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FLUORIMETRIC DETERMINATION OF THE QUATERNARY COMPOUND TROSPIUM AND ITS METABOLITE IN BIOLOGICAL MATERIAL AFTER DERIVATIZATION WITH BENOXAPROFEN CHLORIDE*

GERTRUD SCHLADITZ-KEIL, HILDEGARD SPAHN and E. MUTSCHLER*

Pharmakologisches Institut für Naturwissenschaftler der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Gebäude 75 A, D-6000 Frankfurt am Main 70 (F.R.G.)

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SUMMARY

The quantitative determination of the quaternary compound trospium and the corresponding spiroalcohol is described for human biological material. Analysis is performed by alkaline hydrolysis, ion-pair extraction into chloroform, subsequent derivatization with the fluorophor benoxaprofen chloride and high-performance liquid chromatographic separation on a reversed-phase column. The limit of quantitation is ca. 1 pmol (1.03 pmol = 1 ng of trospium chloride) per millilitre of plasma.

INTRODUCTION

Trospium chloride (3α-benziloyloxynortropane-8-spiro-1′-pyrrolidinium chloride; Spasmex®; Fig. 1a) belongs to the group of anticholinergic agents and is used for the relief of smooth-muscle spasms.

The quantitative determination of trospium in human plasma and urine has not yet been possible, and bioavailability data for trospium obtained from plasma levels and urinary excretion have not been available. In 1983, Albrecht et al. [1] found an oral bioavailability of 5—10% using the so-called pupillometry, i.e. quantifying the marked mydriatic effect of the substance.

In animal experiments, serum levels of trospium were determined spectrophotometrically, measuring the complex of the quaternary compound with picric acid after extraction from serum [2]. The limit of quantitation of

*Part of the PhD thesis of G. Schladitz-Keil, Department of Pharmacology, University of Frankfurt/Main, Frankfurt am Main, F.R.G.

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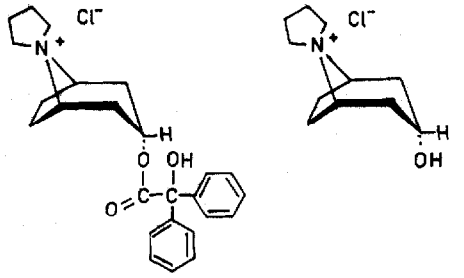

a b

Fig. 1. Structural formulae of (a) trospium chloride and (b) spiroalcohol (chloride salt).

this method was ca. 1 μg/ml. As the therapeutic doses in man are usually very low (0.2—0.5 mg i.v. and 5—10 mg p.o.), more sensitive assay methods are necessary for determination of the drug in human biological material after single or chronic administration.

The drug was assumed to be excreted mainly as the corresponding spiroalcohol (3α-hydroxynortropane-8-spiro-1′-pyrrolidinium; Fig. 1b) [3]. Therefore an assay method was needed for this alcohol because it does not possess sufficient chromophoric properties.

In this paper, the quantitative determination of trospium in plasma after hydrolysis to the corresponding spiroalcohol, ion-pair extraction from biological material and subsequent fluorescent derivatization with benoxaprofen chloride (Fig. 2) is described.

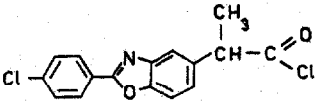

Fig. 2. Structural formula of benoxaprofen chloride.

The metabolite, spiroalcohol, can be detected in urine, and extraction and derivatization were performed with and without previous alkaline hydrolysis. From the difference in the concentrations, measured with and without hydrolysis, trospium levels in urine samples can be calculated.

The chromatographic separation of the spiroalcohol derivative was carried out by high-performance liquid chromatography (HPLC) using reversed-phase ion-pair chromatography with chloride as the counter-ion. To prove the results obtained by this method, the concentrations of trospium in urine were also determined by measuring the UV absorbance of the unchanged molecule after ion-pair extraction and reversed-phase ion-pair chromatography.

