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GAS CHROMATOGRAPHIC METHOD FOR DETERMINATION OF A PIPERAZINE DERIVATIVE (TRELIBET®) AND ITS METABOLITES IN HUMAN PLASMA AND URINE

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SUMMARY

A sensitive gas chromatographic method was developed for the determination of Trelibet®, 1-benzyl-4-(2′-pyridinecarbonyl)piperazine, and of its major metabolites in biological fluids. The compounds were extracted as bases into dichloromethane, and the extracts were analysed by a dimethylsilicone capillary column with a nitrogen–phosphorus flame-ionization detector. The lower limit of detection was 1 ng/ml for Trelibet and 5 ng/ml for the metabolites. Peak-area ratios of the compounds and internal standard were linearly correlated to their plasma concentrations between 1 and 1000 ng/ml. The method was used for quantification of Trelibet and two of its metabolites in depressed patients after oral administration of a single dose of 200 mg of Trelibet. Concentration data measured in plasma and urine showed that the method is sensitive enough to monitor concentrations both for pharmacokinetic studies and for plasma steady-state levels daily.

INTRODUCTION

According to pharmacological studies Trelibet® [TRE; 1-benzyl-4-(2′-pyridinecarbonyl)piperazine], a recently developed compound, seems to have antidepressive effects [1–3]. Its clinical trials have recently been approved. Its chemical structure (Fig. 1, structure 1) is different from the first-generation tricyclic antidepressants. The metabolism of the compound was studied in rats and dogs by Magyar *et al.* [5, 8] and it has been concluded that the main metabolic pathway is the cleavage of the molecule into N-(2′-pyridinecarbonyl)-piperazine (N-PCP) as shown in Fig. 1 (structure 2) and N-benzylpiperazine (N-BP) (Fig. 1, structure 3), followed by further cleavages and hydroxylation.

In earlier pharmacological investigations in animals, the compound and its

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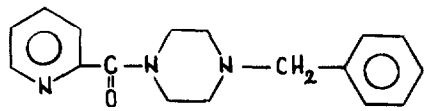

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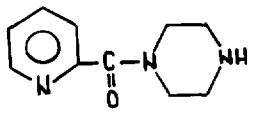

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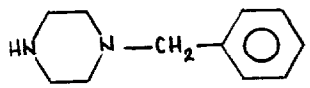

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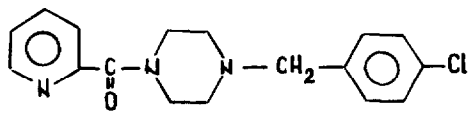

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Fig. 1. Chemical structures of compounds: (1) 1-benzyl-4-(2'-pyridinecarbonyl)piperazine (Trelibet or TRE); (2) N-(2'-pyridinecarbonyl)piperazine (N-PCP); (3) N-benzylpiperazine (N-BP); (4) *p*-chloro derivative of Trelibet (internal standard).

metabolites were determined by thin-layer chromatography (TLC), UV spectrophotometry and by determination of the radioactivity of the molecule labelled on the carbonyl group [4—8]. In our experience, the TLC method has limited accuracy and is time-consuming, and UV spectrophotometry does not prove to be sensitive enough for metabolites. A high-performance liquid chromatographic (HPLC) method with moderate sensitivity was published in this field [9].

The purpose of our work was to develop a simple method by which the piperazine compound as well as its main metabolites could be determined in one step, both in plasma and urine at low concentrations. Since the molecules display good gas chromatographic behaviour and because of their nitrogen content, they have to be determined by a highly sensitive method using specific nitrogen—phosphorus flame-ionization detection (NP-FID). In spite of this behaviour, the measurement and quantification of these molecules in biological samples created a lot of problems because of the great similarity of the measured compounds to several endogenous ones, thus their separation became very difficult.

EXPERIMENTAL

Reagents, solvents and materials

Sodium hydroxide and hydrochloric acid were of analytical grade and products of Reanal (Budapest, Hungary). Dichloromethane and methanol (Reanal) were also of analytical grade, purified carefully by distillation before use. Extrelut®, an aqueous organic phase separator, was obtained from Merck (Darmstadt, F.R.G.). For the separation of compounds, home-made separator

columns were used, filled with 500 mg of Extrelut phase separator (Merck Cat. No. 11738) using a 25 cm × 0.6—0.7 cm I.D. Pasteur pipette.

