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QUANTITATIVE DETERMINATION OF PROPRANOLOL IN PLASMA AND PLASMA WATER FROM NORMAL SUBJECTS AND PATIENTS WITH ANGINA PECTORIS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A precise and sensitive high-performance liquid chromatographic method using a column packed with porous polystyrene gel is described for the determination of propranolol in plasma and plasma water from normal subjects and patients with angina pectoris. Propranolol in the samples was extracted with an *n*-heptane—isoamylalcohol (98.5:1.5) mixture after addition of penbutolol used as an internal standard. The extracts were chromatographed and detected with a spectrofluorophotometer. The quantitative limit of propranolol was 1 ng using 1 ml of plasma or 0.5 ml of plasma water. The present method should be useful for monitoring propranolol concentrations in plasma and plasma water during drug therapy and for pharmacokinetic study of propranolol.

INTRODUCTION

Propranolol (PL), a β-adrenergic receptor antagonist, is widely used for the treatment of patients with angina pectoris, cardiac arrhythmia and essential hypertension. Clinically, PL is used as a racemic mixture. The determination of PL concentration in plasma and plasma water is useful to adjust the PL dose and to study the plasma protein binding of PL during chronic PL therapy. Therefore, it is necessary to have a highly sensitive and precise method to determine the drug concentration in plasma and plasma water.

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PL is a molecule that contains a chiral carbon atom and exists as two optical isomers, (*R*)- and (*S*)-forms, where the (*S*)-form is responsible for almost all of the pharmacological effects [1]. Several methods have been reported concerning the determination of (*R*)- and (*S*)-PL in human plasma [2, 3]. Furthermore, several analytical methods of racemic PL or racemic PL and its metabolite, 4-hydroxypropranolol (4-OHPL), in plasma have been reported. Earlier fluorometric methods of PL [4, 5] were rapid and simple, but they suffer from a disadvantage of selectivity and sensitivity. Gas chromatographic (GC) methods using electron-capture [6, 7] and nitrogen–phosphorus-selective detectors [8] were used to detect 1—10 ng of PL using 0.5—2 ml of plasma, but these methods involve a laborious sequence of clean-up procedures followed by derivatization of PL and its metabolite to the trifluoroacetyl ester. A gas chromatographic—mass spectrometric (GC—MS) method [9] was based on derivatization of PL and 4-OHPL to the trifluoroacetyl ester. The quantitative limit of PL is 1 ng using 1 ml of plasma volume. While several high-performance liquid chromatographic (HPLC) methods [10—18] for determining PL alone or PL and its metabolite in plasma have been reported, most of these methods are based on separation using a reversed-phase column. It was reported that contribution of the pharmacological effect of 4-OHPL (the active metabolite) was small, since 4-OHPL had a shorter half-life than PL and therefore did not accumulate in the body during chronic therapy [15]. So the interference of the 4-OHPL peak with that of PL was only checked in this study.

In this paper, we describe a precise and sensitive HPLC method using a column packed with porous polystyrene-type material for determination of PL concentration in plasma and plasma water from normal subjects and patients receiving the drug.

EXPERIMENTAL

Drugs, reagents and materials

Hydrochloric salts of PL (PL · HCl) and 4-OHPL were kindly supplied by ICI-Pharm (Tokyo, Japan) and penbutolol, used as an internal standard (I.S.), was from Hoechst Japan (Tokyo, Japan). PL tablets (Inderal®) containing 10 mg of PL · HCl were purchased from ICI-Pharm. Column-packaging material, Hitachi Gel® 3013, was kindly supplied by Hitachi Seisakusho (Hitachi, Japan). Drug-free plasma was obtained from healthy subjects. All other solvents and reagents used were of reagent grade (Wako Pure Chemical Industries, Osaka, Japan).

Extraction of PL from biological samples

Extraction of PL from plasma and plasma water was performed according to a minor modification of the method of Shand et al. [5]. To 0.5—1 ml of plasma or 0.5 ml of plasma water in a 20-ml glass-stoppered centrifuge tube were added 6 μg of the I.S. in 50 μl of ethanol and a mixture of 1 ml of 1 *M* sodium hydroxide and 12 ml of *n*-heptane—isoamylalcohol (98.5:1.5). The mixture was shaken with a mechanical shaker for 10 min and centrifuged with a KN-70 centrifuge (Kubota Seisakusho, Tokyo, Japan) at 1680 *g* for

5 min. Most of the upper organic phase was transferred to another tube and evaporated to dryness with a rotary vacuum evaporator at 40°C in a water bath. The residue was dissolved in 50 μl of ethanol. A 10-μl aliquot of this solution was injected into the HPLC column.

