Journal of Chromatography, 131 (1977) 347–355

CHROM. 9456

AUTOMATED HIGH-RESOLUTION GAS CHROMATOGRAPHIC ANALYSIS OF PSYCHOTROPIC DRUGS IN BIOLOGICAL FLUIDS USING OPEN-TUBULAR GLASS CAPILLARY COLUMNS

I. DETERMINATION OF NOMIFENSINE IN HUMAN PLASMA

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(Received May 20th, 1976)

SUMMARY

An automated high-resolution gas chromatographic method has been developed for the determination of low levels of the antidepressant psychotropic drug Nomifensine in human plasma. The drug is extracted from alkalinized plasma with diethyl ether and then back-extracted into an acidic aqueous phase. After subsequent extraction into diethyl ether the drug is analysed by gas–liquid chromatography as its heptafluorobutyrate derivative using an OV-101 open-tubular glass capillary column with a nitrogen-specific detector. The propyl and butyl analogues of Nomifensine are used as internal standards, added to the plasma before extraction. The method is accurate, specific and precise, and capable of measuring plasma concentrations down to a level of 2 ng/ml.

A preliminary study of the pharmacokinetics of the drug, together with steady-state level measurements in normal individuals receiving therapeutic dosages of Nomifensine, has been made.

INTRODUCTION

There is an increasing need for new and improved methods for the routine analysis of drugs and drug metabolites in biological samples suitable for pharmacokinetic studies and the monitoring of steady-state therapeutic levels. Gas–liquid chromatography (GLC) is the most practical and widely used method for determining drugs at low concentration levels. Previous gas chromatographic methods, however, have the disadvantage that conventional packed columns are used which have a limited separating efficiency. As a consequence, in order to achieve specificity and sensitivity, these assay procedures, which invariably utilize electron capture or nitrogen detection, often involve elaborate and extensive purification of the biological extract prior to the gas chromatographic analysis. Many of these methods could be simplified and the specificity and sensitivity improved by the use of glass capillary col-

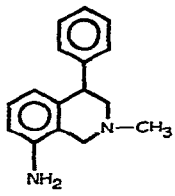
umns of high separating efficiency. High-resolution gas chromatography is finding increasing application in the field of biochemical analysis, but to-date has not been applied to the routine analysis of drugs. We are currently investigating the potential of this technique for the determination of psychotropic drugs in plasma. This publication describes an automated high-resolution gas chromatographic method using an open-tubular glass capillary column with a nitrogen-phosphorous detector (NPD) for the routine analysis of the antidepressant drug Nomifensine in human plasma. A number of studies relating to the pharmacology and clinical properties of this new psychotropic agent have been published[1–6]. During the preparation of this manuscript a GLC method utilizing a packed column with electron capture detection was described for the assay of Nomifensine in human plasma[7].

A similar study of the drug pharmacokinetics in normal individuals as described in this paper has also recently been made[8].

MATERIALS AND METHODS

Reagents

Nomifensine (8-amino-2-methyl-4-phenyl-1,2,3,4-tetrahydroisoquinoline) and its 2-propyl and 2-butyl analogues* were kindly supplied by the manufacturers (Hoechst, Frankfurt, G.F.R.). [4-^{14}C]Nomifensine, with a specific activity of 0.93 μCi/mg, was also a gift from Hoechst. Standard solutions of the drug and its analogues were prepared by dissolving weighed amounts of the salts in methanol. All concentrations were expressed in terms of the free base. The stock standard solution of the two internal standards used for the assay procedure contained 4.79 ng/μl of the propyl and 6.34 ng/μl of the butyl analogue.



Nomifensine

The solvents used were obtained from BDH (Poole, Great Britain) and were of Analar grade. Hexane was washed with conc. H_2SO_4 and water, dried over anhydrous sodium sulphate, and redistilled. Diethyl ether was redistilled twice and then further purified prior to use by passage through a column of aluminium oxide W200 "Woelm" basic (Koch-Light, Colnbrook, Great Britain). Ethyl acetate was redistilled and dried over calcium chloride.

