Journal of Chromatography, 570 (1991) 453–461
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6003

Short Communication

Liquid chromatographic analysis of propranolol enantiomers in human blood using precolumn derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate

A. ROUX*, G. BLANCHOT, A. BAGLIN and B. FLOUVAT

Laboratoire de Toxicologie et de Pharmacocinétique, Hôpital Ambroise Paré, 9 Avenue Charles de Gaulle, 92104 Boulogne Billancourt (France)

(First received March 19th, 1991; revised manuscript received May 13th, 1991)

ABSTRACT

A method for the determination of the *R*-(+) and *S*-(−) enantiomers of propranolol in blood was developed. After extraction with heptane–isopentanol and derivatization with (+)-1-(9-fluorenyl)ethyl chloroformate, excess reagent was removed using solid-phase extraction. The enantiomers were separated on an achiral, reversed-phase, radially compressed column, and detected by fluorescence with excitation and emission wavelengths of 260 and 340 nm, respectively. The limit of quantification was 0.5 ng/ml. This method was used for pharmacokinetic analysis of propranolol enantiomers after administration of immediate-release (80 mg) or sustained-release (160 mg) racemic propranolol.

INTRODUCTION

Propranolol, like most β-blockers, is commercially available as the racemic mixture, although it is now well known that the *R*-(+) and the *S*-(−) enantiomers differ in their pharmacodynamic and pharmacokinetic behaviour. *S*-(−)-Propranolol has a higher binding affinity for the β-receptor: it is a 100-fold more potent β-blocker than the *R*-(+) enantiomer, and is also more active in the management of hypertension.

Single- and multiple-dose pharmacokinetic studies have shown that the disposition of propranolol enantiomers is different: the distribution, plasma protein binding, clearance and metabolism are stereoselectively controlled processes giving rise to different pharmacokinetic parameters [1–4]. For these reasons, in a study of the interference of paracetamol with the pharmacokinetics of propranolol, we preferred to use a method allowing the separation of the enantiomers of propranolol and their assay in the blood.

This paper describes a simple method that involves the preparation of

0378-4347/91/$03.50

diastereoisomers of propranolol with the chiral reagent (+)-1-(9-fluorenyl)ethyl chloroformate (FLEC), followed by achiral reversed-phase high-performance liquid chromatography (HPLC) with fluorimetric detection.

EXPERIMENTAL

Materials and reagents

Acetonitrile (HPLC grade), methylene chloride and heptane (for spectroscopy) and acetone, isopentanol and isooctane (analytical grade) were purchased from Merck (Darmstadt, Germany), methanol (HPLC grade) from Carlo Erba (Milan, Italy) and sodium carbonate (analytical grade) from Prolabo (Paris, France).

Pure water (18 MΩ) was obtained using a MilliQ system (Millipore, Bedford, MA, USA).

Boric acid, potassium chloride and sodium hydroxide (analytical grade) were purchased from Merck. Borate buffer (pH 7.85) was prepared from 50 ml of 0.1 *M* boric acid and potassium chloride: the pH was adjusted with 0.1 *M* sodium hydroxide, and the solution made up to 100 ml with water.

FLEC (EKA Nobel) was obtained from Touzart et Matignon (Vitry, France).

Racemic propranolol (racemic P), *R*-(+)-propranolol [R(+)P] and methyl 4-propranolol (M4P, internal standard) hydrochlorides were supplied by ICI Pharmaceutical Division (Macclesfield, UK); *S*-(−)-propranolol [S(−)P] hydrochloride was obtained from Aldrich (Milwaukee, WI, USA).

Solid-phase extraction columns (1 ml/100 mg C_{18} Bond Elut) and a vacuum manifold (Vac Elut) were purchased from Analytichem International (Harbor City, CA, USA) and supplied by Prolabo (Paris, France).

Chromatographic conditions

HPLC analyses were carried out using a Shimadzu LC-6A pump, a Perkin LS40 spectrofluorimeter set at 260 nm for excitation and 340 nm for emission and at 1 for "fixed factor", a Kontron Instruments HPLC 360 autosampler and a Shimadzu CR4A integrator.

The RCM Waters radial compression system was used for separation, with a Nova-Pak C_{18} cartridge (100 mm × 8 mm I.D.) (Waters Millipore).

