Journal of Chromatography, 421 (1987) 360–366
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3817

Note

Simultaneous quantitative determination of tertatolol and its hydroxylated metabolite in human plasma and urine by gas chromatography–mass spectrometry

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(First received April 9th, 1987; revised manuscript received June 5th, 1987)

4-Hydroxytertatolol (S-10950), *dl*-1-*tert.*-butylamino-3-(4-hydroxythiochroman-8-yloxy)-2-propanol (Fig. 1), is an active metabolite of tertatolol (S-2395, Institut de Recherches Servier, Suresnes, France), a new non-cardioselective β-blocking agent devoid of intrinsic sympathomimetic activity and shown to exert beneficial effects on renal perfusion [1–6].

We have previously reported a gas chromatographic–mass spectrometric (GC–MS) method for the quantitative determination of tertatolol in human biological fluids [7]. We report here an improved modification of this method, which allows the simultaneous quantitative determination of tertatolol and its 4-hydroxylated metabolite in human biological fluids by GC–MS.

EXPERIMENTAL

Chemical and reagents

Pure tertatolol hydrochloride, 4-hydroxytertatolol acetate and [$^2H_9$]4-hydroxytertatolol acetate were supplied by Technologie Servier (Orleans, France) and [$^2H_9$]tertatolol was synthesized by Dr. R. Wolf (Institut de Chimie, Strasbourg, France) (Fig. 1). The isotopic purity of the deuterated derivatives was greater than 99.8%.

N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce, Rotterdam, The Netherlands) was purchased from Spiral (Dijon, France). The *Helix pomatia* extract, purchased from IBF (Clichy, France), contained $1 \cdot 10^5$ F.U./ml (F.U. = Fishman units of glucuronidase activity; 200 F.U. = 1 I.U.) and $1 \cdot 10^6$ R.U./ml (R.U. = Roy units of sulphatase activity).

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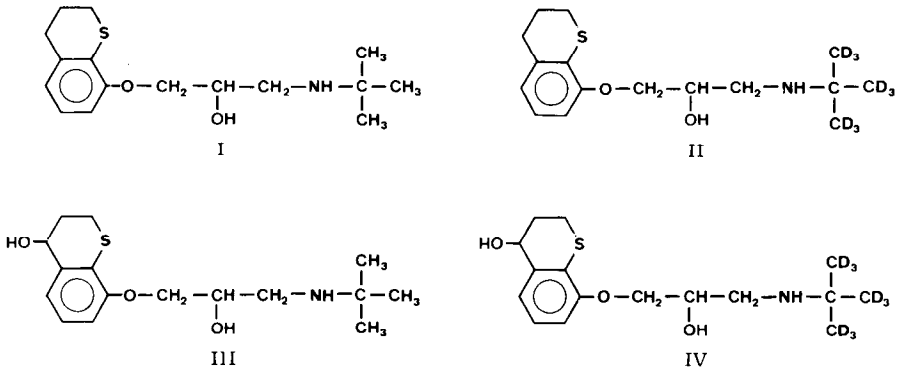

Fig. 1. Structures of tertatolol (I), [$^2H_9$]tertatolol (II), 4-hydroxytertatolol (III) and [$^2H_9$]4-hydroxytertatolol (IV).

All the other solvents and reagents used were analytical-grade products from E. Merck (Darmstadt, F.R.G.).

Instrumentation

A Nermag R-10-10 GC–MS system was used, under the control of a SIDAR data system (Delsi Instruments, Rueil-Malmaison, France).

The gas chromatograph was equipped with a 2.1 m×2 mm I.D. glass column, packed with 3% SE-30 on 100–120 mesh Chromosorb W AW DMCS. Helium was used as the carrier gas at a flow-rate of 20 ml/min. The injector, oven and interface temperatures were set at 270, 260 and 270°C, respectively.

The mass spectrometer was operated in the positive-ion chemical ionization mode with ammonia as the reagent gas. The following ions were monitored: *m/z* 368 for tertatolol, *m/z* 377 for [$^2H_9$]tertatolol, *m/z* 456 for 4-hydroxytertatolol and *m/z* 465 for [$^2H_9$]4-hydroxytertatolol, which correspond to the quasi-molecular ions of the trimethylsilyl (TMS) derivatives of these compounds. All quantifications were based on peak-area calculations.

