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DETERMINATION OF BETAXOLOL ENANTIOMERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

APPLICATION TO PHARMACOKINETIC STUDIES

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SUMMARY

A high-performance liquid chromatographic (HPLC) method is described for the determination of (*R*)- and (*S*)-enantiomers of betaxolol in blood and other biological fluids. Separation of the enantiomers is performed after preparation of diastereomeric derivatives with the chiral reagent *R*(—)-naphthylethylisocyanate by reversed-phase HPLC. Fluorimetric detection allows the quantification of betaxolol enantiomers down to 0.5 ng/ml. This method was used to evaluate the pharmacokinetic profile of the betaxolol enantiomers in three subjects following one single oral dose (20 mg) of racemic betaxolol. No significant difference was observed in blood levels of the isomers.

INTRODUCTION

Betaxolol (Kerlone), a new cardioselective β-adrenoceptor-blocking agent widely used in the treatment of hypertension, is commercially available like most adrenergic β-blocking drugs as a racemic mixture of two isomers, (*R*)- and (*S*)-betaxolol. The pharmacokinetic profile of racemic betaxolol has been evaluated in several studies, following determination of the racemate by gas chromatography (GC) [1, 2] or high-performance liquid chromatography (HPLC) [3].

The pharmacological effects of the enantiomers of β-blockers may be quite different, as demonstrated for propranolol, alprenolol and oxprenolol [4–6]. The (*S*)-isomer, more potent than the (*R*)-isomer, is responsible for virtually all of the β-adrenoceptor-blocking effect. If the enantiomers have different pharmacokinetic profiles, it is necessary to measure concentrations of the active isomer in the blood prior to attempting any correlation between pharma-

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cological effect and blood level. This problem has generated great interest in the chromatographic separation of optical isomers of β-blockers. In general, resolution by chromatography may be approached in three ways [7—10]: (a) separation of the enantiomers on an optically active stationary phase; (b) addition of a chiral reagent to the mobile phase (in HPLC), followed by chromatography on an inactive stationary phase [11, 12]; (c) conversion of the enantiomers into diastereomers by a suitable chiral reagent, followed by chromatography on an inactive stationary phase [13—15].

The aim of the present study was to evaluate the pharmacokinetic profile of the enantiomers of betaxolol. In order to achieve this goal, we had first to develop a method for the quantitative determination of the enantiomers. As the expected blood concentrations are in the nanogram range, derivatization with a chiral reagent that would enhance detection was thought to be necessary. The present report describes an HPLC method for the separation and quantification of the enantiomers of betaxolol, following reaction with a fluorescent reagent.

EXPERIMENTAL

Chemicals and reagents

(*R*)- and (*S*)-betaxolol, racemic betaxolol and *S*(—)-cicloprolol were synthetized by the chemistry department of L.E.R.S. (France). Their structural formulae are given in Fig. 1. *R*(—)-1-(1-Naphthyl)ethylisocyanate [*R*(—)-NEI] was purchased from Aldrich (Milwaukee, WI, U.S.A.) and tetramethylethylenediamine (TEMED) from J.T. Baker (Deventer, The Netherlands).

All other solvents were of analytical or equivalent grade and used without further purification.

