Journal of Chromatography, 491 (1989) 507–512
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4753

Note

Ion-exchange high-performance liquid chromatography in drug assay in biological fluids

V. Propranolol and metabolites

V.G. BELOLIPETSKAJA, V.K. PIOTROVSKII*, V.I. METELITSA and S.A. PAVLINOV

USSR Research Center for Preventive Medicine, 101953 Moscow (U.S.S.R.)

(First received December 7th, 1988; revised manuscript received March 3rd, 1989)

Propranolol (PR) is one of the β-adrenoceptor blockers commonly used in cardiology [1]. Traditional methods of quantitating intact PR in biological samples include fluorimetric procedures [2], gas chromatography [3] and liquid chromatography [4–6]. Several techniques described are suitable for the simultaneous quantitation of some PR metabolites [5,6]. However, glucuronide and sulphate conjugates of PR and its pharmacologically active primary metabolite 4-hydroxypropranolol (4-OH-PR) have been usually assayed indirectly, after enzymic hydrolysis. This entails a number of factors, such as hydrolase activity and reproducibility of the enzymic hydrolysis conditions, that may lead to significant errors and make the analytical techniques insufficiently valid. In view of this, the design of a technique that would permit direct assay of a conjugate without hydrolysis is of importance.

We have suggested the use of cation-exchange liquid chromatography to separate PR and its metabolites [7–10]. The advantage of this procedure relative to the commonly used reversed-phase sorbents is that the former separates drugs of widely different polarity under isocratic conditions. This technique has proved more suitable, since in a good separation the retention times for PR and its metabolites are shorter than in reversed-phase high-performance liquid chromatography (HPLC). Earlier we developed a procedure for PR determination by cation-exchange HPLC. This paper described a highly sensi-

0378-4347/89/$03.50

tive HPLC fluorescence assay that makes possible the simultaneous determination in plasma and urine of PR, and its glucuronide and the sulphate glucuronide of 4-OH-PR.

EXPERIMENTAL

Chromatographic system

The HPLC system consisted of a Model B-100-S2 solvent pump (Eldex, Menlo Park, CA, U.S.A.), a Model 7125 injector (Rheodyne, Cotati, CA, U.S.A.), a Model GM-970 fluorimetric detector (Kratos, Westwood, NJ, U.S.A.), and a Model 100-40 UV detector (Altex, Berkeley, CA, U.S.A.). The columns used were analytical (20 cm×4 mm I.D.) with a cation exchanger Nucleosil 5 SA (Macherey-Nagel, Düren, F.R.G.) and preparative (25 cm×10 mm I.D.)with a cation exchanger Partisil 10-SCX (Alltech, Deerfield, IL, U.S.A.) and with a reversed-phase sorbent Ultrasphere ODS, particle size 5 μm (Beckman, Altex, CA, U.S.A.).

Chemicals

Acetonitrile, diethylamine, orthophosphoric acid, ethyl acetate, potassium hydroxide (E. Merck, Darmstadt, F.R.G.), tetraheptylammonium iodide (Serva, Heidelberg, F.R.G.), tetrabutylammonium phosphate (Regis, Chicago, IL, U.S.A.) and Glusulase (Sigma, F.R.G.) were used as received. The water was purified by means of Milli RO4/Milli Q system (Millipore, Bedford, MA, U.S.A.). Anaprilin (MinChimProm, Kharkov, U.S.S.R.) was used as a standard for PR. Naphthyzine nitrate (0.05% solution, MinChimProm, Kiev, U.S.S.R.) served as the internal standard.

Preparation of samples

Serum of urine (1.0 ml) was transferred into the siliconized tube, followed by the addition of 0.02 ml of 25 μg/ml internal standard solution, 0.02 ml of 10 *M* potassium hydroxide, 0.05 ml of 25 m*M* tetrabutylammonium phosphate and 5 ml of ethyl acetate. The tube was vortexed for 30 s, then centrifuged at 500 *g* for 10 min. The organic phase was transferred into a conical tube and evaporated to dryness at 40°C under air flow. The residue was dissolved in the mobile phase (50 μl) and chromatographed for PR and sulphate of 4-OH-PR. To the aqueous layer in the extraction tube were added 1 ml of water, 0.02 ml of the internal standard solution (25 μg/ml), 0.02 ml of 10 *M* potassium hydroxide, 0.05 ml of 25 m*M* tetraheptylammonium chloride in acetone and 5 ml of ethyl acetate. After vortex-mixing for 30 s and centrifugation at 500 *g* for 10 min, the organic phase was transferred to another tube for evaporation. The solvent was evaporated to dryness at 40°C under air flow, and the residue was dissolved in 0.05 ml of the mobile phase. The solution was subjected to chromatography for glucuronides of *R*- and *S*-PR.

