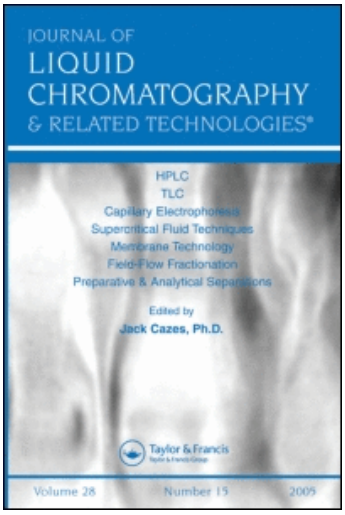

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DETERMINATION OF 4-HYDROXYTERTATOLOL STEREOISOMERS IN RAT AND HUMAN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A stereospecific high-performance liquid chromatographic method for the simultaneous determination of the four stereoisomers of 4-hydroxytertatolol, an active metabolite of tertatolol, in human and rat urine is described. The method is based on solid-phase extraction of urine followed by derivatization with S(+)-naphtylethylisocyanate. The four stereoisomers were resolved by reversed phase high-performance liquid chromatography and detected by fluorescence ($\lambda_{excitation}$ = 210 nm, no cutoff emission filter). The analytical method is suitable for the simultaneous determination of the four stereoisomers of 4-hydroxytertatolol in rat and human urine down to 22.0 ng/ml. Preliminary results in rat suggest stereoselective hydroxylation of tertatolol enantiomers.

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INTRODUCTION

Tertatolol (fig. 1) (± - (hydroxy - 2' - tertbutylamino- 3' -propyloxy) -8 - thiochromane , hydrochloride) is a powerful , long acting and non cardioselective beta-blocker without partial agonistic activity (1). Tertatolol differs from other beta-blockers in that it increases renal blood flow in hypertensive and normotensive patients (2). In human and in rat, about 5 % of the oral administered dose of tertatolol is converted to 4-hydroxytertatolol (fig. 1), (± - (hydroxy - 2' - tertbutylamino- 3' -propyloxy) -8 - hydroxy -4 - thiochromane , acetat). There are two asymmetric carbons in this metabolite, making four stereoisomers to be separated. The beta-adrenoreceptor antagonist activity of this hydroxylated metabolite is closely related to that of tertatolol but 4-hydroxytertatolol is devoid of any significant renal vasodilator effect (3).

Analytical method allowing the determination of the two enantiomers of tertatolol and the simultaneous determination of the sum of both enantiomers as well as the sum of the four stereoisomers of 4-hydroxytertatolol has already been developed (4, 5). In order to evaluate the pharmacokinetics of 4-hydroxytertatolol stereoisomers and the substrate and product stereoselectivity of the metabolism of tertatolol, a stereoselective analytical method allowing the quantitative determination of the four stereoisomers of 4-hydroxytertatolol was needed.

We report here an HPLC assay for the simultaneous determination of the four stereoisomers of 4-hydroxytertatolol in rat and human urine. The method involves derivatization with an optically pure fluorescent reagent [S(+)-naphtylethylisocyanate (S(+)-NEI)], which has already been used for the determination of tertatolol enantiomers (4), and fluorimetric detection to allow quantification of the four stereoisomers in the nanogram range.

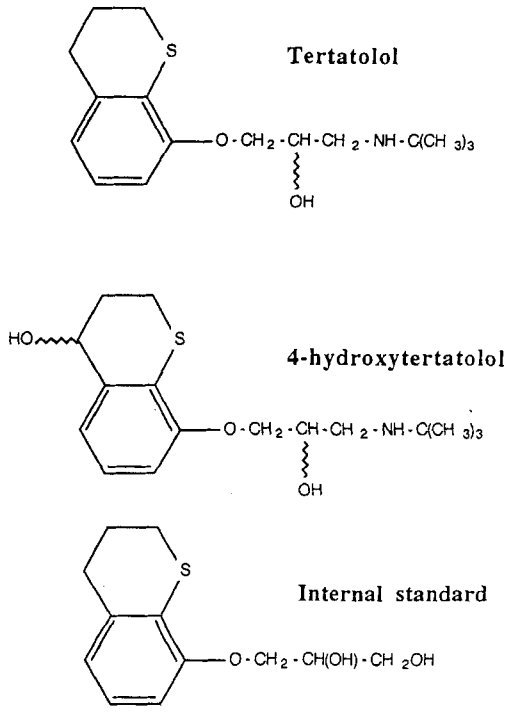

Fig. 1. Structures of tertatolol, 4-hydroxytertatolol and the internal standard. The wavy bonds indicate the presence of both configurations at each asymetric center.

