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Determination of tertatolol enantiomers in biological fluids by high-performance liquid chromatography

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ABSTRACT

A stereospecific high-performance liquid chromatographic method for the quantification of (−)- and (+)-tertatolol in plasma and urine is described. The method involves solid-phase extraction followed by derivatization with *S*(+)-naphthylethylisocyanate to form the urea derivative, which is more sensitive to fluorescence detection. The separation of the diastereomeric derivatives was performed by reversed-phase high-performance liquid chromatography. Fluorimetric detection ($\lambda_{excitation}$ = 220 nm, $\lambda_{emission}$ = 320 nm) allows the quantification of tertatolol enantiomers down to 6 ng/ml. The assay was used to study the pharmacokinetic profile of tertatolol enantiomers following oral administration of racemic tertatolol; preliminary results suggest enantioselective absorption and/or disposition of tertatolol.

INTRODUCTION

Tertatolol [S2395; (±)-hydroxy-2-*tert*.-butylamino-3-propyloxy)-8-thiochromane hydrochloride] is a powerful, long-acting and non-cardioselective β-blocker without partial agonistic activity [1]. Tertatolol is used clinically in the treatment of hypertension [2] and differs from other β-blockers in that it increases renal blood flow in hypertensive and normotensive patients [3]. Tertatolol is marketed as a racemic mixture of the two optical isomers. The (−) isomer is about 100-fold more potent than the (+) isomer; the β-adrenoreceptor blockade and renal vasodilatation induced by tertatolol have been demonstrated to be stereospecific for the (−) isomer [4]. Thus differences in the disposition of the enantiomers of tertatolol, as has been reported for other β-blockers [5,6], could be of therapeutic relevance.

Several analytical techniques facilitating investigation of the disposition of both enantiomers of β-blockers have been reported using radioimmunoassay [7], gas chromatography–mass spectrometry (GC–MS) assays with deuterium-labelled pseudoracemates [8] and high-performance liquid chromatography

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(HPLC) with a chiral stationary phase [9] or with a chiral counter-ion [10] or after chiral derivatization [11,12]. We report here an HPLC assay for the determination of both enantiomers of tertatolol in biological fluids. The method involves derivatization with an optically pure fluorescent reagent [*S*(+)-naphthylethylisocyanate (*S*(+)-NEI)] to enhance detection efficiency and to allow quantification of both enantiomers in the nanogram range.

EXPERIMENTAL

Chemicals and reagents

Racemic tertatolol and (+)-tertatolol (purity >99%), both as hydrochloride salts, were purchased from Institut de Recherches Internationales Servier (Suresnes, France). (−)-Alprenolol, used as internal standard, was purchased from Aldrich (Strasbourg, France). Its optical purity was higher than 99%. *S*(+)-NEI was purchased from Aldrich. The chemical purity of this reagent was higher than 99% and the optical purity higher than 99.5%. A working solution of 0.1% *S*(+)-NEI in dichloromethane was prepared. Other solvents and reagents used (diethyl ether, dichloromethane stabilized with methyl-2-butene, acetonitrile, 1 *M* sodium hydroxide, *tert.*-butylamine) were analytical-grade products from Merck (Darmstadt, Germany).

HPLC instrumentation and conditions

A Beckman Gold HPLC system was used, consisting of a 340 organizer injector, two 112 pumps and an analogical interface. The system was equipped with an HPLC column (Altex Ultrasphere XLODS, 7 cm × 4.6 mm I.D., 3 μm particle size). The detector was a Kratos FS970 fluorimeter which was operated at an excitation wavelength of 220 nm and an emission wavelength of 320 nm. The mobile phase, acetonitrile–water (40:60, v/v) was pumped through the column at a flow-rate of 2 ml/min.

Extraction

In a 10-ml tube 25 ng of alprenolol (internal standard) as an alcoholic solution of the hydrochloride salt were added. After evaporation of the organic solvent, 1.00 ml of plasma or urine and 100 μl of 1 *M* sodium hydroxide were added. The mixture was shaken on a vortex mixer for 1 min, then transferred to an Extrelut (Merck) column and extracted with 2 × 4 ml of diethylether. The ether collected was evaporated under a stream of dry nitrogen.

Derivatization procedure

A 100-μl aliquot of dichloromethane and 10 μl of 0.1% *S*(+)-NEI in dichloromethane were added to the residue. The tube was shaken for 1 min. After reaction at room temperature for 12 h, 10 μl of *tert.*-butylamine was added and the mixture evaporated to dryness under a light nitrogen stream. The residue was redis-

solved in 20 μl of acetonitrile and shaken on a vortex mixer for 1 min. A 20-μl aliquot of the sample was injected into the HPLC column for tertatolol enantiomer quantification.