Chromatography

The mobile phase consisted of acetonitrile–water–diethylamine–orthophosphoric acid (156:300:1.9:1.55, v/v). It was degassed and used at a flow-rate of 1.5 ml/min. Detection with a fluorescent detector was used with excitation at 225 nm and emission through a cut-off filter of 350 nm. The column was maintained at ambient temperature.

Preparation of metabolite standards

The PR metabolites were isolated from the urine of a patient chronically treated with the drug (Anaprilin). After alkalinization the urine was twice extracted with ethyl acetate to remove the intact PR and other organic compounds. The sulphate of 4-OH-PR was extracted with ethyl acetate after the addition of tetrabutylammonium phosphate (25 m*M*), and then reextracted from the organic layer into 0.05 *M* sulphuric acid and isolated by HPLC on the reversed-phase preparative column with acetonitrile–water–acetic acid (90:300:4, v/v) as mobile phase. The flow-rate was 1.5 ml/min. The PR glucuronides were separated from the urine after the extraction of sulphate by column chromatography on XAD-2 resin, following washing with water and elution with methanol. The methanolic effluent was evaporated, and the mixture of glucuronides was isolated by preparative HPLC on a Partisil 10-SCX column. The mobile phase was acetonitrile–water–diethylamine–orthophosphoric acid (100:394:4:3.6, v/v) at a flow-rate of 1.5 ml/min.

Mass spectrometry

The structures of the sulphates and PR were confirmed by mass spectrometry of the secondary ions (SIMS) on the mass spectrometer (Varian MAT 331A, San Jose, CA, U.S.A.).

RESULTS AND DISCUSSION

A typical chromatogram (Fig. 1) demonstrates that the cation exchanger Nucleosil 5-SA under the adjusted conditions provides complete separation of PR (peak 5) and some of its metabolites: the sulphate of 4-OH-PR (peak 1) and the diastereomeric glucuronides *S*- and *R*-PR (peaks 2 and 3, respectively). The identification of the peaks was supported by enzymic hydrolysis in the presence of Glusulase including β-glucuronidase and arylsulphatase (pH 4.7, 37°C, 2 h). When the glucuronide fraction isolated from the urine was incubated, peaks 2 and 3 with retention times 5.2 and 7.8 min, respectively (Table I), were displaced by the peak at 21.5 min, which corresponded to PR. Incubation with *d*-saccharic acid-1,4-lactone, which selectively inhibits β-glucuronidase, did not alter peaks 2 and 3, although peak 1 disappeared. No additional peaks appeared, owing to oxidation of the 4-OH-PR that was formed.