The mobile phase (acetonitrile–water, 75:25, v/v) was degassed through a 0.45-μm filter (Millipore) and used at a flow-rate of 2.0 ml/min, without recycling. The separations were performed at room temperature.

Standards and quality controls

A stock solution (200 μg/ml) of racemic P in water was prepared and stored at 4°C. Aqueous working solutions (5, 10, 20, 30, 40, 50 and 100 ng/ml, *i.e.* 2.5–50 ng/ml for each enantiomer) were prepared immediately prior to use for spiking drug-free blood samples.

Drug-free human blood samples spiked with 6, 12, 36 and 100 ng/ml racemic P (*i.e.* 3–50 ng/ml of each enantiomer) were used as controls. Aliquots (1.2 ml) were stored at −20°C and assayed with each series of samples.

Blood samples

The potential interference of paracetamol with the pharmacokinetics of propranolol was studied. Six healthy volunteers received, in randomized fashion, a single dose of either 80 mg of immediate-release propranolol (Avlocardyl, 40 mg, two capsules, IR), or 160 mg of sustained-release propranolol (Avlocardyl LP, 160 mg, one capsule, SR) on the fifth day of treatment with paracetamol (500 mg three times a day) or placebo. Blood samples were obtained until the 30th hour following the administration.

Extraction procedure

Water or working standard solution (1 ml), internal standard (M4P, 40 ng per 100 μl water) and 1 *M* sodium carbonate (3 ml) were added to whole blood samples, quality controls or drug-free whole blood (1 ml) in 30-ml glass-stoppered tubes. The samples were extracted with 10 ml of heptane–isopentanol (98:2, v/v) on a reciprocating shaker (80 100 strokes per min) for 15 min.

Following centrifugation at 1500 *g* at 4°C for 5 min, the organic phase (7 ml) was transferred to 16-ml glass-stoppered tubes and evaporated to dryness at 50°C under a gently stream of nitrogen.

Derivatization procedure

The dry residue was taken up in acetone (150 μl) and vortex-mixed for 30 s, then 100 μl of borate buffer (pH 7.85) and 50 μl of FLEC solution (500 μg/ml in acetone) were added. After mixing for 30 s, the tubes were left to stand for 5 min at room temperature.

Purifications of the reaction mixture

A C_{18} Bond Elut column was positioned on the vacuum manifold and conditioned with methanol and water (1.5 ml each). The vacuum was removed and 0.8 ml of water followed by 0.3 ml of the reaction mixture were pipetted onto the column. The vacuum was applied again, and the mixture was aspirated through the column to the waste outlet. The column was washed with 1.5 ml of isooctane, then the compounds were eluted with 500 μl of methylene chloride into a clean tube. The solvent was evaporated to dryness and the residue taken up in acetonitrile (35 μl) and vigorously vortex-mixed for 15 s. Water (75 μl) was added, and 100 μl of the acetonitrile–water phase were injected onto the analytical column.

RESULTS

Chromatography

Fig. 1 shows the chromatograms obtained with drug-free blood, blood spiked with 5 ng/ml racemic P and a blood sample taken 4 h after a single oral dose of 160 mg of propranolol SR. The retention times were 10.5, 11.3, 14.5 and 15.5 min, respectively, for the S(−)P, R(+)P and M4P enantiomers. Blank blood showed no interfering peaks from endogenous substances.