Standard solutions and internal standards

The deuterated derivatives in ethanolic solution proved to be stable for a long period (several months) at −20°C. From these stock solutions, a working solution of each internal standard (1 ng/μl) in ethanol was prepared. A 100-μl (100 ng) volume of each of the latter solutions was added to each sample as internal standards.

Standard ethanolic solutions of tertatolol and 4-hydroxytertatolol were prepared at various concentrations and used for the preparation of short-, medium- and wide-range calibration curves, according to the expected sample concentrations.

Extraction and derivatization procedure

Volumes of 100 μl each of both internal standard solutions were added to a disposable 10-ml screw-capped tube with a PTFE washer and the organic solvent

was evaporated under nitrogen. Aliquots of 1 ml of plasma or urine were added. The samples were vortexed, allowed to stand for 10–15 min and rendered alkaline with 500 μl of 28% ammonia solution. Diethyl ether (3 ml) was added and the tubes were shaken manually for 3 min and centrifuged at 1200 *g* for a further 3 min. The organic phase was transferred into a second tube and the extraction was repeated with 3 ml of diethyl ether. The organic phases were combined, 2 ml of 1 *M* hydrochloric acid were added to the combined phases and the extraction was repeated as before. The organic phase was carefully discarded. Finally, the aqueous phase was rendered alkaline with 500 μl of 28% ammonia solution and extracted with 4 ml of diethyl ether as before. The organic phase was transferred into a 5-ml open-top screw-capped tube with a PTFE washer and evaporated to dryness under a gentle flow of nitrogen. A 25-μl volume of BSTFA was then added to the residue and after overnight derivatization at 60 °C, an aliquot of 1–2 μl was finally injected into the GC–MS system.

For the determination of the conjugated drug, 50 μl of 1 *M* hydrochloric acid, 500 μl of 0.1 *M* phosphate buffer (pH 4.1) and 100 μl of *Helix pomatia* extract were successively added to 1 ml of urine. The mixture was incubated at 37 °C for 8 h and then rendered alkaline by the addition of 500 μl of 28% ammonia solution and analysed as described above.

The conjugated drug concentration was calculated from the difference between the free and total drug concentrations after hydrolysis.

Extraction recovery and reproducibility study procedure

In order to study the recovery of tertatolol and 4-hydroxytertatolol from plasma and urine, using diethyl ether as extraction solvent, 1 ml of blank plasma or urine spiked with known amounts of both compounds, was extracted as described above. The internal standards (100 ng of each) were added before derivatization of the samples.

The same procedure as before was followed for the reproducibility study, except that known amounts of both internal standards were added before the extraction procedure.

Calibration curves

Calibration curves were established by spiking blank plasma and urine with various amounts of tertatolol and 4-hydroxytertatolol in the presence of fixed concentrations of the corresponding internal standards. Calibration curves were also established using pure solutions (i.e., without extraction).

RESULTS AND DISCUSSION

GC–MS analysis provides high sensitivity and selectivity and has already been used for the simultaneous determination of other β-blocking drugs and their metabolites [8,9]. 4-Hydroxytertatolol is an active metabolite of tertatolol; therefore an analytical procedure using this method for the simultaneous quantification of these two molecules in biological fluids was investigated.

TABLE I

EXTRACTION YIELDS FOR DIFFERENT CONCENTRATIONS OF TERTATOLOL AND 4-HYDROXYTERTATOLOL USING DIETHYL ETHER AS SOLVENT

Intitial concentration (ng/ml)	Concentration recovered (mean ± S.D., $n = 10$) (ng/ml)	Extraction yield (%)
Tertatolol		
4.5*	3.6 ± 0.6	80.4
90.0*	88.1 ± 3.8	97.9
10.5**	6.7 ± 0.6	63.8
891.3**	657.7 ± 80.1	73.8
4-Hydroxytertatolol		
8.6*	4.9 ± 0.4	57.3
85.5*	59.9 ± 2.6	70.1
9.7**	6.0 ± 0.4	61.9
890.1**	546.3 ± 34.7	61.4

*Concentration initially present in plasma.
**Concentration initially present in urine.

