Journal of Chromatography, 381 (1986) 75—82
Biomedical Applications
Elsevier Science Publishers B V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3203

SIMULTANEOUS DETERMINATION OF TROPATEPINE AND ITS MAJOR METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC—MASS SPECTROMETRIC IDENTIFICATION

APPLICATION TO METABOLIC AND KINETIC STUDIES

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(First received August 22nd, 1985; revised manuscript received April 17th, 1986)

SUMMARY

Tropatepine is used to combat against extrapyramidal syndrome induced by neuroleptic drugs. A high-performance liquid chromatographic method was proposed for the simultaneous determination of tropatepine and its potential metabolites in biological fluids. After double extraction of compounds in hexane and back-extraction in hydrochloric acid, the chromatographic separation was performed on a reversed-phase column with an acetonitrile—perchlorate buffer mixture as mobile phase. Compounds were detected at 229 nm and the detection limit was about 15 ng/ml. The method was applied to bile and urine samples collected in rats, after a single high oral dose of 100 mg/kg of tropatepine hydrochloride. Gas chromatography—mass spectrometry was used for identification of the potential metabolites. Nortropatepine and tropatepine S-oxide were identified in this way, and it seemed that tropatepine was subjected to a large and intense metabolic process. The analytical procedure and the results of the metabolic investigation were applied to a preliminary pharmacokinetic study in patients undergoing long-term oral therapy with tropatepine.

INTRODUCTION

Tropatepine (I), (6*H*-dibenzo[*b*,*e*]thiepinnylidene-11)-3-tropane, is a compound that presents central anticholinergic properties. Tropatepine is usually used over a number of years against neuroleptic-induced extrapyramidal syndrome [1—5]. Since no method of determining I has yet been reported, and

0378-4347/86/$03.50

since the therapeutic concentrations are not known, a high-performance liquid chromatographic (HPLC) method for the determination of unchanged drug was devised and reported recently [6]. Preliminary studies in healthy volunteers showed that after a single oral dose of 20 mg of tropatepine hydrochloride, very low plasma concentrations of unchanged drug were recovered [7]. In contrast, I and its potential metabolites were detected in urine. Metabolic investigation was therefore performed in animals and implied the use of a new method.

This report describes an HPLC procedure for the simultaneous determination of I and its potential metabolites. The method is applied to a metabolic study in rats receiving single high oral doses of tropatepine hydrochloride (100 mg/kg) and in patients undergoing long-term therapy (20 mg per day). With regard to our chromatographic results, the presence of two metabolites was suspected in animal and human urine samples. Their structures were determined by gas chromatography—mass spectrometry (GC—MS).

EXPERIMENTAL

Drug standards

Tropatepine (I), nortropatepine (II) and tropatepine S-oxide (III) hydrochlorides were supplied by Diamant (Paris, France) and clotiapine hydrochloride (used as internal standard, IV) by Sandoz (Paris, France). Compound structures are shown in Fig. 1. Stock solutions of I, II, III and IV, containing 1 mg/ml, were prepared in ethanol and stored at 4°C.

Stock solutions were serially diluted weekly into the working range of 1—1000 ng/ml.

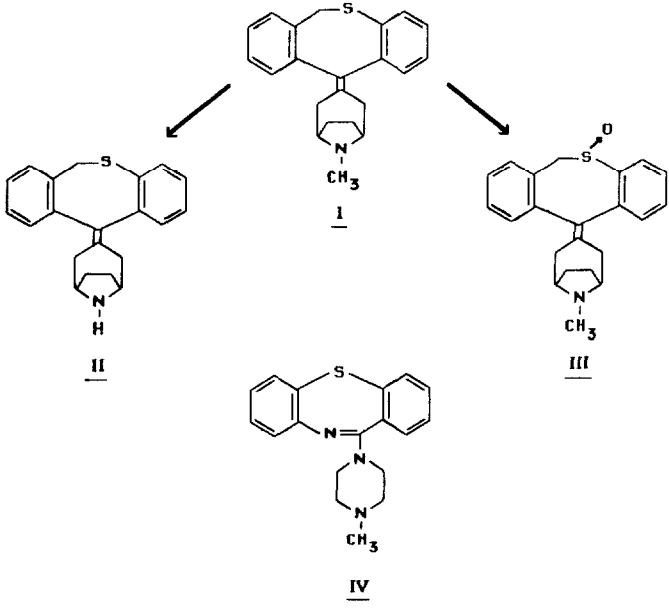


Fig. 1. Chemical structures of tropatepine (I), nortropatepine (II), tropatepine S-oxide (III) and internal standard (IV).