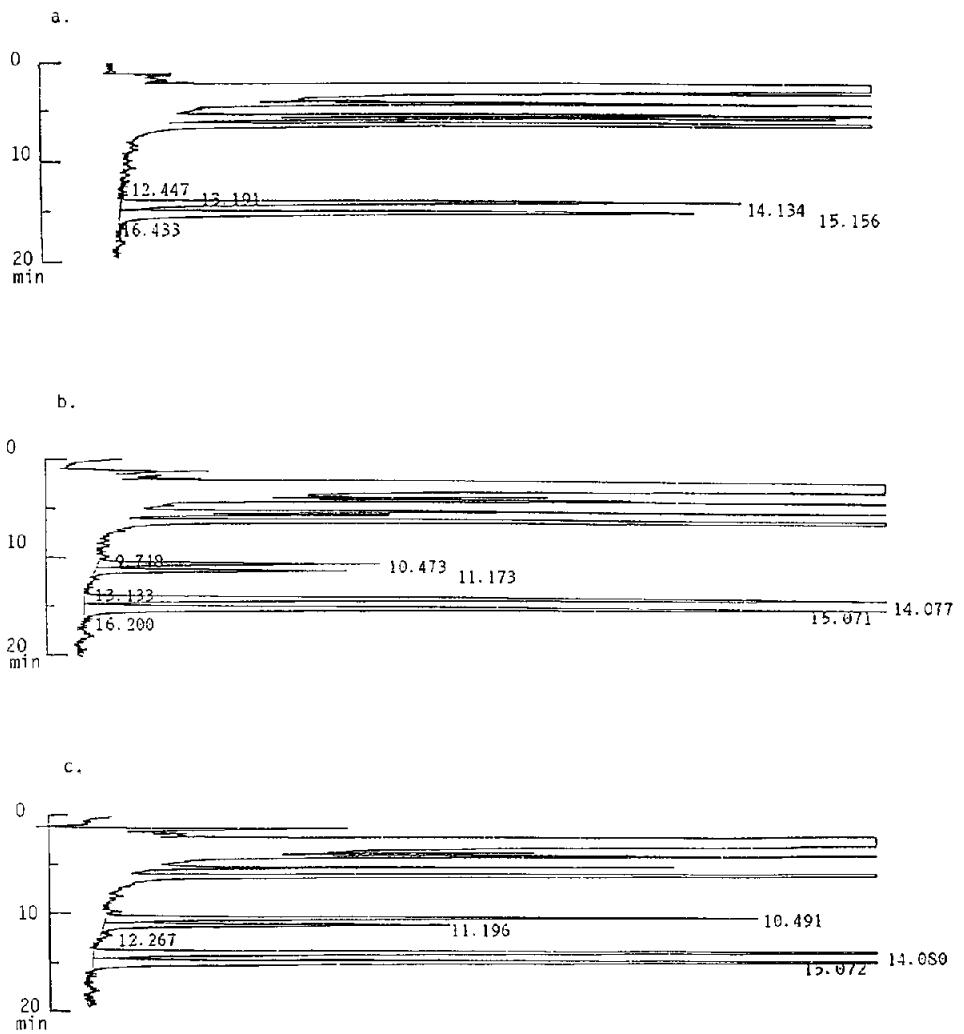

Fig. 1. Chromatograms obtained from extracts of (a) propranolol-free blood with internal standard, (b) blood spiked with 5 ng/ml racemic propranolol and (c) blood sample withdrawn 4 h after the administration of 160 mg of propranolol SR.

The elution order of individual diastereoisomers was established by derivatizing each pure enantiomer of propranolol: the S(−)P derivative formed the first eluted peak. Although we could not verify the identity of the two peaks of M4P, we supposed that the *S* enantiomer was eluted first, as is the case for S(−)P.

The selectivity and resolution factors were 1.07 and 2.74, respectively, for P enantiomers, and 1.08 and 2.62 for M4P enantiomers.

Extraction and derivatization

The recoveries of racemic P and M4P from spiked blood (50 ng/ml) were 72 and 70%, respectively.

The efficiency of derivatization was not improved by increasing the reaction time (5 min to 1 h), and the derivatives were stable at room temperature for at least 24 h. FLEC was always used, according to the manufacturer's instructions, in a large molar excess (20:1) relative to the two (P and MP) β-blockers

The purification of the derivatized products required the elimination of the excess reagent, which generated interfering peaks. Passage through the Bond Elut column permitted this step to be performed rapidly and allowed almost all of the extract to be injected. With no recycling of mobile phase, the injections could be carried out automatically every 25 min without fluctuation of the baseline; a series of assays included *ca.* 30 samples.

Calibration

Two calibration curves were constructed from S(−)P and R(+)P in racemic P. Peak-height ratios were calculated with the corresponding enantiomer of M4P, although the results were similar if the first-eluted peak of M4P was taken as the only internal standard.

