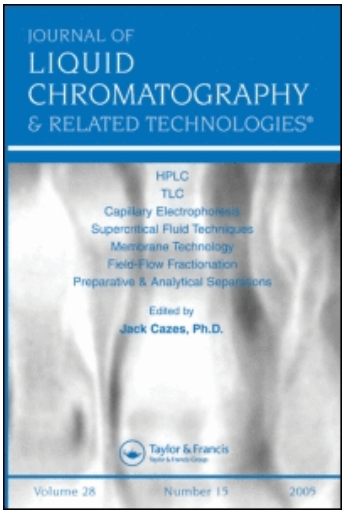

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Determination of Fluoxetine and Norfluoxetine in Serum by Liquid Chromatography with Fluorescence Detection

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Original Article

DETERMINATION OF FLUOXETINE AND NORFLUOXETINE IN SERUM BY LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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ABSTRACT

A reversed phase liquid chromatographic procedure with fluorescence detection for the simultaneous determination of fluoxetine and its active metabolite, norfluoxetine in human serum is described. A 0.5-mL aliquot of the sample after the addition of protriptyline as the internal standard is passed through a 1-mL BondElut C_{18} silica extraction column. The column is selectively washed to remove polar, neutral, acidic and weakly basic compounds. The desired compounds are eluted with a 0.25-mL aliquot of a mixture of 0.1 N perchloric acid + acetonitrile (1:3). A 20-uL aliquot of the eluate is injected onto a 15 cm X 4.6 mm (i.d.) column packed with 5-um C_8-silica particles, which is eluted at ambient temperature with a mobile phase containing tetramethylammonium perchlorate. The peaks are detected with a fluorescence detector (ex = 235 nm; em = 310 nm). In the resulting chromatogram, there are only few extraneous peaks, fluoxetines give sharp peaks which are well resolved from peaks for solvent and internal standard. The extraction recovery of fluoxetines and internal standard is in the range of 85%.

INTRODUCTION

Fluoxetine (Flu) is a new bicylic antidepressant drug which enhances serotoninergic neurotransmission through potent and selective inhibition of neuronal reuptake of serotonin (1). Norfluoxetine (N-Flu) formed in humans by N-demethylation also inhibits serotonin reuptake. Preliminary clinical trials have shown that Flu is similar in therapeutic efficacy to traditional tricyclic antidepressants but has significantly fewer side effects. No significant correlation has been observed between serum fluoxetines concentrations and therapeutic response (2). However, the demand for serum Flu assay is increasing with the increased use of the drug to monitor compliance or unexpected toxic concentrations after chronic use of Flu.

Fluoxetines have been determined in plasma by gas chromatography with electron capture detection at therapeutic concentrations (3,4), by flame ionization or nitrogen selective detection at toxic concentrations (5,6). There is an increasing trend to use liquid chromatography (LC) for monitoring therapeutic concentrations of antidepressants (7) and LC procedures for the determination of fluoxetines have also been described (2,8,9). The purpose of this study was to select conditions which may further improve the specificity and sensitivity for the LC determination of fluoxetines.

EXPERIMENTAL

Materials

All reagents were of analytical grade. De-ionized water was distilled in an all glass still.

Stock fluoxetines solution (1 mg/mL) was prepared by dissolving 11.2 mg of fluoxetine hydrochloride and 14 mg of norfluoxetine maleate (both from Eli Lilly & Co., Indianapolis, IN 46285) in 10 mL of methanol. The solution was stored in 0.2-mL aliquots at -20°C in tightly capped glass tubes. Serum standard, 1000 ng/mL was prepared by diluting 25 uL of the stock solution with pooled serum to 25 mL in a volumetric flask. Standards of 31.25, 62.5, 125, 250, and 500 ng/mL were prepared by serial dilution with pooled serum. The standards were stored at -20°C in 1-mL portions.

Stock internal standard (IS) solution (1 mg/mL) was prepared by dissolving 11.4 mg of protriptyline HCl (Merck Sharp & Dohme, Kirkland, QC) in 10 mL of methanol. The solution was stored at -20°C in 0.2-mL aliquots. Working (IS) solution was prepared just before use by diluting 5 uL of the stock solution with 5 mL of water.