EXPERIMENTAL

Chemicals and reagent solutions

Solvents (analytical-reagent grade or LiChrosolv®) and reagents were obtained from Merck (Darmstadt, F.R.G.). Dipicrylamine contained 50% water. Ingotest® water (Boehringer Ingelheim, Ingelheim, F.R.G.) was used for HPLC. Trospium chloride and the metabolite 3α-hydroxynortropane-8-spiro-1′-

pyrrolidinium chloride were made available by Dr. Robert Pfleger (Chemische Fabrik GmbH, Bamberg, F.R.G.). Sodium heptane sulphonate (p.a.) was purchased from Serva (Heidelberg, F.R.G.).

Benoxaprofen was made available by Eli Lilly (Bad Homburg, F.R.G.). Benoxaprofen chloride was synthesized according to Spahn et al. [4].

The benoxaprofen chloride solution was prepared by dissolving 10 mg of benoxaprofen chloride in 1 ml of dried acetonitrile. For the assay methods, three different dipicrylamine solutions were used. Solution I: 98.7 mg dipicrylamine containing 50% water + 10 ml of 0.1 mol/l sodium hydroxide solution + 600 mg anhydrous sodium carbonate; solution II: 32.9 mg dipicrylamine containing 50% water + 10 ml of 0.1 mol/l sodium hydroxide solution + 200 mg anhydrous sodium carbonate; solution III: 32.9 mg dipicrylamine containing 50% water + 10 ml of 0.1 mol/l sodium hydroxide solution.

Instruments

Melting points were estimated with a Büchi apparatus and are uncorrected. Infrared (IR) spectra were obtained in nujol with a Beckman Acculab 2 spectrophotometer.

An LC 601 chromatograph with a 605-10 S fluorescence detector (both from Perkin Elmer, Überlingen, F.R.G.) or with a variable-wavelength UV spectrophotometer (DuPont, Wilmington, DE, U.S.A.) and a 56 recorder (Perkin Elmer) were used for HPLC.

Synthesis of the reference compound

Benoxaprofen chloride (1200 mg) was dissolved in 30 ml of dried acetonitrile. Then 3α-hydroxynortropane-8-spiro-1′-pyrrolidinium chloride (60 mg) was added with stirring and the mixture was heated up to 140—145°C in a glycerine bath and refluxed for 1—1.5 h. The solvent was evaporated and a white crystalline solid was recovered. The residue was washed three times with 20 ml of ethyl acetate.

The ester obtained was recrystallized from water—acetone (1:30) by dissolving it in water at 50°C and adding 30 ml of acetone at the same temperature. Melting points: benoxaprofen 191°C, benoxaprofen chloride 91.5°C, ester 244°C (under decomposition). Yield (after recrystallization) 46%. R_F Values on high-performance thin-layer chromatographic plates (silica gel 60 with fluorescent indicator, 5 × 5 cm, Merck) using acetone—methanol—formic acid (42.5:42.5:15) as mobile phase: benoxaprofen 0.95, spiroalcohol 0.26, ester 0.38.

Hydrolysis and extraction procedures for trospium and spiroalcohol

Trospium in plasma. In a screw-capped centrifuge tube, 1 ml of 1 mol/l sodium hydroxide solution is added to 5 ml of plasma. The screwed tube is heated up to 140—145°C in a glycerine bath for 105 min. After cooling to about 40°C, 0.2 ml of hydrochloric acid (25%) are added and the mixture is centrifuged for 10 min (5000 *g*) and again heated up to 120°C (10 min) and centrifuged after cooling.

A 5-ml volume of the hydrolysed plasma phase is mixed with 0.5 ml of dipicrylamine solution I. To 5 ml of this mixture, 4.8 ml of chloroform (LiChrosolv) are added. The tubes are intensively shaken for 3 min. After

centrifugation (30 min, 6000 *g*) the plasma phase is removed. A 4-ml volume of the organic phase is intensively shaken with 2.3 ml of 0.1 mol/l hydrochloric acid and centrifuged for 10 min (5000 *g*). A 2-ml volume of the inorganic phase is mixed with 2 ml of methanol and evaporated to dryness using a vacuum centrifuge. The residue is washed with methanol several times (10—11 ml in total) in order to remove the hydrochloric acid completely; each time, the methanol is evaporated to dryness under nitrogen at 80—90°C.