Mass spectrometry

An LKB 2091 mass spectrometer operated under direct-probe electron-impact conditions was used. The mass spectrometer was scanned repetitively from 35 to 600 a.m.u. using an ionizing energy of 70 eV.

Standard solutions

A solution of (−)-alprenolol (1 ng/μl) in ethanol was prepared. A 25-μl aliquot of this solution was added to each sample as internal standard.

Solutions of the individual enantiomers as well as of racemic tertatolol were prepared to a concentration of 1 ng/μl hydrochloride salt in ethanol.

These solutions were stored at −20°C.

Calibration curves

Calibration curves were established by spiking blank plasma and urine with various quantities of racemic tertatolol and 25 ng/ml (−)-alprenolol. The prepared plasma and urine standards contained 6.4–128.3 ng/ml of each enantiomer.

Recovery from extraction

In order to study the recovery of each enantiomer of tertatolol from plasma and urine, 1 ml of blank plasma or urine spiked with known amounts of each enantiomer was extracted as described in the Extraction section. The internal standard was added after the extraction procedure and before derivatization of the samples.

Chromatographic characteristics of the assay

Selectivity (α) and resolution (R) factors were calculated for the peaks of interest based upon the following equations:

$$\alpha = (t_{R2} - t_{R0})/(t_{R1} - t_{R0})$$
$$R = 2(t_{R2} - t_{R1})/(w_1 + w_2)$$

where t_R and w are retention time and band width, respectively. Subscripts 0, 1 and 2 refer to the solvent peak and the first and second eluting enantiomers of tertatolol, respectively.

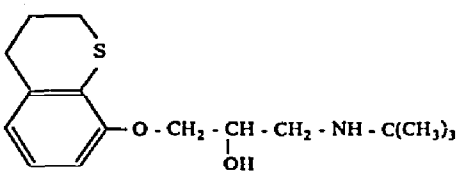
RESULTS AND DISCUSSION

In preliminary experiments GITC (2,3,4,6-tetra-O-acetylglucopyranosyl isothiocyanate) and PEI [S(−)-phenylethylisocyanate] were used as chiral derivatiz-

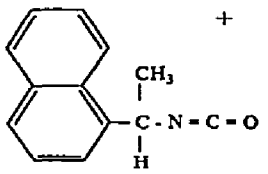
ing reagents. Separation of tertatolol enantiomers with GITC was not satisfactory. Furthermore, this reagent does not allow the quantification of tertatolol at low concentrations since it has no chromophore in its structure. Separation of tertatolol enantiomers after derivatization with PEI was satisfactory, but the quantification limit (100 ng/ml) was still inappropriate for any clinical pharmacokinetic analysis because maximum plasma concentrations of racemic tertatolol after administration of 5 mg of tertatolol are generally about 100 ng/ml [13]. Thus NEI derivatives were prepared with the expectation that the detection limit would be lower than with PEI. Chiral derivatization with this reagent has already been reported to increase the fluorimetric detection limits of other β-blockers [12].

The derivatization of tertatolol was carried out at room temperature for 12 h and led to the formation of the diastereomeric urea (Fig. 1).

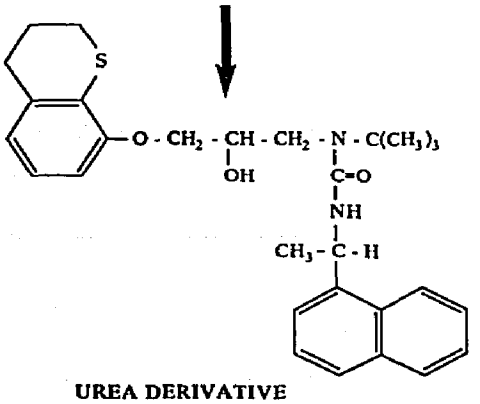
An indirect proof that NEI reacts with the secondary amino group rather than with the hydroxy group is that the derivative was not acid-extractable. To obtain definite proof of the nature of the derivatives they were first collected by HPLC



TERTATOLOL



S(+)NAPHTHYLETHYLISOCYANATE



UREA DERIVATIVE

Fig. 1. Reaction of tertatolol with *S*(+)-NEI leading to the urea derivative.