Reference drug stock solutions and standards

TRE, N-PCP and N-BP were synthesized and donated by EGIS Pharmaceutical Works (Budapest, Hungary). The *p*-chloro derivative of Trelibet (Fig. 1, structure 4) used as internal standard (I.S.) was donated by the Department of Pharmacodynamics, Semmelweis University of Medicine (Budapest, Hungary). Primary stock solutions of the drug, the metabolites and the I.S. were prepared in methanol at concentrations of 1 g/l. The solutions were stable for at least six months. Working solutions (10 ng/μl) were occasionally diluted for standard curves and samples down to 10 mg/l. Control samples of plasma were prepared for precision studies with a concentration of 100 ng/ml of each compound.

Extraction procedure for plasma and urine

To 1 ml of heparine-treated plasma or urine stored at −20° C until analysed, 200 ng of I.S. for plasma and 1000 ng for urine were added (20 and 100 μl of working stock solution). Samples were alkalized to pH 9 by adding 0.2 ml of 0.1 *M* sodium hydroxide. As TRE seems to have some effect on the secretion and on the pH of the urine, sometimes acidification was necessary for urine instead of alkalization, because of its high pH value. In such cases, 0.1 *M* hydrochloric acid was added drop by drop until it reached pH 9. The prepared plasma or urine samples were applied to the top of the separator column. The aqueous phase was allowed to soak on the Extrelut bed for 30 min, then 10 ml of dichloromethane were passed through the column. After elution, the organic phase was evaporated in a gentle stream of nitrogen. The residue was dissolved in 100 μl of methanol and aliquots of 1—3 μl were injected into the gas chromatograph.

Gas chromatography

A Hewlett-Packard 5840 A gas chromatograph equipped with a 18835 B capillary inlet system and a nitrogen—phosphorus-selective flame-ionization detector was used. The flexible fused-silica capillary column was 25 m × 0.20 mm I.D. with a 0.33-μm film of dimethyl silicone stationary phase (Hewlett-Packard, U.S.A.).

Nitrogen of high purity was used as both carrier and auxiliary gas.

TABLE I

RECOVERIES OF THE COMPOUNDS FROM PLASMA USING SOLID—LIQUID EXTRACTION

Values are means (± S.D.) of three plasma samples extracted and chromatographed.

Concentration added (ng/ml)	Concentration determined (ng/ml)			Recovery (%)		
	TRE	N-PCP	N-BP	TRE	N-PCP	N-BP
50	42.6 ± 2.4	26.8 ± 1.4	22.8 ± 0.9	85.2 ± 0.9	53.6 ± 0.7	45.6 ± 0.2
100	86.2 ± 1.8	51.2 ± 1.1	44.9 ± 1.1	86.2 ± 1.0	51.2 ± 0.4	44.9 ± 0.7
500	459.1 ± 3.4	258.3 ± 3.2	219.3 ± 2.0	91.8 ± 1.1	51.7 ± 0.9	43.9 ± 1.1
1000	882.1 ± 4.2	569.2 ± 2.9	488.5 ± 3.0	88.2 ± 1.8	56.9 ± 1.9	48.8 ± 1.7

The temperature programme was from 120°C at 20°C/min to 255°C. Column pressure was 0.196 MPa and the auxiliary gas flow-rate was 30 ml/min. Hydrogen and air gas flow-rates were 3 and 50 ml/min, respectively. The temperature settings were as follows: capillary injection port in split mode, 260°C; NP-FID system, 270°C. The alkali metal salt-bead heating voltage was 16.5 V; the split ratio was 10:1.

The reaction times for TRE, N-BP, N-PCP and I.S. were 9.85, 3.45, 5.39 and 13.05 min, respectively.