Heptafluorobutyric anhydride (Pierce, Rockford, Ill., U.S.A.) was redistilled over P_2O_5 and then stored at 0° in a dark bottle.

Glassware

All glassware used in the extraction and in the evaporation of the extracts was first cleaned by soaking in a solution of Pyroneg (Diversey) and then silanized using

* Nomifensine and its 2-propyl analogue were obtained as their hydrogen maleinate salts, the 2-butyl analogue as its hydrogen chloride.

a 5% solution of dimethyldichlorosilane in toluene. After drying in an oven at 100°, the glassware was rinsed several times with methanol before use.

Plasma samples

Samples of 10 ml blood in heparinized tubes were centrifuged immediately after collection and the plasma stored at −4° until analysed.

Extraction procedure and derivative formation

To a 2-ml aliquot of plasma contained in a 30-ml glass-stoppered tube were added 15 μl of the internal standard solution. The plasma was made alkaline (pH 12) with 0.5 ml 5 *N* NaOH and extracted with 8 ml diethyl ether by shaking the mixture for 10 min on an automatic horizontal shaker. After centrifugation at 2000 × *g* for 10 min, the diethyl ether layer was transferred to a similar glass-stoppered tube containing 2.4 ml 1 *N* HCl and the mixture shaken for 15 min. The solvent layer was removed by aspiration after centrifugation and the aqueous phase washed with 6 ml hexane followed by 6 ml diethyl ether. Eight millilitres diethyl ether and 0.8 ml 5 *N* NaOH were added to the acid extract and the tube shaken for 10 min and centrifuged. Six millilitres of the diethyl ether fraction were transferred into a tube fitted with a PTFE-lined cap and then taken to dryness under dried nitrogen at 40°. The heptafluorobutyrate derivatives were prepared by dissolving the dried extract in 100 μl ethyl acetate and reacting at 60° for 10 min with 8 μl heptafluorobutyric anhydride. The reaction mixture was then taken to dryness at room temperature under dried nitrogen and finally reconstituted to 50 μl with hexane containing 0.5% heptafluorobutyric anhydride. Aliquots of this solution were taken for analysis by GLC.

Gas–liquid chromatography

Analyses were performed on a Pye Unicam Series 106 automatic analytical gas chromatograph equipped with dual flame ionization detectors (FIDs) and a tape programmer. One of the FIDs was replaced by a Perkin-Elmer nitrogen–phosphorus detection system. Samples were injected into the capillary column using a Pye Unicam autosolid injector which was modified in order to eliminate contact of the sample with metal during vaporization in the injection port. Details of this modification will be given in a future publication.

The procedure used to prepare the 50 m × 0.25 mm OV-101 coated glass capillary column and the method of connecting the column in the gas chromatographic oven have been described previously[9]. Additional flow restrictors were placed in the hydrogen and air lines to the NPD and a helium make-up gas was used to optimize flow conditions to this detector.

The injection and temperature recycling operations, controlled automatically by the chromatograph tape programmer, are given in Table I. Helium was used as carrier gas with a column flow-rate of 2 ml/min, measured at 200°. The injection port and detectors were maintained at a temperature of 280°. The optimized flow-rates to the FID of hydrogen, air, and make-up gas were 30, 220, and 40 ml/min, respectively. The corresponding flow-rates to the NPD were 5, 150, and 20 ml/min. The ionization amplifier was operated at an attenuation of either 2×10^{-11} or 1×10^{-11} A and results were recorded on a two-pen Telsec 700T recorder set at 20 and 50 mV with a chart speed of 20 cm/h.