then extracted with dichloromethane and finally analyzed by direct-probe electron-impact mass spectrometry. The molecular ion was not observed, suggesting the loss of the tertiary butyl group [$C(CH_3)_3$] from the tertatolol structure: thus we obtained the fragment at m/z 435 (Fig. 2). The loss of the derivatizing group gave fragment 2, corresponding to tertatolol at m/z = 295, and fragment 4 at m/z = 198, corresponding to the entire derivatizing group. Two characteristic fragments of the derivatizing group are obtained at m/z 170 (fragmentation 5) and at m/z 155 (fragmentation 7). Moreover, two other characteristic ions were fragments 3 and 6 at m/z 213 and 166, respectively. In the case of fragments 2, 3 and 6, a rearrangement with H^+ had occurred in the ion source of the mass spectrometer. Thus there was an increase in one mass unit for these fragments. Fragment 3 was the one that confirmed the site of attachment of NEI on the tertatolol structure.

The derivatives were stable at room temperature for at least 24 h. The extent of derivatization established by analyzing the residual tertatolol was about 70% for each enantiomer.

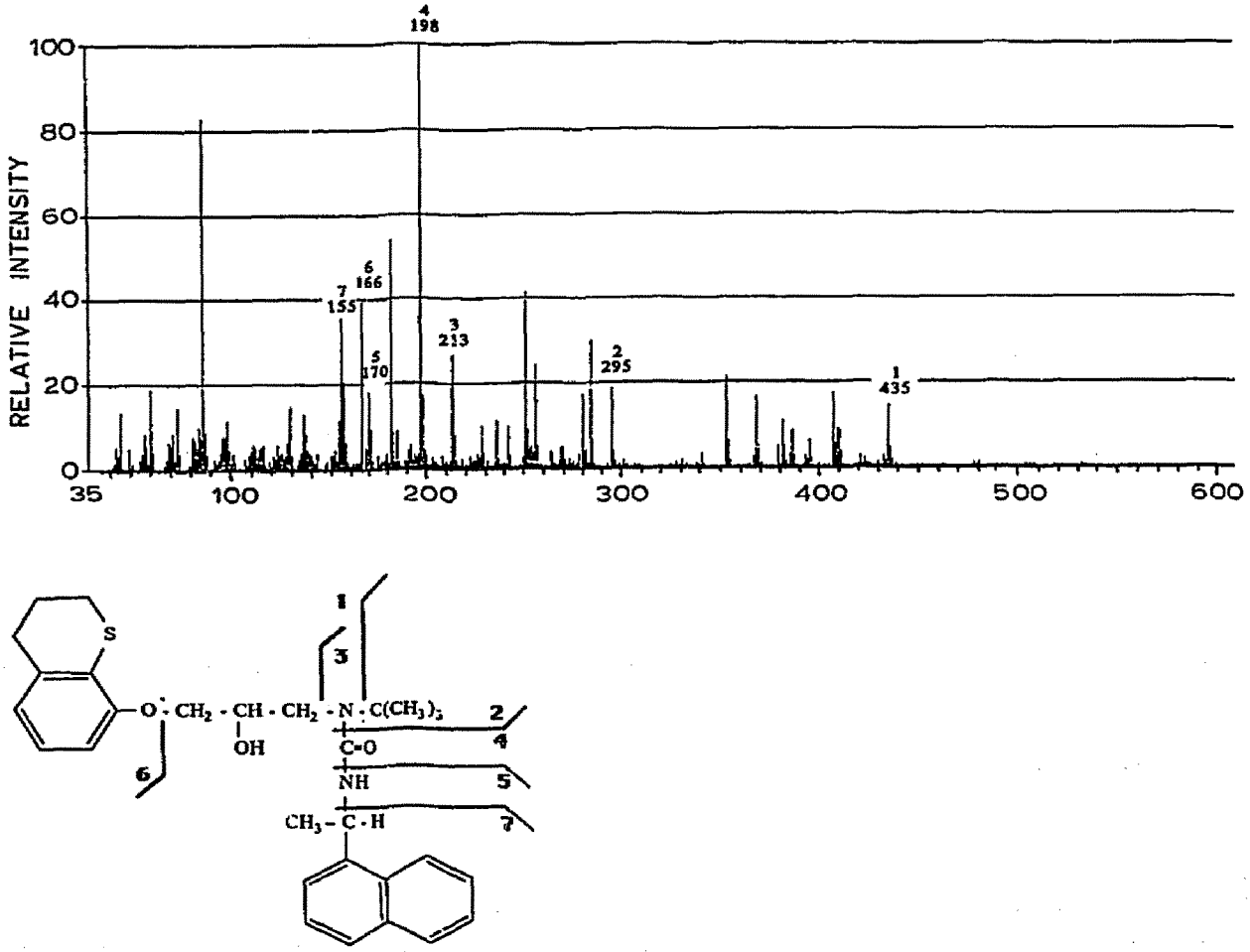


Fig. 2. NEI derivative mass spectrum: numbers from 1 to 7 indicate the sites of fragmentation.