RESULTS

Extraction procedures

Because of the chemical similarity of endogenous substances and the compounds investigated, extremely high efforts were made to separate them and find the optimum extraction procedure. For the investigations, water solutions were used containing 200 ng/ml of each compound. For liquid—liquid extraction from water solutions, *n*-hexane, benzene, diethyl ether, chloroform and dichloromethane were examined at pH 1, 3, 5, 7, 9 and 11. Maximum recoveries were obtained with dichloromethane at pH 9, but these values were lower than those at solid—liquid separations using Extrelut, which is why we chose to build our whole methology on the latter procedure. Data obtained by solid—liquid separations are shown in Table I.

Calibration of the method

Known amounts of the compounds were added in increasing concentrations to plasma blanks. The concentration of the I.S. was 200 ng/ml. The correlation coefficients and equations were calculated by the least-squares method. The value y represents the ratio of peak areas of the measured compounds to peak area of the I.S., and x the concentrations of the measured compounds, in ng/ml.

The equations are: TRE, $y = 0.0077x - 0.0047$, $r = 0.999$; N-BP, $y = 0.0010x + 0.0039$, $r = 0.996$; N-PCP, $y = 0.0035x + 0.068$, $r = 0.997$. The ratio

TABLE II

ACCURACY OF THE METHOD

Peak-area ratios of the compounds and the I.S. were linearly correlated to their plasma concentrations between 1 and 1000 ng/ml. Equations for calibration curves are given in the text.

Concentration added (ng/ml)	Concentration determined (mean ± S.D., $n = 3$) (ng/ml)		
	TRE	N-PCP	N-BP
1	1.0 ± 0.1	1.9 ± 0.2	0.5 ± 0.4
10	9.9 ± 0.1	13.3 ± 0.8	6.3 ± 0.3
50	49.5 ± 0.5	52.6 ± 1.7	51.5 ± 0.2
100	100.3 ± 0.5	100.5 ± 2.1	105.2 ± 4.1
200	196.7 ± 5.5	210.6 ± 3.6	187.4 ± 5.2
500	492.2 ± 3.2	514.7 ± 12.7	507.8 ± 4.1
1000	1000.6 ± 1.9	1011.1 ± 3.2	1006.0 ± 5.5

of the peak area of TRE to the I.S. was linear in concentration range 1—1000 ng/ml. Calibration curves for N-BP and N-PCP were not linear below 10 ng/ml; however, the measurements were reproducible (see Table II).

Unknown concentrations of TRE, N-PCP and N-BP were calculated from the results obtained with an area under the curve (AUC) value of several standards relating to the AUC value of the I.S.

Precision studies of control plasma samples gave acceptable coefficients of variation as outlined in Table III.

For checking possible chromatographic interferences, the retention times of some commonly used drugs were established under the extraction and chromatographic conditions applied. Of the drugs examined, only chlorpromazine interfered with TRE; however, it is unlikely that they would be combined in the same treatment. Retention times are given in Table IV.

TABLE III

PRECISION DATA OF TRE, N-PCP AND N-BP ASSAYS MEASURED IN PLASMA

Compound	Mean concentration (ng/ml)	Coefficient of variation (%)
Within-run precision (n = 6)		
TRE	104.0 ± 3.9	3.73
N-PCP	96.5 ± 3.1	3.21
N-BP	101.1 ± 9.3	9.29
Day-to-day precision (n = 15)		
TRE	97.2 ± 3.9	4.08
N-PCP	99.9 ± 5.2	5.3
N-BP	103.3 ± 11.5	11.1

TABLE IV

RETENTION TIMES OF SOME DRUGS ON MEASUREMENT FOR TRE AND ITS METABOLITES

Drug	Retention time (min)	Drug	Retention time (min)
Amitriptyline	7.44	Chlorpromazine	9.84
Nortriptyline	7.56	Thioridazine	11.52
Imipramine	7.61	Trifluperidol	12.06
Deispramine	7.73	Clozapine	16.36
Maprotiline	8.55	Phenobarbital	6.95
Tofisopam	9.44	Diazepam	9.30
Promazine	8.36	Medazepam	7.70

DISCUSSION

The new method was developed to determine TRE and N-BP precisely, as biologically active compounds and to obtain information on the quantity of N-PCP as the other major metabolite, being biologically inactive according to preclinical studies.