Spiroalcohol in urine. A 5-ml volume of urine is mixed with 0.6 ml of dipicrylamine solution II. The mixture is centrifuged (10 min, 5000 *g*) and 5 ml of the urine phase are extracted with 4.5 ml of chloroform by shaking for 3 min. After centrifugation (20 min, 5000 *g*), the urine phase is removed. A 4-ml volume of the organic phase is shaken intensively for 3 min with 2.3 ml of 0.1 mol/l hydrochloric acid and centrifuged (10 min, 5000 *g*). A 2-ml volume of the acid phase is mixed with 2 ml of methanol and evaporated to dryness in a vacuum centrifuge. As described for trospium in plasma, the residue is washed with methanol (6—7 ml in total).

Trospium in urine with previous hydrolysis. A 5-ml volume of urine is hydrolysed for 90 min in the same way as described for trospium in plasma, except for heating up again (to 120°C). After cooling, 0.2 ml of hydrochloric acid (25%) and 0.6 ml of dipicrylamine solution III are added. After shaking, this mixture is centrifuged for 10 min (5000 *g*). The further extraction procedure is the same as described for spiroalcohol in urine.

Native trospium in urine without derivatization. The extraction procedure is the same as described for spiroalcohol in urine.

Derivatization

For all the assay methods that include a derivatization step, the following procedure is used.

After extraction, 0.2 ml of benoxaprofen chloride solution is added to the residue and the screwed tube is heated up to 140—145°C (30 min) in a glycerine bath. The solvent is then evaporated in a vacuum centrifuge. A 1-ml volume of ethyl acetate and 1.2 ml of water are added. After shaking for 5 min, using a mechanical shaker, and centrifugation (10 min, 5000 *g*), the organic phase is removed. Again, the aqueous phase is extracted with 1 ml of ethyl acetate. After centrifugation, 1 ml of the aqueous phase is evaporated to dryness in a vacuum.

Chromatographic conditions

Trospium in plasma. The residue after derivatization is dissolved in 100 µl of water—acetonitrile (69:31). Injection volume: 20 µl; column: analytical column (Knauer), 12.5 cm × 4.6 mm I.D., filled with Nucleosil C_8, particle size 5 µm; column temperature: 55°C; mobile phase: water—acetonitrile (69:31) containing 0.08 mol/l sodium chloride and 0.031 mol/l choline chloride; to 100 ml of this mixture 1 ml of 1 mol/l hydrochloric acid is added; flow-rate: 2 ml/min (91 bar); retention time of the derivative: 7.5 min.

Spiroalcohol in urine, trospium in urine. The derivatized extract is dissolved in 100 µl of water—acetonitrile (60:40). Injection volume: 20 µl; column: analytical column (Knauer), 12.5 cm × 4.6 mm I.D., filled with LiChrosorb®

RP-8, particle size 5 μm; column temperature: 50°C; mobile phase: water—acetonitrile (60:40) containing 0.08 mol/l sodium chloride and 0.031 mol/l choline chloride; to 100 ml of this mixture 1 ml of 1 mol/l hydrochloric acid is added; flow-rate: 2 ml/min (105 bar); retention time of the derivative: 6 min.

Native trospium in urine without derivatization. The residue after extraction is dissolved in 100 μl of water—acetonitrile (60:40). Injection volume and column: same as above; mobile phase: 0.01 mol/l sodium heptane sulphonate solution—acetonitrile (60:40) containing 0.01 mol/l choline chloride; to 1 l of this mixture 1.5 ml of phosphoric acid (85%) are added; column temperature and flow-rate: same as above; retention time of trospium: 3.4 min.

Detection

(1) Assay methods that include a derivatization step: excitation wavelength 313 nm, emission wavelength 370 nm;

(2) Native trospium: UV absorbance at 210 nm.