Tertatolol was extracted from plasma and urine by solid-phase extraction, the recovery of extraction being >98% for (−)- and (+)-tertatolol from plasma and urine. Thus the efficiency of extraction of the method reported here is better than a previously described liquid extraction for GC–MS analysis of tertatolol [14]. The solid extraction was carried out with diethyl ether, which was found to be superior to dichloromethane, ethyl acetate and cyclohexane.

The analysis of the NEI-tertatolol derivatives performed on a 3-μm packing reversed-phase HPLC column revealed the presence of two peaks corresponding to the derivatives of the two enantiomers of tertatolol. The elution order was established by derivatization with *S*(+)-NEI of optically pure (+)-tertatolol: the diasteroisomer derived from the (−) enantiomer eluted first.

(−)-Alprenolol used as internal standard eluted just before the two enantiomers of tertatolol and was well resolved from the derivatives of (−)- and (+)-tertatolol. The peaks of the derivatives of tertatolol were resolved in about 20 min with baseline separation (selectivity = 1.12, resolution = 1.56). No substances interfering with either tertatolol diastereoisomers or with the internal standard [(−)-alprenolol] were found after either urine or plasma extraction (Fig. 3).

The good chromatographic separation of the diastereoisomers may be due to the pronounced conformational differences between them. The naphthyl group (a bulky and planar substituent) and the presence of the urea function lead to a good chromatographic selectivity [15].

Calculation of the concentration of (−)- and (+)-tertatolol was obtained from calibration curves established by spiking blank plasma and urine as described in the Experimental section. Good linear relationships ($r > 0.986$) were obtained between peak-area ratios [of (−) and (+) and enantiomers of tertatolol and internal standard] and corresponding concentrations in both plasma and urine. These standard curves gave the following equations: $y = (1.48 \pm 0.07) \cdot$

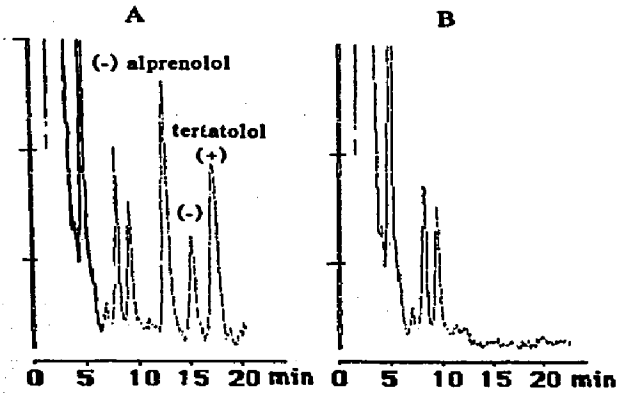


Fig. 3. Chromatograms obtained after extraction of racemic tertatolol followed by derivatization with *S*(+)-naphthylethylisocyanate. (A) Plasma sample containing 250 ng/ml racemic tertatolol; (B) blank plasma sample.

TABLE I

PRECISION (COEFFICIENT OF VARIATION, C.V.) AND ACCURACY (ERROR) OF THE ASSAY FOR PLASMA ($n=6$)

Added concentration (of each enantiomer) (ng/ml)	(−)-Tertatolol			(+)-Tertatolol		
	Calculated (ng/ml)	C.V. (%)	Error (%)	Calculated (ng/ml)	C.V. (%)	Error (%)
6.4	6.6±0.4	5.6	2.4	5.7±0.4	7.6	11.5
12.8	13.6±1.2	9.0	6.0	14.0±0.6	4.2	9.5
25.7	24.9±1.8	7.2	3.1	22.0±2.8	12.6	14.2
51.3	54.4±4.2	7.8	5.9	51.6±2.7	5.3	0.5
128.3	123.9±11.4	9.2	3.4	116.5±6.2	5.3	9.2

$10^{-4}\,x+(6.93\pm1.66)\cdot10^{-4}$ for the (−) enantiomer and $y=(2.10\pm0.10)\cdot10^{-4}\,x+(0.97\pm3.58)\cdot10^{-4}$ for the (+) enantiomer in plasma and $y=(2.34\pm0.19)\cdot10^{-4}\,x+(1.91\pm3.26)\cdot10^{-3}$ for the (−) enantiomer and $y=(3.50\pm0.43)\cdot10^{-4}x+(-1.62\pm4.40)\cdot10^{-3}$ for the (+) enantiomer in urine. The intercept value was not significantly different from 0. The results of assay precision and accuracy are reported for plasma and urine in Tables I and II. The quantification limit was about 6 ng/ml.

