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Determination of the quaternary compound ciclotropium in human biological material after hydrolysis and derivatization with the fluorophor flunoxaprofen chloride[a,b]

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ABSTRACT

The quantitative determination of the quaternary spasmolytic compound ciclotropium and its metabolite N-isopropyltropinium is described for human plasma and urine. The analytical procedure consists of ion-pair extraction from biological material, alkaline hydrolysis, subsequent derivatization with the fluorophor flunoxaprofen chloride and separation by high-performance liquid chromatography on a reversed-phase column with fluorimetric monitoring. The detection limits of 0.5 ng/ml in plasma and 10 ng/ml in urine at signal-to-noise ratios higher than 3 permit the determination of pharmacokinetic parameters after therapeutic doses.

INTRODUCTION

Ciclotropium bromide [(8*R*)-3α-hydroxy-8-isopropyl-1αH,5αH-tropanium bromide α-phenylciclopentaneacetate; Fig. 1a] is a new parasympatholytic agent with a quaternary nitrogen which can be used to relieve spasm of the smooth muscle of the gastrointestinal tract, biliary colic and dysmenorrhoea.

First estimates of pharmacokinetic data were obtained from a study with ^{14}C-labelled drug that was administered to five healthy male volunteers [1]. Yet, until now the quantitative determination of the unlabelled drug in human biological material has not been possible. As the therapeutic doses in man are usually low (20 mg orally), and since it is known that other quaternary compounds such as N-butylscopolaminium bromide [2,3] or trospium chloride [4] are poorly absorbed from the human gastrointestinal tract, low plasma and urine concentrations of ciclotropium were to be expected. Therefore, it was necessary to develop a sensitive assay method that would permit the quantification of concentrations

[a] Dedicated to Professor Dr. rer. nat. Dr.h.c. mult. Herbert Oelschläger on the occasion of his 70th birthday.

[b] Part of the Ph.D. thesis of B. Liebmann.

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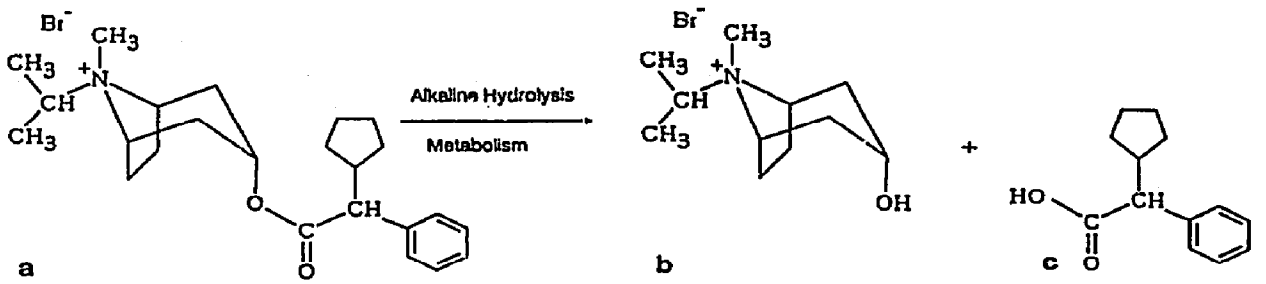

Fig. 1. Structures of ciclotropium and its (metabolic) hydrolysis products. (a) Ciclotropium bromide; (b) N-isopropyltropinium; (c) α-phenylciclopentaneacetic acid.

in the low nanogram range. This was achieved in a similar way as previously described for trospium [5]. It was assumed that the drug is metabolized to the corresponding alcohol, N-isopropyltropinium [(8*R*)-3α-hydroxy-8-isopropyl-1αH,5αH-tropanium; Fig. 1b], and the carboxylic acid (α-phenylciclopentaneacetic acid; Fig. 1c) [6]. Similar behaviour has been described for related drugs such as ipratropium bromide [7] and trospium chloride [4]. Hence an assay method for the metabolite N-isopropyltropinium was needed as well. Ciclotropium and this metabolite do not possess sufficient chromophoric properties (poor UV absorbance at 211 nm), therefore a derivatization appeared advantageous.

In this communication the quantitative determination of ciclotropium in human plasma and urine after ion-pair extraction from biological material, alkaline hydrolysis to the corresponding alcohol, N-isopropyltropinium (the drug itself does not possess reactive groups for a direct covalent labelling), and subsequent derivatization with the fluorophor flunoxaprofen chloride (Fig. 2a) to the corresponding ester (Fig. 2b) is described. The metabolite N-isopropyltropinium itself can also be assayed fluorimetrically after ion-pair extraction without previous hydrolysis and after reaction of the hydroxy moiety with flunoxaprofen chloride.