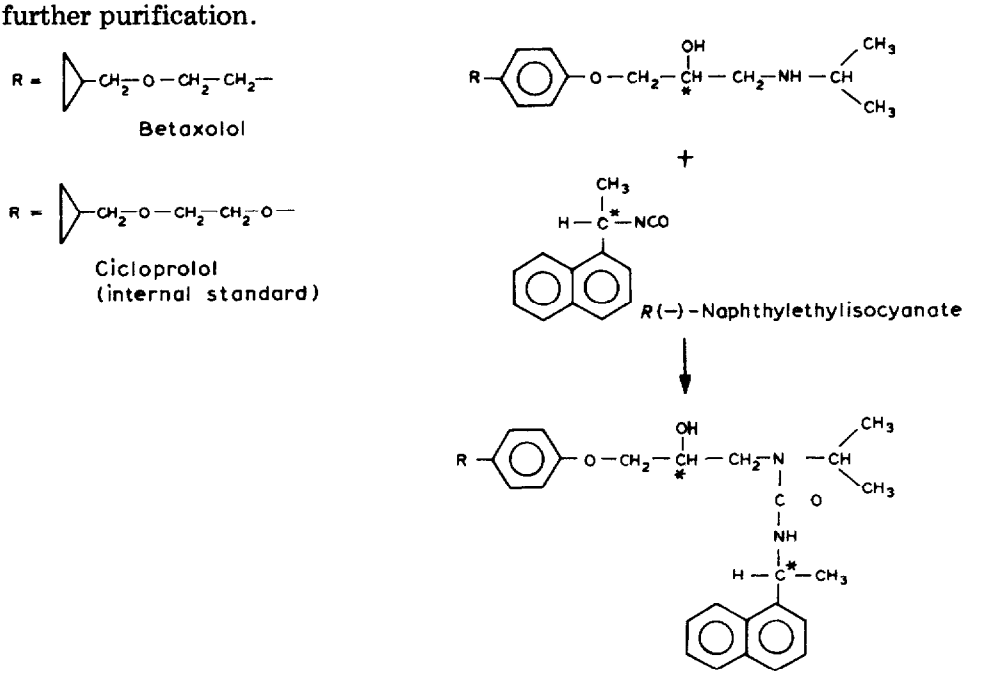

Fig. 1. Reaction of betaxolol, and its internal standard *S*(—)-cicloprolol, with *R*(—)-naphthylethylisocyanate to give the urea derivative.

HPLC instrumentation and conditions

The liquid chromatographic system consisted of a Sopares Constametric II G pump and a WISP 710B injector (Water Assoc.). A Kontron SFM 23 B fluorimeter was used as the detector. It was operated with an excitation wavelength of 285 nm and an emission wavelength of 330 nm.

The separation was carried out on a Hypersil ODS 3-μm column (150 × 4.6 mm I.D.; Shandon, Runcorn, U.K.) maintained at 36°C.

The mobile phase, 0.4% TEMED in water (pH 3)—methanol—tetrahydrofuran (34:52:14) was pumped through the column at a flow-rate of 1 ml/min; it was degassed in an ultrasonic bath for 2 min prior to analysis and was kept under helium.

Chromatographic peaks were integrated by a laboratory automation system (LAS) implemented on a Hewlett-Packard HP 1000 computer.

Extraction and derivatization procedure

Water (1 ml), 2 *M* sodium hydroxide (200 μl) and the internal standard *S*(−)-cicloprolol in methanol (75 μl) were added to 1 ml of blood. Betaxolol was then extracted with 7 ml of diethyl ether for 15 min. The tubes were centrifuged for 5 min at 1000 *g* (−20°C). The ether layer was collected and evaporated at 37°C under a stream of dry nitrogen. Then, 25 μl of 0.1% *R*(−)-NEI in methanol were added to the residue. After reaction at room temperature for 1.5 h, excess reagent was evaporated at 37°C. The betaxolol-*R*(−)-NEI derivatives, dissolved in 2 ml of Clark and Lubs buffer (boric acid—potassium chloride—sodium hydroxide; pH 10), were purified by extraction with chloroform (7 ml). After extraction for 5 min, and centrifugation for 5 min at 1000 *g* (4°C), the aqueous layer was discarded and the organic layer evaporated to dryness at 37°C under nitrogen. The samples were dissolved in 150 μl of 0.4% TEMED (pH 3)—methanol (50:50) and 100 μl were injected into the column.

RESULTS

Derivatization procedure

In preliminary experiments, *S*(−)-phenylethylisocyanate was used as the chiral reagent but, with this reagent, the limit of detection was not low enough to evaluate with sufficient precision the pharmacokinetics of betaxolol enantiomers. Diastereomeric derivatives of betaxolol were therefore prepared by reaction with *R*(−)-NEI (Fig. 1). This reagent, commercially available as a pure compound, increases fluorimetric detection, owing to its naphthyl moiety [10].

We studied the influence of temperature on the reaction kinetics of betaxolol with *R*(−)-NEI. At room temperature, the reaction seemed complete within 60 min but at 60°C a lower yield was obtained (Fig. 2). The amino group, and not the alcohol, reacts at room temperature to form the urea derivative since the derivative is not acid-extractable.

The nature of the derivative was confirmed by mass spectrometry. Under chemical-ionization mode with ammonia as the reagent gas, the MH^+ ion at *m/z* 505 corresponds to the addition of one NEI group (mol-wt. of betaxolol =

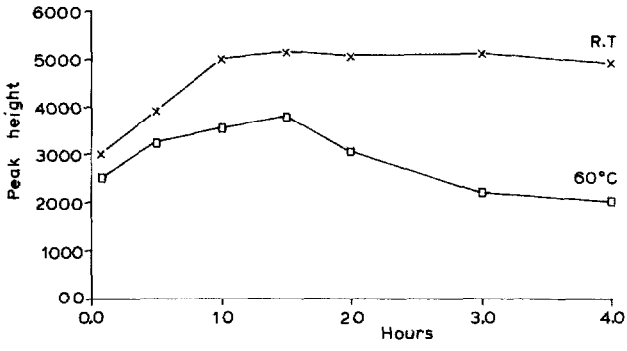

Fig. 2. Reaction kinetics of betaxolol with *R*(—)-naphthylethylisocyanate at room temperature (R.T.) and at 60°C.

