evaporated at 37°C under nitrogen.

Chiral derivatization

The residue after extraction and evaporation was dissolved in 0.5 ml of chloroform; 30 μl of triethylamine and 2 μl of *R*-(+)-PEIC were added and the tubes were shaken and left for 30 min at room temperature, tightly capped. The samples were then evaporated to dryness at room temperature under nitrogen and the residue was reconstituted in 300 μl of mobile phase. A 50-μl aliquot was injected into the HPLC system. After enzymatic cleavage of the glucuronidase and sulfate conjugates only 30 μl were injected into the HPLC system.

Analysis of conjugates of propranolol and 4-hydroxypropranolol

To 0.5 ml of plasma 5 mg of ascorbic acid and 100 μl of sodium acetate buffer (1 *M*, pH 5.5) were added. The mixture was shaken and treated with 100 μl of β-glucuronidase/sulfatase (80 mg/ml) for 4 h at 37°C. After extraction (pH 10.5) with ethyl acetate, the extract was derivatized with *R*-(+)-PEIC as described above. For the exclusive determination of the sulfate conjugate saccharo-1,4-lactone (2 mg), a specific β-glucuronidase inhibitor, was added before the treatment with β-glucuronidase/sulfatase.

Assay validation

Calibration curves were obtained after analyzing plasma standards, which were prepared by adding racemic propranolol hydrochloride (2.5–200 ng, calculated as base) and racemic 4-hydroxypropranolol (10–300 ng) to 0.5 ml of drug-free plasma. Six concentrations were used for the calibration curve. Absolute peak heights were measured and plotted against the concentration of each enantiomer. The slopes and intercepts were determined using linear-least squares regression. Reproducibility measurements (intra- and inter-day variability) were based upon a minimum of five replicates on three different concentrations.

Application

One healthy, male subject received 60 mg racemic propranolol (Inderal®) three times daily for three days. On the fourth day only one dose was administered and 10-ml blood samples were drawn before dosing and 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h after administration. Blood was allowed to clot for 20 min. After centrifugation the serum was removed, 40 mg of ascorbic acid were added to the serum and the samples were frozen (−70°C) until assayed.

RESULTS AND DISCUSSION

The chiral derivatization of propranolol and 4-hydroxypropranolol results in the exclusive formation of two sets of equally sized peaks (Fig. 2A). The first set of peaks, at retention times of 27 and 31 min, corresponds to the diastereomeric derivatives of propranolol. The second set of peaks, at retention times of 37 and 42 min, results from the derivatization of 4-hydroxy propranolol. The two peaks were assumed to be the derivatives after reaction at both the amino and the phenolic groups (di-PEIC derivatives), because the mono-PEIC derivatives, which are more polar than the di-PEIC and the propranolol derivatives, have shown shorter retention times in comparison to the propranolol derivatives, whereas the di-PEIC derivatives have higher retention times using reversed-phase HPLC [14]. Chiral derivatization in chloroform without any basic catalyst gave a mixture of the mono- and di-PEIC derivatives of 4-hydroxypropranolol [14]. The reaction conditions in the present study (chlo-

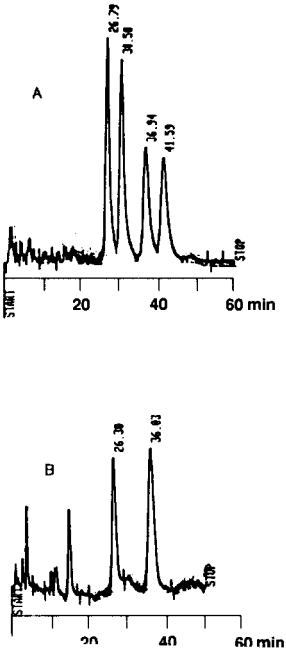

Fig. 2. (A) Chromatogram of a plasma samples spiked with (±)-propranolol and (±)-4-hydroxypropranolol. (B) Chromatogram of a dog urine sample after oral administration of *S*-(−)-propranolol and enzymatic cleavage of the glucuronic and sulfate conjugates.

roform, triethylamine, 40 min at room temperature) result in the quantitative formation of the di-PEIC derivatives. The peak symmetry as well as the resolution (R_s) of the propranolol and the 4-hydroxypropranolol derivatives is good ($R_s = 1.35$ and 1.7, respectively).

Identification of the individual diastereomers derived from 4-hydroxypropranolol was possible after oral administration of 60 mg *S*-(−)-propranolol to one dog. Urine was collected for 4 h. After enzymatic cleavage of the conjugates, extraction and derivatization, the chromatogram shown in Fig. 2B was obtained. For both propranolol and 4-hydroxypropranolol the derivative of the (−)-enantiomer is eluted first. These results were verified for propranolol using pure enantiomers for the derivatization.