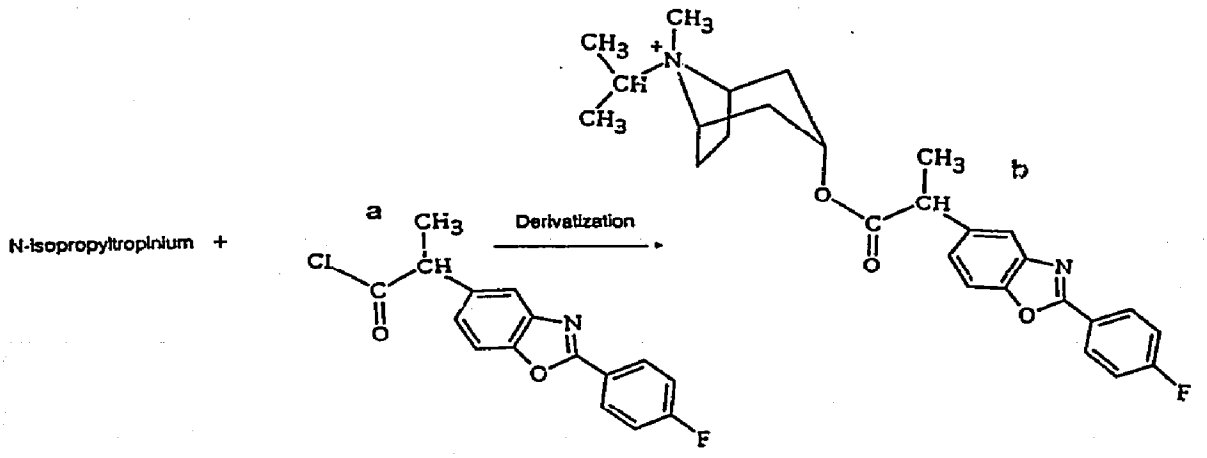

Fig. 2. Derivatization of hydrolysed ciclotropium (N-isopropyltropinium) with flunoxaprofen chloride (a) yielding the corresponding ester (b).

EXPERIMENTAL

Chemicals and reagent solutions

Solvents (analytical-reagent-grade or LiChrosolv) and reagents were obtained from Merck (Darmstadt, Germany). Double-distilled water was used for the preparation of aqueous solutions and the high-performance liquid chromatography (HPLC) mobile phase. Dipicrylamine, moistened with 50% water, was purchased from Fluka (Buchs, Switzerland). For the assay methods, two different dipicrylamine solutions were used. Their compositions were as follows: solution I = 100 mg of dipicrylamine + 600 mg of anhydrous sodium carbonate + 10 ml of water; solution II = 35 mg of dipicrylamine + 10 ml of 0.1 *M* sodium hydroxide solution.

Ciclotropium bromide, N-isopropyltropinium bromide and N-desmethylciclotropium were made available by Helopharm (Berlin, Germany). N-Butyltropinium bromide [(8*R*)-3α-hydroxy-8-(*n*)butyl-1αH,5αH-tropanium] was provided by Pfleger (Bamberg, Germany). S-Flunoxaprofen was kindly donated by Ravizza (Milan, Italy). Flunoxaprofen chloride was synthesized as described previously [8]. Flunoxaprofen chloride solution was freshly prepared by dissolving 10 mg of flunoxaprofen chloride in 1 ml of anhydrous acetonitrile.

Instruments

The solvents were evaporated in a Speed-Vac concentrator (suction power 5 m^3/h, 1725 *g*, 45°C) (Bachofer, Reutlingen, Germany). The HPLC system consisted of a Knauer 64 pump (Berlin, Germany), a Rheodyne 7125 injection valve (Cotati, CA, USA) with a 20-μl loop, a Shimadzu RF-535 fluorescence HPLC monitor (Duisburg, Germany) and a Knauer TY recorder. Stainless-steel tubing was used.

Extraction from plasma and urine

Ciclotropium. In a screw-capped glass centrifuge tube, 4 ml of heparinized plasma were extracted with 5.5 ml of chloroform after addition of 500 μl of dipicrylamine solution I and 250 μl of 1.0 *M* hydrochloric acid (pH in the aqueous phase 8.5–9.5). The tube was intensively shaken for 5 min, and after centrifugation (30 min, 2500 *g*, 10°C) the plasma layer was carefully removed. A 4-ml volume of the organic layer was transferred into another tube, shaken with 2.4 ml of 0.1 *M* hydrochloric acid for 15 min and centrifuged for 10 min (2500 *g*, 20°C). A 2-ml sample of the resulting aqueous layer was mixed with 1 ml of 1.0 *M* hydroxide solution, and then by heating in a screw-capped tube (140–145°C, 90 min) in a paraffin bath ciclotropium was hydrolysed. After cooling the sample to room temperature, 500 μl of dipicrylamine solution I were added and the pH was adjusted to 8.5–9.5 with hydrochloric acid (5 *M*). Then chloroform (2.6 ml) was added, and the tube shaken for 15 min and centrifuged (10 min, 2500 *g*, 20°C). A 2-ml aliquot of the chloroform phase was mixed with 1.4 ml of 0.1 *M* hydrochlo-

ric acid, again shaken for 15 min and centrifuged (10 min, 2500 *g*, 20°C). Then 1 ml of the aqueous layer was diluted with 1 ml of methanol (azeotropic mixture) and the mixture was evaporated to dryness using a vacuum centrifuge. As a neutral pH was necessary for the subsequent derivatization procedure [5], the residue was "treated" three times by adding 3 ml of methanol and subsequently evaporating to dryness in order completely to remove traces of hydrochloric acid.