Chromatographic conditions

The HPLC apparatus used in this study consisted of a Shimadzu liquid chromatograph LC-3A and a Shimadzu spectrofluorophotometer RF-500 (Shimadzu, Kyoto, Japan). Excitation and emission wavelengths of the detector were set at 285 and 340 nm, respectively. The column was a stainless-steel tube (250 × 4 mm I.D.) packed with Hitachi Gel 3013 using a column-packaging apparatus (Senshukagaku, Tokyo, Japan). For column packaging, 30 ml of the mobile phase were added to 2 g of Hitachi Gel 3013 (5-μm spherical porous particles of styrene—divinylbenzene). To thoroughly wet the gel, the mixture was shaken for 30 min and allowed to stand overnight at ambient temperature. The upper aqueous phase, containing small particles of the materials, was aspirated off. The lower phase, i.e. slurry, was put into a column packer connected with an empty stainless-steel tube, and a back-pressure was maintained at 160 kg/cm^2 with the same solution for ca. 2 h. The pressure was reduced over 15 min. The column was connected to the HPLC apparatus and its temperature was maintained at 30°C by a column jacket connected to a water bath. The mobile phase was ethanol—0.02 *M* $HClO_4$—$NaClO_4$ (pH 2.0) solution (65:35), degassed by sonication before use. The flow-rate of the mobile phase was set at 0.2 ml/min (at a pressure of ca. 40 kg/cm^2).

Analysis of chromatographic peak of PL by GC—MS

Ten plasma samples, each containing a known amount of PL (200 ng), were prepared. Extraction of PL from each sample was performed by the method described above, except for adding the I.S. After evaporation, each dried residue was dissolved in 60 μl of ethanol; 10 μl of this solution were injected into the HPLC column. About 1 ml of the chromatographic mobile phase was collected, per injection, during elution of the PL peak. By repeating this procedure, five times per sample, ca. 5 ml of mobile phase per sample were collected. Extraction of PL from each mobile phase collected was performed as described above. After the extraction, PL in each upper organic phase was back-extracted with 1 ml of 0.1 *M* hydrochloric acid according to the method of Suzuki et al. [19]. The aqueous phase was transferred to another tube. Then, 1 ml of 1 *M* sodium hydroxide and 12 ml of *n*-heptane—isoamylalcohol (98.5:1.5) were added and the mixture was shaken and centrifuged. Each upper organic phase was combined and evaporated to dryness. The residue was treated with trifluoroacetic anhydride (TFAA) according to the method of Walle et al. [9] and analysed by GC—MS. The electron-impact (EI) mass spectrum of the trifluoroacetyl (TFA) derivative of PL was obtained with a JGC-20K gas chromatograph—JMS D-300 mass spectrometer equipped with a JMA-2000 mass data system (JEOL, Tokyo, Japan).

Calibration curves, reproducibility and extraction recovery

Calibration curves. Plasma and plasma water samples were prepared by adding 1 ml of human plasma or 0.5 ml of plasma water to 1 ml of each aqueous solution containing 1, 5, 10, 25, 50 and 100 ng/ml PL. Plasma water was obtained from human plasma by ultrafiltration as described in *Application of HPLC* (see later). These samples were assayed according to the described method. The peak-height ratio was calculated by dividing the peak height of PL by that of the I.S. and was plotted against a known amount of PL.

Reproducibility. Plasma and plasma water samples containing 1, 5, 10, 50 and 100 ng/ml PL were prepared and assayed as described above. Reproducibility was determined by repeating the procedure five times for each sample and calculating the standard deviation.

Extraction recovery. The extraction recoveries of PL from plasma and plasma water were determined at three different concentrations (10, 50 and 100 ng/ml). The I.S. was added to 10 ml of the *n*-heptane—isoamylalcohol phase, transferred after completion of the extraction. Peak-height ratio was calculated and compared to the peak-height ratio of an injected amount of PL and I.S., equivalent to a 100% extraction yield.