TABLE I

CALIBRATION OF PROPRANOLOL ENANTIOMERS IN SPIKED BLOOD SAMPLES

Racemic propranolol spiked (ng/ml)	*S*-(−)-Propranolol		*R*-(+)-Propranolol	
	Measured (ng/ml)	R.E.[a] (%)	Measured (ng/ml)	R.E.[a] (%)
5	2.57	2.92	2.46	−1.56
10	5.25	5.06	5.24	4.74
20	9.99	−0.09	10.02	0.23
30	14.59	−2.69	14.52	−3.17
40	19.75	−1.26	19.94	−0.30
50	25.59	2.36	25.28	1.12
100	49.89	−0.22	49.99	−0.01

[a] Relative error = (measured concentration − theoretical concentration) × 100/theoretical concentration.

TABLE II

INTER-ASSAY REPRODUCIBILITY OF PROPRANOL ENANTIOMERS

Racemic propranolol spiked (ng/ml)	*S*-(−)-propranolol		*R*-(+)-propranolol	
	Measured (mean ± S.D.) (ng/ml)	C.V.[a] (%)	Measured (mean ± S.D.) (ng/ml)	C.V.[a] (%)
6	3.1 ± 0.3	11	3.3 ± 0.5	14.3
12	5.9 ± 0.8	14.1	6 ± 0.8	13.9
36	18 ± 1	5.7	18.3 ± 1.1	6.1
100	51 ± 3.8	7.5	50.2 ± 3.9	7.8

[a] Coefficient of variation.

An example of calibration is shown in Table I: the equations were: $C = 8.5160R - 0.13686$ ($r = 0.999816$) for S(−)P and $C = 8.6222R + 0.04134$ ($r = 0.999900$) for R(+)P where C is the calculated concentration and R the peak-height ratio.

The linearity was good up to 60 ng/ml for each enantiomer.

TABLE III

RESULTS OF BLOOD CONCENTRATIONS IN A SUBJECT AFTER A SINGLE ORAL DOSE OF 80 mg OF PROPRANOLOL (P) IR

Time (h)	Measured concentation (ng/ml)				R.E.[a] (%)
	S(−)P	R(+)P	Racemic P	S(−)P plus R(+)P	
0.5	11.4	7.1	17.9	18.5	3.35
1	24.1	15.3	39.6	39.4	−0.51
1.5	23.6	14.4	37.6	38.0	1.06
2	24.0	14.0	35.4	38.0	7.34
3	18.4	10.9	28.6	29.3	2.45
4	14.0	8.3	21.5	22.3	3.72
6	7.8	4.7	11.8	12.5	5.93
8	5.1	3.1	8.7	8.2	−5.75
10	3.3	2.1	5.4	5.4	0
12	1.7	1.7	4.1	3.4	0
24	NQ[b]	NQ	0.7	NQ	
30	NQ	NQ	0.4	NQ	

[a] Relative error.

[b] Non-quantifiable, *i.e.* below the quantification limit (see text).

Reproducibility and accuracy

The between-run variability was studied using quality controls assayed each day, and varied from 5.7 to 14.3% according to the concentration (Table II). The quantification limit was 0.5 ng/ml for both enantiomers, evaluated as three times the S.D. of the mean intercept for fifteen calibration curves.

We checked that the sum of the concentrations of the enantiomers measured corresponded to the concentration of racemic propranolol: 2 ml of the remaining heptane–isopentanol phase were evaporated to dryness, and racemic P was determined by means of ion-pair reversed-phase chromatography with fluorimetric detection. The inter-assay precision was 8.1–5.6% for 8–150 ng/ml, and the limit of detection was 0.2 ng/ml. Results are shown in Table III using data obtained for a subject who received a single oral dose of 80 mg of propranolol IR.

DISCUSSION

The presently available methods for the quantification of propranolol enantiomers in biological fluids for pharmacokinetic studies, with the exception of gas chromatography–mass spectrometry, are all based on HPLC. Enantioselective chromatography using either a chiral stationary phase or a chiral mobile phase can be used. When chiral stationary phase is used, derivatization is sometimes necessary; thus condensation with phosgene to produce cyclic 2-oxazolidone derivatives of *R*- and *S*-propranolol has been used prior to separation on a Pirkle-type chiral stationary phase [5] or on a α_1-acid glycoprotein chiral phase [6]. In the latter case, it was a question of preventing interference by endogenous compounds. This preliminary step makes the manipulations long and laborious.