Determination of plasma levels and urinary excretion after intravenous and oral administration of trospium chloride

After collection of plasma and urine control samples, two healthy male volunteers were given trospium chloride, one received 0.5 mg intravenously and the other received 10 mg orally. Blood (12 ml) was taken at the following times after administration: i.v.: 5, 10, 20, 30, 60, 120, 180 and 240 min; p.o.: 20, 40, 60, 120, 180, 240, 360 and 480 min.

The blood samples were collected in heparin-treated tubes and the plasma was separated by centrifugation. The samples were stored at −20°C until analysis. Urine was collected over 48 h. The urine samples were tested for pH and total volume. Fractions of 50 ml were stored at −20°C until analysis.

RESULTS

The derivatization procedure described in this paper can be performed in a similar way using the UV marker dinitrobenzoyl chloride. But, on measuring the fluorescence of the benoxaprofen derivative, the limit of quantitation obtained was about five times lower than measuring the UV absorbance of the dinitrobenzoyl derivative (244 nm) and also of the benoxaprofen chloride product (313 nm). Thus, only fluorescence measurement makes the determination of lower urine, and even plasma, concentrations of trospium possible.

Spectral and chromatographic properties of the benoxaprofen derivatization product

The derivatization of trospium (after hydrolysis) and its metabolite leads to a stable product which can be separated from constituents of plasma and urine (e.g. choline) by reversed-phase ion-pair chromatography. Fig. 3 shows the excitation and emission spectra of the benoxaprofen—spiroalcohol—ester. The excitation and emission maxima were determined to be 313 and 365 nm, respectively. The maxima observed are almost identical with those described for benoxaprofen chloride [4].

Figs. 4 and 5 show chromatographic separations of trospium hydrolysed to

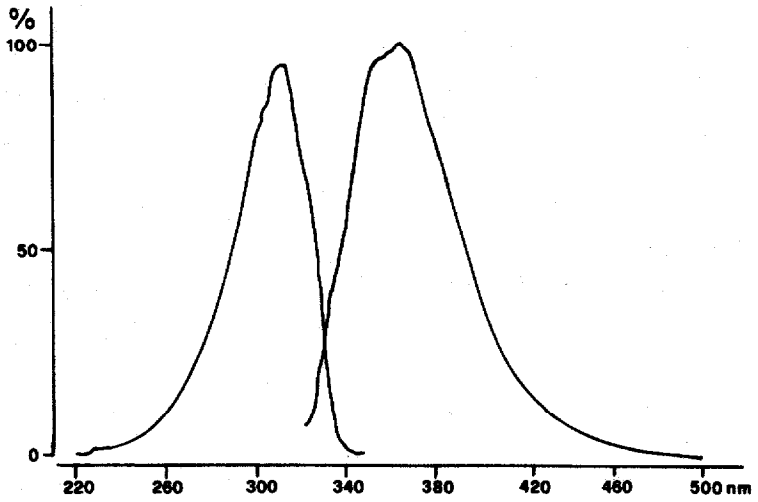

Fig. 3. Fluorescence excitation and emission spectra of benoxaprofen—spiroalcohol—ester measured during HPLC. Excitation maximum, 313 nm; emission maximum, 365 nm.

