Journal of Chromatography, 490 (1989) 81–90
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4658

DETERMINATION OF THE GABAERGIC ANTIDEPRESSANT DRUG FENGABINE AND SOME OF ITS METABOLITES IN PLASMA BY CAPILLARY GAS CHROMATOGRAPHY WITH ELECTRON-CAPTURE DETECTION

V. ASCALONE* and L. DAL BO

Pharmacokinetic Laboratory, Clinical Research Unit, LERS Synthelabo, Via N. Rivoltana 35, 20090 Limito-Milan (Italy)

and

N. DESCHAMPS and P. GUINEBAULT

Pharmacokinetic Laboratory, Clinical Research Department, LERS Synthelabo, 23-25 Avenue Morane Saulnier, 92366 Meudon la Foret (France)

(First received October 31st, 1988; revised manuscript received December 19th, 1988)

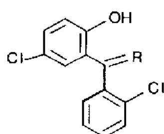
SUMMARY

A sensitive capillary gas chromatographic method was developed for the determination of fengabine (a GABAergic antidepressant drug) and some of its metabolites in plasma samples. The method involves a single and rapid liquid–liquid extraction of the parent drug and metabolites from plasma buffered at pH 5, evaporation of the organic phase under nitrogen, derivatization to *tert*-butyldimethylsilyl ethers and esters and automatic gas chromatography on a fused-silica, silicone-bonded capillary column coupled to an electron-capture detector. The detection limit for fengabine and other compounds is lower than 1 ng/ml in plasma; the method was successfully applied to pharmacokinetic and drug monitoring clinical studies and tested on more than 2000 biological samples and was found not to suffer from endogenous or exogenous interferences.

INTRODUCTION

Recent research on the pharmacology of GABAergic transmission, indicating an important role of this amino acid in affective disorders [1–3], led to the development of new GABAergic antidepressant drugs, one of which is fengabine. This drug, 2-[butylimino(2-chlorophenyl)methyl]-4-chlorophenol, is a

0378-4347/89/$03.50 © 1989 Elsevier Science Publishers B.V.



Compound	R	
Fengabine	N-(CH_2)$_3$-CH_3	
(SL 81 0196)	O	Metabolite 1
(SL 81 0013)	N-CH_2-CH(OH)-CH_2-CH_3	Metabolite 2
(SL 81 0048)	N-$(CH_2)_2$-CH(OH)-CH_3	Metabolite 3
(SL 78 0622)	N-$(CH_2)_3$-COOH	Metabolite 4
(SL 87 0221)	N-$(CH_2)_4$-CH_2OH	Int Stand

Fig. 1. Structure of fengabine, some metabolites and the internal standard.

novel benzylidene derivative active in behavioural models of depression [3] without having cardiovascular and anticholinergic effects [4], typical of tricyclic antidepressants. In order to perform pharmacokinetic and clinical pharmacology studies in man, a sensitive capillary gas chromatographic (GC) method with electron-capture detection (ECD) was developed in our laboratory; the method is suitable for the determination of fengabine and some metabolites (as silyl derivatives) whose pharmacological activity was assessed in animal models: SL 81.0048, SL 81.0013, SL 78.622 and SL 81.0196 (benzophenone derivative), which does not possess antidepressant activity but should be monitored because of its potential toxic effects on the liver (see Fig. 1).

In previous studies a capillary CG method with ECD for fengabine and SL 78.622 (as the methyl derivative) was developed; the method was simple and gave good results, but it did not allow the determination of the alcoholic metabolites which were not eluted from the chromatographic column. This method has now been abandoned and replaced by the method reported here.

EXPERIMENTAL

Reagents, chemicals and standards

Sodium acetate [high-performance liquid chromatography (HPLC) grade], acetic acid (HPLC grade), *n*-hexane (Nanograde) and ethyl acetate (Nanograde) were obtained from J.T. Baker (Deventer, The Netherlands). Dimethylformamide (DMF) (silylation grade) was obtained from Pierce (via Prodotti Gianni, Milan, Italy), *tert.*-butyldimethylsilyl chloride (TBDMSCl, 97% purity) from Aldrich (via Prodotti Gianni) and N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) from Pierce (via Prodotti Gianni).

The derivatization mixture was MTBSTFA–DMF–TBDMSCl (catalyst) (49:49:2, w/v) and was stable for one week at −20°C.