TABLE 1

INJECTION AND TEMPERATURE RECYCLING OPERATIONS FOR AUTOMATED GAS CHROMATOGRAPHIC ANALYSIS

Channel No.	*Time (min)*	*Operations*
1	0	Heater off, oven door opened (oven temperature down)
2	7	Oven door closed
3	8	Sample injected (oven temperature 120°)
4	10	Heater on (oven temperature increases rapidly to 200°)
5	12	Temperature programme started at 2°/min
6	32	Final temperature held at 240° for 5 min
7	0	Cycle repeated

The silanized glass injection tubes were loaded into the injector magazine and aliquots of the samples were transferred into the tubes using a microlitre syringe. The solvent was allowed to evaporate at room temperature and the magazine then placed in the injector for automatic analysis. This injector, when used with the automatic gas chromatograph, is capable of the continuous unattended programmed analysis of up to 36 samples.

Methylene unit (*MU*) values were determined under temperature-programmed conditions by co-injecting *n*-alkanes with the derivatized samples. The efficiency of the capillary column was determined for *n*-octacosane at 240° using liquid injection with a splitting system. The measured theoretical plate number was 96,400.

Gas chromatography–mass spectrometry

The mass spectrometer used was a MS-1075 double-beam, double-focussing instrument (AEI Scientific Apparatus Division) interfaced to a Pye Unicam Series 104 gas chromatograph with a single-stage glass jet separator. A helium make-up gas of 15–20 ml/min introduced at the outlet end of the column was used to optimize flow conditions in the separator. The mass spectrometer was operated with a nominal electron energy of 70 eV with an ion source temperature of 230° and a separator temperature of 240°.

Quantitation

Peak height measurements were used and quantitation made by reference to a standard calibration curve prepared with each batch of plasma samples analysed. This standard curve was obtained by analysing 2-ml aliquots of pooled blank plasma each containing the two internal standards and from 10–30 ng/ml Nomifensine. The ratio peak height Nomifensine to mean peak height of the two internal standards was plotted against the concentration of the drug in plasma. A typical calibration curve is shown in Fig. 1.

RESULTS AND DISCUSSION

Although Nomifensine and the two analogues used as internal standards can be gas chromatographed as free bases, the gas chromatographic properties of these compounds are considerably improved by derivative formation. For example, the trifluoroacetate (TFA), pentafluoropropionate (PFP) and heptafluorobutyrate (HFB)

TABLE II

MU VALUES OF THE ACYL DERIVATIVES OF NOMIFENSINE AND ITS PROPYL AND BUTYL ANALOGUES

Compound	*MU value*		
	TFA	*PFP*	*HFB*
Nomifensine	20.95	21.25	21.48
Propyl analogue	21.80	22.07	22.32
Butyl analogue	22.67	22.86	23.14

derivatives each exhibit excellent gas chromatographic properties and they can also readily be prepared in quantitative yield. Listed in Table II are the *MU* values of these acyl derivatives on the OV-101 coated glass capillary column. The HFB derivatives were selected for use in the assay procedure because of their good chemical and thermal stability and being the least volatile of these acyl derivatives were the most suitable for use with solid injection. Derivatized extracts when dissolved in hexane containing 0.5% of the derivatizing reagent were stable at 0° for periods of over a week and when dried on the glass injection tubes could be stored in the solid injection system for up to three days without undergoing any measurable losses due to volatilization or decomposition.

The chromatogram shown in Fig. 2 illustrates the high separating efficiency of the capillary column. This tracing was obtained using FID from the analysis of authentic Nomifensine and its analogues as their HFB derivatives, co-injected with a mixture of standard hydrocarbons. The minimum amount of the derivatized drug that could be detected by FID was approximately 1 ng.

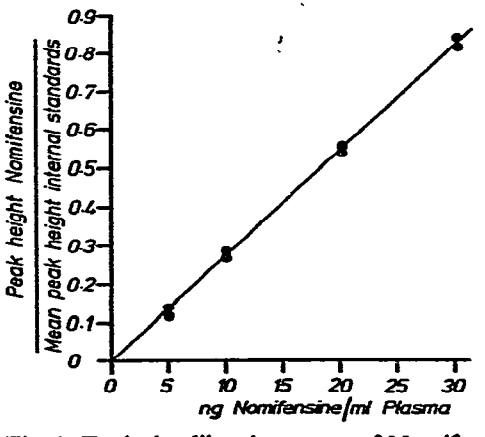


Fig. 1. Typical calibration curve of Nomifensine in plasma.