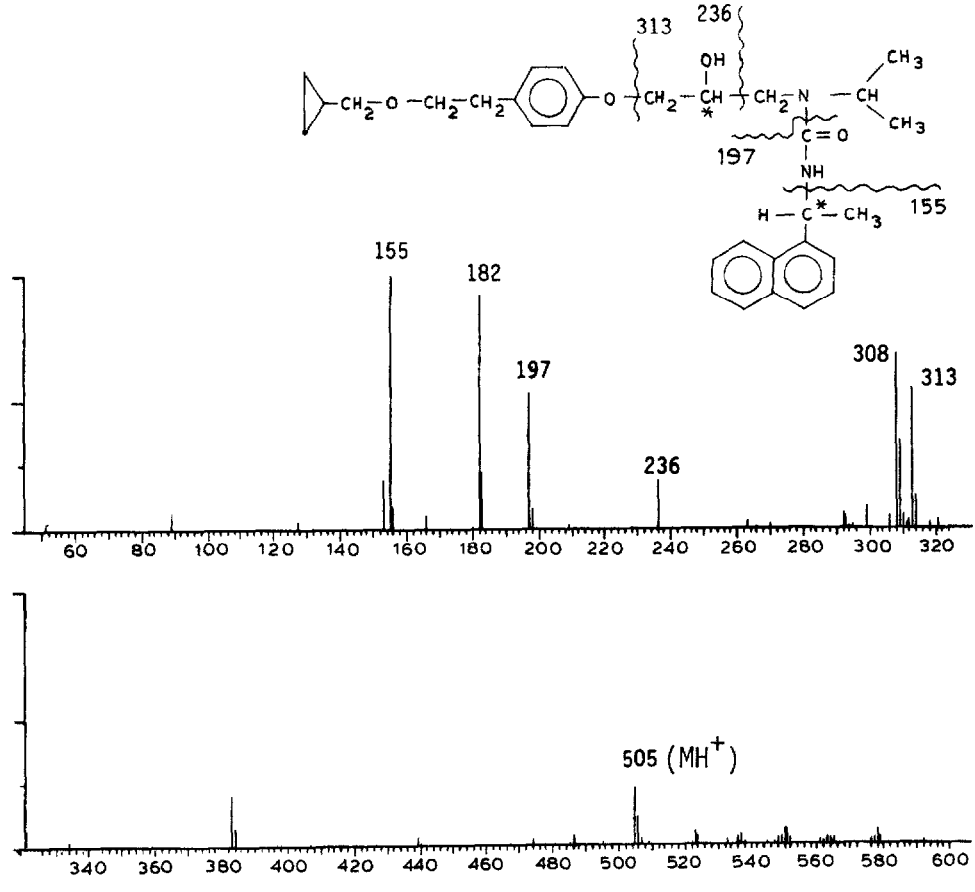

Fig. 3. Mass spectrum of the reaction product of betaxolol with *R*(—)-naphthylethylisocyanate obtained under chemical-ionization mode with ammonia as the reagent gas.

307). The fragment at *m/z* 236 and the absence of a fragment at *m/z* 72 (the latter being indicative of a free isopropylamino group) are in favour of a reaction occurring on the nitrogen. The fragmentation pattern is shown in Fig. 3.

Chromatographic separation

Baseline resolution of the betaxolol diastereomers was achieved with the chromatographic conditions described in the experimental section (Fig. 4).

A binary solvent system (water—methanol) was capable of separating the racemate, but the retention time was too long to be useful. Adjunction of tetrahydrofuran allowed the separation in about 10 min and addition of TEMED to the solvent further improved the chromatographic behaviour of the betaxolol derivatives. In order to avoid any untoward rise of head pressure observed with a 3-μm packing, separation was performed at 36° C.

The order of elution of the individual diastereomers was established by separate derivatization of each optically pure enantiomer. The (*S*)-enantiomer of betaxolol is eluted prior to the (*R*)-isomer.