Peak heights of the propranolol and 4-hydroxypropranolol derivatives correlated linearly ($r > 0.99$) with concentrations of both enantiomers in the concentration ranges 5–400 and 20–600 ng/ml, respectively. Tables I and II show the intra- and inter-day variability of the method. The coefficients of variation (C.V.) were less than 12%.

We tested a number of other β-blockers, tricyclic antidepressants and N-ω-methyltryptamine as internal standard. Only N-ω-methyltryptamine was use-

TABLE I

INTRA-DAY VARIABILITY

Concentration added (ng/ml)	n	Concentration assayed (ng/ml)	C.V. (%)	Accuracy (%)
(−)-Propranolol				
50	5	47.15	8.3	94.3
100	5	93.3	5.1	93.3
150	5	144.2	4.0	96.2
(+)-Propranolol				
50	5	47.7	8.4	95.4
100	5	96.6	2.9	96.6
150	5	146.3	4.1	97.5
(−)-4-Hydroxypropranolol				
100	7	104.6	9.5	104.6
150	4	160.9	9.4	107.2
250	4	245.5	8.6	98.2
(+)-4-Hydroxypropranolol				
100	5	100.5	10.8	100.5
150	5	150.3	8.6	100.1
250	10	249.9	7.0	100.0

ful. It reacts also with *R*-(+)-PEIC and the derivative shows a retention time of about 6 min. However, the coefficients of variation during intra- and inter-day variability studies were higher when N-ω-methyltryptamine was used as internal standard. This may be caused by partial degradation of N-ω-methyltryptamine or less reproducible reaction with *R*-(+)-PEIC. Hence, no internal standard was used in this assay.

The method was then applied to determine the stereochemical composition of unconjugated as well as glucuronic acid- and sulfate-conjugated propranolol and 4-hydroxypropranolol in human plasma at steady state.

The enzyme preparation used has glucuronidase and sulfatase activity and hydrolyzed both conjugates. After addition of the specific glucuronidase inhibitor, saccharo-1,4-lactone, the concentration of the sulfate conjugates can be measured [17]. The use of a partially purified enzyme preparation is necessary because unpurified enzymes have fluorescence impurities, which interfere with this assay.

The plasma concentrations of unconjugated propranolol enantiomers were less than 30 ng/ml with higher concentrations for the *S*-(−)-enantiomer (Fig. 3) [18]. No unconjugated 4-hydroxypropranolol could be detected.

Further, the steady-state concentrations of the *S*-(−)-propranolol glucu-

TABLE II

INTER-DAY VARIABILITY

Concentration added (ng/ml)	n	Concentration assayed (ng/ml)	C.V. (%)	Accuracy (%)
(−)-Propranolol				
50	5	48.1	12.0	96.13
100	5	102.2	9.7	102.2
150	5	149.9	6.9	99.9
(+)-Propranolol				
50	5	49.2	8.5	98.5
100	5	96.8	11.2	96.8
150	6	149.5	7.4	99.7
(−)-4-Hydroxypropranolol				
100	5	103.6	8.6	103.6
150	6	148.9	6.6	99.2
250	6	242.7	6.5	97.1
(+)-4-Hydroxypropranolol				
100	5	107.9	12.3	107.8
150	5	148.3	7.4	98.9
250	5	249.2	5.1	99.7

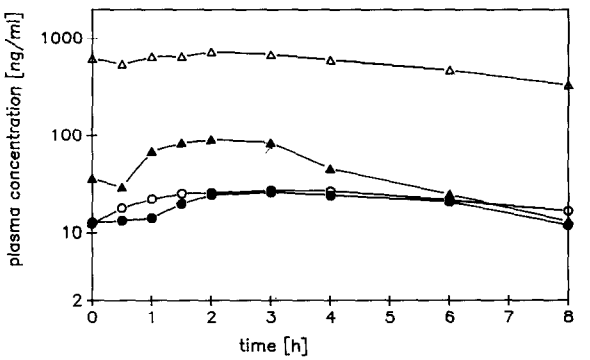

Fig. 3. Plasma concentrations of conjugated and unconjugated propranolol enantiomers after oral administration of 60 mg racemic propranolol three times daily to one subject: △, (−)-propranolol glucuronide; ▲, (+)-propranolol glucuronide; ○, (−)-propranolol (unconjugated); ●, (+)-propranolol (unconjugated).