Urine (1 ml) was mixed with 2 ml of 0.5 *M* sodium hydroxide solution and 100 ng of the internal standard (N-butyltropinium, dissolved in 100 µl water) and kept at 140-145°C for 90 min. After cooling to room temperature 500 µl of dipicrylamine solution II were added and the pH was adjusted to 8.5–9.5. The further extraction procedure was the same as described above.

N-Isopropyltropinium. Heparinized plasma (4 ml) was treated in a similar way as described for cicloptropium in plasma. Yet, no alkaline hydrolysis was performed. Instead, a 2-ml volume of the aqueous layer was mixed with 2 ml of methanol, evaporated to dryness and "treated" with 3 ml of methanol three times. [As the maximum concentrations of the metabolite in plasma were beyond the detection limit in a single-dose study (< 0.3 ng/ml), no further studies were performed to quantify the metabolite in plasma samples.]

In order to quantify the metabolite N-isopropyltropinium in urine, 1 ml was extracted in a same way as for parent ciclotropium in urine, including the addition of internal standard, but without previous alkaline hydrolysis.

Derivatization procedure

To the extraction residue 200 µl of flunoxaprofen chloride solution were added and the screw-capped tube was heated to 110°C for 15 min in a paraffin bath. After evaporating the solvent in a vacuum centrifuge, 1 ml of ethyl acetate and 1.3 ml of 0.01 *M* hydrochloric acid were added. After intensively shaking for 15 min and centrifugation (10 min, 2500 *g*, 5°C) the organic layer was removed and discarded. This extraction with ethyl acetate was performed twice, in order to remove excess reagent. Finally 1 ml of the aqueous layer was evaporated to dryness in a vacuum centrifuge.

Chromatographic conditions

The residue was dissolved in 100 µl of acetonitrile–methanol–water (1:1:1, v/v; better solubility than in the mobile phase), and in 500 µl of this mixture when urine samples were analysed. The injection volume was 20 µl (loop filling); the stationary phase was a Waters Nova-Pak Resolve C_{18} (4 µm) HPLC column (150 mm × 3.9 mm I.D.) (Waters-Millipore, Eschborn, Germany). The mobile phase consisted of water–acetonitrile (13:7, v/v) containing 1.0 ml of phosphoric acid (85%) and 2.5 g of choline chloride per litre, yielding pH 2.2; its flow-rate was 1.2 ml/min and the mean pressure 12.2 MPa. The retention time of the N-isopropyltropinium-flunoxaprofen ester was 8.9 min; the derivative of the internal standard eluted after 12.3 min. A xenon lamp was used as a light source for fluo-

rescence detection with the detection wavelengths set at 310 nm for excitation and 365 nm for emission (two monochromators); the slit widths of the fluorimeter were 13 nm for excitation and 15 nm for emission.

An alternative stationary phase was a Supelco Suplex pkb-100 (5 μm) HPLC column (150 mm × 4.6 mm I.D.) (Supelco Deutschland, Bad Homburg, Germany) with acetonitrile–water (11:9, v/v) containing 1.0 ml of phosphoric acid (50%) and 0.6 g of dodecylsulphate per litre as mobile phase. Here the retention times of the derivatives were 8.0 min for ciclotropium and 11.1 min for the internal standard (flow-rate of the mobile phase 1.0 ml/min).

As ciclotropium had to be hydrolysed before determination, the assay recorded the "total quaternary compounds", *i.e.* the measured concentration was the sum of ciclotropium and its metabolite N-isopropyltropinium. The amount of ciclotropium was calculated from the difference between the concentrations of the "total quaternary compounds" and of N-isopropyltropinium.

Extraction rate and relative recovery

In order to define the extraction rate and the relative recovery for ciclotropium and N-isopropyltropinium, spiked plasma and urine samples of different concentrations (Table II) were analysed and the peak areas compared with those of directly derivatized N-isopropyltropinium.

Reaction yield of hydrolysis and derivatization in dependence of time and temperature

In order to investigate optimal conditions for hydrolysis and derivatization the dependence of reaction yield on time and temperature was tested. Hydrolysis was carried out at 20, 50, 80, 100, 120 and 140°C for 90 min, and for 20, 40, 60, 80, 100 and 120 min at 140°C. The corresponding peak areas were compared with those of samples spiked with N-isopropyltropinium reference compound. Hence the resulting data are absolute data. The optimal temperature for derivatization was evaluated by reacting the compounds at different temperatures (20, 50, 80, 110, 125, 140 and 170°C) for 30 min. At 110°C the time course of the product yield within 60 min was studied. For each experimental point, three samples, containing 100 ng of ciclotropium, were investigated.

