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DETERMINATION OF PHENELZINE IN HUMAN PLASMA WITH GAS CHROMATOGRAPHY—MASS SPECTROMETRY USING AN ISOTOPE LABELED INTERNAL STANDARD

SATYA P. JINDAL*, THERESA LUTZ and THOMAS B. COOPER

Rockland Research Institute, Orangeburg, NY 10962 (U.S.A.)

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SUMMARY

A quantitative gas chromatographic—mass spectrometric assay was developed for the determination of phenelzine in human plasma. Phenelzine, in aqueous solution or in plasma reacts at room temperature with pentafluorobenzaldehyde to form quantitatively a hydrazone derivative. The derivative has good gas chromatographic characteristics. The assay utilizes selected ion monitoring in a gas chromatographic effluent, the molecular ion generated by electron impact ionization of phenelzine derivative. Phenelzine-d, was synthesized and used as an internal standard. The assay can measure 2 ng/ml of the drug with about 10% precision.

The method was used for the determination of steady state levels of phenelzine in the plasma of patients taking a therapeutic dose of the drug.

INTRODUCTION

Several aralkyl hydrazines, synthesized by Biel et al. [1], have been shown to be potent central nervous system stimulants. Phenelzine, β-phenylethylhydrazine, is a powerful monoamine oxidase inhibitor [2] and is of considerable value in the treatment of neurotic but not endogenous depression [2, 3]. The drug has been shown to be more effective than amitriptyline in non-endogenous depression [4]. Clinical efficacy and incidence of side effects seem to be related to acetylator status [5—7], obviously acetylation is an important biotransformation pathway for phenelzine.

Phenelzine is readily susceptible to auto-oxidation; decomposition occurs when basified solutions are extracted into organic solvents [8, 9]. Recently Caddy et al. [10] reported a gas chromatographic [GC] method for the analysis of phenelzine in human urine. The analysis is based on the reaction of phenelzine with acetone in basic solution to form phenelzine hydrazone and

the GC quantitation of the resulting hydrazone using a suitable internal standard. The method lacks sensitivity and could not be used for phenelzine assay in the plasma of patients on normal dose of the drug.

This paper describes a gas chromatographic—mass spectrometric (GC—MS) assay of phenelzine in human plasma. Phenelzine in aqueous solution reacts with pentafluorobenzaldehyde to form quantitatively the corresponding hydrazone. The derivative can be extracted with organic solvents from aqueous solutions and has excellent GC and MS characteristics. Selective ion monitoring (SIM), the technique built on combined GC—MS [11,12] was used to develop a sensitive and specific assay for phenelzine in human plasma with site-specific deuterium labeled phenelzine as internal standard. The method was used to measure phenelzine in the plasma of patients receiving 60 mg phenelzine sulphate (30 mg twice daily) for 28 days.

MATERIALS AND METHODS

Reagents

Analytical grade phenelzine sulphate (Warner-Lambert Research Institute, Morris Plains, NJ, U.S.A.), phenylacetic acid-d_7 (Merck, Sharp & Dohme, Montreal, Canada) (96.4 atom % deuterium), lithium aluminium hydride (Pfaltz & Bauer, Flushing, NY, U.S.A.), triphenylphosphine (Aldrich, Milwaukee, WI, U.S.A.), hydrazine monohydrate (64% in water), pentafluorobenzaldehyde (Eastman Organic Chemicals, Rochester, NY, U.S.A.), Sephadex LH-20 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) were used without further purification. All solvents were of analytical grade (Fisher Scientific, Pittsburgh, PA, U.S.A.), silanized tubes (10 ml) with screw caps were used for plasma extraction, final solvent evaporation was performed in 5-ml glass stoppered centrifuge tubes (Kimble, Owens, Illinois, Toledo, OH, U.S.A.). Pasteur pipets with hand drawn constricted tips were utilized for all solution transfers.

All blood samples were collected in ethylenediaminetetraacetic acid using glass free collection, the plasma was frozen and stored till analysis.

