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QUANTITATIVE DETERMINATION OF TERTATOLOL IN BIOLOGICAL FLUIDS BY GAS CHROMATOGRAPHY—MASS SPECTROMETRY

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SUMMARY

Quantitative determination of tertatolol concentrations in plasma and urine was performed by gas chromatography—mass spectrometry in the chemical-ionization mode with ammonia after successive extractions of the β-blocking drug in alkaline, acid and final alkaline medium. [$^2H_9$]Tertatolol, isotopically stable under the operating conditions employed, was used as an internal standard, thus allowing quantities of 1 ng/ml to be specifically determined. Overall analytical error was less than 10%.

Prior to isothermal chromatography at 240°C on a column packed with 3% SE-30, both compounds were silylated with bis(trimethylsilyl)trifluoroacetamide. Detection was performed by monitoring the quasimolecular ions of tertatolol, *m/z* 368 and *m/z* 377, for the [$^2H_9$]tertatolol in the chemical-ionization mode with ammonia.

The calibration curves obtained had linear characteristics for the concentration range 1—1125 ng/ml.

INTRODUCTION

Tertatolol (S-2395), *dl*-8-(2-hydroxy-3-terbutylaminepropyloxy)thiochromane (I, Fig. 1) is a new β-blocking agent synthesized at I.d.R.S. (Institut de Recherches Servier, Suresnes, France) [1]. Its pharmacological properties have been established both in animals [2] and in man [3].

As the doses administered to man were low, the daily dose being 5 mg, gas chromatography—mass spectrometry (GC—MS) was chosen as the assay method for tertatolol in biological fluids. This method, which combines sensitivity with selectivity, has already been used in assaying other β-blockers such as timolol [4], betaxolol [5] and bufuralol [6].

Tertatolol (pK_a = 9.8) was extracted by cyclohexane after alkalinization of

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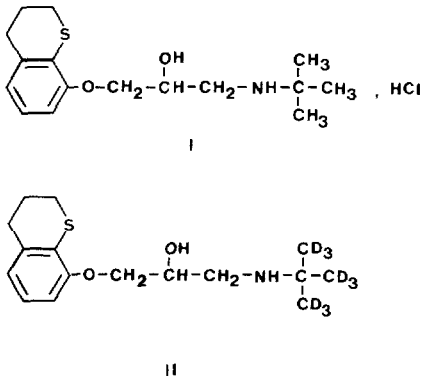

Fig. 1. Chemical formulae of tertatolol (I) and [$^2H_9$]tertatolol (II).

plasma or urine. After addition of 1 *M* hydrochloric acid the tertatolol remained in the aqueous acid phase and impurities in the organic phase which was discarded. Tertatolol was then back-extracted into cyclohexane following alkalinization. After this treatment there were no impurities which interfered in the analysis. Tertatolol was transformed into the trimethylsilyl (TMS) ether before GC—MS analysis. The hydrolysis of conjugated tertatolol derivatives from urine was accomplished through the action of *Helix pomatia* extract at an acidic pH.

The analytical method employed included the use of the $^2H_9$ derivative of tertatolol (II, Fig. 1) as an internal standard for GC—MS analysis. Chemical ionization was used to increase the sensitivity of the method [7, 8].

EXPERIMENTAL

Reagents

Pure tertatolol, compound I, supplied by I.d.R.S., was used without further purification. [$^2H_9$]Tertatolol, compound II, was synthesized by Dr. R. Wolf; its purity was higher than 99.8%. Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Pierce, Rotterdam, The Netherlands) was purchased from Spiral (Dijon, France). Extract from *Helix pomatia* was purchased from IBF (Clichy, France) and contained 100 000 F.U./ml (F.U. = Fishman units of glucuronidase activity, 200 F.U. = 1 I.U.) and 1 000 000 R.U./ml (R.U. = Roy units of sulphatase activity). Other solvents and reagents used (cyclohexane, ammonium hydroxide, hydrochloric acid) were analytical-grade products from E. Merck (Darmstadt, F.R.G.).

Instrumentation

A Nermag R 10-10 GC—MS system (Delsi, Rueil-Malmaison, France) was used for tertatolol analysis. The chromatograph was equipped with a 2.1 m × 2 mm I.D. glass column packed with 3% SE-30 on 100—120 mesh W AW DMCS Chromosorb. The oven temperature was set at 240°C, injector and interface temperatures were 260°C and 270°C, respectively. Helium was used as the carrier gas at a flow-rate of 20 ml/min.