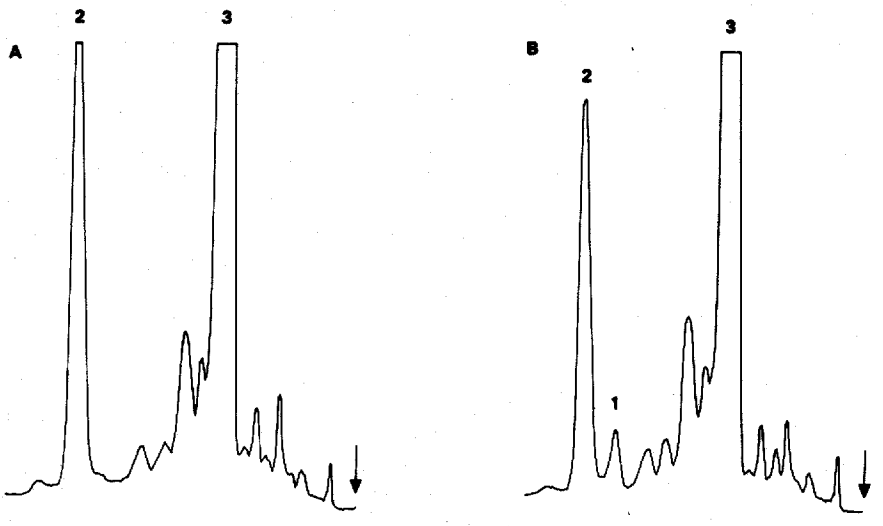

Fig. 4. Chromatographic separations of plasma samples after hydrolysis, extraction and fluorescent derivatization (subject W.P.). (A) Blank plasma sample; (B) concentration of trospium 10 min after intravenous administration of 0.5 mg of trospium chloride. Peaks: 1 = spiroalcohol—benoxaprofen derivative, 2 = benoxaprofen, 3 = "choline" peak, i.e. benoxaprofen—choline derivative.

spiroalcohol or of spiroalcohol itself after extraction and derivatization. If urine standards contain trospium chloride only, and if no hydrolysis is carried out, the chromatograms obtained are the same as for blank urine samples, i.e. trospium is not hydrolysed during the extraction and derivatization procedure.

Extraction from biological material

The extraction of both compounds from biological material via ion-pair formation is almost complete if the solution of the counter-ion dipicrylamine