Procedures

Sample collection. Blood was collected in 10-mL Vacutainer Tubes (Cat.No. 6430; Becton Dickinson; Mississauga, ON) containing no additive. Serum was

separated within 2 hours of sample collection and stored frozen at -20°C until analysis.

Extraction. Wash 1-mL BondElut C_{18} columns (Analytical International Inc. Harbor City, CA 90710) once with 1 N HCl, twice with methanol, each time aspirating the liquid with a VacElut device. Apply 0.25 mL of 0.1 N sodium carbonate, 0.25 mL of working IS solution, and 0.5 mL of sample (standard or unknown) to labelled washed columns. Let the liquid pass through the column at a rate of <1 mL/min. Then wash the columns twice with water, once with 50% aqueous methanol and once with acetonitrile.

Transfer the columns to correspondingly labelled 12 X 75 mm glass tubes washed with methanol. Apply 0.25 mL of a mixture of 0.1 N perchloric acid and acetonitrile (1:3) to each column and elute by centrifugation. Vortex mix the eluate and inject a 20-uL aliquot into the liquid chromatograph.

Chromatography. A modular chromatographic system comprising a model 100A pump (Beckman Instruments, Berkely, CA 94928); a 15 cm X 4.6 mm(i.d.) Ultrasphere Octyl reversed phase column (5-um particles, Beckman) protected by a RP-18 1.5 cm guard cartridge (7-um particles, Brownlee Labs, Santa Clara, CA 95050); a model RF535 fluorescence detector (Shimadzu Scientific Instrument, Columbia, Md 21046) and a model C-R6A integrator plotter (Shimadzu) was used. The

chromatography was performed at ambient temperature. The mobile phase consisting of acetonitrile (375 mL) + water (625 mL) + tetramethylammonium perchlorate (Sigma Chemical Co.,St.Louis, Mo 63178-9916) (1.5 g) + 70% perchloric acid (0.1 mL) was pumped at a flow rate of 2 mL/min with an operating pressure of 12.4 MPa. The fluorescence was monitored at 310 nm (excitation at 235 nm).

RESULTS AND DISCUSSION

Detection. Fluoxetine dissolved in the mobile phase has an absorption maximum at 227 nm with fair absorptivity (A^{1}_{1} = 400). In the earlier LC procedures described for the assay of fluoxetines detection has been carried out at around 225 nm. However, in a recently published procedure detection has been carried out at 214 nm to make the assay applicable to fixed wavelength detectors (9). The detection of fluoxetines at 227 or 214 nm is non specific as a number of commonly prescribed antidepressant drugs and their metabolites and benzodiazepines elute close to Flu or N-Flu. Native fluorescence of fluoxetines was examined in an attempt to find a selective mode of detection for these compounds. It was observed that both Flu and N-Flu showed intense fluorescence at ex = 235 nm and em = 310 nm. The areas of fluorescence peaks of fluoxetines are approximately 30 times the areas of absorbance peaks at 227 nm. The

baseline of fluorescence detector at these settings was, however, quite noisy and required plotter attenuation of 5 for a smooth baseline. On the other hand the baseline of the absorbance detector (Model SPD-6AV, Shimadzu) is smooth at the plotter attenuation of 2. The integrator plotter is connected to 1 V output of the fluorescence detector and to 0.8 V output of the absorbance detector. Figures 1A and 1B show absorbance and fluorescence peaks respectively when 20 uL of a 500 ng/mL of fluoxetines and IS were injected on the column. The heights of fluorescence peaks are still approximately 4 times than those of absorbance peaks. Protriptyline, maprotiline, and desmethylmaprotiline are the only compounds among the traditional antidepressant drugs and benzodiazepines which show fluorescence response under the conditions selected for Flu detection.

Internal Standard. Our object was to find a commercially available non-drug compound for use as an IS for the assay of fluoxetines. For this purpose we obtained 3,3-diphenylpropylamine, 2,2-diphenylpropylamine, and 2,2-diphenylethylamine (Aldrich Chemical Co., Milwaukee, WIS 53233). These compounds show weak fluorescence response under the conditions for Flu detection and elute close to the solvent peaks. Tomoxetine (LY 139603, Eli Lilly & Co.), a compound of somewhat similar structure to that of Flu and used as IS by Nash et al.(3) for their GC procedure, could not be used as IS for the present LC procedure as it did not