Application of HPLC

In the preliminary study, five male subjects (age 22–27 years, body weight 52.4—75.4 kg) were orally administered one Inderal tablet. They were informed about the nature of the trial and their written consents were obtained. They were shown to be healthy by clinical examination. Blood samples were drawn through an indwelling cannula 0.5, 1, 1.5, 2, 3, 4, 6 and 8 h after the dose. The blood was transferred into heparinized tubes. Plasma was separated by centrifugation at 1680 *g* for 5 min and stored at −20°C until analysis.

Five patients (two males, age 31 and 57 years; three females, age 47—68 years) with angina pectoris, receiving therapeutic doses (60 mg/day, three times a day) of Inderal for periods longer than three months, were studied. Blood samples were drawn 2 h after the dose in a morning during chronic therapy, and plasma was separated as described above. To determine the percentage of plasma protein binding of PL, ultrafiltration was carried out using a cellulose acetate tubing (Type 20/32, 130 mm length, Visking, U.S.A.) as follows. A 5-ml aliquot of plasma was pipetted into the tubing. Then 500 µl of ultrafiltrate, i.e. plasma water, were obtained by centrifugation (1680 *g*) for ca. 1 h at 37 ± 0.5°C. The plasma before filtration and the ultrafiltrate were stored at −20°C until analysis. In a preliminary study on the adsorption of PL to the cellulose acetate tubing using 1/15 *M* phosphate buffer (pH 7.4) solution containing 10 and 50 ng/ml PL, adsorption was less than 3 and 1%, respectively.

RESULTS AND DISCUSSION

Chromatographic conditions and extraction

In a preliminary study, the fluorescent intensity of PL was stable in ethanol and increased with increasing acidity of solution. Therefore, the mixture ethanol—0.02 *M* $HClO_4$—$NaClO_4$ (pH 2.0) solution (65:35) was used as the

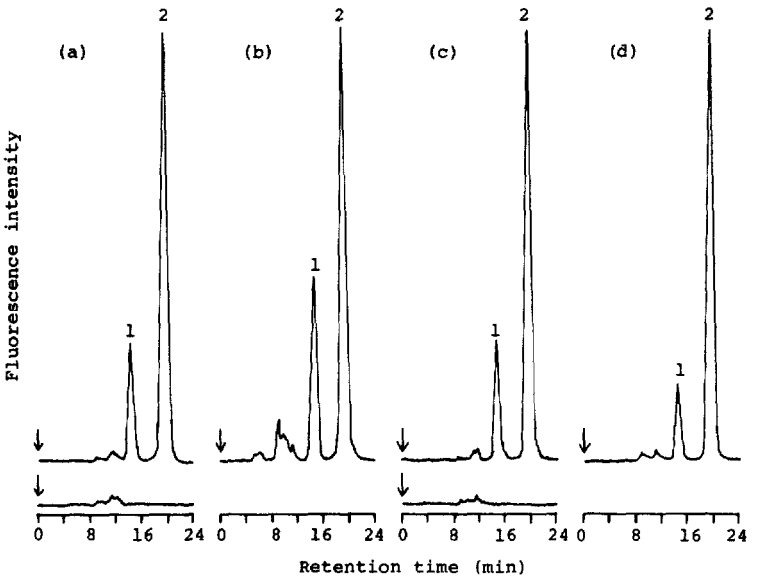

Fig. 1. Chromatograms obtained from: (a) plasma of normal subject containing 10 ng of propranolol (PL) (upper trace) and blank human plasma (lower trace); (b) plasma of patient during chronic therapy of PL; (c) plasma water of normal subject containing 10 ng of PL (upper trace) and blank plasma water of normal subject (lower trace); (d) plasma water of patient during chronic therapy of PL. Chromatographic conditions were as described in Experimental. Peaks: 1 = PL; 2 = penbutolol, the internal standard (6 μg).

mobile phase. The fluorescent intensity of PL in this mobile phase was greater than that of previous studies on HPLC analysis [10–18]. Among acetonitrile, cyclohexane and various mixtures of *n*-heptane and isoamylalcohol (98.5:1.5, 98:2, and 97:3) tested for the extraction solvent of PL, *n*-heptane–isoamylalcohol (98.5:1.5) proved to be the most suitable as a practical extraction solvent of this drug, since it produced the smallest endogenous peak on the chromatogram under HPLC conditions. Penbutolol, selected as an internal standard, was suitable for the analysis of PL since it shows similar behaviour to that of PL in the procedure.