Linearity studies and limit of quantification

The linear behaviour of the calibration curve was tested with spiked standards of different concentrations. For ciclotropium in plasma a range up to 3 μg/ml and in urine a range up to 10 μg/ml was investigated. For the metabolite N-isopropyltropinium in urine, linearity was verified up to 5 μg/ml (Table I).

The detection limits were determined with plasma and urine standards of decreasing concentrations; the lower limit of quantification was the concentration producing a peak with a signal-to-noise ratio of $\geqslant 3$.

TABLE I

LINEARITY STUDIES

For the calculation of the S.D. values (in parentheses, %), six calibration curves were used.

Compound	Concentration range	*n*	Correlation coefficient (*r*)	Regression	
				Slope	Intercept
Plasma					
Ciclotropium	0.5–10 ng/ml	7	0.9997 (0.02)	0.081 (19.4)	0.002 ng (38.9)
	1–3000 ng/ml	7	0.9999 (0.00)	0.088 (12.3)	0.009 ng (44.7)
Urine[a]					
Ciclotropium	0.01–10 μg/ml	10	0.9997 (0.01)	6.429 (10.0)	0.207 μg (36.0)
N-Isopropyltropinium	0.01–5 μg/ml	7	0.9995 (0.01)	5.128 (9.3)	−0.041 μg (37.9)

[a] N-Butyltropinium was used as internal standard.

Accuracy and reproducibility

Reproducibility experiments for ciclotropium were performed at five different concentrations in both plasma (2, 5, 10, 20 and 500 ng/ml) and urine samples (10, 50, 500, 1000 and 10 000 ng/ml). For N-isopropyltropinium the coefficients of variation were determined in urine samples at three concentrations (50, 1000 and 2500 ng/ml). The number of samples was ≥ 6 for each concentration (Table II).

TABLE II

INTRA-DAY COEFFICIENTS OF VARIATION AND RECOVERY

Concentration (ng/ml)	Number of data points (*n*)	S.D. (%)	Recovery (%)
Ciclotropium in plasma			
500	8	2.8	89
20	10	4.4	86
10	8	3.3	87
5	10	5.5	81
2	8	7.7	83
Ciclotropium in urine			
10000	7	1.7	93
1000	7	3.4	92
500	7	3.8	90
50	7	5.0	89
10	6	7.6	91
N-Isopropyltropinium in urine			
2500	8	2.4	94
1000	6	3.3	93
50	8	6.5	89

In order to investigate the inter-day variability of the assay method, three different concentrations in plasma (5, 10 and 20 ng/ml) and two in urine (500 and 1000 ng/ml for ciclotropium, 50 and 100 ng/ml for N-isopropyltropinium) were measured on six different days (Table III).

Short- and long-term stability of ciclotropium in biological samples upon storage

Plasma and urine samples that contained ciclotropium were assayed immediately after sampling, after one month and after twelve months storage at −20°C.

Interference of metabolites

For two further potential metabolites, the two demethylated compounds N-desmethylciclotropium (Fig. 3a) and N-isopropylnortropine (Fig. 3b), it was determined whether there was interference with the ciclotropium and N-isopropyltropinium assay method.

In addition to the above-mentioned mobile phase, where the derivatives of N-isopropyltropinium and N-isopropylnortropine exhibited almost similar retention behaviour, a second mobile phase was used. The latter differed from the usually used mobile phase in that the pH of the aqueous phase was neutral. Then, the retention behaviour of the two compounds (the one with the quaternary and the one with the tertiary nitrogen) was different.

Preliminary pharmacokinetic study

In order to test the applicability of this assay method for pharmacokinetic studies, plasma levels and urinary excretion of ciclotropium and its metabolite N-isopropyltropinium after intravenous and oral administration of ciclotropium were determined. Two healthy male volunteers received an intravenous dose of 10

TABLE III

INTER-DAY COEFFICIENTS OF VARIATION

Concentration (ng/ml)	Number of days (n)	S.D. (%)
Ciclotropium in plasma		
5	6	6.2
10	6	6.2
20	6	4.2
Ciclotropium in urine		
500	6	4.3
1000	6	6.2
N-Isopropyltropinium in urine		
50	6	5.6
100	6	3.2