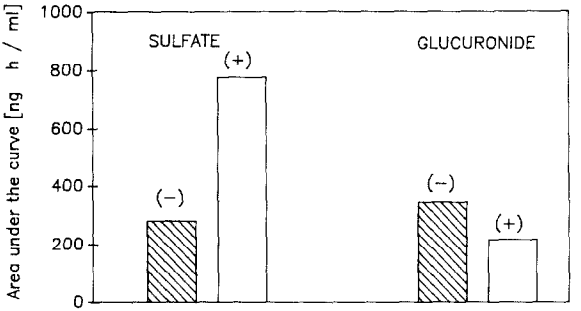

Fig. 4. Area under the plasma concentration–time curve during one dosing interval (AUC_{0-8h}) for the 4-hydroxypropranolol conjugates after oral administration of 60 mg racemic propranolol three times daily to one subject.

ronide conjugate were much higher than those for the *R*-(+)-enantiomer (Fig. 3). Less than 1% of the propranolol conjugates are sulfate conjugates. The *R*-(+)-propranolol glucuronide has a shorter half-life than the glucuronide of the *S*-(−)-enantiomer [4].

As shown in Fig. 4 glucuronidation of 4-hydroxypropranolol favours the *S*-(−)-enantiomer, whereas the sulfatation shows a large preference for the *R*-(+)-enantiomer; the area under the concentration–time curve during one dosing interval (AUC_{0-8h}) is about three times higher for the *R*-(+)-enantiomer than for the *S*-(−)-enantiomer (Fig. 4). Results from other studies on urine samples [19,20] showed similar differences between the enantiomers. Therefore, these differences in plasma concentrations of the sulfate and glucuronide conjugates seem to be due to stereospecific formation of the conjugates and are not due to differences in renal clearance.

In summary, the described method can be used to determine the stereochemical composition of unconjugated and conjugated propranolol and 4-hydroxypropranolol in human plasma. This method is presently used in our laboratory to evaluate the extent and mechanism of pharmacokinetic interactions between calcium antagonists and propranolol.

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REFERENCES

1 G. Johnsson and C.G. Regardh, Clin. Pharmacokin., 1 (1976) 223.
2 B. Silber and S. Riegelman, J. Pharmacol. Exp. Ther., 215 (1980) 643.
3 T. Walle, M.J. Wilson, U.K. Walle and St. A. Bai, Drug Metab. Dispos., 11 (1983) 544.
4 B. Silber, N.H.G. Holford and S. Riegelman, J. Pharm. Sci., 71 (1982) 699.

5 W. Lindner, M. Rath, K. Stochitzky and H.J. Semmelrock, Chirality, 1 (1989) 10.
6 T. Walle and U.K. Walle, Res. Commun. Chem. Pathol. Pharmacol., 23 (1979) 453.
7 D.B. Barnett, M. Batta, B. Davies and S.R. Nahorski, Eur. J. Clin. Pharmacol., 17 (1980) 349.
8 J. Hermansson, J. Chromatogr., 298 (1984) 67.
9 H. Takahashi, S. Kanno, H. Ogata, K. Kashiwada. M. Ohira and K. Someya, J. Pharm. Sci., 77 (1988) 993.
10 W. Lindner and M. Rath, J. Chromatogr., 487 (1989) 375.
11 H. Schmitthenner, M. Fedorchuk and D.J. Walter, J. Chromatogr., 487 (1989) 197.
12 S. Laganiere, E. Kwong and D.D. Shen, J. Chromatogr., 488 (1989) 407.
13 J.A. Thompson, J.L. Holtzman, M. Tsuru, Ch.L. Lerman and J.L. Holtzman, J. Chromatogr., 238 (1982) 470.
14 M.J. Wilson and T. Walle, J. Chromatogr., 310 (1984) 424.
15 J.D. Fitzgerald and S.R. O'Donnell, Br. J. Pharmacol., 43 (1971) 222.
16 P. Huyu and K.M. Giacomini, J. Pharm. Sci., 75 (1986) 601.
17 E.C. Kwong and D.D. Shen, J. Chromatogr., 414 (1987) 365.
18 C. von Bahr, J. Hermansson and K. Tawara, Br. J. Clin. Pharmacol., 14 (1982) 79.
19 T. Walle, U.K. Walle, S. Olanoff and E.C. Conradi, Br. J. Clin. Pharmacol., 22 (1986) 317.
20 T. Walle, U.K. Walle, M.J. Wilson, T.C. Fagan and T.E. Gaffney, Br. J. Clin. Pharmacol., 18 (1984) 741.