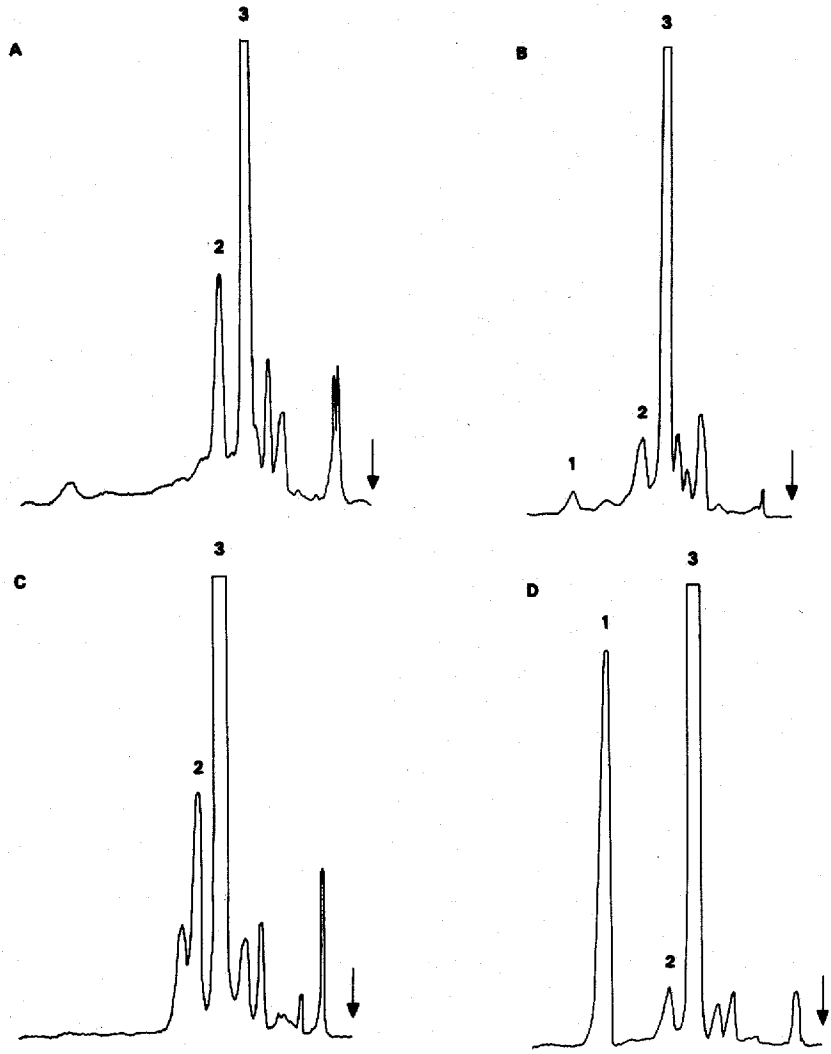

Fig. 5. Chromatographic separations of urine samples of one subject after extraction and fluorescent derivatization. (A) Blank urine sample without previous hydrolysis; (B) spiro-alcohol in a urine sample (sampling interval: 2—4 h) after intravenous administration of 0.5 mg of trospium chloride; (C) blank urine sample with previous hydrolysis; (D) urine sample (2—4 h after 0.5 mg intravenously, see B) with previous hydrolysis. For peak identification, see Fig. 4.

contains not less than 0.005 mol/l for urine samples (pH 8—9) and not less than 0.015 mol/l for plasma samples. One disturbing peak after extraction from plasma and urine must be completely separated from the benoxaprofen—spiro-alcohol—ester, especially if the concentrations are low. This peak is probably caused by choline, another quaternary compound which is extractable from human urine by ion-pair formation with dipicrylamine [5]. The benoxaprofen derivatization product of choline [4] has the same chromatographic behaviour as the disturbing peak in all of the chromatographic systems tested (thin-layer chromatography and HPLC). This "choline peak" turned out to be significantly higher in urine after alkaline hydrolysis than without hydrolysis, and even higher in plasma than in urine.

Limit of quantitation

The limit of quantitation for the pure reference compound was 131 pg benoxaprofen—spiroalcohol—ester chloride (containing water) under the chromatographic conditions described for urine samples. This is equivalent to 56.9 pg of spiroalcohol chloride and to 111.8 pg of trospium chloride. In plasma, the limit of quantitation for trospium chloride is 0.5—1 ng/ml. In urine, it is 3 ng/ml for trospium chloride with previous hydrolysis and 0.6—1 ng/ml for spiroalcohol chloride.

Hydrolysis

Under the conditions described in this paper, trospium is almost entirely hydrolysed to spiroalcohol. In order to show that the higher fluorescence signal after alkaline hydrolysis in urine is caused by native trospium, two additional experiments were performed.

(1) After ion-pair extraction of a urine sample from one volunteer supposed to contain a high amount of trospium (intravenous administration), the residue was investigated qualitatively via fast atom bombardment (FAB) mass spectrometry. A substance with a molecular weight of m/z 392 (molecular peak of the trospium cation) was present in the spectra of the trospium standard urine and in the urine sample from the volunteer. This peak was not found in the blank urine. If an internal standard is added, this procedure can also be used for quantitative analysis. Further investigations will be described elsewhere.

(2) The HPLC determination of native trospium was performed in order to prove the data from the fluorescent derivatization. Native spiroalcohol could not be detected using this method. The investigations showed that trospium is excreted into urine in an unchanged form. Under the conditions described, 25 ng/ml trospium chloride in urine can be quantified. This limit of quantitation is not sufficient for routine analysis of the drug. However, nearly identical concentrations were found in urine samples of volunteers, e.g. 2271 ng/ml after derivatization and 2201 ng/ml by measuring the UV absorbance of the parent molecule.

Recovery and linearity

Using the synthesized reference substance, recovery could be determined to be almost 100% for trospium and for spiroalcohol after extraction from biological material. But this recovery could only be obtained if the pH of the

TABLE I

DEPENDENCE OF RECOVERY OF TROSPIUM AND SPIROALCOHOL ON THE pH OF THE BENOXAPROFEN CHLORIDE SOLUTION

Sample	Recovery (%)			
	pH 5	pH 4—4.5	pH 3.5—4	pH 3—3.5
Trospium in plasma	100	100	100	68
Trospium in urine	100	85	75	75
Spiroalcohol in urine	100	78	24	24

benoxaprofen chloride solution (in acetonitrile) measured with a wet pH-stick (Acilit) is ca. 5. An acidic reaction of this solution is probably caused by traces of thionyl chloride. The dependence of recovery rates on the pH of the benoxaprofen chloride solution is given in Table I.