Applications

The enantioselective assay was used to quantify therapeutic concentrations of tertatolol enantiomers in the plasma of one healthy subjet after a single oral dose of 5 mg of racemic tertatolol. The plasma concentrations of (−)- and (+)-tertatolol showed that the concentration of the (−) enantiomer was always higher than that of the (+) enantiomer in this subject (Fig. 4).

TABLE II

PRECISION (COEFFICIENT OF VARIATION, C.V.) AND ACCURACY (ERROR) OF THE ASSAY FOR URINE ($n=6$)

Added concentration (of each enantiomer) (ng/ml)	(−)-Tertatolol			(+)-Tertatolol		
	Calculated (ng/ml)	C.V. (%)	Error (%)	Calculated (ng/ml)	C.V. (%)	Error (%)
6.4	5.6±0.9	15.4	12.5	6.0±0.8	13.9	5.7
12.8	13.4±1.1	8.6	4.6	12.8±0.2	1.2	0.3
25.7	23.3±1.2	5.2	9.3	23.6±1.4	5.8	8.2
51.3	55.1±5.6	10.2	7.2	52.1±5.4	10.4	1.6
128.3	126.9±5.2	4.1	1.05	128.1±2.6	2.0	0.1

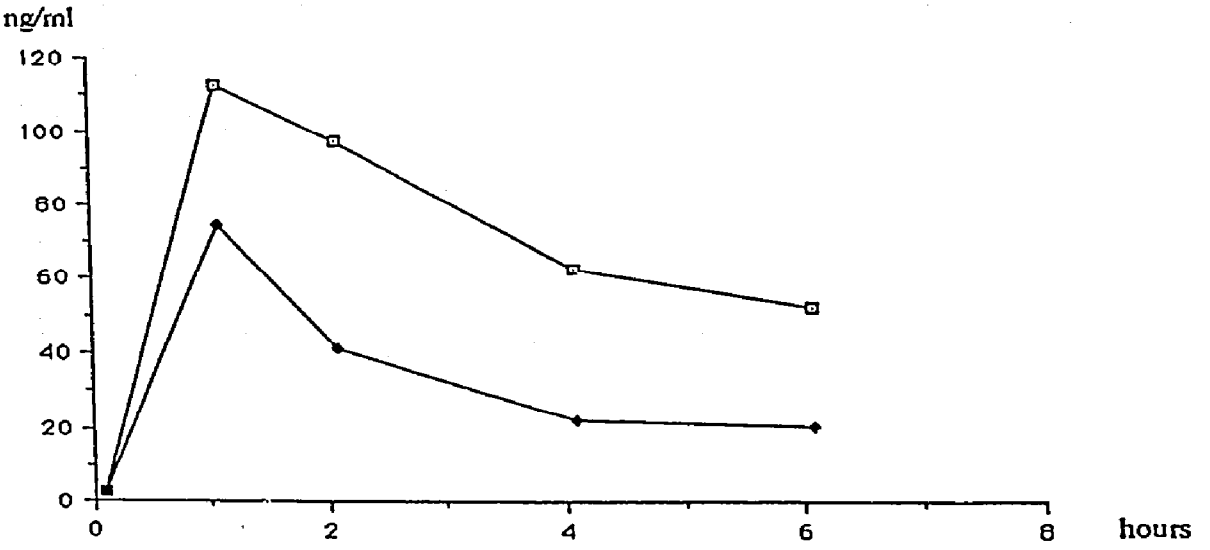


Fig. 4. Plasma concentrations of (−)-tertatolol (□) and (+)-tertatolol (◆) in one healthy subject after a single oral dose of 5 mg of racemic tertatolol.

Such stereoselective pharmacokinetics has been reported for the most lipophilic β-blocking drugs [16].

In conclusion, a sensitive indirect enantioselective HPLC assay for the analysis of tertatolol is now available. In a preliminary study, pharmacokinetic differences between (+) and (−) enantiomers of tertatolol in plasma and urine have been found, suggesting faster elimination and/or decreased availability for the (+) enantiomer.

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