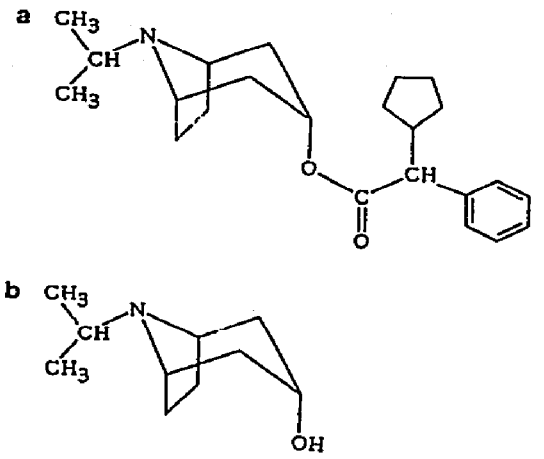

Fig. 3. Structures of potential N-demethylated metabolites. (a) N-Desmethylciclotropium; (b) N-isopropylnortropine.

mg (short infusion over 15 min) and an oral dose of 40 mg of ciclotropium bromide in a cross-over study with a wash-out period of one week. Before starting the study blank plasma and urine samples were taken. For the intravenous application every 5 min until the end of the short infusion (15 min) and then after 5, 10, 15, 30, 45 min and 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 24.0 and 28.0 h venous blood was taken, transferred into heparin-treated tubes, and plasma was separated by centrifugation. For oral dosage the sampling scheme was even, starting at 0.5 h after administration of the drug. The samples were stored at −20°C until analysis. Urine was fractionally collected over a 48-h period.

From the plasma concentration–time curves preliminary pharmacokinetic parameters were estimated: C_{max} represents the maximum concentration detected in plasma and t_{max} the corresponding time value. $t_{1/2}$ is the half-life of the terminal phase calculated from λ_z, the terminal disposition rate constant, which is again calculated from the terminal log-linear phase by regression analysis. The area under the concentration–time curve (AUC) was calculated via the linear trapezoidal method and extrapolated to infinity. The AUC was used to determine the renal clearance (Cl_R) from the total amount excreted into urine ($Ae_{0\to\infty}$) as $Cl_R = Ae_{0\to\infty}/\mathrm{AUC}$.

RESULTS AND DISCUSSION

The extraction yield of ciclotropium and N-isopropyltropinium is acceptable for an assay of biological material (Table II). The extraction yield is highest at a pH range of 8.5–9.5. Repeated extractions and back-extractions lead to relatively "clean" (*i.e.* low-noise) chromatograms. Other physiological quaternary compounds in human plasma and urine such as choline and acetylcholine are coextracted with dipicrylamine as well [9], but can be separated by HPLC. Using

spiked plasma and urine samples it was found that ciclotropium is not hydrolysed to N-isopropyltropinium during the extraction and derivatization step.

Ciclotropium was found to be completely hydrolysed to N-isopropyltropinium during incubation at 140°C for 90 min (Fig. 4a and b), which is in accordance with results described for trospium [5]. For the derivatization the optimal temperature ranges between 110 and 140°C. At this temperature, 10 min after starting the reaction the amount of reaction product does not increase any more (Fig. 4c and d). Similar results were found for the internal standard.

The derivatization of N-isopropyltropinium produced by metabolism as well as by hydrolysis of ciclotropium leads to a stable product, which was separated from plasma and urine constituents (*e.g.* choline) and from excess reagent (flunoxaprofen) by reversed-phase HPLC. The excitation and emission maxima were determined to be 310 and 365 nm. The maxima observed were almost identical to those described for flunoxaprofen [8]. Fig. 5A and B depict chromatograms which were obtained from plasma and urine samples.