HPLC system

The chromatographic system consisted of an HP1084B solvent delivery system (Hewlett-Packard, Les Ulis, France). The compounds were detected with a Waters Model 481 spectrophotometer set at 229 nm (Waters Assoc., Milford, MA, U.S.A.).

The analytical column (300 mm × 4.0 mm I.D.) was a stainless-steel μBondapak C_{18} (10 μm) column obtained from Waters. The mobile phase consisted of a degassed and filtered mixture of 0.05 *M* perchlorate buffer (pH 2.9) containing 30% acetonitrile, at a flow-rate of 1.7 ml/min. The percentage of acetonitrile of the mobile phase was linearly increased after injection, with a gradient steepness of 9%/min from 30 to 39% at 6 min. The column was regenerated, after the gradient run, to the initial mobile phase conditions by running a 10-min reversed gradient at a flow-rate of 1.7 ml/min, followed by 10-min of isocratic operation at initial mobile phase conditions before injection of the next sample.

Gas chromatography—mass spectrometry

Samples were analysed by an HP5985 gas chromatograph—mass spectrometer, operated in the positive chemical-ionization mode. GC separations were carried out using a fused-silica wall-coated open tubular capillary column (10 m × 0.5 mm I.D.) with polydimethylsiloxane (CP-Sil 5 Chrompack, Orsay, France) as the stationary phase. For analysis, the oven temperature was programmed from 200 to 290°C at a step-rate of 15°C/min. The solid injector, the ion source and the GC—MS interface were held at 280°C. The mass spectrometer was operated with an emission current of 350 μA and an electron energy of 230 eV.

Reagents

Reagent-grade chemicals and HPLC-grade solvents were used throughout. The perchlorate solution was prepared by mixing 0.005 *M* perchloric acid and 0.045 *M* sodium perchlorate in distilled water. All the HPLC-grade solvents were filtered through organic—aqueous filter paper (pore size 0.5 μm; Millipore FH-UP 04700) and degassed in an ultrasonic bath for 45 min before use.

Animal study

The metabolic studies were conducted in healthy male Wistar rats, weighing 200—250 g. After cannulation of the bile duct under anaesthesia, the rats orally received 100 mg/kg I. Bile and urine samples were collected over a period of 32 h following administration.

Sample preparation

Plasma was separated from oxalated whole blood by centrifugation at 3000 *g* for 10 min. A plasma sample (1.0 ml), spiked with IV (12.5 μl, 10 ng/ml) as internal standard, was made alkaline with 0.5 ml of 5 *M* sodium hydroxide and then extracted into 30-ml cylindro-conical tubes with 4.0 ml of hexane for 15 min using an inversion mixer. After centrifugation at 2000 *g* for 5 min, the organic layer was removed to a 10-ml cylindro-conical tube. A second extraction was operated on the same sample, with 4.0 ml of hexane. After

mixing and centrifuging as before, the combined organic layers were back-extracted with 75 μl of 0.15 *M* hydrochloric acid for 5 min using a Vortex mixer. Aliquots (20—50 μl) of the acidic phase were then injected onto the HPLC column.

The same procedure was used for urine (1.0—5.0 ml) and bile (0.2—0.5 ml) samples.

For GC—MS determination, the HPLC procedure was used for separation and purification of unchanged drug and potential metabolites. On each collection of chromatographic eluent, corresponding to a peak elution, compounds were extracted with 5.0 ml of hexane—5 *M* sodium hydroxide (4:1) mixture. After centrifugation, the organic layers were dried under nitrogen at 37°C. The dried residues were dissolved in 50 μl of ethanol, immediately prior to analysis.