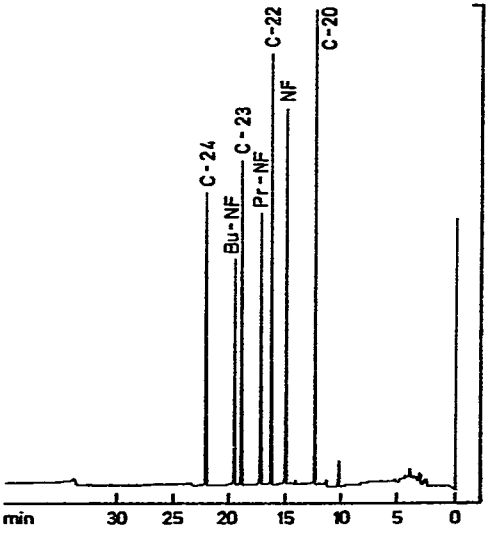
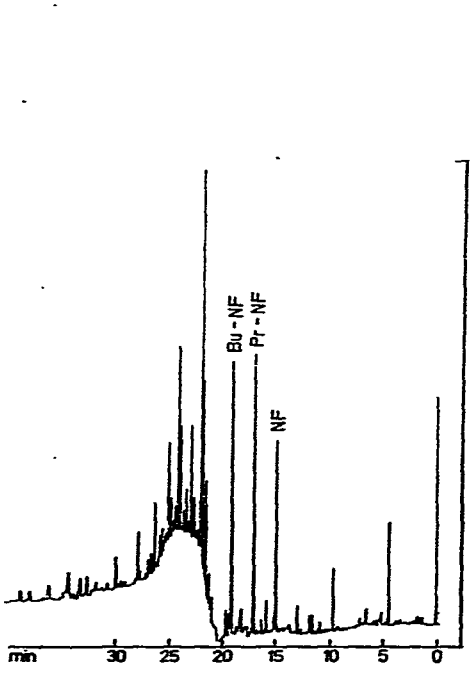


Fig. 2. Gas chromatogram with FID of authentic Nomifensine (NF) and its propyl (Pr-NF) and butyl (Bu-NF) analogues as their HFB derivatives together with a standard mixture of hydrocarbons. Each peak corresponds to 20 ng of compound injected.

An evaluation of high-resolution GLC for the determination of Nomifensine in plasma was initially made using this non-specific detector. Provided that extreme care was taken to ensure that the solvents, reagents, and glassware were free from impurities, quantitation of the drug down to 20 ng/ml plasma was possible. Fig. 3 shows a typical chromatogram from the analysis of a plasma sample containing this drug concentration level. When, however, attempts were made to extend the sensitivity of the method to measure therapeutic plasma levels, it was found to be unreliable due to lack of specificity. This problem was overcome by using a nitrogen-sensitive thermionic detector. Gas chromatographic methods developed for the analysis of nitrogen-containing drugs employing this type of detection system have previously been considered as unsuitable for routine application due to the lack of control over many of the variables involved in the operation, optimization, and stability of the detector. In contrast the new NPD used in this study has been shown to exhibit long-term stability and reliability comparable with the FID[10,11]. Also, its suitability for use with capillary columns has recently been demonstrated[12,13]. Fig. 4 shows a chromatogram of a standard mixture on the capillary column coupled to the NPD and Fig. 5 shows a typical tracing on this GLC system from the analysis of plasma Nomifensine.