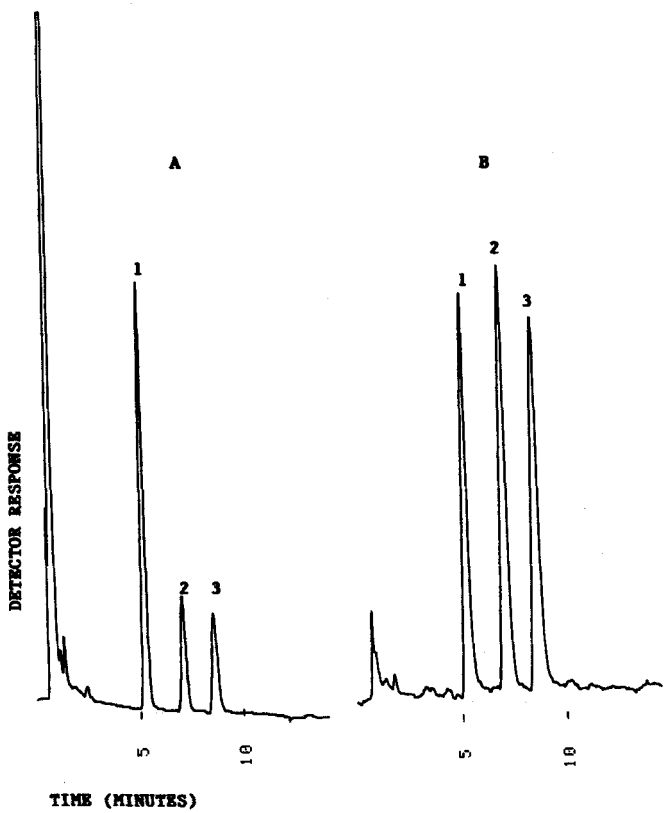

Figure 1. Chromatograms of 20 uL injections of 500 ng/mL of each of protriptyline, norfluoxetine and fluoxetine in 0.1 N $HClO_4$ + acetonitrile (1:3).
(A) Absorbance detection (227 nm); detector output: 0.8 V; Plotter attenuation: 2.
(B) Fluorescence detection (ex = 235 nm; em = 310 nm); detector output: 1.0 V; Plotter attenuation: 5.
Peak 1 = protriptyline, 2 = norfluoxetine, 3 = fluoxetine.

show any fluorescence response. Consequently, we selected protriptyline as the IS. It shows fluorescence similar to that of Flu, is well separated from solvent and fluoxetines peaks, and is a rarely prescribed drug. Protriptyline has been used as IS by Kelly et al. for their LC procedure with absorbance detection for the assay of fluoxetines (2).

Extraction. Fluoxetines have been extracted from plasma by 4-step liquid-liquid extraction for LC (2,8) and GC (3) procedures. In the most recent report, the extraction procedure has been simplified by avoiding the second extraction into organic phase. The acidic layer obtained after back extraction of the 1st organic extract was injected into the liquid chromatograph directly (9). In our initial experiments we also used liquid-liquid extraction. To minimize use of glassware, we washed the serum after the addition of IS at acidic pH with pentane. Serum was then made alkaline and extracted into pentane (10) which was evaporated easily at 45°C without any vacuum or a stream of nitrogen. The recovery of fluoxetines was 50-60% and the chromatograms obtained with absorbance or fluorescence detection were clean. However, the procedure is tedious and slow as it requires silylation of extraction and evaporation tubes.

Solid phase extraction with the use of C_{18} silica columns was then attempted. Fluoxetines and protriptylines were strongly retained by the extraction columns. A number