Phenelzine-d_7

Phenylacetic acid-d_7 in tetrahydrofuran was reduced with lithium aluminum hydride to give quantitatively β-phenylethanol-d_7 [13]. Equimolar amounts of β-phenylethanol-d_7 and triphenyl phosphine in carbon tetrachloride were heated under reflux for 12 h [14]. The resulting β-phenylethyl chloride-d_7, 65% yield, was chromatographically identical to the authentic unlabeled chloride. The labeled chloride on hydrazinolysis with aqueous hydrazine [1] gave phenelzine-d_7 (Fig. 1). A selected ion detection analysis of phenelzine-d_7 showed the presence of an ion equivalent to 99.1% ± 0.2% (n = 5) phenelzine-d_7 and an ion equivalent to 0.9% ± 0.3% (n = 5) phenelzine-d_0.

Instrumentation

Preliminary GC was performed on a Perkin-Elmer instrument, Model 3920 (Norwalk, CT, U.S.A.) equipped with a silanized 1.8-m column packed with 1% OV-17, maintained at 190°C with a detector temperature of 300°C. The carrier

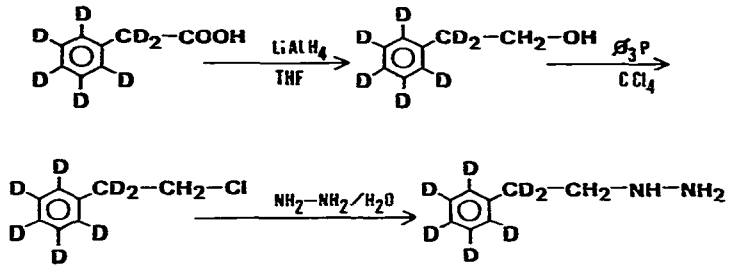

Fig. 1. An outline for the synthesis of phenelzine-d_7.

gas (nitrogen) flow-rate was 30 ml/min. MS was done on LKB-9000 instrument (Stockholm, Sweden) equipped with a multiple ion detector peak matcher accessory (MID/PM) [11, 12]. GC column temperature was 190°C, the flash heater was at 220°C, the separator was at 235°C and the ion source was at 250°C, the helium flow-rate was 20 ml/min.

Derivatization method with pentafluorobenzaldehyde

A 50-µl aliquot of phenelzine stock solution (200 ng/µl in 0.2 *M* aqueous sulphuric acid) was taken in 10-ml centrifuge tubes. To this were added 1 ml of pH 6.7 phosphate buffer and 25 µl of pentafluorobenzaldehyde solution (20 µg/µl in dimethylformamide). The solution was shaken for 5 min, 3 ml of benzene were added and again shaken for 10 min and centrifuged. The organic phase was separated, 50 µl of *n*-tetracosane stock solution (400 ng/µl in benzene) were added and the material was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 50 µl of benzene, 1 µl of the solution was injected into the gas chromatograph (Fig. 2A). The electron impact (EI) mass spectrum of the material (Fig. 3) is consistent with the structure of phenylethylhydrazone of pentafluorbenzaldehyde (Fig. 4).

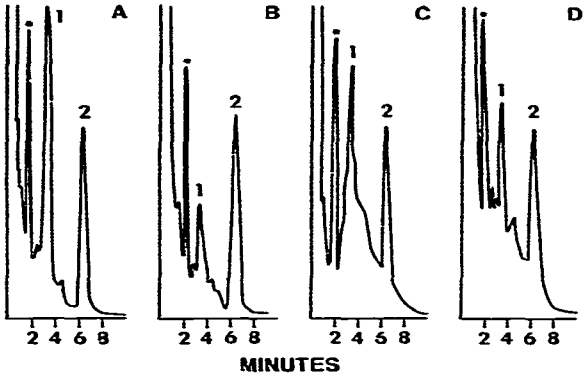

Fig. 2. Gas chromatogram with flame ionization detection of phenelzine derivative (1) with internal standard (2). Phenelzine extracted from (A) water and subsequently derivatized with pentafluorobenzaldehyde; (B) spiked plasma and subsequently derivatized; (C) phenelzine derivatized in spiked plasma and the derivative subsequently extracted; (D) the crude phenelzine derivative extracted and further chromatographed on LH-20 and eluted with benzene. The peak marked with an asterisk arises from excess of pentafluorobenzaldehyde present in the extract.