Standard solutions and internal standards

The [$^2H_9$]tertatolol, stocked in an ethanolic solution, was prepared at a concentration of 400 μg/ml. The deuterated derivative was stable for at least two years at −20°C. Working standard solutions were prepared from it at 2 ng/μl. A 50-μl volume of this latter solution was added to each sample as an internal standard.

Analytical procedure

Free tertatolol. To a disposable 10-ml screw-cap tube with a PTFE washer, 100 ng (50 μl) of [$^2H_9$]tertatolol (internal standard) were added. After evaporation of the organic solvent, 1 ml of plasma or urine was added and the mixture was allowed to stand for 15—30 min. After addition of 300 μl of 28% ammonium hydroxide and 3 ml of cyclohexane, the mixture was shaken for 3 min and centrifuged at 1200 *g* for 5 min. After transfer of the cyclohexane to a second tube the extraction was repeated with 3 ml of cyclohexane and the organic phases were combined. Then 2 ml of 1 *M* hydrochloric acid were added to the combined phases which were extracted for 3 min, and then centrifuged at 1200 *g* for 2 min, after which the organic phase was carefully discarded. The remaining acidic aqueous phase was rinsed twice with 1 ml of cyclohexane to ensure removal of all the cyclohexane extract. Then 500 μl of 28% ammonium hydroxide were used to alkalinize this last phase which was extracted with 4 ml of cyclohexane for 3 min and then centrifuged for 3 min at 1200 *g*. The cyclohexane was transferred into a 5-ml open-top screw-cap tube with PTFE washer. After evaporation of the cyclohexane under a gentle nitrogen stream, 25 μl of BSTFA were added to the residue and 1—3 μl of the mixture were injected into the GC—MS system.

Conjugated tertatolol. To 1 ml of urine, 50 μl of 1 *M* hydrochloric acid, 500 μl of 0.1 *M* phosphate buffer at pH 4.1 and 100 μl of *Helix pomatia* extract were added. After 8 h incubation at 37°C, 500 μl of 28% ammonium hydroxide were added to alkalinize the medium. The analytical procedure used was identical to that described above.

The amount of conjugated tertatolol was obtained by the difference between free tertatolol and total tertatolol after hydrolysis.

RESULTS AND DISCUSSION

Plasma and urine assay

For all quantifications the Nermag/Sidar automated computing system was used, and based on peak area calculations.

The extraction recovery of tertatolol from plasma by cyclohexane [9] was

TABLE I

EXTRACTION YIELD OF TERTATOLOL FROM PLASMA USING CYCLOHEXANE

Quantity (ng/ml)	Quantity recovered (ng/ml, mean ± S.D., $n = 6$)	Extraction yield (%)
5.625	3.41 ± 0.4	60.6
1125.0	903.88 ± 52.76	80.3

tested by spiking 1 ml of blank plasma with known quantities of tertatolol. After extraction, internal standard (100 ng/ml) was added and the samples were derivatized and analysed. In Table I are shown the extraction yields. Cyclohexane was a sufficiently good solvent. Many interfering substances were not extracted by the cyclohexane from the biological fluids because of its low polarity.

The overall analytical method (extraction, derivatization and GC—MS analysis) was tested by analysing samples at two different concentrations submitted to the total analytical procedure ten times. Results obtained (Table II) showed a good reproducibility and sensitivity of the method.

The isotopic stability of the [$^2H_9$]tertatolol in aqueous medium was also tested by adding 100 ng of [$^2H_9$]tertatolol in 1 ml of water, containing 100 ng of tertatolol. Contact with water was maintained at room temperature for variable periods. A parallel control assay was performed in ethanol. After analysis and quantification, the quantities recovered (Table III) from the aqueous or the ethanolic solutions were the same. For this assay the [$^2H_9$]-tertatolol was used as the internal standard. The reverse procedure, where tertatolol was the internal standard, gave identical results. [$^2H_9$]Tertatolol was thus considered stable for at least 5 h in aqueous medium.