The water used for the preparation of reagent solutions was of HPLC grade, produced with a Milli Q-4 system (Millipore, Bedford, MA, U.S.A.) and filtered through a 0.22-μm filter. The extraction buffer (acetate, pH 5, 2 *M*) was prepared from a 2 *M* sodium acetate solution adjusted to pH 5 with glacial acetic acid. Fengabine, metabolite 1 (SL 81.0196), metabolite 2 (SL 81.0013), metabolite 3 (SL 81.0048), metabolite 4 (SL 78.622) and the internal standard (SL 87.0221) were of pharmaceutical grade and obtained from LERS Synthelabo (Meudon la Foret, France).

Apparatus

A Carlo Erba Mega series gas chromatograph with a Model 400 electron-capture detector and an automatic sample injector were used. The fused-silica capillary column (25 m×0.32 mm I.D., 0.21 μm film thickness), coated with cross-linked silicone mixture, CP Sil 19 CB, was obtained from Chrompack (Middelburg, The Netherlands). The carrier gas was helium at an inlet pressure of 48 kPa, corresponding to a linear flow-rate of about 26 cm/s, the make-up gas was argon–methane (90:10) supplied at a flow-rate of 50 ml/min and the gas for pressuring the micro-vials for the automatic sample injector was nitrogen. The gas used for secondary cooling (cooling of the injector zone) and for the automatic devices of the autosampler was compressed air.

The oven temperature, initially held at 160°C for 1 min after injection, was heated at 20°C/min to 285°C and maintained at this final temperature for 25 min. The detector, heated at 330°C, was operated in the constant-current mode with a pulse amplitude voltage of 13.5 V, the corresponding reference current was 1 nA and the pulse width was 0.1 μs; under these conditions, with a clean and equilibrated detector, the detector frequency was about 5 kHz. The injections were performed in the cold split–splitless mode with a Carlo Erba MFA 515 module; the split gas was helium supplied at 10 ml/min. This injection system allows the introduction of the liquid sample into a vaporizing chamber maintained at a suitable initial temperature (60°C); after elimination of the solvent by splitting, the temperature of the injection zone was increased to 300°C (the splitting valve remained closed) and the compounds of interest, reaching their boiling points, were vaporized and transferred to the top of the analytical column where the chromatographic process took place.

Stock and standard solutions

Stock solutions of fengabine, its metabolites and the internal standard were prepared by dissolving 10 mg of each compound in 10 ml of methanol. Working standard solutions were prepared from the stock solutions by suitable dilutions with methanol (see Table I) and used for instrument calibration. The stock

and standard solutions are stable for at least two months and two weeks, respectively, if stored in a refrigerator (0–5°C).

Procedure for plasma samples

Frozen plasma samples (unknowns and pre-dose) were thawed at room temperature before pipetting, 1 ml (or less) of plasma was transferred into a set of screw-capped test-tubes for each unknown and for pre-dose plasma samples (future plasma standards), 20-μl aliquots of methanolic standard solutions (Table I) were added to pre-dose plasma tubes and mixed well, and 30-μl aliquots of internal standard solution (Table I) were added to all samples and mixed well.

All the samples were treated with 1 ml of acetate buffer (2 *M*, pH 5) and 6 ml of *n*-hexane–ethyl acetate (90:10). The tubes were shaken on a tumble extractor for 5 min at 40 rpm, then centrifuged at 2500 *g* for 3 min at 5°C on a refrigerated Model K 110 centrifuge (Jouan, Saint Nazaire, France).

After centrifugation, the tubes were immersed in a cryogenic bath (−70°C) to freeze the aqueous phase, then the upper organic phase was transferred into conical test-tubes for evaporation to dryness under a gentle stream of pure nitrogen in a thermostated water-bath at 37±2°C.

Derivatization

MTBSTFA, a new derivatizing agent for GC, derivatizes hydroxy, carboxy, thiol and primary and secondary amine groups with yields greater than 96% [5]; *tert.*-butyldimethylsilyl ethers (t-BDMS) are 10^4 times more stable than trimethylsilyl (TMS) ethers and less susceptible to solvolysis [5,6].

Each residue was dissolved in 100 μl of derivatization mixture and held in a water-bath at 70±2°C for 1 h. The extracts, cooled at room temperature, were transferred into glass autosampler vials with conical inserts (Chrompack, Cat. No. 10375) for automatic sample injection. A 0.3-μl volume was injected.