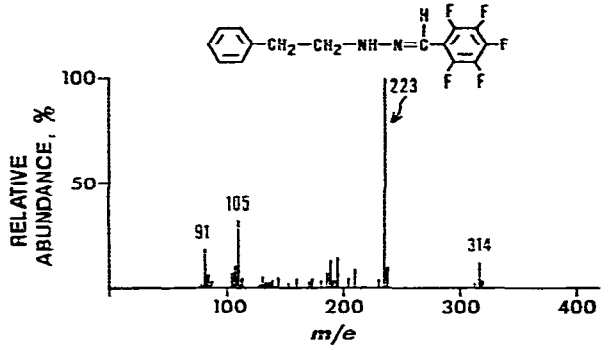

Fig. 3. EI (70 eV) mass spectrum of phenelzine derivative.

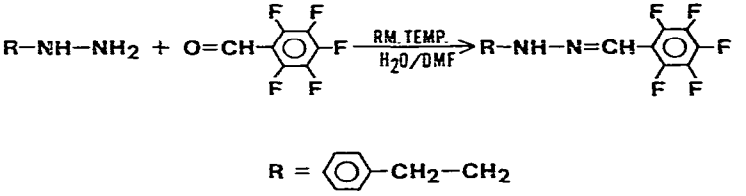

Fig. 4. Derivatization reaction of phenelzine with pentafluorobenzaldehyde.

Extraction and derivatization of phenelzine from plasma

Method A. Drug-free plasma (2 ml) and 50 µl of the phenelzine stock solution were taken in a centrifuge tube. To this were added 2 ml of pH 6.7 phosphate buffer, the solution was thoroughly mixed on a vortex mixer and extracted with 10 ml of benzene—ethyl acetate (4:1, v/v). The organic layer was separated, 1 ml of 0.1 *N* sulphuric acid was added and the mixture was shaken for 15 min. The organic layer was discarded, the aqueous phase was adjusted to pH 6.7 and 25 µl of pentafluorobenzaldehyde solution were added. The material was vigorously shaken, phenelzine derivative was extracted with 3 ml of benzene, 50 µl of the *n*-tetracosane stock solution were added and the material was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was constituted in 50 µl of benzene, ca. 1 µl of the solution was injected into the gas chromatograph. The gas chromatogram (Fig. 2B) is fairly clean, nevertheless the recovery of phenelzine, compared with the standard (Fig. 2A) is only 20% ± 9% ($n = 4$).

Method B. Drug-free plasma (2 ml) and 50 µl of phenelzine stock solution were taken in a centrifuge tube. To this were added 2 ml of pH 6.7 phosphate buffer, the material was thoroughly mixed and 25 µl of the pentafluorobenzaldehyde solution were added. The solution was vigorously shaken for 15 min and extracted with 10 ml of benzene. The organic layer was separated, 50 µl of the *n*-tetracosane stock solution were added to it and the material was evaporated to dryness at 40°C under a gentle stream of nitrogen. The residue was reconstituted in 50 µl of benzene, ca. 1 µl of the solution was injected into the gas chromatograph (Fig. 2C), the chromatogram shows phenelzine overlapping with other extraneous peaks. The crude phenelzine derivative was chromatographed on a 10 cm × 0.5 cm I.D. Sephadex LH-20 column. The

column was eluted with benzene, first 5 ml of the eluate were collected and evaporated to dryness. The residue was reconstituted in 50 μl of benzene, an aliquot (1 μl) of this solution was injected into the gas chromatograph. GC analysis (Fig. 2D) shows phenelzine cleanly separated from contaminants and indicating 65% + 7% (n = 4) recovery of the drug in the spiked plasma.