The tris-(3.5-dimethylphenylcarbamate) cellulose chiral phase (Chiralcel OD) [7,8] allows the determination of underivatized enantiomers of propranolol in plasma, with a detection limit of 3 ng/ml and a resolution factor as high as 3.75. The cost of this phase, together with the limited number of injections that it tolerates without loss of performance, dissuaded us from using it.

Ion-pair chromatography using (+)-10-camphorsulphonic acid [9,10] or benzoxycarbonylglycyl L-proline (ZGP) has also been proposed. This chiral counterion in the mobile phase, although optically impure, can be used for the separation and quantification of propranolol enantiomers [11], and gives a detection limit of 0.13 ng of propranolol in plasma [12]. We tried this method but were unable to obtain satisfactory results.

We, therefore, used a more classical approach, *i.e.* the conversion of propranolol enantiomers into diastereoisomers, followed by achiral reversed-phase HPLC with fluorescence detection. Among the chiral reagents previously used, N-trifluoroacetyl-l-prolylchloride (TPC) [13,14] has the disadvantage of being contaminated by the (+)-enantiomer, and can racemize on storage; (*R,R*)-O,O-diacetyltartaric acid anhydride (DATAAN), proposed by Lindner *et al.* [15], permits pharmacokinetic determinations (detection limit 0.5–1 ng/ml) but the reaction is

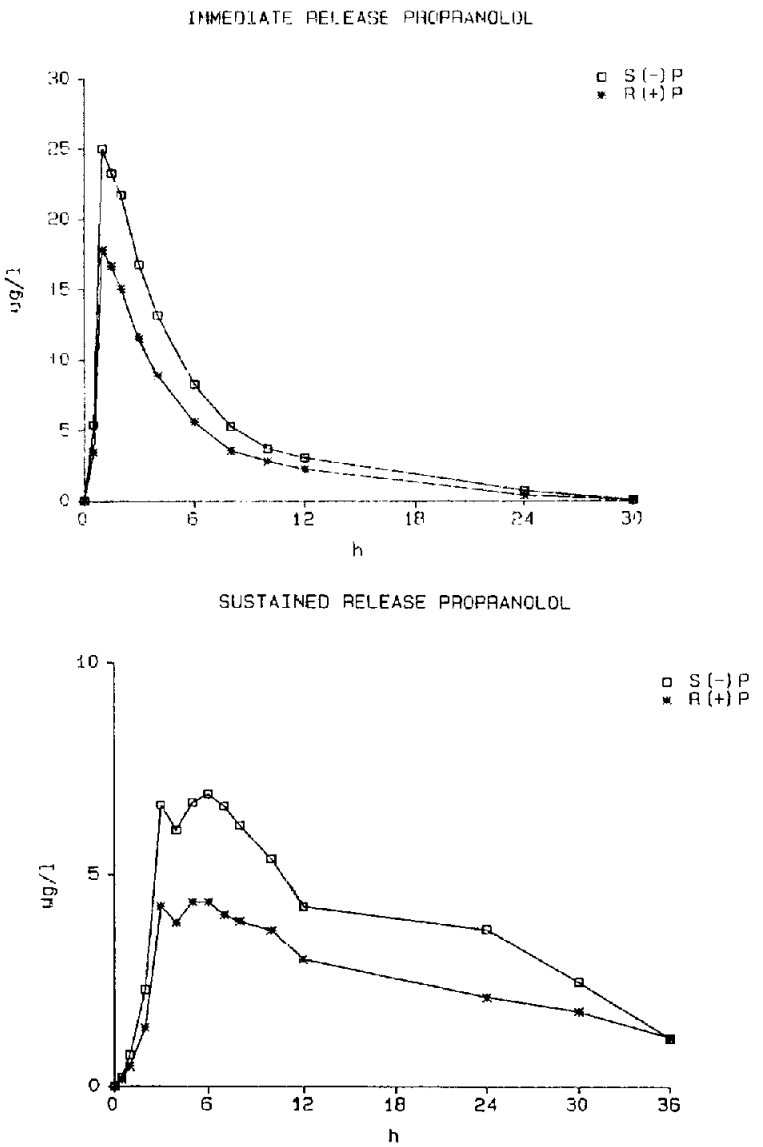

Fig. 2. Mean blood concentrations of S(−)P and R(+)P after oral administration of 80 mg of propranolol IR or 160 mg of propranolol SR to six subjects.

time-consuming (4 h) and water must be excluded in order to ensure quantitative derivatization reactions.