TABLE II

REPRODUCIBILITY OF THE OVERALL ANALYTICAL METHOD USING BLANK URINE SAMPLES

Theoretical concentration (ng/ml)	Concentration recovered (mean ± S.D., $n = 10$) (ng/ml)	Coefficient of variation (%)
Tertatolol		
9.0	8.7 ± 0.5	5.5
99.0	99.8 ± 3.2	3.2
700.0	696.8 ± 38.6	5.5
4-Hydroxytertatolol		
10.0	10.5 ± 0.5	4.9
100.0	96.7 ± 2.1	2.2
1000.0	1014.5 ± 34.0	3.4

The difference in lipophilicity between the parent molecule and its hydroxylated metabolite requires an extraction solvent which sufficiently extracts both molecules from the biological fluids with minimal amounts of interfering substances. Of several solvents tested [10] (e.g. benzene, ethyl acetate, chloroform and dichloromethane) and different mixtures of them, diethyl ether proved to be the best. In addition, diethyl ether has already been used for the extraction of metabolites of other β-blocking agents from biological fluids [11]. Extraction by Extrelut (Merck) was also tested using the same solvents, but the triple manual extraction gave better results (less interfering substances).

The extraction yields for different concentrations of tertatolol and 4-hydroxytertatolol, using diethyl ether as the solvent, are shown in Table I. The greater

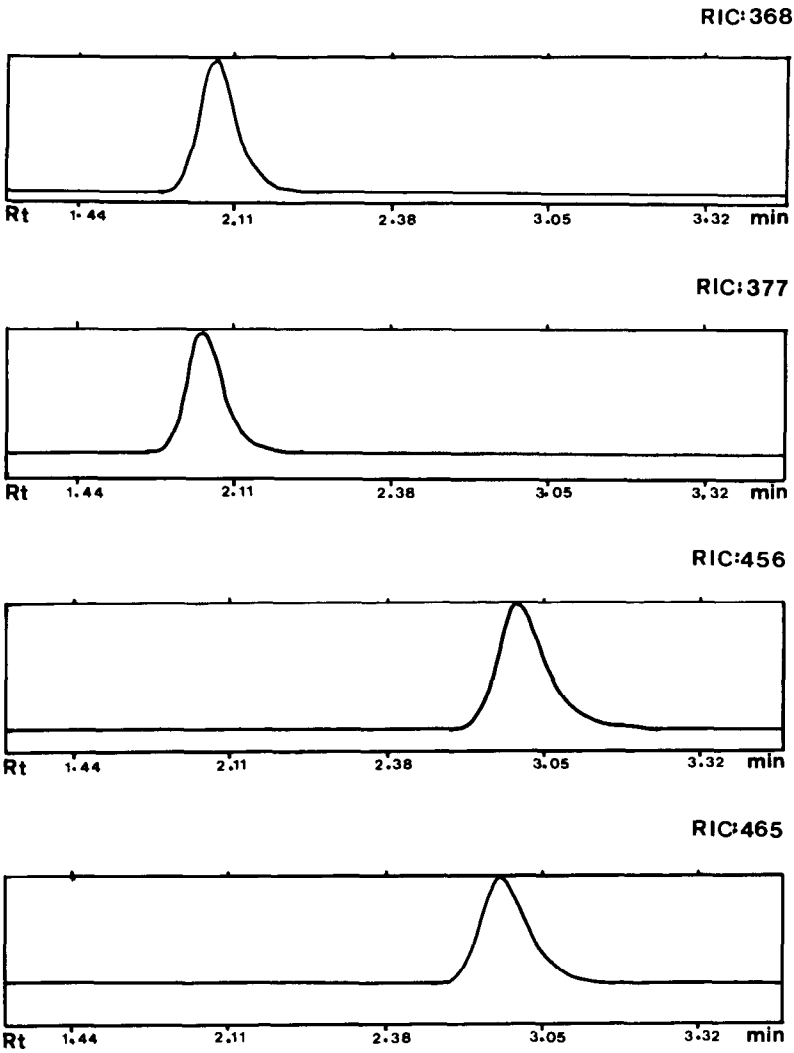

Fig. 2. Reconstructed ion chromatograms (RIC) of the silylated derivatives of tertatolol (m/z 368), [$^2H_9$]tertatolol (m/z 377), 4-hydroxytertatolol (m/z 456) and [$^2H_9$]4-hydroxytertatolol (m/z 465).

retention of diethyl ether by the more aqueous urine medium could account for the difference in extraction yields observed. The same phenomenon has already been observed in the determination of pindolol by high-performance liquid chromatography [12].