Quantitation

Standard curves were prepared by addition of known concentrations of I, II and III to control plasma, bile or urine, and then analysing a set of these standards with each batch of samples. Least-squares linear regression curves were employed to fit plots of peak-area ratios (drug or metabolite/IV) versus plasma concentration.

RESULTS AND DISCUSSION

Performance of the HPLC system

The extraction procedure gives a good HPLC baseline for both plasma, urine and bile, and there are no interferences from endogenous compounds.

Fig. 2 shows the UV spectra of I, II and III in ethanol. No difference was observed between the UV spectra of I and II.

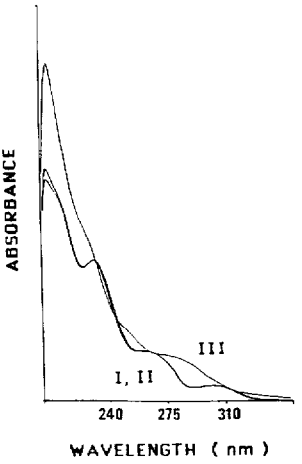
Fig. 3 shows the chromatograms of a drug-free plasma extract and a plasma sample extract from a patient chronically treated with I (20 mg per day). Compounds I, II and III and the internal standard (IV) had, under the chromatographic conditions, retention times of 16, 14, 5 and 11 min, respectively, and were resolved satisfactorily from the peaks owing to the endogenous plasma components.

Recovery

Recovery was calculated by comparing the measured peak-area ratios of I, II and III to IV (used as external standard) of spiked serum to those of standard aqueous solutions. Under these conditions, the recoveries of I, II and III in the range of 50—500 ng/ml were ca. 70—80%. These extraction yields were found to be satisfactory, taking into account the extraction complexity and the likely glass adsorption.

Reproducibility and precision

Repeated extractions of plasma samples spiked with I, II and III at different concentrations, indicated that the procedure's reproducibility was good. The data presented in Table I show the accuracy (defined by the difference between found and added concentration) and the precision of the intra- and inter-assays.



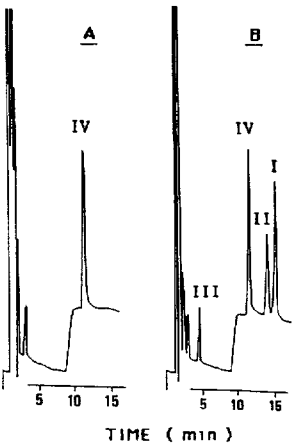


Fig. 2. UV spectra of tropatepine (I; 0.01 μg/ml), nortropatepine (II; 0.01 μg/ml) and tropatepine S-oxide (III; 0 01 μg/ml) in ethanol.

Fig. 3. Chromatograms of: (A) control plasma spiked with internal standard (IV; 125 ng/ml); (B) plasma obtained 1 h after the daily oral administration of 20 mg of tropatepine hydrochloride to a patient in long-term therapy. Peaks: I = tropatepine (100 ng/ml); II = nortropatepine (53 ng/ml); III = tropatepine S-oxide (40 ng/ml); IV = clotiapine (internal standard; 125 ng/ml).

TABLE I

REPRODUCIBILITY AND PRECISION

Compound	Concentration added (ng/ml)	Concentration found (mean ± S.D.) (ng/ml)	Coefficient of variation (%)	Accuracy (%)
Intra-assay precision (repeatability) (n = 6)				
I	50	52 ± 5.1	9.9	+3.8
	250	240 ± 11.5	4.8	−4.2
	500	497 ± 3.9	0.8	−0.6
II	50	55 ± 3.6	6.5	+10.0
	250	241 ± 12.0	5.0	−3.7
	500	503 ± 7.5	1.5	+0.1
III	50	52 ± 0.5	1.0	+3.8
	250	247 ± 9.6	3 9	−1.2
	500	490 ± 9.8	2.0	−2.0
Inter-assay precision (reproducibility) (n = 3)				
I	50	54 ± 4.0	7.4	+7.4
	250	240 ± 12.3	5.1	−4.2
	500	533 ± 10.1	1.9	+6.2
II	50	54 ± 1.9	3.4	+7.4
	250	260 ± 8.7	3.5	+3.8
	500	498 ± 3.8	0.1	−0.1
III	50	47 ± 3.5	1.0	−3.8
	250	263 ± 21.7	8.2	+4.9
	500	506 ± 2.8	0.1	+1.2