In addition to betaxolol, other racemic β-blocking drugs, such as propranolol, metoprolol and alprenolol, can be resolved by this HPLC method with a slight modification of the tetrahydrofuran ratio in the mobile phase. In the chromatographic conditions described above, the retention times of the enantiomers were 7 and 7.5 min for metoprolol, 8 and 8.5 min for alprenolol and 11 and 12 min for propranolol.

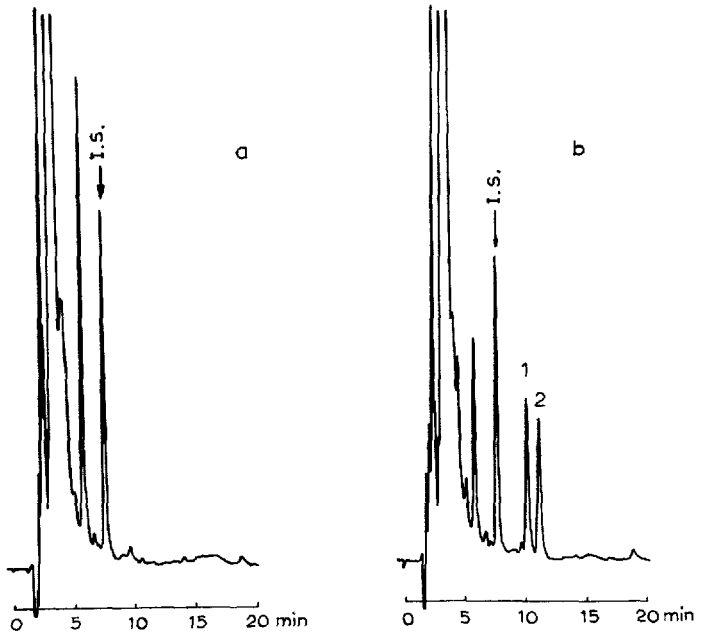

Fig. 4. HPLC separation of blood extracts derivatized with *R*(—)-naphthylethylisocyanate. (a) Blood spiked with 75 ng of the internal standard (I.S.), *S*(—)-cicloprolol; (b) blood spiked with the internal standard as in (a) and with 20 ng/ml of each of the betaxolol enantiomers. Peaks: 1 = *S*(—); 2 = *R*(+).

Determination of (R)- and (S)-betaxolol in human blood

The blood concentrations of (*R*)- and (*S*)-betaxolol were determined using the method described above. Optically pure (*S*)-cicloprolol was chosen as an internal standard in order to obtain only one reference peak.

No endogenous substance interferes with betaxolol or the internal standard

TABLE I

PRECISION AND ACCURACY OF THE DETERMINATION OF (*S*)- AND (*R*)-BETAXOLOL

Sample	Concentration found (ng/ml)			
	5 ng/ml added		25 ng/ml added	
	(*S*)	(*R*)	(*S*)	(*R*)
1	4.9	4.8	24.9	25.0
2	5.2	4.9	25.0	25.0
3	5.1	5.1	24.8	23.9
4	4.9	5.2	25.3	25.1
5	5.0	5.1	25.6	25.4
6	4.8	5.0	24.5	24.8
7	5.1	4.8	25.1	25.3
8	5.1	4.9	25.5	25.4
9	4.9	5.3	23.8	24.0
10	5.0	5.1	25.1	25.3
Mean	5.0	5.0	24.9	24.9
S.D.	0.12	0.17	0.52	0.54
S.E.M.*	0.04	0.05	0.16	0.17
C.V.** (%)	2.5	3.4	2.1	2.2

*S.E.M. = Standard error of the mean.
**C.V. = Coefficient of variation.

derivatives. Calibration curves used for quantitation of the enantiomers in blood, obtained by plotting peak heights against sample concentrations, were linear in the concentration range evaluated (1–50 ng/ml). Correlation coefficients were higher than 0.9992 for the two derivatives.

Accuracy and precision of the method were measured at two concentrations: 5 and 25 ng/ml. Accuracy is excellent, as shown in Table I, and precision varies between 2.1 and 3.4%.

Application to the pharmacokinetic profile of the enantiomers

A preliminary study on three subjects was carried out to demonstrate the applicability of the method. The subjects received an oral dose of 20 mg of racemic betaxolol in association with a diuretic, chlortalidone.

The blood concentrations of (*R*)- and (*S*)-betaxolol measured in the three subjects are shown in Fig. 5. No significant difference between the pharmacokinetic profiles of the two betaxolol enantiomers can be observed. It should be noted that 48 and 72 h after administration, concentrations are close to the limit of detection of the method (0.5 ng/ml) and the differences shown may be due to analytical variability. These results will be confirmed in another study.