Typical chromatograms of PL as determined in extracts of plasma and plasma water from a normal subject and a patient, and blank plasma and plasma water, are shown in Fig. 1. Peaks of PL and the I.S. were well separated from the endogenous peak in every chromatogram, and their retention times were approximately 14 and 18 min, respectively. Each of these peaks was sharp and symmetric. The peak of 4-OHPL was not found on the chromatograms, since this compound was decomposed during the extraction of PL in the present method. The column prepared with Hitachi Gel 3013 was very stable in the mobile phase and maintained constant resolution after ca. 2000 injections. For this reason, the column used in this study is suitable for routine analysis of PL in the clinical laboratory.

Analysis of chromatographic peak of PL by GC–MS

The purity of the chromatographic peak of PL was ascertained by GC–MS. As shown in Fig. 2, the mass spectrum of the TFA derivative of the sample obtained by HPLC separation was identical to that of authentic PL.

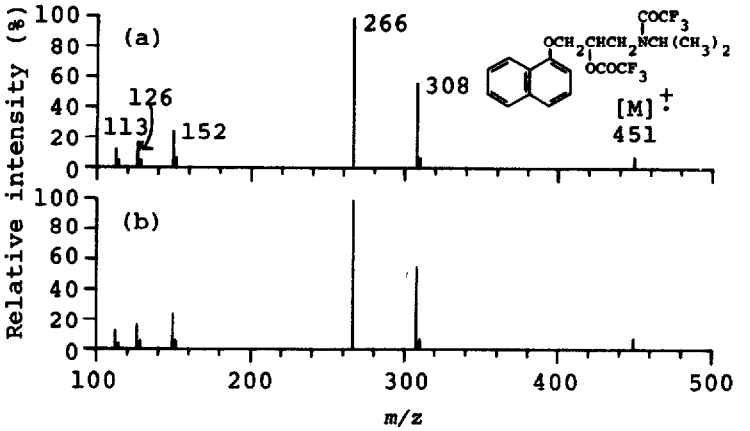

Fig. 2. Electron-impact mass spectra of TFA derivatives of authentic propranolol (a) and sample (b) from the present HPLC method. GC—MS conditions: column, 1% OV-17 Chromosorb W (1 m × 2 mm I.D., 80—100 mesh); column and injection port temperatures, 181 and 220°C; flow-rate of carrier gas (helium), 40 ml/min; ionization energy, 70 eV; emission current, 0.3 mA; scan speed, *m/z* 50–500 in 2.5 s; separator and ion source temperatures, 210 and 200°C.

Calibration curves, precision and extraction recovery

The calibration curve for plasma assay was linear in the range 1—100 ng using a 1-ml sample and could be expressed by the equation $y = 0.0281x + 0.0041$ ($r = 0.9997$). Similarly, the calibration curve for plasma water assay was linear in the range 1—100 ng using a 0.5-ml sample and could be expressed by the equation $y = 0.0286x + 0.0142$ ($r = 0.9995$). The precision of analysis of PL in plasma and plasma water was determined at five different concentrations of the drug (1, 5, 10, 50 and 100 ng/ml). The coefficients of variation (C.V.) obtained by repeating the procedure five times for each sample were less than 3.5% in plasma and 4.1% in plasma water (Table I). These

TABLE I

REPRODUCIBILITY OF ANALYSIS OF PROPRANOLOL IN HUMAN PLASMA AND PLASMA WATER

Amount added (ng)	Peak-height ratio (mean ± S.D., $n = 5$)	Coefficient of variation ($n = 5$) (%)
Plasma		
1	0.031 ± 0.001	3.2
5	0.143 ± 0.005	3.5
10	0.281 ± 0.003	1.1
50	1.426 ± 0.020	1.4
100	2.804 ± 0.061	2.2
Plasma water		
1	0.035 ± 0.001	2.9
5	0.147 ± 0.006	4.1
10	0.317 ± 0.006	1.9
50	1.430 ± 0.010	0.7
100	2.890 ± 0.026	0.9