MATERIAL AND METHODS

Chemicals and reagents

4-hydroxytertatolol and the four stereoisomers, as acetate salts, were purchased from Institut de Recherches Internationales Servier (Suresnes, France). The chemical purity were higher than 99% and 95% for racemic 4-hydroxytertatolol and the four stereoisomers, respectively. The optical purity of the four stereoisomers was higher than 90%. The four stereoisomers are designated (1, -) OHT, (2, -)

OHT for the two stereoisomers coming from (-)-tertatolol and (2, +) OHT and (1, +) OHT for the two stereoisomers coming from (+)-tertatolol ; as the configuration of the carbon in position 4 is unknown, the two possible configurations were arbitrarily designated 1 and 2 (fig. 1). Racemic (thiochromannyl-8 oxy)-3 propanediol-2,3, purchased from Institut de Recherches Internationales Servier (Suresnes, France) was used as internal standard (fig. 1). Its chemical purity was higher than 99.5 %. 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 0.03% S(+)-NEI in dichloromethane was prepared. Other solvents and reagents used (diethylether, dichloromethane stabilized with methyl-2-butene, acetonitrile, 1M sodium hydroxide, *tert.*-butylamine) were analytical-grade products from Merck (Darmstadt, Germany).

HPLC instrumentation and conditions

A Beckmann 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 (5 µm Licrospher, 15 cm x 4.6 mm I.D., Merck, Darmstadt, Germany). The detector was a Kratos FS970 fluorimeter which was operated at an excitation wavelength of 210 nm. No cutoff filter was used. The stereoisomers were eluted in a gradient of acetonitrile-water (10:90, v/v to 40:60, v/v in 15 min). The mobile phase was pumped through the column at a flow rate of 1.5 ml/min. Chromatography was carried out at room temperature (≈ 20°C).

Extraction

In a 10-ml tube, 100 ng of internal standard as an alcoholic solution were added. After evaporation of the organic solvent, 0.5 ml of rat urine or 1.00 ml of human

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, Darmstadt, Germany) column and extracted with 6 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 0.03% S(+)-NEI in dichloromethane were added to the residue. The tube was shaken during 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 redissolved 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 4-hydroxytertatolol stereoisomer quantification.

Standard solutions

A solution of internal standard (10 ng/µl) in ethanol was prepared. A 10-µl aliquot of this solution was added to each sample as internal standard.

Solutions of the individual stereoisomers of 4-hydroxytertatolol, as their acetate salts, were prepared to a concentration of 1 ng/ml (of the free base) in ethanol.

The stereoisomers and the internal standard in ethanolic solutions were stable for several months at -20°C.

Calibration curves

Calibration curves were established by spiking blank urine with various quantities of the individual stereoisomer of 4-hydroxytertatolol and 100 ng/ml internal standard. The prepared urine standards contained 22.0 - 440.0 ng/ml of each stereoisomer.

Extraction recovery

In order to study the recovery of each stereoisomer of 4-hydroxytertatolol from urine, 1 ml of blank urine with known amounts of each stereoisomer was extracted as described above. The internal standard was added after the extraction procedure of the samples.

Application to rat urine samples

Male wistar rats (n = 6), eight weeks old and weighing about 250 g, were used. The rats were acclimated to the laboratory conditions one week before the beginning of the study. They had free access to water during the study, but were fasted overnight before dosing and during the study. Racemic tertatolol in aqueous solution was given orally at a dose of 30 mg/kg by gavage. For urine collection, the rats were put in different metabolic cages and urine and feces were collected separately over a 24 h period.

RESULTS AND DISCUSSION

HPLC for the simultaneous determination of tertatolol enantiomers after derivatization with S(+)-NEI, has already been described (4). 4-hydroxytertatolol is an active metabolite of tertatolol with two chiral centers making four possible stereoisomers to be separated. Therefore an analytical procedure using HPLC for the simultaneous quantification of these four stereoisomers was investigated.