DISCUSSION

The method described here allows the separation and determination of betaxolol enantiomers down to 0.5 ng/ml. Derivatization with NEI rather than

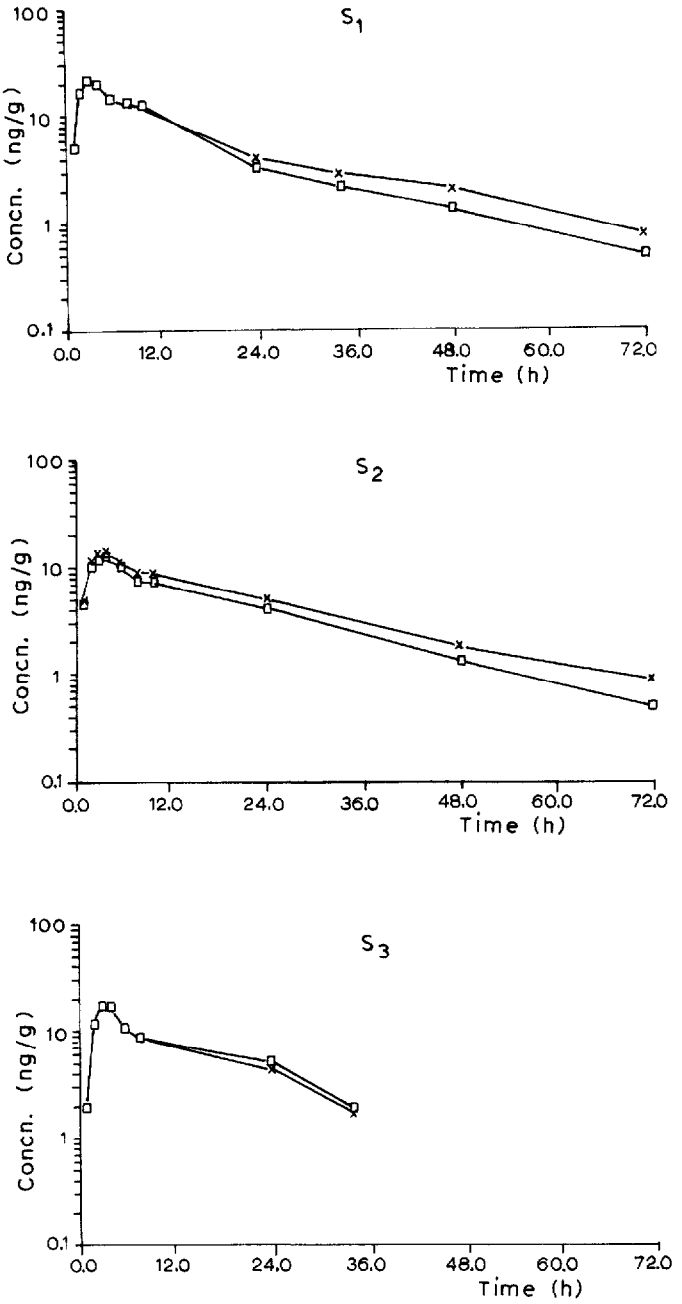

Fig. 5. Blood concentrations of (*R*)-betaxolol (□) and (*S*)-betaxolol (×) in three subjects S_1, S_2 and S_3 following oral administration of 20 mg of racemic betaxolol. Concentrations are expressed in ng/g, as blood samples were weighed.

phenylethylisocyanate makes it easier to achieve this sensitivity by fluorescence detection.

It is not yet clear why reaction at 60°C gives a lower derivatizaton yield. Both the amino and hydroxy groups of betaxolol may react with the

isocyanate and a di-derivative may be formed at 60°C as suggested by Wilson and Walle [13] with phenylethylisocyanate. Furthermore, following reaction at 60°C, two peaks were eluted with retention times of about 40 min. If these were the di-derivatives, they would be eluted later than the mono-derivative under reversed-phase chromatography.

The present method is applicable to the determination of other β-blockers, with a minimum adjustment in the composition of the mobile phase.

This preliminary study in three subjects indicates that blood concentrations of betaxolol enantiomers are virtually equal and there is no particular need for measurement of the (*S*)-isomer in blood since both enantiomers have the same pharmacokinetic profile. This is at variance with results observed with propranolol [14, 15], alprenolol and metoprolol [16], for which plasma concentrations of the (*S*)-enantiomer are higher than those of the (*R*)-enantiomer.

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