The reproducibility of the overall analytical procedure (extraction, derivatization and GC–MS analysis) was tested and the results obtained using blank urine samples are presented in Table II. Similar results were obtained using blank plasma samples. Concentrations as low as 1 ng tertatolol and 4 ng 4-hydroxytertatolol per ml of biological fluid can be accurately determined.

Although the derivatization reaction is complete within a few minutes [13], it was preferred to analyse the samples after overnight derivatization at 60 °C. The

TABLE III

URINARY CONCENTRATIONS OF FREE AND TOTAL (AFTER HYDROLYSIS) TERTATOLOL AND 4-HYDROXYTERTATOLOL AFTER A SINGLE 5-mg DOSE OF TERTATOLOL TO ONE SUBJECT

Time after administration (h)	Urine volume (ml)	Before hydrolysis*		After hydrolysis**	
		Concentration (ng/ml)	Cumulated amount (μg)	Concentration (ng/ml)	Cumulated amount (μg)
Tertatolol					
0- 4	160	133.5	21.36	1431.8	229.09
4- 8	270	38.8	31.84	428.3	344.73
8-12	300	21.1	38.17	160.2	329.79
12-24	1070	20.2	59.78	45.8	441.80
24-30	410	5.9	62.20	25.5	452.25
4-Hydroxytertatolol					
0- 4	160	1098.8	175.81	1438.6	230.18
4- 8	270	406.6	285.59	596.2	391.15
8-12	300	165.0	335.09	245.4	464.77
12-24	1070	68.8	408.71	113.9	586.64
24-30	410	56.9	432.04	85.7	621.78

*Free molecules.
**Free + conjugated molecules.

stability of the derivatized samples was also tested. No alteration was found after a minimum of one week.

The isotopic stability of the [$^2H_9$]tertatolol in aqueous medium was tested previously [7] and the derivative found to be stable for at least 5 h in this medium. The isotopic stability of the deuterated 4-hydroxytertatolol was considered to be the same.

The calibration curves were linear in the range 1–1000 ng/ml for tertatolol and 4–1000 ng/ml for 4-hydroxytertatolol. The general equation was $y = ax + b$, where $a = 0.986$ (± 0.024) and $b = 0.019$ (± 0.022) for tertatolol and $a = 1.109$ (± 0.063) and $b = -0.031$ (± 0.043) for 4-hydroxytertatolol ($n = 10$). Calibration curves established using pure solutions had identical linear characteristics. The correlation coefficient was always greater than 0.999. The small b values and the absence of a signal in the analysis of blank samples demonstrated the selectivity of the method.

Reconstructed ion chromatograms of the quasi-molecular ions monitored are shown in Fig. 2. Under the analytical conditions described above, the retention times for tertatolol and 4-hydroxytertatolol were 2 min 8 s and 2 min 55 s, respectively. Attempts to use only one deuterated derivative ([$^2H_9$]tertatolol) as the internal standard for the analysis gave less satisfactory results.

The method was applied successfully to the simultaneous determination of tertatolol and 4-hydroxytertatolol in plasma and urine samples for pharmacokinetic studies in man. Table III gives the urinary concentrations of unchanged and total

(free + conjugated) tertatolol and 4-hydroxyltertatolol in one healthy volunteer after a single 5-mg oral dose of tertatolol.

CONCLUSIONS

The GC–MS method described permits the simultaneous determination in biological fluids of tertatolol and 4-hydroxytertatolol, one of its metabolites with β-blocking activity. The use of diethyl ether as the extraction solvent ensures a sufficient recovery of both molecules. The purification of the samples by successive alkali and acid extractions, the use of deuterated derivatives as internal standards and the GC–MS selected-ion monitoring in the CI/NH_3 mode provides a highly selective and sensitive method by which concentrations as low as 1 ng/ml for tertatolol and 4 ng/ml for 4-hydroxytertatolol in biological fluids can be accurately determined. The method is suitable for pharmacokinetic studies, the complete results of which will be published later.

ACKNOWLEDGEMENTS

We thank Dr. R. Wolf for the gift of [$^2H_9$]tertatolol and Miss N. Heintz for typing the manuscript.

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