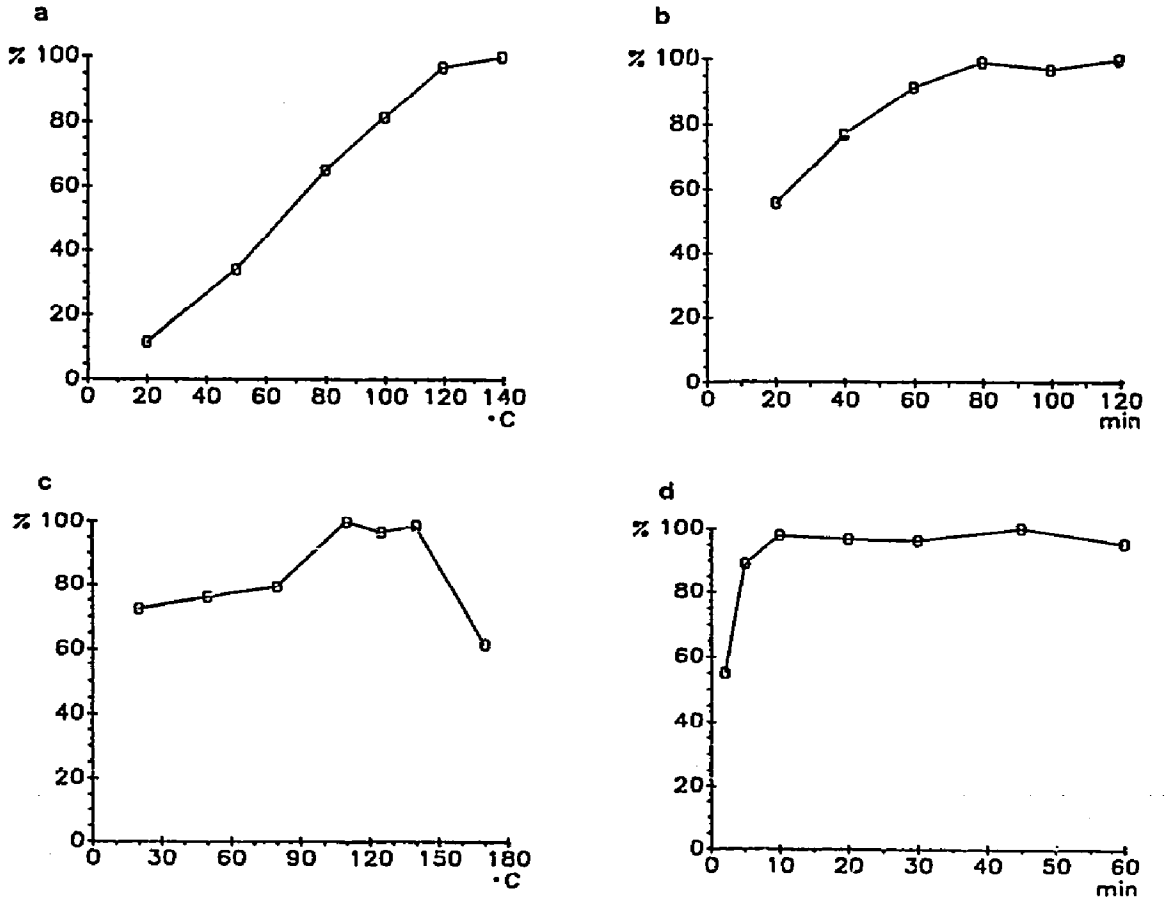

Fig. 4. Dependence of reaction yield of ciclotropium hydrolysis on (a) temperature and (b) time. Dependence of derivatization yield on (c) temperature and (d) time. Experimental details are given in the experimental section.