Some endogenous substances coextracting from plasma can hinder the de-

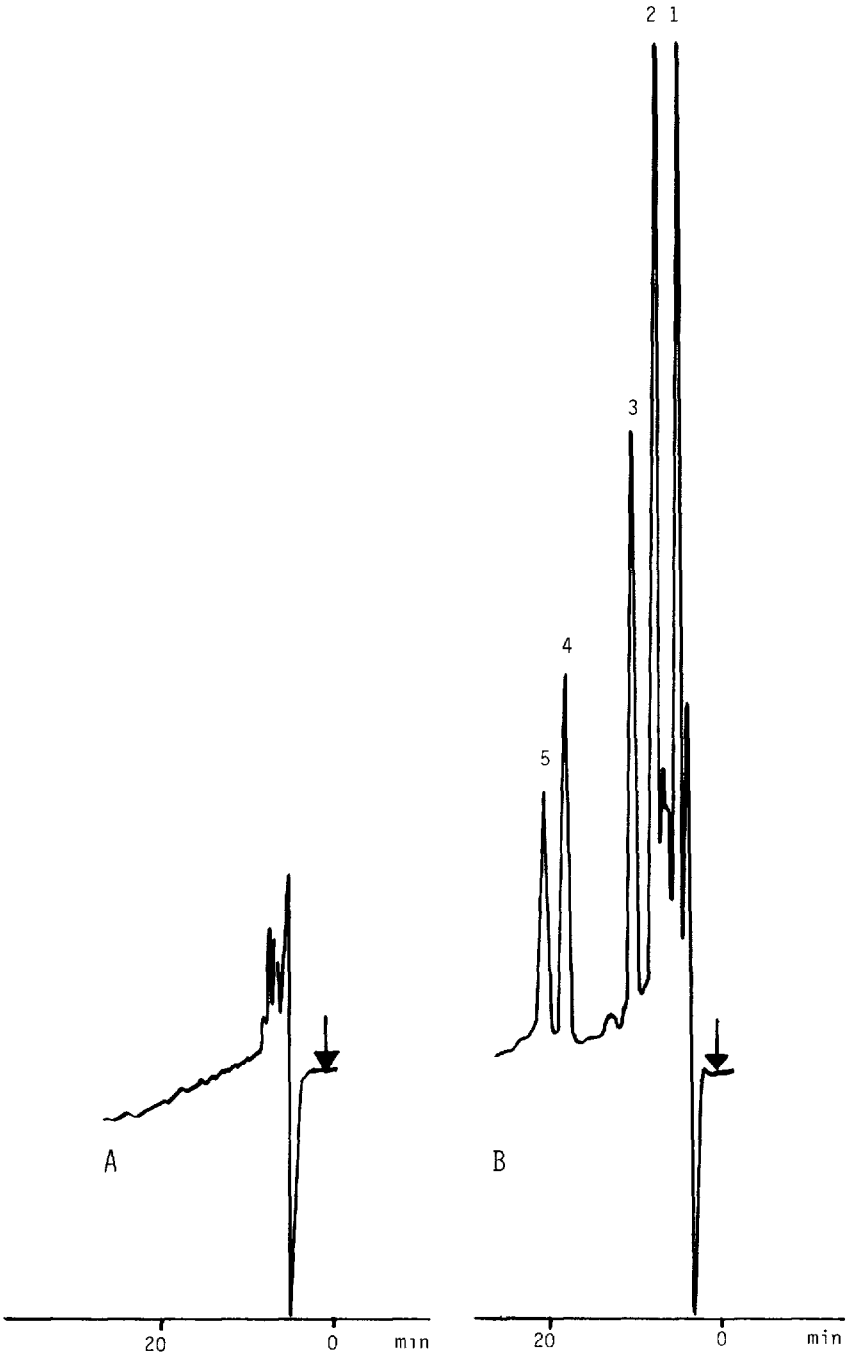

Fig. 1. Determination of propranolol and its metabolites in serum taken from patients (A) before and (B) after drug administration. Peaks: 1 = 4-hydroxypropranolol sulphate; 2 = *S*-propranolol glucuronide; 3 = *R*-propranolol glucuronide; 4 = naphthyzine (internal standard); 5 = propranolol.

TABLE I

RETENTION TIMES OF PROPRANOLOL AND ITS METABOLITES

Peak No.	Compound	Retention time (min)
1	4-Hydroxypropranolol sulphate	2.3
2	*S*-Propranolol glucoronide	5.2
3	*R*-Propranolol glucoronide	7.8
4	Naphthyzine (internal standard)	17.8
5	Propranolol	21.5

TABLE II

CALIBRATION DATA FOR PROPRANOLOL AND METABOLITES IN BLOOD SERUM

Compound	Concentration range (μM)	Recovery (%)	Correlation coefficient	Calibration equation
Propranolol	0.077–0.77	90	0.999	$y=0.0328+0.0084x$
4-Hydroxypropranolol sulphate	0.324–1.62	95	0.9998	$y=0.059+0.6x$
S-Propranolol glucuronide	0.56 –1.69	70	0.978	$y=1.15+4.17x$
R-Propranolol glucuronide	0.28 –0.85	70	0.975	$y=0.377+2.61x$