In preliminary experiments we attempted a simultaneous separation of tertatolol enantiomers and 4-hydroxytertatolol stereoisomers. Due to the difference in lipophilicity between tertatolol and 4-hydroxytertatolol, it has not been possible to

attain our goal with the octadecyl column used for the separation of tertatolol enantiomers. A cyano column gave better results allowing the simultaneous separation of the two enantiomers of tertatolol and a partial separation of two peaks corresponding to 4-hydroxytertatolol containing (1, -) OHT and (2, -) OHT for the first peak and (2, +) OHT and (1, +) OHT for the second one (fig. 2). The two additional peaks eluting before the two peaks of 4-hydroxytertatolol were corresponding to the tertatolol sulfoxide metabolites. This separation of the stereoisomers of 4-hydroxytertatolol was not sufficient to study the stereoselectivity of hydroxylation of tertatolol enantiomers in position 4. Thus, a procedure similar to that describe for tertatolol enantiomers was developed. The method is based on the extraction of urine by Extrelut®. The extraction yields for the four stereoisomers of 4-hydroxytertatolol was about 98% using diethylether as the extraction solvent. The derivatization of the stereoisomers was carried out with S(+)-NEI at room temperature for 12 h as described for tertatolol enantiomers but with three times lower amounts of reagent in order to avoid chromatographic interferences with the stereoisomers of 4-hydroxytertatolol. Nevertheless, the extent of derivatization of each stereoisomer, determined after analyzing residual 4-hydroxytertatolol by GC/MS (5), was still acceptable (≈ 70%) and close to that obtain for tertatolol enantiomers (4). The internal standard, with a secondary and primary alcoholic functions, did not react with S(+)-NEI in the derivatization conditions.

The separation was achieved by gradient elution with acetonitrile and water. Under the chromatographic conditions described above, the retention times of the four derivatives of 4-hydroxytertatolol were respectively 25.3, 26.1, 27.0 and 28.8 min for (1, -) OHT, (2, -) OHT, (2, +) OHT and (1, +) OHT ; the retention time of the internal standard being about 12 min. The chromatograms of a drug-free rat

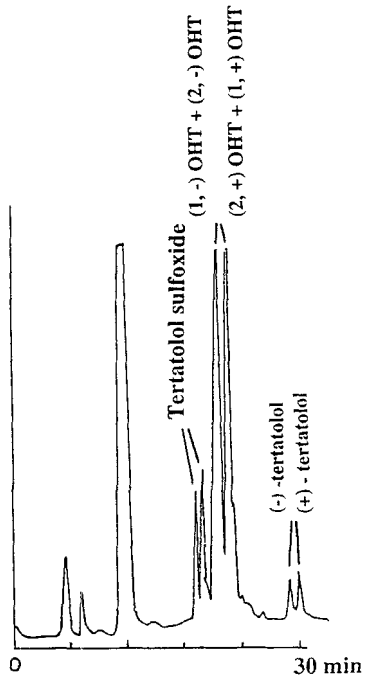

Fig. 2. Separation of the NEI derivatives of tertatolol, 4-hydroxytertatolol and tertatolol sulfoxide on a cyano column (3μm Ultrasphere cyano 7 cm x 4.6 mm i.d., Beckman); mobile phase : acetonitrile-water (10:90, v/v to 35:65, v/v in 30 min, flow rate = 1.5 ml/min). The chromatogram was obtained using pure solutions.

urine sample (0.5 ml) and a sample (0.5 ml), containing 500 ng/ml of 4-hydroxytertatolol, are shown in fig. 3. The extract of the blank urine sample contained no interfering peak.

The accuracy and precision of the method were evaluated from six calibration curves obtained by analysing spiked urine samples. Five different concentrations were used for each calibration curve. The calibration curves were linear ($r > 0.995$) in the range 22.0 - 440.0 ng/ml for the four stereoisomers of 4-hydroxytertatolol.

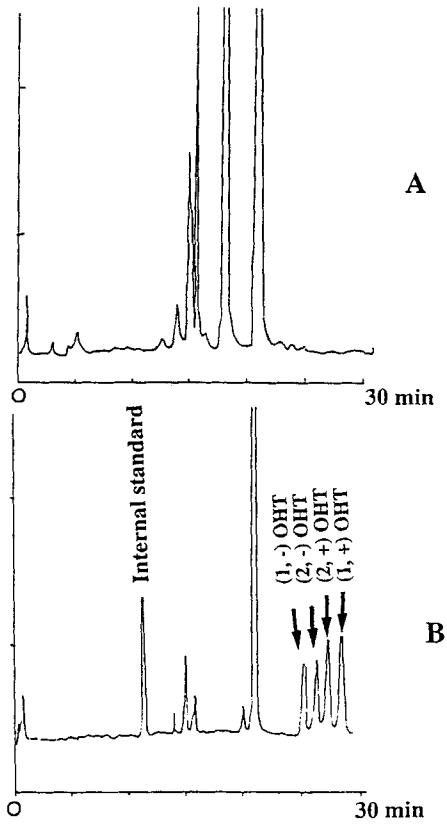

Fig. 3. Chromatograms of extract of drug-free rat urine sample (A) and of 0.5 ml rat urine sample containing 500 ng/ml 4-hydroxytertatolol (**B**). The samples were processed as described under "Experimental".