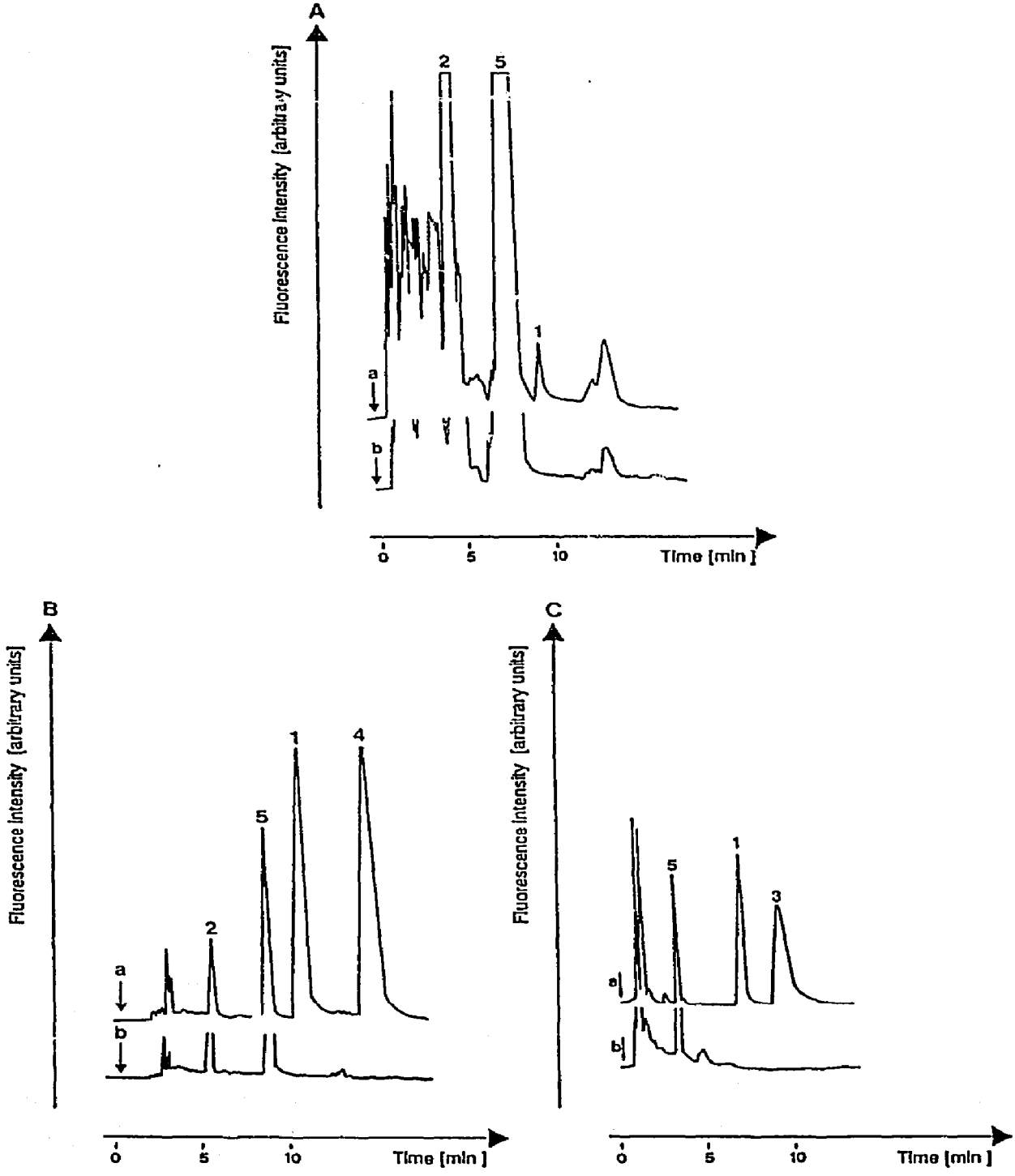

Fig. 5. Chromatograms obtained from plasma and urine samples. The top of the chromatogram always represents the sample (a), the bottom chromatogram the blank (b). (A) Plasma (0.9 ng/ml ciclotropium bromide), volunteer 2, 24 after intravenous application of 10 mg of ciclotropium bromide. (B) Urine (115 ng/ml ciclotropium bromide), volunteer 2, 32–38 h collection period. (C) N-Desmethylciclotropium in urine (25 ng/ml), spiked sample. Peaks: 1 = ciclotropium; 2 = choline; 3 = N-desmethylciclotropium; 4 = N-butyltropinium; 5 = flunoxaprofen.

The calibration curves obtained from ciclotropium in plasma and urine as well as for N-isopropyltropinium in urine are linear over a range wider than necessary to cover the clinically relevant concentrations (the correlation coefficients, regression slopes and intercepts including relative standard deviations are given in Table I).

The detection limit of ciclotropium is 0.5 ng/ml for plasma and 10 ng/ml for urine samples. The detection limit for urine can easily be further improved by increasing the sample volumes. As the pilot study demonstrated, ciclotropium concentrations in urine are much higher than those in plasma, and the urine assay is sufficiently sensitive without further modifications. The detection limit for N-isopropyltropinium in urine is 2 ng/ml.

The intra-day variabilities for the ciclotropium assay in plasma and urine and for N-isopropyltropinium in urine at different concentrations are given in Table II. The inter-day variability of the assay method is summarized in Table III. The intra- and inter-day coefficients of variation were always below 8%, even though no internal standard was used for the determination of plasma samples. N-Butyltropinium can be used as an internal standard for plasma samples as well, but as the baseline noise is higher in plasma than in urine samples and interfering peaks sometimes occur, the coefficients of variation do not improve significantly. Overall linearity and accuracy studies indicate a high reliability.

The concentrations of ciclotropium remained constant throughout the observation periods for short- and long-term stability.

As Fig. 5C shows, the derivative of N-isopropyltropinium can be separated from the derivative of the demethylated compound N-isopropylnortropine when the alternative mobile phase is used. In human plasma and urine these two potential metabolites were not detected.

The amount of ciclotropium has to be calculated from the difference between the concentrations of the "total quaternary compounds" and N-isopropyltropinium, but as the amount of this metabolite appearing in urine is very low (about 1% of the "total quaternary compounds") and its plasma concentrations are beyond the detection limit it is negligible.

The plasma concentration–time curves for ciclotropium in two healthy male volunteers are shown in Fig. 6A and B for intravenous and oral administration, respectively. After oral dosage of 40 mg of drug, maximum plasma concentrations (C_{max}) of 9.3 and 5.7 ng/ml were found after 3.0 and 2.5 h (t_{max}) in the two volunteers. The oral bioavailability of 5.3 and 5.9% was in the same range as described for other quaternary compounds (3% for trospium chloride [4]). The average $t_{1/2}$ values were found to be 10.8 h for the intravenous and 10.4 h for the oral administration. The cumulative renal excretion, *Ae*, for these volunteers is given in Table IV. The unchanged renal clearance was calculated as 566.7 and 379.8 ml/min for intravenous and 667.7 and 691.6 ml/min for oral administration of the drug.

