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Short Communication

Determination of fluoxetine and norfluoxetine in human plasma by capillary gas chromatography with electron-capture detection

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ABSTRACT

A capillary gas chromatographic method with ^{63}Ni electron-capture detection is reported for the determination of fluoxetine (Prozac) and its metabolite norfluoxetine in human plasma. A liquid–liquid extraction is used, followed by derivatization with heptafluorobutyric anhydride to increase the sensitivity of detection. A 30 m × 0.25 mm I.D. DB-17 capillary column resolves the compounds from endogenous matrix interferences. The limit of quantitation by this method is 5 ng/ml for each compound. Stability studies show that fluoxetine and norfluoxetine are stable in human plasma for up to 96 h at room temperature and up to one year at −20°C.

INTRODUCTION

Fluoxetine hydrochloride is currently marketed as an antidepressant under the trade name of Prozac. The metabolism of fluoxetine yields its desmethyl metabolite, norfluoxetine. Several HPLC methods for the determination of fluoxetine and norfluoxetine in serum or plasma have been described [1–3]. However, these same reports indicate that certain tricyclic antidepressants can interfere with the HPLC assays. A GC–electron-capture detection (ECD) method utilizing solid-phase extraction [4] has been described using pentafluoropropionic anhydride (PFPA) as the derivitizing agent with a reported limit of quantitation (LOQ) of 20 ng/ml. We have previously reported a packed column GC method where nortriptyline, imipramine, desipramine, amitriptyline, doxepin, diazepam, chlordiazepoxide, prochlorperazine, and chlorpromazine did not interfere with the assay [5]. That procedure included the derivatization of the analytes, dissolved in benzene, also with PFPA. This report describes an improved capillary GC assay using heptafluorobutyric anhydride (HFBA) as the derivatization agent for increased sensitivity. Additionally, we have replaced the use of benzene, a known carcinogen, with toluene. This method has been used for the determination of fluoxetine and norfluoxetine in human plasma in support of clinical trials. The lower detection limit of this method has facilitated pharmacokinetic analyses.

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EXPERIMENTAL

Chromatography

The chromatographic system consisted of a Hewlett Packard Model 5890 gas chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with a split–splitless inlet, a ^{63}Ni electron-capture detector, and a Hewlett Packard Model HP7673A automatic sample injector. The capillary column used was a DB-17 (50% phenyl, 50% methyl silicone), 30 m × 0.25 mm I.D., 0.15 μm film thickness (J & W Scientific, Folsom, CA, USA).

Chemicals and reagents

Fluoxetine hydrochloride or (±)-N-methyl-3-phenyl-3-[(α,α,α-trifluoro-*p*-tolyl)oxy]propylamine hydrochloride, norfluoxetine hydrochloride (desmethyl metabolite of fluoxetine), and 4,4-bis-(*p*-fluorophenyl)-N-ethylbutylamine hydrochloride (internal standard) were obtained from Lilly Research Labs. (Eli Lilly and Co., Indianapolis, IN, USA). The toluene and butyl chloride were HPLC-grade solvents (Burdick and Jackson, Division of Baxter Healthcare, Muskegon, MI, USA). All other reagents were analytical grade. HFBA was used as the derivatizing agent (Sigma, St. Louis, MO, USA). Purified water (Milli-Q system, Waters Assoc., Millipore, Milford, MA, USA) was used in all aqueous solutions. Human heparinized plasma was obtained from healthy volunteers.

Hydrochloric acid solutions were prepared at concentrations of 0.01 and 0.2 *M*. A carbonate buffer of pH 10 was prepared by adding equal parts of sodium carbonate (1.0 *M*) and sodium bicarbonate (1.0 *M*).