Linearity

Linearity proved satisfactory for plasma, urine and bile. In plasma, the least-squares linear regression curves for I, II and III have the following respective equations: $y = 0.0047x - 0.006$ ($r = 0.9998$); $y = 0.0057x - 0.171$ ($r = 0.9999$); $y = 0.0019x + 0.062$ ($r = 0.9999$), for the concentration range 50–500 ng/ml.

Sensitivity

The limit of detection using a 1.0-ml plasma sample and a 70-μl injection was 15 ng/ml, with a signal-to-noise ratio greater than 3:1.

Specificity

Samples of plasma spiked with a variety of neuroleptic drugs (such as haloperidol, pipothiazine, levomepromazine, flupenthixol and chlorpromazine) responsible for the appearance of extrapyramidal syndrome, and anti-Parkinson drugs (such as atropine, ponalide, artane and mantadix) were tested by the HPLC procedure. No compounds interfered with the tropatepine retention time. Other basic compounds such as benzodiazepines or tricyclic antidepressants were not tested. Indeed, the patients recruited for the pharmacokinetic study had never been treated with these types of compounds.

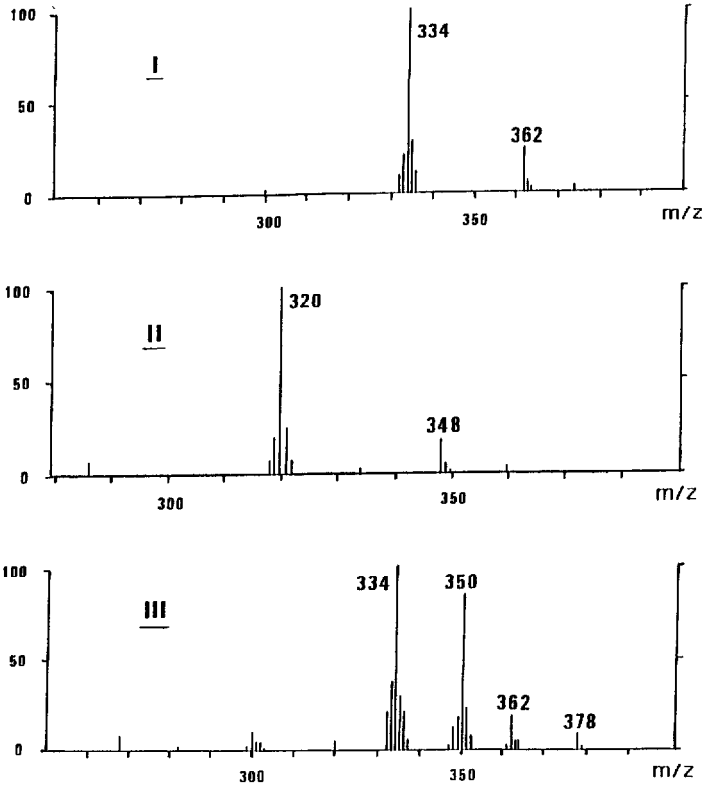


Fig. 4. Molecular mass spectra of compounds obtained from urine samples of rat following a single high oral dose of tropatepine (100 mg/kg), after separation by HPLC, extraction and GC—MS identification. I, tropatepine; II, nortropatepine; III, tropatepine S-oxide.

GC—MS analysis

The analyses of I, II and III were performed with the HPLC procedure upon urine samples of rats treated with a single high oral dose of tropatepine hydrochloride (100 mg/kg).

Determination of the structure of each compound was carried out by GC combined with an identification by MS. Under the GC—MS conditions described previously, three mass spectra were obtained (Fig. 4) and compared with those acquired under the same conditions with different pure compounds. The mass spectra were characterized by base isotopic peaks at *m/z* 334 (M^+), 320 ($M - CH_2$) and 350 (M + O), corresponding to I, II and III, respectively.