The linearity of the assays was investigated by preparing standards of different concentrations. In plasma, six different concentrations up to 100 ng/ml trospium chloride (= 50.9 ng/ml spiroalcohol chloride) were determined. In this range, the calibration curve is linear ($r = 0.9999$). For the determination of trospium chloride in urine (after alkaline hydrolysis), the linearity of the calibration curve could be observed up to 3000 ng/ml ($\approx$ 1526.1 ng/ml spiroalcohol chloride; $r = 0.9998$, $n = 12$). For spiroalcohol chloride in urine, linearity was proved up to 800 ng/ml ($r = 0.9996$, $n = 10$).

Standard deviation

The relative standard deviations at different concentrations in plasma and urine are given in Table II.

Determination of plasma levels and urinary excretion

Plasma levels found in healthy volunteers after intravenous (0.5 mg) and oral (10 mg) administration are shown in Fig. 6. Urinary excretion of trospium and spiroalcohol within 48 h after administration is given in Table III.

TABLE II

RELATIVE STANDARD DEVIATIONS OF TROSPIUM IN PLASMA AND URINE AND OF SPIROALCOHOL IN URINE

Sample	Standard concentration (ng/ml)	n	S.D. (%)
Trospium chloride in plasma	2	8	8.9
	5	9	6.2
	20	8	5.4
Trospium chloride in urine	10	12	4.5
	40	11	2.6
	200	11	4.1
	1000	11	4.9
Spiroalcohol chloride in urine	5	8	4.5
	20	6	3.2
	100	10	4.7

TABLE III

URINARY EXCRETION OF TROSPIUM AND SPIROALCOHOL (BOTH CALCULATED AS TROSPIUM CHLORIDE) WITHIN 48 h AFTER ADMINISTRATION OF TROSPIUM CHLORIDE IN MAN

Subject	Trospium		Spiroalcohol		Total (nmol)	Percentage of given dose
	(μg)	(nmol)	(μg)	(nmol)		
W.P. (0.5 mg i.v.)	263.03	614.5	8.82	40.5	655.0	56.1
M.M. (10 mg orally)	141.10	329.6	29.20	134.2	463.8	2.0