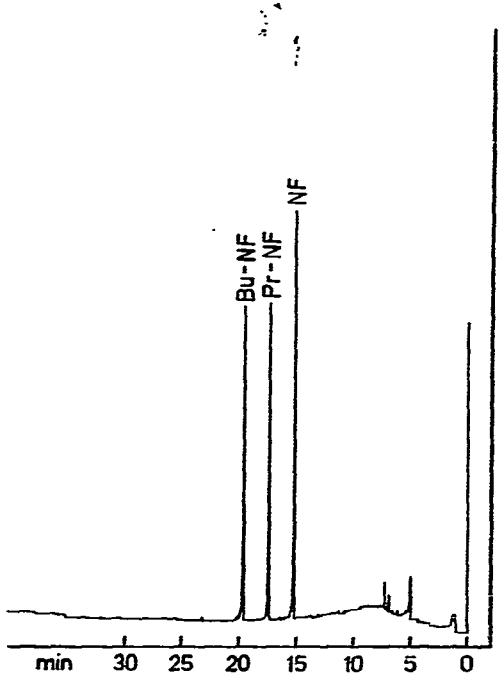
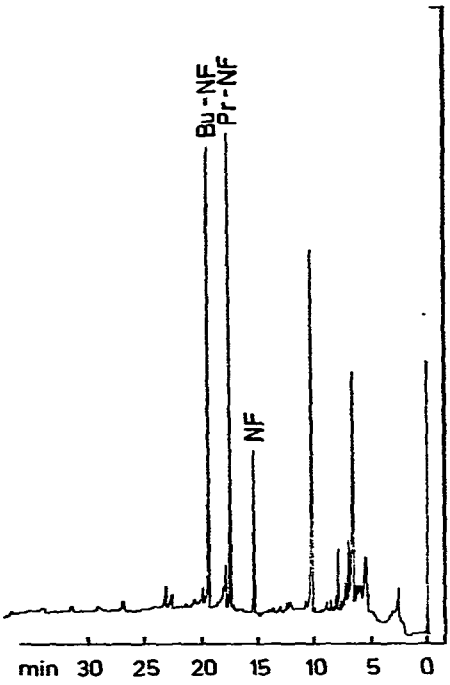


Fig. 3. Gas chromatogram with FID of an extract from a plasma sample containing 22.4 ng of NF/ml plasma.

Fig. 4. Gas chromatogram with NPD of the same standard mixture used to obtain Fig. 2. The absence of the hydrocarbon peaks demonstrates the selectivity of this detection system for the nitrogen-containing compounds. Each peak corresponds to 10 ng of compound injected.



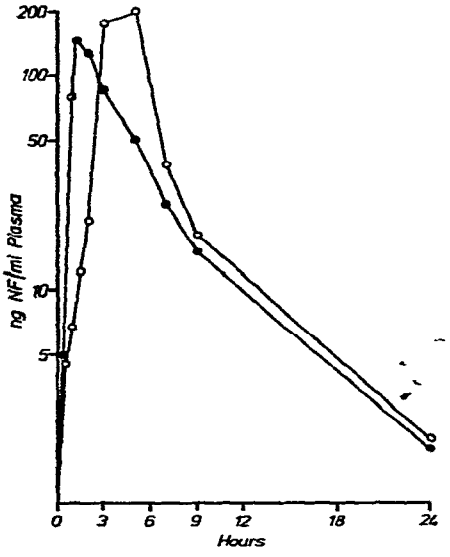


Fig. 5. Gas chromatogram with NPD of an extract from a plasma sample containing 12.0 ng of NF/ml plasma.

Fig. 6. Plasma levels of Nomifensine in two normal individuals after a single 75-mg oral dose of the drug.

Evaluation of method

Recovery studies. To achieve the quantitative extraction of the drug and the two internal standards from plasma, uncontrollable losses due to adsorption of these basic compounds on the glassware had to be avoided. Careful cleaning of the glassware with the detergent Pyroneg and subsequent silanization of the glass surfaces prevented these losses. The mean determined recovery of the ^{14}C-labelled drug from plasma in eight experiments was 87.10% (S.D. $\pm$ 4.56).

Accuracy and precision. The accuracy of the method was determined from the analysis of 2-ml aliquots of blank plasma containing from 10–60 ng/ml of added authentic Nomifensine. The calculated recoveries over this range varied from 88.3–97.6% with a mean of 91.3% (S.D. $\pm$ 5.40).