This GC—MS procedure has not been envisaged for routine use, bearing in mind the insufficient sensitivity obtained for each compound.

Application

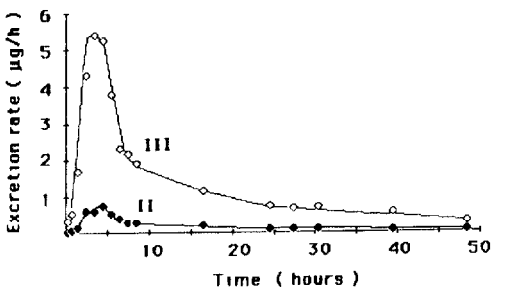
The method was applied to metabolic studies in male Wistar rats receiving a single oral dose of I (100 mg/kg) and where bile and urine were continuously sampled.

In bile (Fig. 5), no measurable levels of unchanged drug were noted, while II and III were observed shortly after oral administration (45 and 15 min, respectively). Metabolite III was produced and eliminated in larger proportions. These results show biliary excretion of II and III, and suggest a rapid and intense metabolism process in rat, and may in part explain the low concentrations of I observed in man following a single oral dose of 20 mg of tropatepine hydrochloride [7].

Bile elimination was at a maximum at around 4 h for the two compounds, and then declined, remaining measurable beyond 50 h for compound III.

Compounds I, II and III were noted in larger amounts in urine than in bile, and III was the major metabolite (Fig. 6).

In man, the desalkylation (II) and sulphoxidation (III) of I was confirmed in a pharmacokinetic study performed with patients orally dosed with tropatepine (20 mg per day) in long-term therapy. A plasma concentration—time curve of I, II and III is shown in Fig. 7. Here, unchanged drug (I) was the major plasmatic compound. Plasma levels of I did not increase significantly (80—140



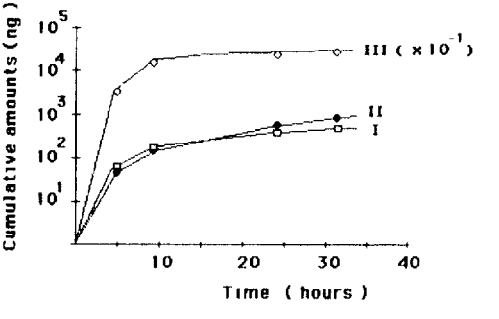


Fig. 5. Bile extraction rate of nortropatepine (II) and tropatepine S-oxide (III) following a single oral dose of 100 mg/kg tropatepine to a male Wistar rat.

Fig. 6. Urinary cumulative amounts of tropatepine (I), nortropatepine (II) and tropatepine S-oxide (III) following a single oral dose of I (100 mg/kg) to a male Wistar rat.

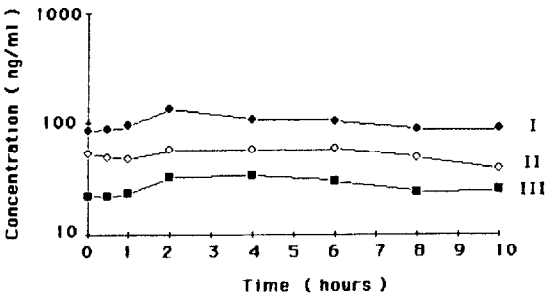


Fig. 7. Semilogarithmic plot of plasma tropatepine (I), nortropatepine (II) and tropatepine S-oxide (III) concentrations against time, in a patient treated in long-term oral therapy, after a single daily tropatepine hydrochloride administration of 20 mg.

ng/ml) after chronic oral administration. Compounds II and III reached lower concentrations. In practice, these results seem to confirm the necessity of chronic administration of tropatepine hydrochloride in order to achieve efficacy against extrapyramidal syndrome induced by neuroleptic drugs.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. P. Coassolo for his helpful suggestions. This work was supported, in part, by INSERM-Diamant Grant No. 84038.

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