We chose FLEC, originally proposed by Enarsson *et al.* [16], as a stable reagent with high optical purity for the separation of amino acid enantiomers and chiral amines. As stated by Ahnoff *et al.* [17], chloroformates are attractive be-

cause their reactivity with primary and secondary amines allows derivatization under mild conditions in the presence of water.

During our study, another reagent of the same series, (−)-methyl chloroformate, was used to assay enantiomers of atenolol [18] and propranolol [19], and the reaction appears to be applicable to other β-blockers [20]. Derivatization occurs on the nitrogen of the secondary amine function of the side-chain [18,19].

The assay is sufficiently sensitive for application to pharmacokinetic studies. The mean blood concentrations of propranolol enantiomers assayed in six subjects after the administration of 80 mg of propranolol IR or 160 mg propranolol SR are shown in Fig. 2. The AUC ratios, $S(-)/R(+)$, were not significantly different between the two treatments (1.54 ± 0.28 for propranolol IR and 1.64 ± 0.40 for propranolol SR); similar values have been reported elsewhere [1,4,19].

The method we describe is low cost, relatively simple and fast, and has been used in our laboratory for more than 300 assays.

REFERENCES

1 T. Walle, U. K. Walle, M. J. Wilson, T. C. Fagan and T. E. Gaffney, *Br. J. Clin. Pharmacol.*, 18 (1984) 741.
2 R. L. Lalonde, M. B. Bottorff, R. J. Straka, D. M. Tenero, J. A. Pieper and I. W. Wainer, *Br. J. Clin. Pharmacol.*, 265 (1988) 100.
3 T. Walle, J. G. Webb, E. E. Bagwell, U. K. Walle, H. B. Daniell and T. E. Gaffney, *Biochem. Pharmacol.*, 37 (1988) 115.
4 W. Lindner, M. Rath, K. Stoschitzky and H. J. Semmelrock, *Chirality*, 1 (1989) 10.
5 I. W. Wainer, T. D. Doyle, K. H. Donn and J. R. Powell, *J. Chromatogr.*, 306 (1984) 405.
6 J. Hermansson, *J. Chromatogr.*, 325 (1985) 379.
7 R. J. Straka, R. L. Lalonde and I. W. Wainer, *Pharm. Res.*, 5 (1988) 187.
8 H. Takahashi, S. Kanno, H. Ogata, K. Kasiwada, M. Ohira and K. Someya, *J. Pharm. Sci.*, 77 (1988) 993.
9 C. Pettersson and G. Schill, *J. Liq. Chromatogr.*, 9 (1986) 269.
10 M. B. Gupta, J. W. Hubbard and K. K. Midha, *J. Chromatogr.*, 424 (1988) 189.
11 C. Pettersson, A. Karlsson and C. Gioeli, *J. Chromatogr.*, 407 (1987) 217.
12 C. Pettersson and M. Osefsson, *Chromatographia*, 21 (1986) 321.
13 J. Hermansson and C. Von Bahr, *J. Chromatogr.*, 221 (1980) 109.
14 B. Silber and S. Riegelman, *J. Pharmacol. Exp. Ther.*, 215 (1980) 643.
15 W. Lindner, M. Rath, K. Stoschitzky and G. Uray, *J. Chromatogr.*, 487 (1989) 375.
16 S. Einarsson, B. Josefsson, P. Moller and D. Sanchez, *Anal. Chem.*, 59 (1987) 1191.
17 M. Ahnoff, S. Chen, A. Green and I. Grundevik, *J. Chromatogr.*, 506 (1990) 593.
18 R. Mehvar, *J. Pharm. Sci.*, 78 (1989) 1035.
19 C. Prakash, R. P. Koshakji, A. J. Wood and I. A. Blair, *J. Pharm. Sci.*, 78 (1989) 771.
20 R. Mehvar, *J. Chromatogr.*, 493 (1989) 402.