The concentrations of the metabolite N-isopropyltropinium in urine were in

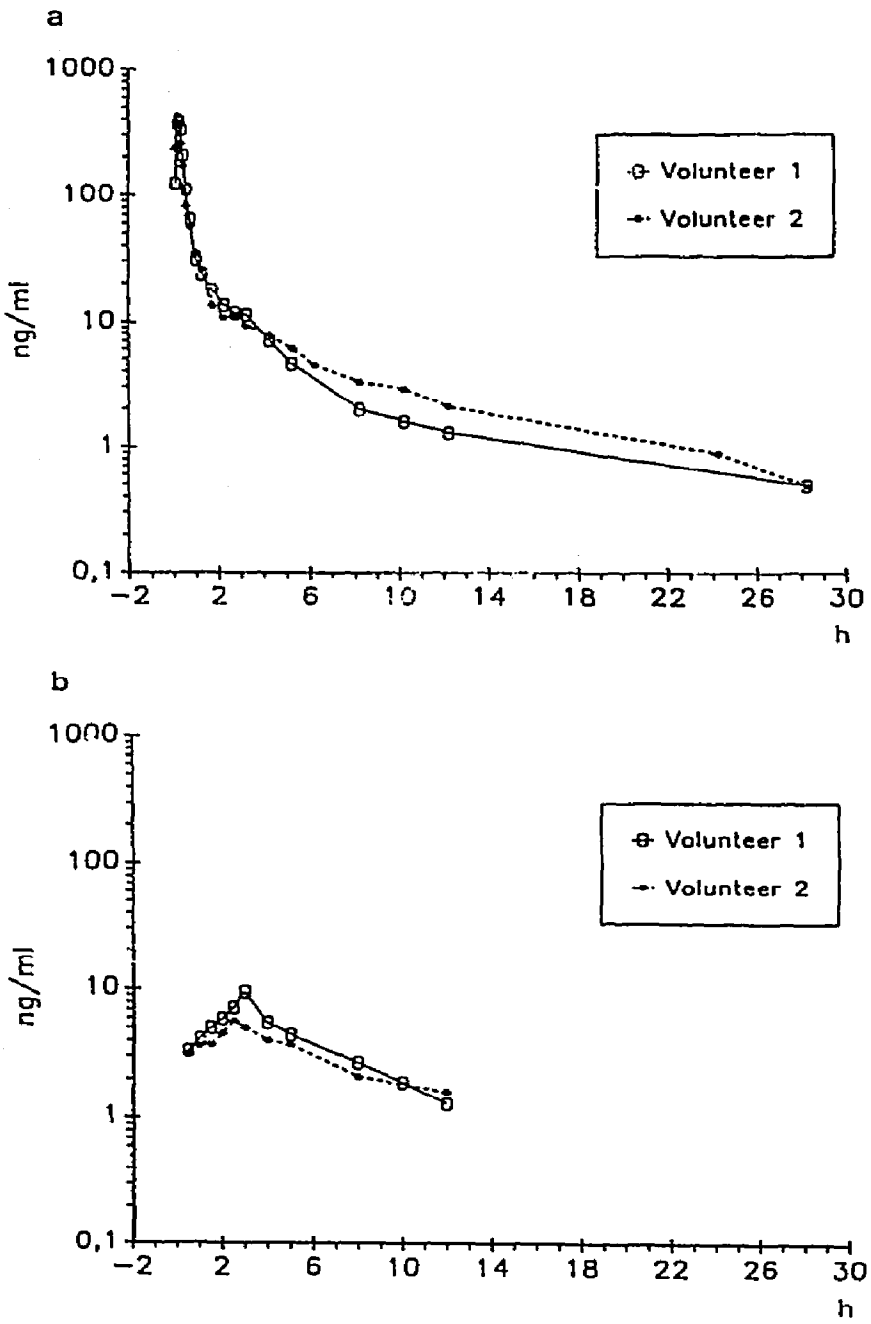

Fig. 6. Plasma concentration–time curves of ciclotropium in two healthy volunteers after (a) intravenous dosage of 10 mg as a short infusion for 15 min and (b) oral dosage of 40 mg ciclotropium bromide.

all cases (intravenous and oral administration) about 1% of the "total quaternary compounds". The two potential demethylated metabolites could not be detected.

TABLE IV

CUMULATIVE RENAL EXCRETION $Ae_{0\to\infty}$ FOR CICLOTROPIUM IN MAN

Volunteer	Mode of administration	Amount excreted (μg)	Fraction of the dose (%)
1	10 mg intravenously	8463.5	84.6
2	10 mg intravenously	5806.9	58.1
1	40 mg orally	2115.1	5.3
2	40 mg orally	2473.3	6.2

CONCLUSION

Quantification of unlabelled quaternary drugs such as N-butylscopolaminium bromide or trospium chloride in biological material employing conventional chromatographic techniques is known to be rather difficult. Owing to the low bioavailability, plasma concentrations after oral dosage are low and, in addition, the chromophoric properties of these atropine derivatives are poor. Using the assay method described in this paper it was possible to measure plasma and urine concentrations of ciclotropium and its metabolite N-isopropyltropinium in man after intravenous and oral administration of the drug. In spite of involving two extractions, one hydrolysis and one derivatization step, the reproducibility and reliability of the assay method are high.

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