TABLE II

REPRODUCIBILITY OF THE TOTAL METHOD

Quantity injected (ng/ml)	Quantity recovered (ng/ml, mean ± S.D., n = 10)	C.V. (%)
2	2.07 ± 0.14	6.7
100	107.70 ± 6.03	5.6

TABLE III

ISOTOPIC STABILITY OF [$^2H_9$]TERTATOLOL AFTER VARIOUS CONTACT PERIODS WITH AQUEOUS OR ETHANOLIC MEDIUM

Contact time	Tertatolol in distilled water (n = 4)		Tertatolol in ethanol (n = 4)	
	ng/ml, mean ± S.D.	C.V. (%)	ng/ml, mean ± S.D.	C.V. (%)
10 min	97.4 ± 2.9	2.9	99.6 ± 2.0	2.0
5 h	99.6 ± 1.4	1.4	98.6 ± 1.2	1.2

Following the above considerations, calibration curves established by spiking blank plasma and urine with various quantities of tertatolol and 100 ng/ml [$^2H_9$]tertatolol gave linear characteristics in the range 1—1125 ng/ml. Calibration curves established daily in different ranges (depending on the expected sample concentrations) gave identical linear characteristics in the low, intermediate and high concentration ranges. The general equation was $y = ax + b$ where a = 1.03 (± 0.08) and b = 0.059 (± 0.03) for n = 15. The characteristics of the calibration curves established using pure solutions were

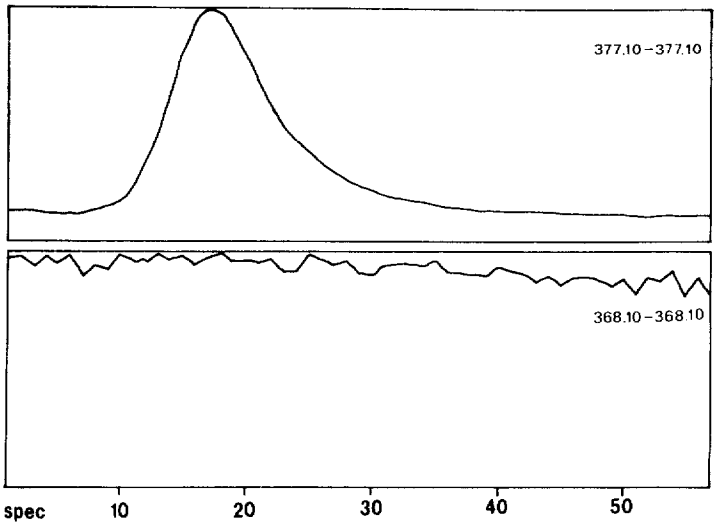

Fig. 2. Reconstructed ion chromatogram for *m/z* 368.10 and 377.10 from a blank plasma. Urine blanks gave similar results.

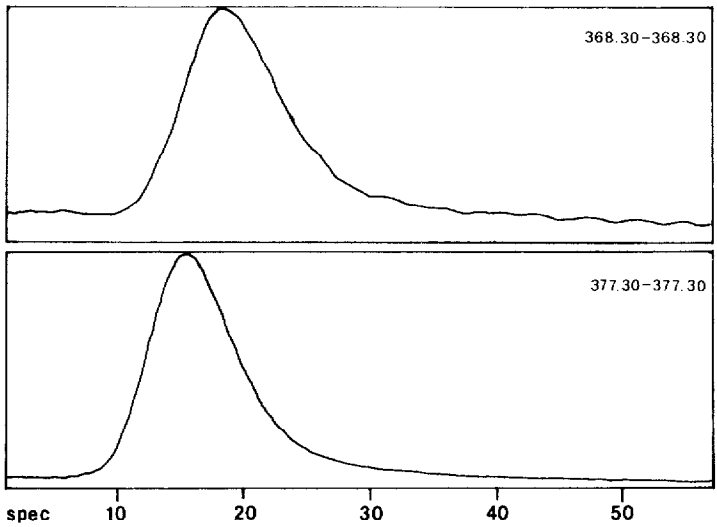

Fig. 3. Reconstructed ion chromatogram for *m/z* 368.30 and 377.30 from a sample containing tertatolol and [$^2H_9$]tertatolol, respectively, after GC—MS analysis in the chemical-ionization mode with ammonia.

identical. For all calibration curves the correlation coefficient was better than 0.999. The selectivity of the method was demonstrated since both the calibration curves exhibited low *b* terms and the absence of signal was observed in the analysis of blanks (Fig. 2). The O-TMS derivatives used for the GC—MS analyses were very stable. Fig. 3 shows a reconstructed ion chromatogram of the quasimolecular ions monitored: *m/z* 368.30 for tertatolol and *m/z* 377.30 for [$^2H_9$]tertatolol. The retention times in the conditions described above were 2 min 37 sec for tertatolol and 2 min 35 sec for [$^2H_9$]tertatolol.