Instrument conditions

Argon–methane (95:5, v/v) was used for the carrier and make-up gas to the electron-capture detector. A gas purifier (Alltech Assoc. Applied Science Labs., Deerfield, IL, USA) and an oxygen scrubber (J&W Scientific, Folsom, CA, USA) were used to remove trace impurities and oxygen from the carrier gas. The column head pressure was set at 0.69 bar and the make-up gas to the detector was set at 60 ml/min. Detector temperature was set at 325°C and the inlet temperature was set at 250°C. The settings for purge time off and on were 0.0 and 0.5 min, respectively. The temperature program was as follows: the initial oven temperature was held at 130°C for 1 min, then increased at a rate of 25°C/min to 235°C and held for 10 min. The temperature was further increased at a rate of 25°C/min to 270°C and held there for 5 min.

Preparation of standard solutions

A standard stock solution containing both fluoxetine hydrochloride and norfluoxetine hydrochloride was prepared in 0.01 *M* HCl at a concentration of 100 μg/ml (free base) of each compound. The internal standard was prepared in 0.01 *M* HCl at a concentration of 1 μg/ml.

Plasma standards were prepared at concentrations of 10, 25, 50, 100, and 150 ng/ml of each compound by diluting the appropriate aliquots of the stock solution with drug-free plasma.

Preparation of samples

A 1-ml aliquot of each plasma sample was transferred into a 15-ml disposable centrifuge tube and 100 μl internal standard were added. A 1-ml pH 10 sodium carbonate buffer solution (1 *M*) and 6 ml of butyl chloride were added and the phases were mixed by shaking for 6 min. The samples were centrifuged at 829 *g* for 5 min and the butyl chloride layers were transferred to clean 15-ml disposable tubes. A 2-ml aliquot of 0.2 *M* HCl was added to the butyl chloride and shaken for 3 min. The samples were again centrifuged for 5 min and the butyl chloride was discarded. A 2-ml aliquot of the pH 10 sodium carbonate buffer was added to the acid layer and vortex-mixed. A 5-ml aliquot of toluene was then added to each tube and the samples were shaken for 3 min. The samples were centrifuged for 5 min, the toluene layer was transferred to a clean 15-ml centrifuge tube, and 25 μl of HFBA were added to each tube. The tubes were capped and heated at 88°C for 30 min. The samples were evaporated to dryness under nitrogen. Each sample was reconstituted in 200 μl of toluene, vortex-mixed, trans-

ferred to glass autoinjector vials, and sealed. A 2-μl aliquot of each solution was injected on the GC system.

Determination of precision and accuracy

The precision and accuracy of the method were determined by performing replicate analyses of four pools of plasma spiked with known concentrations of the compounds. Five replicates of each pool were run on three separate days. The concentrations were selected to cover the range of the standard curve and included the limit of quantitation. The limit of quantitation was chosen to be the smallest concentration within acceptable limits of variance. The results were evaluated for both variance and accuracy.

Determination of recovery and stability

The relative recoveries of fluoxetine and norfluoxetine from the plasma matrix were determined by comparing the peak heights of plasma and aqueous samples spiked with equivalent concentrations of the compounds. The water standards were processed through the same extraction and derivatization procedure as the plasma samples.

The stability of the compounds was determined by preparing pooled plasma with known amounts of the compounds. The pools were aliquoted (1 ml) into capped glass tubes and stored at −20°C, 40°C, and at room temperature. These pools were assayed at various times following initial storage.

RESULTS AND DISCUSSION

Chromatography

Fig. 1A is a chromatogram of blank plasma indicating the absence of interfering compounds at the retention times of the compounds of interest. A chromatogram of blank plasma spiked with fluoxetine and norfluoxetine shows good resolution between the compounds and the internal standard (Fig. 1B). The retention times of norfluoxetine, fluoxetine, and the internal standard were approximately 9.6, 10.2, and 12.1 min,