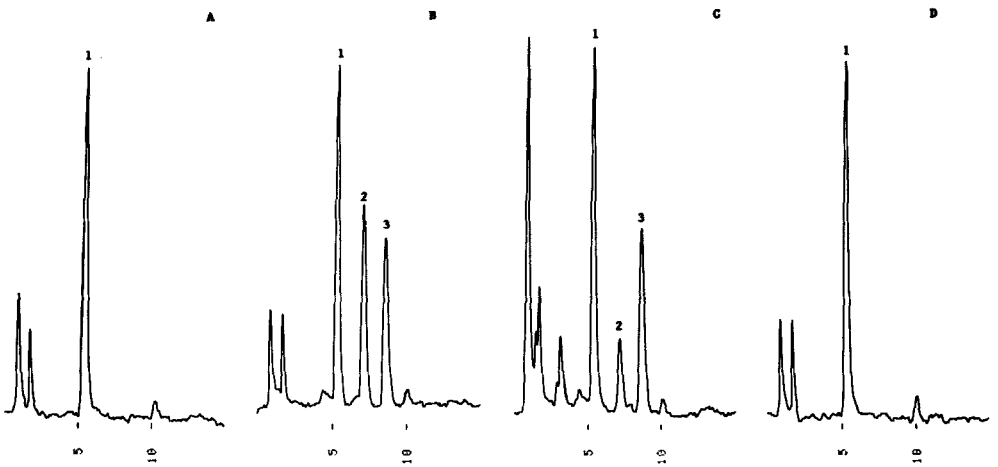

Figure 2. Chromatograms of 20 uL injections of extracts of serum.Fluorescence detection (A) drug free serum; (B) serum standard of 250 ng/mL of each fluoxetine; (C) serum of a patient receiving fluoxetine (norfluoxetine = 80 ng/mL;fluoxetine =260 ng/mL); (D) serum standard of 400 ng/mL each of nortriptyline and amitriptyline.
See Fig. 1 for conditions and peak identification. Unlabelled peaks are extraneous.

of solvents were tried to selectively wash the column to remove extraneous compounds and to elute the desired compounds in a minimum volume of solvent. The described procedure meets our objectives. A large number of acidic, neutral, and weakly basic compounds are removed during washes with 50% methanol and acetonitrile. However, strongly basic compounds including tricyclic antidepressants are co-extracted with fluoxetines. Fluoxetines and protriptyline are eluted in a convenient volume in about 85% yield. The extract is clean by both fluorescence (Fig.2A) and

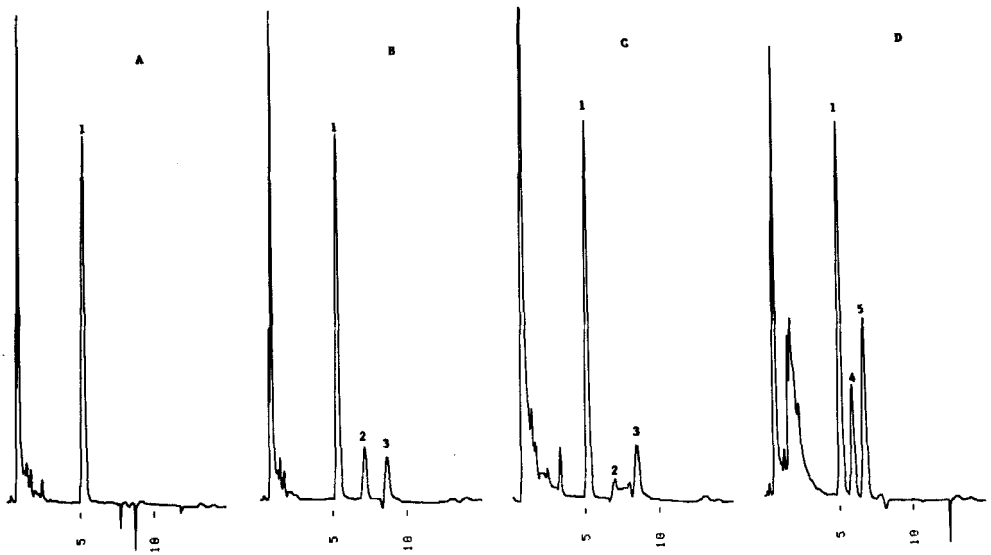

Figure 3. Chromatograms of 20 uL injections of extracts of serum.Absorbance detection.
See Fig. 2 for chromatogram identification.
Peak 4 = nortriptyline; 5 = amitriptyline

absorbance (Fig.3A) detection. There is no change in the ratio of Flu/IS or N-Flu/IS when aliquots of an aqueous mixture of the compounds are injected before and after extraction with the described procedure. The extraction columns have been used twice without significant effect on recovery or the cleanliness of the extract.

Separation of benzodiazepines and antidepressant drugs by solid phase extraction has recently been described (11). A reagent kit based on such an extraction scheme for the determination of benzodizepines and antidepressant drugs in

the same portion of plasma sample is being marketed by Bio-Rad Laboratories (Hercules, CA 94547).