RESULTS AND DISCUSSION

Extremely poor and variable recovery of phenelzine (in Method A) from spiked plasma is in accord with earlier studies [10] and is attributed to its reported instability. However if it is derivatized first in the plasma and/or aqueous solution, the derivative, unlike native phenelzine, is perfectly stable and can be successfully extracted and purified by conventional techniques (Method B). The mass spectrum of the hydrazone (Fig. 3) shows a molecular ion at *m/e* 314, base peak at *m/e* 223 ($M-C_6H_5C^{\cdot}H_2$) and ions of modest intensity at *m/e* 105 (phenylethyl cation) and at *m/e* 91 (benzyl cation). The fragmentation pattern depicted in Fig. 5 is readily discernible. The molecular

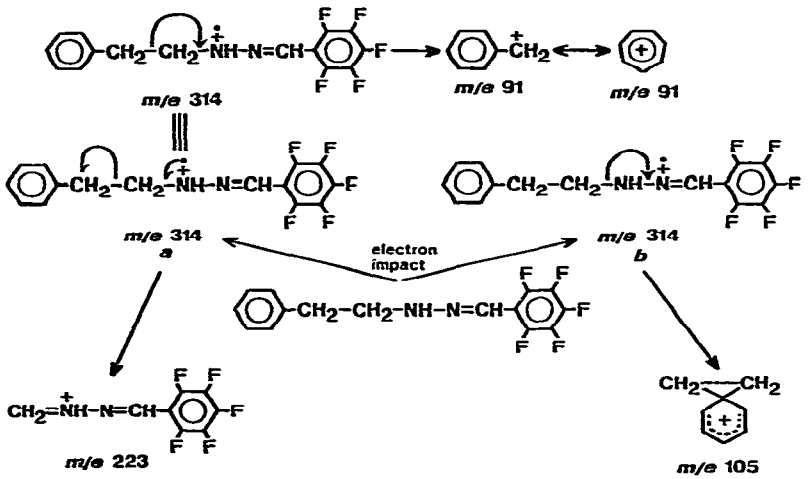

Fig. 5. Proposed fragmentation of phenelzine derivative under electron impact ionization.

ion formed by loss of a lone-pair electron from either nitrogen atom undergoes a typical β-cleavage fragmentation process [15, 16] to give the observed ions. The mass spectrum of phenelzine-d_7 derivative (Fig. 6) shows a molecular ion at *m/e* 321 and is similar to that of phenelzine derivative. Most ions are shifted to a higher mass by 7 a.m.u., except the ion at *m/e* 223, which is a common ion from both phenelzine derivative and phenelzine-d_7 derivative.

Selected ion monitoring assay

The ion at *m/e* 314 is specific for the phenelzine derivative (*m/e* 321 for phenelzine-d_7 derivative) and is a convenient working mass for SIM assay. Furthermore control human plasma, subjected to the described procedure for phenelzine, showed no significant background ions at *m/e* 314 and 321. Although the ion intensity at *m/e* 314 in the spectrum of phenelzine derivative

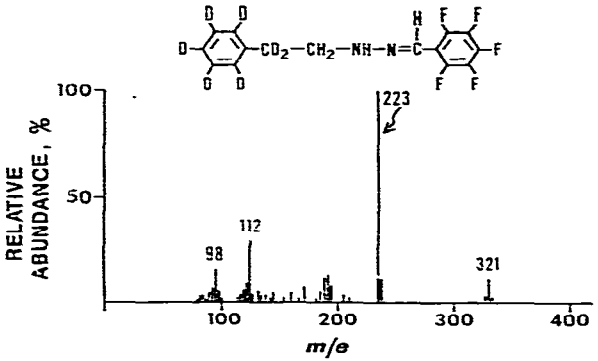

Fig. 6. EI (70 eV) mass spectrum of phenelzine-d_7 derivative.

is 12% of the base peak at 70 eV (Fig. 3), it is increased to 52% of the base peak at 20 eV. Consequently plasma phenelzine extract along with labeled phenelzine-d_7 was processed as described above (Method B), an aliquot of the material was injected into the gas chromatograph—mass spectrometer, phenelzine was quantitated by measuring the ion intensities at *m/e* 314 and 321 respectively. Known amounts of phenelzine along with its isotopic analog in fixed amount, were added to control plasma and processed as described. Phenelzine was quantitated from the ratios of ion intensities at *m/e* 314 and 321. Analysis of the data gave a slope of 0.98 ± 0.01 and an intercept of 0.16 ± 0.2 ng. These data affirm a simple linear relationship between the appropriate ion intensity ratios and concentration of phenelzine and exclude any isotopic exchange or any significant kinetic isotope effect in the fragmentation process.