Since it was necessary to measure great amounts of human biological samples, the analyses had to be quick and less time-consuming. Quantification of TRE and of N-BP concentrations in plasma and urine was achieved. However, an unidentified endogenous substance was eluted from all plasma and urine blanks, with the same retention time as N-PCP. Great efforts were made to separate the interfering substance from N-PCP, but without success.

We also investigated background concentrations of endogenous substances corresponding to N-PCP concentrations in ten different patients who were not receiving TRE. Furthermore, the concentration of the interfering substance was examined in the same patient both in plasma and urine during a 24-h period. The amount of this endogenous substance found for blank plasma was, on average, 3.3 ± 0.9 ng/ml (n = 50) and 24.2 ± 1.5 ng/ml (n = 42) for urine. Although the separation of N-PCP has not yet been solved, and the background interference is relatively high (primarily in urine), our data give some possibilities of assessing the relative magnitude of its plasma and urine levels. N-PCP values were neglected if the observed plasma concentration was only 5 ng/ml or less, and the urine concentration was 30 ng/ml or less.

Thus, these results may be considered to be a compromise, but more than a thousand measurements of the points of human pharmacokinetic curves proved it to be useful.

Fig. 2 shows chromatograms of the extracts of drug-free plasma and urine with the unidentified peak at 5.38 min.

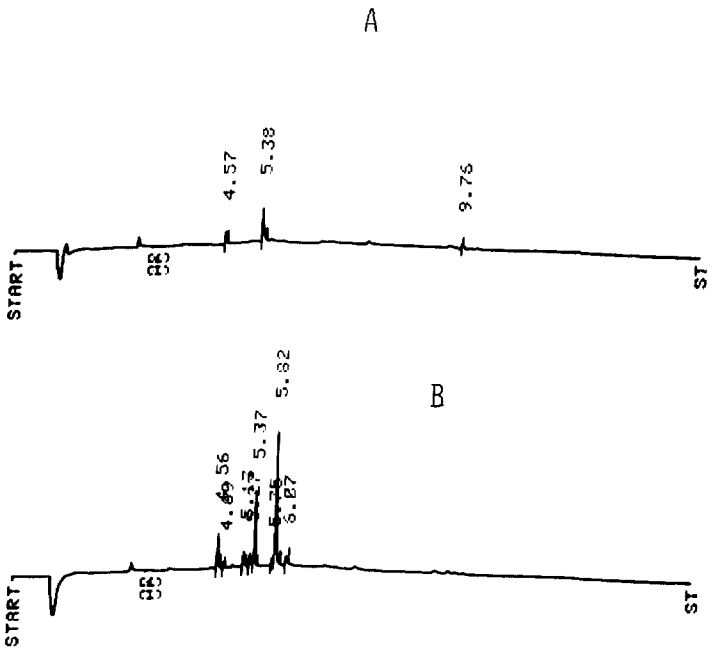

Fig. 2. (A) Chromatogram of extract from drug-free plasma samples (without internal standard). (B) Chromatogram of extract from drug-free urine sample (without internal standard). Impurity peaks at retention times 5.37 (A) and 5.38 min (B) are unknown, found in all the plasma and urine samples. Their amounts were statistically determined and considered in the measurements of N-PCP.