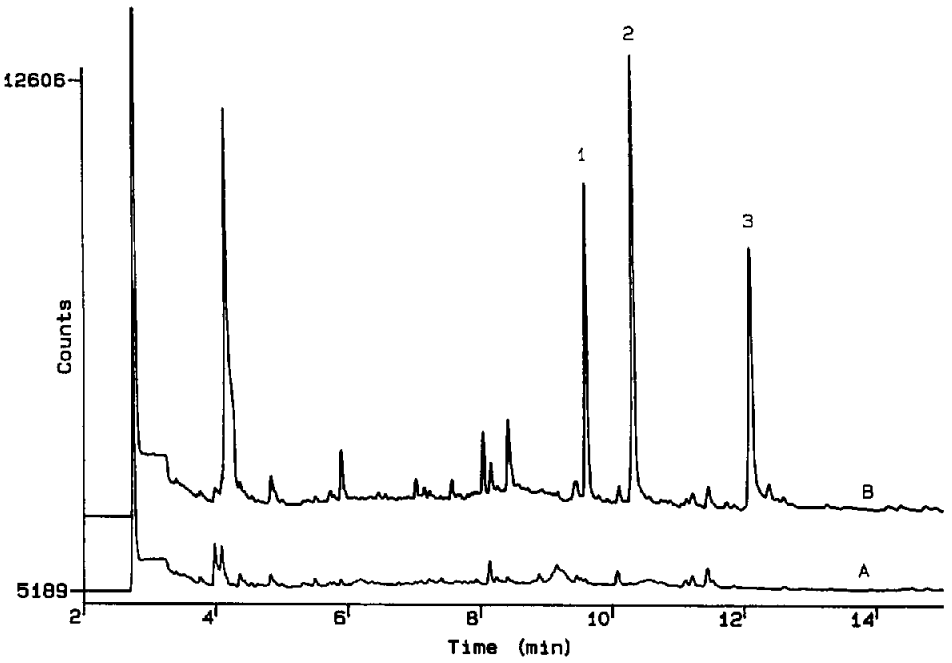

Fig. 1. Chromatograms of plasma extracts. (A) Blank plasma; (B) blank plasma spiked with fluoxetine, norfluoxetine, and internal standard, each at a concentration of 100 ng/ml. Peaks: 1 = norfluoxetine; 2 = fluoxetine; 3 = internal standard.

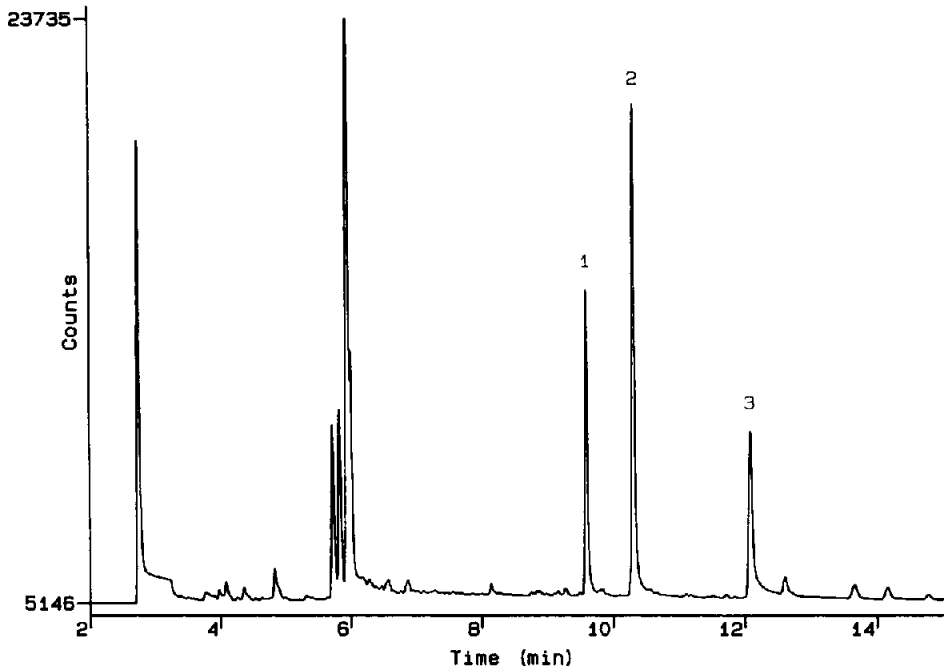

Fig. 2. Chromatogram of a plasma sample from a patient receiving 60 mg of fluoxetine per day. Peaks: 1 = norfluoxetine; 2 = fluoxetine; 3 = internal standard.

respectively. Fig. 2 is a chromatogram of a patient following administration of 60 mg of fluoxetine per day in a Phase II study.