Plasma concentration—time profiles and urinary levels

Fig. 4 shows the plasma concentration—time profiles of unchanged tertatolol after oral or intravenous administration of 2.5 mg of tertatolol to one subject. The maximum concentration was reached within 1 h after oral administration and concentrations of the unchanged drug could reliably be determined 15 h after administration.

The urinary concentrations after 2.5 mg oral and intravenous administrations to the same subject (Table IV) were generally low, and elimination of unchanged tertatolol was approximately 1% of the administered dose.

The total (free + conjugated) eliminated tertatolol (Table V) obtained by

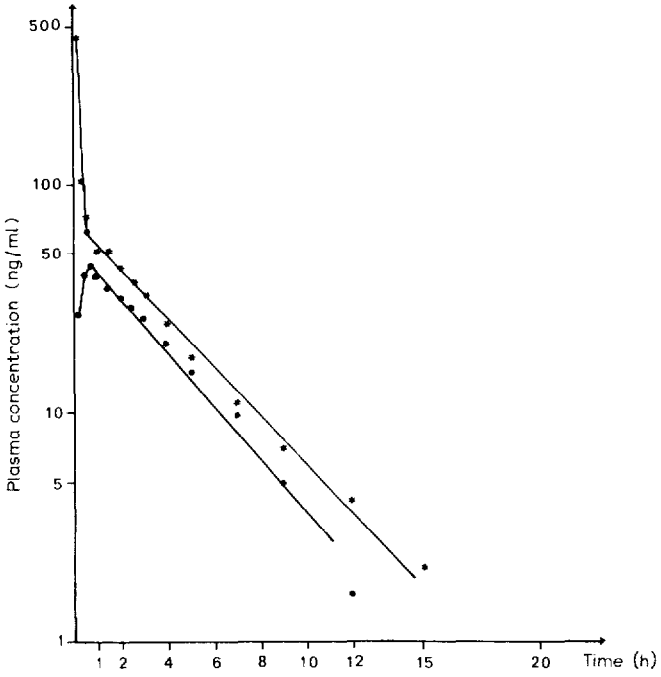

Fig. 4. Plasma concentration—time profiles of tertatolol in one subject after a single 2.5-mg intravenous (*) or oral (•) administration.

TABLE IV

EXCRETION OF UNCHANGED TERTATOLOL IN THE URINE OF ONE SUBJECT AFTER A SINGLE 2.5-mg ORAL OR INTRAVENOUS DOSE

Time (h)	Oral route			Intravenous route		
	Urinary volume (ml)	Quantity (ng/ml)	Cumulative dose (%)	Urinary volume (ml)	Quantity (ng/ml)	Cumulative dose (%)
4	100	129.3	0.52	170	105.3	0.72
9	150	36.2	0.73	580	3.1	0.79
12	180	20.3	0.88	410	1.2	0.81
24	515	8.5	1.05	575	10.4	1.05
48	1230	1.7	1.14	1140	2.4	1.16

TABLE V

URINARY EXCRETION OF TOTAL (FREE + CONJUGATED) TERTATOLOL IN ONE SUBJECT AFTER A SINGLE 2.5-mg ORAL OR INTRAVENOUS DOSE

Time (h)	Oral route			Intravenous route		
	Urinary volume (ml)	Quantity (ng/ml)	Cumulative dose (%)	Urinary volume (ml)	Quantity (ng/ml)	Cumulative dose (%)
4	100	757.5	3.03	170	461.4	3.14
9	150	515.7	6.12	580	100.0	5.46
12	180	300.1	8.28	410	71.4	6.63
24	515	71.0	9.75	575	95.1	8.81
48	1230	23.0	10.80	1140	22.5	9.84

enzymatic hydrolysis of urine, was about ten times more than the unchanged tertatolol eliminated in urine. Approximately 10% tertatolol was eliminated as free plus conjugated drug in the urine of the subject examined.

CONCLUSIONS

The described method enabled us to monitor tertatolol in plasma and urine. Concentrations as low as 1 ng/ml could be determined accurately.

The selectivity and sensitivity of the method depend on the purification of the biological samples by successive extractions, the use of a deuterated internal standard and GC—MS analysis in the chemical-ionization mode. Selected-ion monitoring of the quasimolecular ions of the TMS derivatives allowed standardization and quantification in the range 1—1125 ng/ml.

This method enabled tertatolol to be rapidly and accurately assayed in biological fluids after administration of low doses of this drug for a period of time sufficiently long to allow clinical pharmacokinetic studies.

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