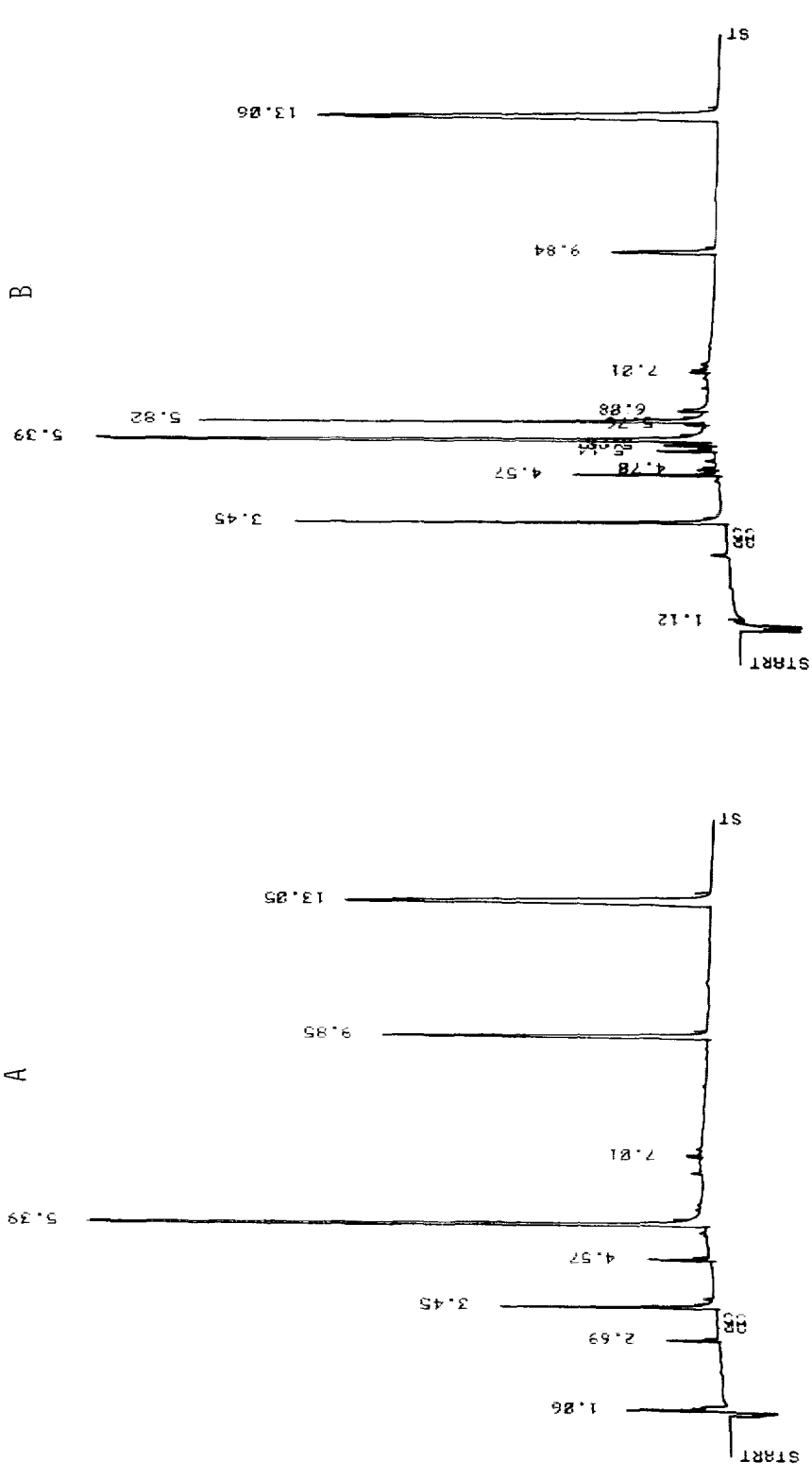

Fig. 3. (A) Chromatogram of extract from patient plasma sample taken 1 h after administration of a single oral dose of 200 mg Trelibet. Concentrations of the compounds are: TRE (9.85 min), 16.2 ng/ml; N-BP (3.45 min), 263.2 ng/ml; N-PCP (5.39 min), 408 ng/ml. (B) Chromatogram of extract from patient urine sample. Urine was collected 4—7 h after receiving a single oral dose of 200 mg Trelibet. Concentrations are: TRE (9.84 min), 5.9 ng/ml; N-BP (3.45 min), 951.1 ng/ml; N-PCP (5.39 min), 1821 ng/ml.

Fig. 3 shows plasma and urine chromatograms of a patient who received a single oral dose of 200 mg of TRE. The concentrations of compounds were found in the plasma 1 h after dosing with TRE. Urine samples were collected during 24 h. The chromatogram shows concentrations of compounds in the third fraction (220 ml) collected 4—7 h after dosing with TRE.

CONCLUSION

The above-proposed method, with some compromising, seems to be sensitive enough for both the determination of Trelibet and its main metabolites in biological samples and for measuring the blood levels of the molecules in pharmacokinetic studies. The procedure is also relatively quick to use for routine measurements.

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