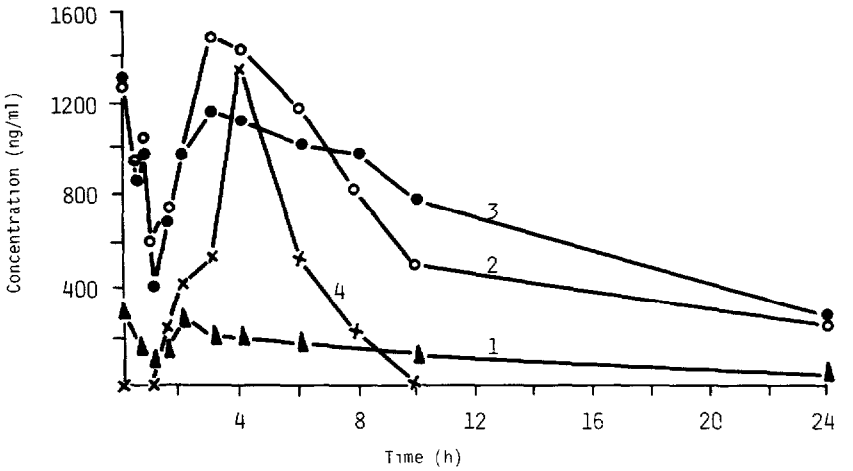

Fig. 2. Pharmacokinetic profiles of (1) propranolol, (2) 4-hydroxypropranolol sulphate, (3) *S*-propranolol glucuronide and (4) *R*-propranolol glucuronide in the serum of a patient treated with propranolol (40 mg four times daily) after the last course dose.

termination of the sulphate of 4-OH-PR. In order to improve the validity of the determination we applied a two-stage extraction. Since we have no pure standards for 4-OH-PR sulphate and PR glucuronides, solutions of these metabolites were obtained from the urine of patients taking PR and used to construct graphs. The concentrations of the solutions were determined in the following way. Glucuronides were incubated with Glusulase and the concentration of resulting PR was measured as above. The concentrations of the sulphate solutions were established photometrically and suggested that the molecular extinctions of PR and 4-OH-PR sulphate are equal. This is supported by the coincidence of the UV spectra for these substances [6].

The calibration data are presented in Table II. They were linear in the ranges of the concentrations of the compounds that could be observed in the plasma and urine of the patients treated with PR in therapeutical doses.

Fig. 2 shows the profiles of changes in concentrations of PR and its metabolites in the blood serum of a patient who was on a prolonged medication with this drug.

We have thus developed a method for the quantitation of PR with some of its metabolites, which needs no hydrolysis procedures for the assay of sulphate and glucuronide conjugates. At present the method is in use for pharmacokinetic studies of PR in humans.

ACKNOWLEDGEMENT

The authors thank Olga Kuznetsova for preparing the English version of the manuscript.

REFERENCES

1 F. Albani, R. Riva and A. Baruzzi, J. Chromatogr., 228 (1982) 362.
2 M. Schafer, H.E. Geissler and E. Mutschler, J. Chromatogr., 143 (1977) 607.
3 T. Walle, J. Morrison, U.K. Walle and E. Conradi, J. Chromatogr., 114 (1975) 351.
4 M. Lo and S. Riegelman, J. Chromatogr., 183 (1980) 213.
5 T. Walle, U.K. Walle, D.R. Knapp, E.C. Conradi and E.M. Bargar, Drug Metab. Dispos.: Biol. Fate Chem., 11 (1983) 344.
6 T. Walle, U.K. Walle and L.S. Olanoff, Drug Metab. Dispos., 13 (1985) 204.
7 V.K. Piotrovskii and V.I. Metelitsa, J. Chromatogr., 231 (1982) 205.
8 V.K. Piotrovskii, D.O. Rumjantsev and V.I. Metelitsa, J. Chromatogr., 275 (1983) 195.
9 V K. Piotrovskii, V.G. Belolipetskaja, A.R. El'man and V.I. Metelitsa, J. Chromatogr., 278 (1983) 469.
10 V.K. Piotrovskii, Yu.A. Zhirkov and V.I. Metelitsa, J. Chromatogr., 309 (1984) 421.