The precision of the method was determined from duplicate analyses using the method of Snedecor[14]. The findings are given in Table III.

Specificity. The extent of interference from endogenous peaks was dependent on the cleanliness of the glassware and the purity of the solvents used in the extraction procedure rather than by constituents of plasma. Interfering peaks, as shown from the analysis of blank plasma samples, were eliminated by careful control of these factors. Combined gas chromatography–mass spectrometry was carried out on a number of plasma extracts and the mass spectra obtained from scans taken of the appropriate peaks were identical to those of the authentic standards.

TABLE III

PRECISION OF THE METHOD FROM ESTIMATES OF DUPLICATE DETERMINATIONS

	Range (ng/ml)		
	5–25	*25–100*	*100–200*
Number of duplicates	10	9	10
Mean, ng/ml	12.37	66.35	159.43
Standard deviation	1.24	4.92	8.56
Coefficient of variation, %	10.0	7.42	5.37

Sensitivity. The sensitivity of detection of the derivatized drug with NPD was approximately 0.5 ng. By the direct injection of a large fraction of the plasma extract, made possible by solid injection, plasma concentrations of the drug down to the 2 ng/ml level could be determined.

Practicability. The simplicity of the extraction procedure enabled up to 36 samples to be processed in a working day and with the fully automated gas chromatographic system this number of samples could be analysed unattended.

Application

Two healthy individuals in a fasting state were given 75 mg Nomifensine orally in capsule form. During the 24 h after administration nine blood collections were taken. The plasma Nomifensine levels determined in these samples are given in Table IV. The drug concentration curves for the two subjects (see Fig. 6) show the initial rapid adsorption of the drug and its rate of elimination from the circulation. The peak plasma concentrations of 134.4 and 199.9 ng/ml were attained at 1.5 and 5.0 h, respectively. The half-life ($t_{\frac{1}{2}}$) values calculated from the terminal slopes and the apparent volumes of distribution (V_D) given in Table IV are similar to those reported recently by Vereczkey *et al.*[9]. Steady-state plasma levels were determined in a group of nine healthy individuals (age range 20–43) who received 25 mg Nomifensine t.d.s.

TABLE IV

PLASMA NOMIFENSINE LEVELS IN TWO SUBJECTS FOLLOWING A SINGLE ORAL DOSE (75 mg) AND THE CALCULATED APPARENT HALF-LIFE ($t_{\frac{1}{2}}$) AND APPARENT VOLUME OF DISTRIBUTION (V_D) VALUES

Hours after dose (h)	*Concentration (ng/ml plasma)*	
	Subject 1	*Subject 2*
0.5	4.2	4.5
1.0	84.0	6.7
1.5	134.4	12.0
2.0	132.7	22.4
3.0	85.7	174.7
5.0	54.6	199.9
7.0	25.2	38.1
9.0	15.1	17.9
24.0	2.0	2.8
$t_{\frac{1}{2}}$, h	4.82	4.86
V_D, l/kg	14.0	8.7

for six days. Six hours after the last dose blood samples were taken and the plasma drug concentrations determined. The values obtained varied from 5.33–16.99 ng/ml with a mean of 10.67 ng/ml.

CONCLUSIONS

This study has shown that an open-tubular glass capillary column with a nitrogen-specific detector can be effectively used for the precise and accurate gas chromatographic determination of low levels of Nomifensine in plasma. The described automation of the gas chromatographic analysis makes this method ideally suited for routine application.

The advantages of improved specificity and sensitivity of high-resolution gas chromatography will undoubtedly lead to its widespread application in the field of drug analysis.

ACKNOWLEDGEMENTS

We wish to thank Hoechst U.K. Ltd., Pharmaceuticals Division, for financial support and Dr. J. R. Chapman, A.E.I. Scientific Apparatus Ltd., Urmston, Manchester, for analysis of samples by combined gas chromatography–mass spectrometry.

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