The standard curves gave the following equations : $y = (1.58 \pm 0.29).10^{-3}x + (-5.76 \pm 14.8).10^{-3}$ for (1, -) OHT, $y = (2.31 \pm 0.13).10^{-3}x + (-1.79 \pm 0.95).10^{-2}$ for (2, -) OHT, $y = (2.66 \pm 0.17).10^{-3}x + (-1.52 \pm 0.60).10^{-2}$ for (2, +) OHT and $y = (3.85 \pm 0.28).10^{-3}x + (0.12 \pm 0.22).10^{-2}$ for (1, +) OHT. The results of assay precision and accuracy are given in tables I and II for human urine. The small intercept value and the absence of a signal in the analysis of blank samples demonstrated the selectivity of the method.

TABLE 1

Precision (coefficient of variation, C.V.) and accuracy (error, E.R.) of the assay of human urine for (1, -) OHT and (2, -) OHT (n = 6)

Added concentration of each stereoisomer (ng/ml)	(1, -) OHT			(2, -) OHT		
	Calculated (ng/ml)	C.V. (%)	E.R. (%)	Calculated (ng/ml)	C.V. (%)	E.R. (%)
22.0	22.2 ± 0.2	1.0	0.9	21.8 ± 0.9	4.3	0.7
55.0	54.1 ± 3.0	5.6	1.5	53.0 ± 5.8	11.0	3.6
110.0	104.9 ± 4.7	4.5	4.6	111.1 ± 8.2	7.4	1.0
220.0	236.6 ± 16.6	7.0	7.5	206.5 ± 11.8	5.7	6.1
440.0	438.6 ± 18.2	4.0	0.3	436.5 ± 13.4	3.1	0.8

TABLE 2

Precision (coefficient of variation, C.V.) and accuracy (error, E.R.) of the assay of human urine for (1, +) OHT and (2, +) OHT (n = 6)

Added concentration of each stereoisomer (ng/ml)	(1, +) OHT			(2, +) OHT		
	Calculated (ng/ml)	C.V. (%)	E.R. (%)	Calculated (ng/ml)	C.V. (%)	E.R. (%)
22.0	22.4 ± 0.4	1.9	1.7	21.5 ± 1.0	4.8	2.1
55.0	51.9 ± 3.4	6.6	5.5	51.4 ± 8.3	16.1	6.5
110.0	114.5 ± 9.5	8.2	4.1	115.6 ± 8.3	7.2	5.1
220.0	224.8 ± 12.1	5.4	2.1	231.6 ± 29.0	12.5	5.3
440.0	444.6 ± 16.4	3.7	1.0	441.6 ± 19.0	4.3	0.4

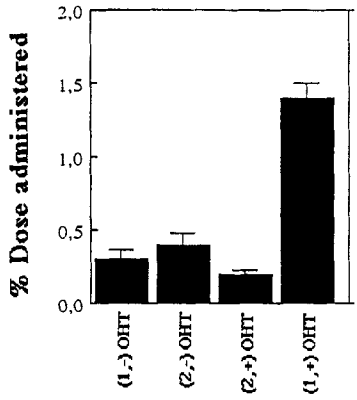

Fig. 4. Stereochemical composition of 4-hydroxytertatolol in rat urine after a single oral dose of 30 mg/kg of racemic tertatolol. The amount of each stereoisomer excreted in urine is expressed as the percentage (mean ± SD, n = 6) of the dose of racemic tertatolol administered.

The method was applied successfully to the simultaneous determination of the four stereoisomers in human and rat urine samples for pharmacokinetic and metabolic studies. The stereochemical composition of 4-hydroxytertatolol in rat urine is presented in fig. 4. As can be seen, the introduction of the hydroxyl group was stereoselective, favouring (1, +)-OHT with a high stereoselectivity [(1, +)-OHT / (2, +)-OHT ≈ 6]. The hydroxylation of (-)-tertatolol was only slightly stereoselective in rats [(1, -)-OHT / (2, -)-OHT ≈ 0.6].

In summary, the analytical method described permits the simultaneous determination of the four stereoisomers of 4-hydroxytertatolol in human and rat urine at concentrations down to 22.0 ng/ml. The method is suitable for pharmacokinetic and metabolic studies, the complete of which will be published later. In a preliminary study, (1, +) OHT seems to be quantitatively the major stereoisomer of 4-hydroxytertatolol in rat urine.

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