Calibration curves

The peak heights of fluoxetine and norfluoxetine were divided by the peak height of the internal standard to obtain the peak-height ratios. The calibration curve for each compound was constructed from a linear-squares regression of the peak-height ratios of the standards *versus* the concentrations. Typical correlation coefficients were greater than 0.99. The linearity of the curve has been demonstrated from 5 to 200 ng/ml.

Recovery of extraction

The extraction efficiency was determined by comparing the peak heights of the compounds extracted from plasma and water and derivatized. The recovery of fluoxetine and norfluoxetine from plasma was 76 and 88%, respectively, relative to water.

Stability

Fluoxetine and norfluoxetine were stable in human plasma at room temperature for up to 96 h. At 40°C, both compounds were stable up to 72 h but showed some losses at 96 h. Fluoxetine had a loss of about 17%, while norfluoxetine had lost about 8%. In human plasma, fluoxetine and norfluoxetine were stable for a least one year stored at −20°C. These studies indicate that samples containing fluoxetine and norfluoxetine can be handled under normal laboratory conditions without significant loss of compounds.

Precision and accuracy

The analysis of fluoxetine and norfluoxetine was evaluated for precision and accuracy (Tables I and II) by analyzing replicate determinations of plasma pools at concentrations of 5, 40, 125, and 200 ng/ml. Overall, the precision and accuracy was acceptable for both compounds. The 40, 125, and 200 ng/ml pools each had a total relative standard deviation (R.S.D.) and relative error

TABLE I

SUMMARY OF PRECISION AND ACCURACY DATA FOR FLUOXETINE

Theoretical concentration (ng/ml)	Day 1 (n = 5)		Day 2 (n = 5)		Day 3 (n = 5)		Overall (n = 15)		
	Mean found (ng/ml)	R.S.D. (%)	Mean found (ng/ml)	R.S.D. (%)	Mean found (ng/ml)	R.S.D. (%)	Mean found (ng/ml)	R.S.D. (%)	R.E. (%)
5.0	7.62	25	6.21	15	5.69	16	6.43	21	29
40	41.6	4.2	43.0	2.8	41.9	4.8	42.2	4.0	5.5
125	127	3.5	129	2.8	121	2.4	126	4.0	0.54
200	194	11	198	2.4	183	4.4	191	7.3	−4.3

TABLE II

SUMMARY OF PRECISION AND ACCURACY DATA FOR NORFLUOXETINE

Theoretical concentration (ng/ml)	Day 1 (n = 5)		Day 2 (n = 5)		Day 3 (n = 5)		Overall (n = 15)		
	Mean found (ng/ml)	R.S.D. (%)	Mean found (ng/ml)	R.S.D. (%)	Mean found (ng/ml)	R.S.D. (%)	Mean found (ng/ml)	R.S.D. (%)	R.E. (%)
5.0	6.42	7.7	6.13	8.9	5.73	12	6.09	10	22
40	35.6	9.6	40.9	1.4	39.4	8.5	38.6	9.5	−3.5
125	121	3.8	123	5.4	116	3.6	120	4.8	−4.0
200	191	14	205	3.6	181	7.4	193	8.4	−3.6

(R.E.) of less than 10%. At the quantitation limit of 5 ng/ml, norfluoxetine showed slightly better precision than fluoxetine. Norfluoxetine had a total R.S.D. of 10% while fluoxetine had an R.S.D. of 21%. In terms of accuracy at the LOQ, the R.E. for both compounds was less than 30%.

CONCLUSIONS

The procedures reported here provide a sensitive and selective method for the determination of fluoxetine and its metabolite, norfluoxetine, in human plasma. Tricyclic antidepressants have been shown not to interfere with the GC methods. While the details have not been included in this report, similar procedures have also been used for the determination of fluoxetine and norfluoxetine in human serum, urine and also tissue samples from toxicological studies. The stability of these compounds in human plasma allows for ease in sample handling. The methodology presented here has been applied in several laboratories, verifying its ruggedness.

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