TABLE I

STANDARD SOLUTION USED IN THE ANALYSIS

Standard solution	Fengabine (ng per 20 μl)	Metabolite 1 (ng per 20 μl)	Metabolite 2 (ng per 20 μl)	Metabolite 3 (ng per 20 μl)	Metabolite 4 (ng per 20 μl)	Internal standard (ng per 30 μl)
A	300.00	60.00	150 00	150.00	600 0	–
B	100 00	20.00	50 00	50.00	200 0	–
C	50.00	10.00	25.00	25.00	100.0	–
D	25 00	5.00	12 50	12.50	50 0	–
E	12.50	2.50	6.25	6.25	25.0	–
F	6.25	1.25	3.12	3 12	12 5	–
G	–	–	–	–	–	45

Calculation

Peak-height ratios between the five different compounds and the internal standard, obtained from plasma extracts by means of an SP 4270 data system (Spectra-Physics, San Jose, CA, U.S.A.), plotted against their concentrations, are used to generate the linear least-squares regression line. The concentrations of fengabine and its metabolites in unknowns were determined by interpolation on the calibration graph using peak-height ratios obtained from unknown specimens. (The results do not change if peak-area ratios are used instead of peak-height ratios).

RESULTS

Recovery

The overall absolute recovery of fengabine and its metabolites from pre-dose human plasma spiked with fengabine and metabolites in the concentration range 5–600 ng/ml ($n=6$) was investigated and found about 90%; the overall relative recovery was found close to 100% ($n=7$).

A linear correlation between the peak-height ratios of the five different compounds and the internal standard versus their concentration was found in the investigated range (see Table I). The linear least-squares regression performed on the peak-height ratios versus concentration gave the following equations:

fengabine, $y=12.5x-2.2$; $r^2=1$

metabolite 1, $y=9.7x-1.8$; $r^2=1$

metabolite 2, $y=20.7x-1$; $r^2=1$

metabolite 3, $y=27.7x-1.8$; $r^2=1$

metabolite 4, $y=50.3x+1.3$; $r^2=1$

Limit of detection

The detection limit of the method was about 0.5 ng/ml for fengabine and metabolite 1, 0.8 ng/ml for metabolites 2 and 3 and 1 ng/ml for metabolite 4, with a signal-to-noise ratio of about 3:1.

Specificity and selectivity

Several pre-dose plasma samples from different subjects were tested for the absence of interfering endogenous compounds; in most instances no endogenous chromatographic interference was found at the retention time of any of the compounds of interest; Fig. 2 shows a typical chromatogram of a drug-free plasma extract derivatized containing the internal standard.

The possible interference due to other co-administered drugs was evaluated;

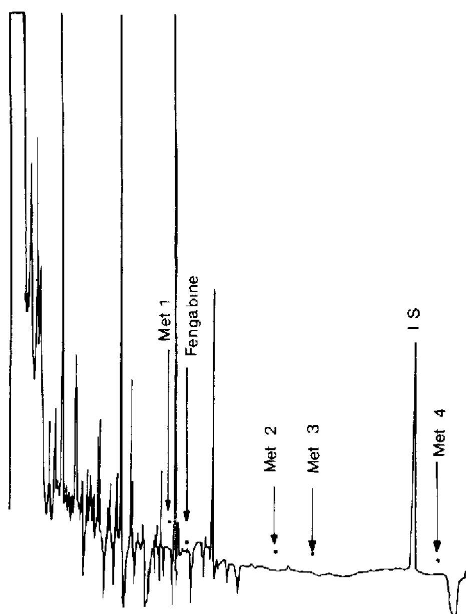


Fig. 2. Chromatogram of a pre-dose plasma extract containing the internal standard (I.S.).

the compounds examined were amitriptyline, imipramine and their demethylated metabolites, clonazepam, carbamazepine, phenytoin and caffeine at the plasma concentrations considered therapeutic. An individual substance, placed in plasma tubes containing fengabine and metabolites, was processed according to the described method, then the final extracts were injected into the gas chromatograph. Amitriptyline and imipramine and their metabolites and caffeine were almost unextracted from the biological matrix at pH 5; all these substances gave no chromatographic peaks. Phenytoin (or its derivative or degradation product) gave a peak at about 13 min (non-interfering), clonazepam gave a peak at about 19 min (non-interfering) and carbamazepine gave a peak close to that of fengabine but was well separated and with a longer retention time.

Stability of the samples after derivatization

The stability of the sample extracts after derivatization was investigated in order to establish how long the samples could remain at ordinary laboratory conditions, before chromatography, without appreciable degradation. The results showed that fengabine and its metabolites, over a wide range of concentrations, were stable for at least four days, when compared with similar freshly prepared plasma standards.