Recovery and precision

Six samples containing 7 ng/ml of phenelzine were analyzed by Method B using 5.4 ng/ml of phenelzine-d_7 as internal standard. The results for these samples were 6.6 ± 0.34 ng/ml. These samples were assayed in duplicate, in this set exactly the same amounts were taken as above but the internal standard (as hydrazone) was added after the extraction. The recoveries for these samples, based on comparison of the ion intensity ratios of the two sets were 62 ± 7.5%. The wide range of recoveries observed is expected in trace analysis and is attributed to variable glassware, GC column adsorption and to susceptibility of phenelzine to oxidative and thermal decomposition. The sensitivity of the assay, being a function of extraction efficiencies, GC column conditions and the ion source, cannot be quoted in absolute terms. With good mass spectrometer performance, clean and freshly silanized GC column and glassware, and better than 50% recoveries an assay sensitivity of approximately 1—2 ng of phenelzine per ml is possible.

The method described above was used for the analysis of the free drug in the plasma of patients maintained on therapeutic dose of phenelzine. The mass fragmentograms obtained from biological extracts (Fig. 7) are clean and symmetrical peaks. The levels of phenelzine (Table I) in the plasma of 25 patients covered in this study varied from 1 to 10 ng/ml; obviously, most of the drug is either eliminated and/or is extensively metabolised in the biological

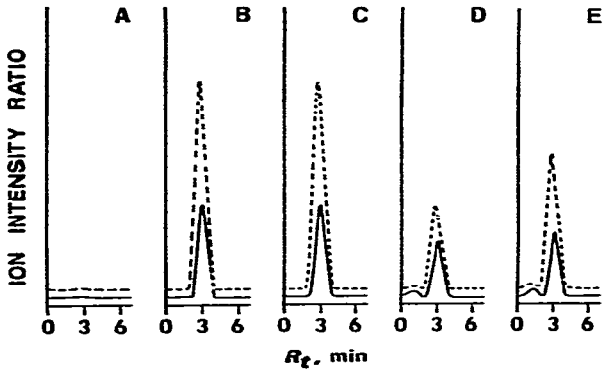

Fig. 7. Selected mass fragmentograms for phenelzine derivative (*m/e* 314) (——) along with phenelzine-d_7 derivative (*m/e* 321) (— — —). (A) Drug-free 2-ml control plasma was processed as in Method B, 2 μl of aliquot (total 10 μl) was injected into the GC—MS system; (B) 2 ml control plasma, spiked with 44 ng/ml of phenelzine-d_7 and 18.3 ng/ml of phenelzine, was processed as in Method B, 1 μl of aliquot (total 25 μl) was injected; (C) 2 ml plasma of a patient, 22 ng/ml of phenelzine-d_7 was added as internal standard and processed as in Method B, 2 μl of aliquot (total 25 μl) was injected and phenelzine concentration was found to be 9.0 ng/ml; (D) 2 ml plasma of a patient, 5.4 ng/ml phenelzine-d_7 was added as an internal standard, and processed as in Method B, 1.5 μl of aliquot (total 15 μl) was injected, phenelzine concentration was found to be 2.9 ng/ml; (E) 2 ml plasma of a patient, 5.4 ng/ml of phenelzine-d_7 was added and processed as in Method B, 1.5 μl of aliquot (total 15 μl) was injected, phenelzine concentration was found to be 2.0 ng/ml.

TABLE I

ANALYSES OF PHENELZINE IN PLASMA OF PATIENTS

Analysis of duplicate runs, using 2 ml of plasma for phenelzine determination; 25 plasma samples were processed as described; 16 samples showed phenelzine concentration 3—6 ng/ml, while 5 samples gave phenelzine concentration 1—2 ng/ml and 4 samples gave phenelzine concentration 9—10 ng/ml.

Subject No.	Phenelzine found (ng/ml)
1	9.6 ± 0.3
2	9.12 ± 0.31
3	5.3 ± 0.29
4	4.9 ± 0.31
5	3.4 ± 0.28
6	1.89 ± 0.2

system. The analytical method described here will permit further detailed assessment of the plasma phenelzine concentration time profiles of the drug and its clinical efficacy.

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