Figure 2B shows a representative chromatogram of an extract of a 250 ng/mL serum standard and figure 2C is the chromatogram of an extract of serum of a patient receiving 20 mg of Flu per day for 6 weeks. Figures 3B and 3C are the corresponding chromatograms obtained with absorbance detection. The relation between the ratios of peak areas of Flu/IS and N-Flu/IS and concentrations of Flu and N-Flu respectively is linear for the range tested (30 to 1000 ng/mL) and the curves pass through the origin. The procedure shows a high degree of specificity. An extract of Therachem TDM high level control (Fisher Scientific, Orangeburg, Ny 10962) which contains 28 drugs in toxic concentrations did not show any peak after the solvent

Table 1. Precision Of The Method

	Fluoxetine		Norfluoxetine	
	Mean ng/mL	CV%	Mean ng/mL	CV%
Within batch (N = 10)				
Low	125	9.1	125	5.8
High	1000	4.7	1000	4.4
Between batch (N = 8)				
Low	128	6.9	127	6.7
High	1028	5.2	1033	4.8

peaks. Figure 2D is the chromatogram of serum spiked with 400 ng/mL each of amitriptyline and nortriptyline and shows the absence of any peak other than that of the IS. Figure 3D is the corresponding chromatogram obtained with absorbance detection and shows the presence of nortriptyline and amitriptyline in the serum extract. Analysis of serum spiked with low (125 ng/mL) and high (1000 ng/mL) Flu and N-Flu showed acceptable precision (Table 1).

In conclusion, the described procedure is quite simple and suitable for use in clinical laboratories. The procedure allows the detection of 20 ng/mL of either of the fluoxetines using only 0.5 mL of the sample. In the absence of a fluorescence detector, the extracts can be analyzed using an absorbance detector at 227 nm provided other antidepressants are not coadministered. Aliquots of 50 uL of the serum extract are injected with absorbance detection to detect low concentrations of fluoxetines.

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REFERENCES

1. Benfield, P., Heel, R.C. and Lewis, S.P. Fluoxetine: A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic efficacy in depressive illness. Drugs 32, 481, 1986.

2. Kelly, M.W., Perry, P.J., Holstad, S.G., and Garvey, M.J. Serum fluoxetine and norfluoxetine concentrations and antidepressant response. Ther. Drug Monit. 11, 165, 1989.

3. Nash, J.F., Bopp, R.J., Carmichael, R.H., Farid, K.Z., and Lemberger, L. Determination of fluoxetine and norfluoxetine in plasma by gas chromatography with electron-capture detection. Clin. Chem. 28, 2100, 1982.

4. Caccia, S., Cappi, M., Fracasso, C., and Garatlini. Influence of dose and route of administration on the kinetics of fluoxetine and its metabolite norfluoxetine in the rat. Phsychopharmacology, 100, 509, 1990.

5. Rohrig, T.P. and Prouty, R.W. Fluoxetine overdose: A case report. J. Anal. Toxicol. 13, 305, 1989.

6. Roettger, J.R. The importance of blood collection site for the determination of basic drugs: A case with fluoxetine and diphenhydramine overdose. J. Anal. Toxicol. 14, 191, 1990.

7. Wong, S.H.Y. Measurement of antidepressants by liquid chromatography: A review of current methodology. Clin. Chem. 34, 848, 1988.

8. Orsulak, P.J., Kenny J.T., Debus, J.R., Crowley, G., and Wittman P.D. Determination of the antidepressant fluoxetine and its metabolite norfluoxetine in serum by reversed-phase HPLC, with ultraviolet detection. Clin. Chem. 34, 1875, 1988.

9. Wong, S.H.Y., Dellafera, S.S., Fernandes, R. and Kranzler, H. Determination of fluoxetine and norfluoxetine by high performance liquid chromatography. J. Chromatogr. 499, 601, 1990.

10. Gupta, R.N., Stefanee, M. and Eng, F. Determination of tricyclic antidepressant drugs by gas chromatography with the use of a capillary column. Clin. Biochem. 16, 94, 1983.

11. Mazhar, M. and Binder, S.R. Analysis of benzodiazepines and tricyclic antidepressants in serum using a common solid-phase clean-up and a common mobile phase. J. Chromatogr. 497, 201, 1989.

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