TABLE II

REPRODUCIBILITY FOR PLASMA SAMPLES SPIKED WITH FENGABINE AND METABOLITES (INTRA-ASSAY)

Compound	Amount added (ng/ml)	*n*	Recovery (%)	Coefficient of variation (%)
Fengabine	300.0	4	104.7	1.8
	50.0	4	94.3	4.0
	12.5	4	96.0	3.7
Metabolite 1	60.0	4	101.0	3.9
	10.0	4	91.0	3.9
	2.5	4	88.0	13.4
Metabolite 2	150.0	4	104.5	5.5
	25.0	4	95.9	4.5
	6.2	4	96.5	6.2
Metabolite 3	150.0	4	107.0	2.6
	25.0	4	85.2	4.7
	6.2	4	85.2	9.5
Metabolite 4	600.0	4	102.2	2.7
	100.0	4	106.3	5.2
	25.0	4	96.0	5.8

TABLE III

REPRODUCIBILITY FOR PLASMA SAMPLES SPIKED WITH FENGABINE AND METABOLITES (INTER-ASSAY)

Compound	Amount added (ng/ml)	*n*	Recovery (%)	Coefficient of variation (%)
Fengabine	300.0	9	93.3	7.3
	100.0	13	99.0	6.8
	12.5	9	106.0	12.8
Metabolite 1	60.0	9	93.0	10.6
	20.0	9	94.5	7.8
	2.5	5	104.0	6.7
Metabolite 2	150.0	9	98.3	8.8
	50.0	13	99.6	7.2
	6.2	9	104.1	10.5
Metabolite 3	150.0	9	100.2	4.4
	50.0	13	98.8	10.0
	6.2	9	97.6	11.4
Metabolite 4	600.0	9	103.5	4.6
	200.0	13	97.6	7.8
	25.0	9	102.0	12.5

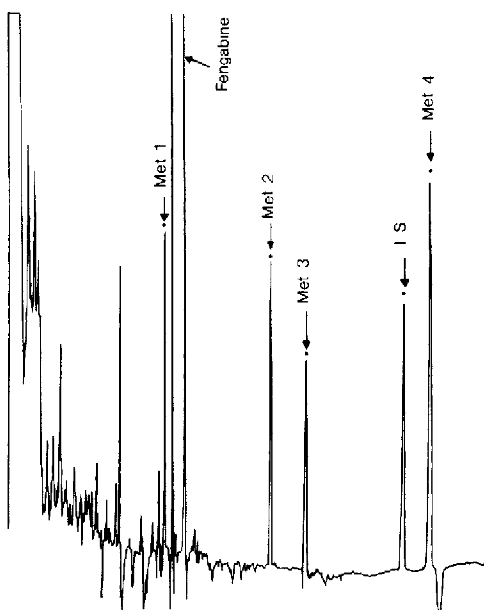


Fig. 3. Chromatogram of authentic standards recovered from control plasma spiked with fengabine and metabolites. The nominal concentrations of fengabine and metabolites 1, 2, 3 and 4 were 100, 20, 50, 50 and 200 ng/ml, respectively.

Statistical validation of the method

Intra-assay precision studies were performed on control plasma spiked with different amounts of fengabine and its metabolites. The intra-assay precision (within-day) was obtained by replicate analysis of plasma samples on the same day. The inter-assay precision (between-day) was determined by analysing pre-dose plasma samples spiked with fengabine and its metabolites over a four-month period in order to obtain long-term reproducibility data.

During the long-term reproducibility study, the capillary column was replaced with a virtually identical one. The results reported in Tables II and III demonstrate acceptable reproducibility of the method over a wide range of concentrations. Fig. 3 shows a representative chromatogram.

DISCUSSION

The method described has been used extensively for the determination of plasma levels of fengabine and its metabolites in clinical pharmacokinetics, drug monitoring and drug-interaction studies. A typical chromatogram is shown in Fig. 4, and Fig. 5 shows the plasma concentration–time course of fengabine and its metabolites in a volunteer (after an oral dose of 600 mg of drug). The method was tested on about 2000 samples with satisfactory results. The cap-