TABLE II

EXTRACTION RECOVERIES OF PROPRANOLOL FROM HUMAN PLASMA AND PLASMA WATER

Concentration (ng/ml)	Recovery (mean ± S.D., n = 5) (%)
Plasma	
10	55.4 ± 4.2
50	56.5 ± 5.1
100	56.0 ± 2.3
Plasma water	
10	58.1 ± 1.4
50	57.3 ± 1.3
100	57.7 ± 0.5

reproducibility data were better than these of the other HPLC assay [10—12]. The quantitative limit of PL in plasma and plasma water was 1 ng (signal-to-noise ratio = 10), which was similar to that reported for other HPLC [10] and GC—MS methods [9]. The extraction recovery of PL was in the range 55.4—56.5% in plasma and 57.3—58.1% in plasma water, and these were virtually constant (Table II).

Check for interference

Drugs that were frequently used together with PL in patients with essential hypertension and angina pectoris were examined from the prescription. Such drugs were disopyramide, diltiazem hydrochloride, quinidine sulphate, dipyridamole, nifedipine, isosorbide dinitrate, digoxin, verapamil hydrochloride, diazepam, nitrazepam, maprotiline hydrochloride, furosemide, trichlormethiazide, reserpine, allopurinol and benzbromarone. These drugs were added to drug-free plasma and assayed in order to detect whether these drugs interfered with the chromatograms of PL. The peaks of reserpine and quinidine sulphate were detected on the chromatogram. However, their chromatographic peaks did not interfere with the measurement of peaks corresponding to PL and the I.S., since the retention times of reserpine and quinidine sulphate were 8.5 and 9.2 min, respectively. The other drugs examined were not detected under the present HPLC conditions.

Application of the method

The method described in this study was applied to the determination of plasma and plasma water concentration of PL in humans. The mean concentration—time course of PL in plasma after a single oral administration of one Inderal tablet to five normal subjects is shown in Fig. 3. Plasma concentrations of PL reached a maximum within 2 h after administration and their mean was 3.2 ng/ml. Thereafter, the concentrations decreased gradually to 1.2 ng/ml after 8 h. The results of determination of plasma protein binding of PL in patients receiving a chronic dose of PL are shown in Table III. The total plasma concentration of PL ranged from 45.4 to 111.4 ng/ml. The concentrations were found to be in almost the same therapeutic ranges as those suggested

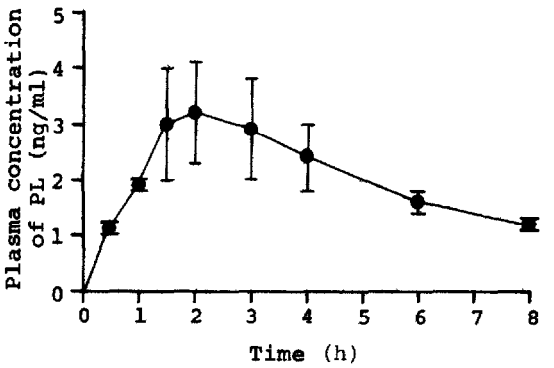

Fig. 3. Plasma concentration—time course of propranolol (PL) in five normal subjects following a single oral administration of a 10-mg dose of PL · HCl. Each point represents the mean of five determinations and the vertical bar indicates the standard error.

TABLE III

PLASMA PROTEIN BINDING DATA OF PROPRANOLOL (PL) IN PATIENTS WITH ANGINA PECTORIS

The plasma protein binding of PL is calculated using the equation: Protein binding of PL (%) = (total plasma concentration of PL − unbound plasma concentration of PL)/(total plasma concentration of PL) × 100.

Patient	Concentration (ng/ml)		Protein binding of PL (%)
	Total	Unbound	
S.T.	111.4	24.0	78.5
M.T.	70.3	14.8	78.9
I.Y.	45.4	8.6	81.1
M.O.	55.2	8.2	85.1
H.O.	89.7	8.6	90.4

by other investigators [20—22]. The percentage of plasma protein binding ranged from 78.5 to 90.4%, and these values varied among the patients.

In conclusion, a sensitive, specific and precise method for the determination of PL in plasma and plasma water by HPLC analysis was established. PL in these biological fluids could be precisely determined at quantities as low as 1 ng by the present method using a 0.5—1 ml sample. The described method should be useful for drug level monitoring of PL during therapy and for pharmacokinetic study following low doses of PL.

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