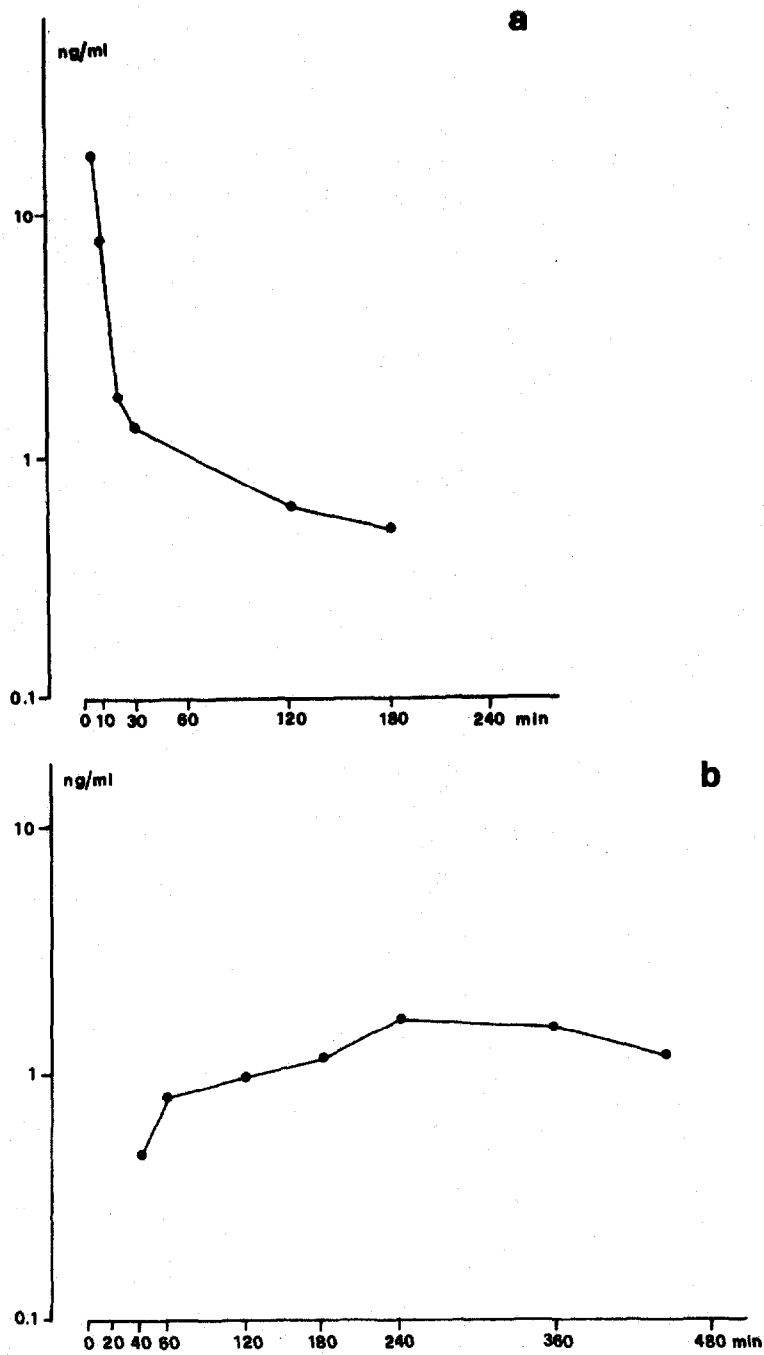

Fig. 6. Plasma concentration—time curves after administration of trospium chloride; (a) 0.5 mg intravenously (subject W.P.); (b) 10 mg orally (subject M.M.).

DISCUSSION

The determination of quaternary compounds in biological fluids are known

to be difficult as the plasma concentrations are low. Although the described procedure is rather time-consuming, containing several extraction steps and one derivatization step, the standard deviations are sufficient and the recovery rate is high. But, using this method it was possible for the first time to determine plasma levels after intravenous and even after oral administration of trospium chloride in man.

Spiroalcohol could not be determined in unhydrolysed plasma samples, as plasma components disturbed the extraction procedure. Therefore, the "trospium concentrations" in plasma are total concentrations and the portion of spiroalcohol remains unknown. But, as trospium is excreted virtually unchanged into urine, the concentrations of spiroalcohol are assumed to be rather low. On average, 0.3% of the given dose is excreted in the form of spiroalcohol within 48 h after oral administration of 10 mg of trospium chloride [6].

The plasma concentration—time curve after intravenous administration can be described by a two-compartment open model. The half-life ($t_{½z}$) from subject W.P. (Table III) was estimated to be 1.8 h. The trospium plasma concentrations after oral administration are in the limit of quantitation of the method. Therefore, this parameter is not yet sufficient to perform complete pharmacokinetic studies, especially in order to determine the plasma level—time curves after oral administration and the bioavailability from plasma data. It might be possible to improve the limit of quantitation in plasma by optimizing the chromatographic system (e.g. stationary phase).

The derivatization procedure described for trospium is also applicable to other quaternary compounds, from which choline has already been mentioned in this paper. For example, scopolamine-N-butyl bromide and its product from alkaline hydrolysis can also be extracted from urine and can easily be derivatized with benoxaprofen chloride. Chromatographic separations are possible by thin-layer chromatography on silica gel plates and by HPLC on RP-8 material [7]. This shows that ion-pair extraction and the use of benoxaprofen chloride for fluorescent derivatization is an approach to the development of analytical methods for quaternary anticholinergic agents — from the analytical point of view, a problematical group — without using radioactivity.

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