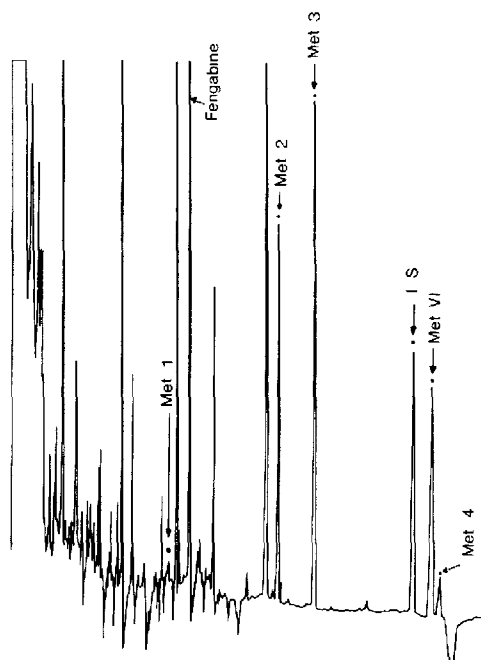


Fig. 4. Chromatogram of a plasma extract from a patient administered orally a single dose of 600 mg of fengabine; sample taken 3 h after drug intake. The peak marked Met. VI belongs to an unknown metabolite.

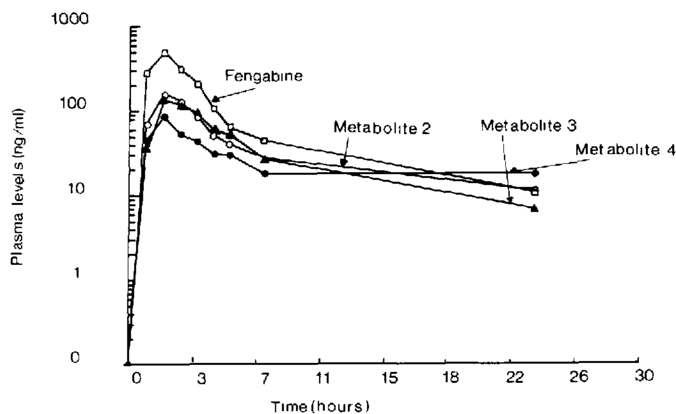


Fig. 5. Plasma concentration–time course of fengabine and its metabolites in a volunteer administered 600 mg in a single dose. □, Fengabine; ○, metabolite 2; ▲, metabolite 3; ●, metabolite 4.

illary column was replaced after about 1000 biological samples had been processed because of a decrease in reproducibility; however, simply rinsing the column with solvent restored the original performance.

Preliminary experiments using BSTFA as the derivatizing agent gave negative results owing to the instability of TMS derivatives and the poisoning of the detector, which decreased in sensitivity, resulting in chromatograms with interfering peaks at the retention times of the relevant compounds.

An advantage of TBDMS over TMS derivatives is that the former are much more stable, the reagent (MTBSTFA) does not affect the ECD performance and the chromatograms do not show interfering peaks. As the sensitivity of the method is very high, in the most instances we started with 0.5 ml of plasma and injected a volume of 0.3 μl. From the beginning of the method development to the present time, three capillary GC columns, from different batches, have been tested, giving comparable results. It is advisable to replace the glass liner inside the injection chamber after about 500 injections.

During pharmacokinetic studies (acute and chronic treatment), an unknown metabolite was observed eluting between the internal standard and metabolite 4 (see Fig. 4).

Recently, the method has also been used, with minor modifications, for the determination of fengabine and its metabolites in human breast milk; under these conditions the drug extracts (not yet derivatized) were dissolved in 1 ml of acetonitrile and then washed with 2 ml of *n*-heptane, the *n*-heptane fraction was discarded and the acetonitrile phase, after evaporation, was reacted with the derivatization mixture. This sample purification procedure gave good and reproducible results.

ACKNOWLEDGEMENTS

We thank Dr. J. Burke for help with the choice of the derivatization mixture and Mrs. C. Gambalonga for the assistance in drafting and typing the manuscript.

REFERENCES

1 G. Bartholini, K.G. Lloyd, B. Scatton, B. Zivkovic and P.L. Morselli, Psychopharmacol. Bull., 21 (1985) 385.
2 R.H. Gerner and L. Fairbanks, in C. Shagrass, R.C. Josiassen and W.H. Bridger (Editors), Biological Psychiatry, Elsevier, New York, 1986, p. 853.
3 K.G. Lloyd, P.L. Morselli and G. Bartholini, Med. Biol., 65 (1987) 159.
4 B. Musch and M. Garreau, in C. Shagrass, R.C. Josiassen and W.H. Bridger (Editors), Biological Psychiatry, Elsevier, New York, 1986, p. 920.
5 Handbook and General Catalog, Pierce, Rockford, IL, 1985-86, p. 100.
6 F. Poole, in K. Blau and G. King (Editors), Handbook of Derivatives for Chromatography, Heyden, London, 1978, p. 152.