study and one by Technicon showed similar results. In the Technicon study, the regression line crosses the 45° line at a uric acid concentration of 61 mg/L (Table 2), which is within the reference interval. Thus, the SMAC uricase value will exceed the phosphotungstate value when the uric acid concentration is <61 mg/L. The results from our study show a similar pattern but the regression line crosses the 45° line at a uric acid concentration of 21 mg/L. A previous study of the SMAC PTA method for determination of uric acid showed a positive bias when compared with a uricase continuous-flow method (10). In this study paired uric acid analyses were done on 1185 unselected specimens. The difference between the means of values obtained by each of the two methods was 4.6 mg/L, the higher value being obtained with the SMAC PTA method. The equation of the regression line was $y = 6.2 + 0.967x$, where y was the SMAC result and x the uricase result. Here the SMAC PTA method is plotted on the ordinate, but in our study it is plotted on the abscissa. The regression line crosses the 45° line at a uric acid concentration of 187 mg/L. Thus, the results for this study show that the PTA method gave higher results than the uric acid method over the entire physiologic range. As was mentioned previously, there is a documented positive systematic bias for the PTA method compared with the uricase method for uric acid of approximately 3 mg/L (9). Because our study and that of Technicon show that the SMAC uricase method produces higher results than the PTA method at concentrations within the reference interval and below, we conclude that the SMAC uricase method needs additional study to resolve this discrepancy.

References

1. Howorth, P. J. N., and Zilva, J. F., Determination of uric acid levels in uraemia by enzymatic and colorimetric techniques. *J. Clin. Pathol.* 21, 192–195 (1968).

2. Itiaba, K., Hsiung, N. W., and Crawhall, J. C., Uric acid estimation: A comparison of the manual uricase UV and the phosphotungstate Auto-Analyzer methods. *Clin. Biochem.* 8, 316–319 (1975).

3. Slaunwhite, W. D., Pachla, L. A., Wenke, D. C., and Kissinger, P. T., Colorimetric, enzymatic, and liquid-chromatographic methods for serum uric acid compared. *Clin. Chem.* 21, 1427–1429 (1975).

4. Duncan, P. H., Gochman, N., Cooper, T., et al., Development and evaluation of a candidate Reference Method for uric acid in serum. U.S. Dept. of HEW, Public Health Service, 1980. (Copies may be obtained from the Clinical Chemistry Division, Centers for Disease Control, Bldg. 17, Rm. 302, Atlanta, GA 30333.)

5. Duncan, P. H., Gochman, N., Cooper, T., et al., A candidate Reference Method for uric acid in serum. I. Optimization and evaluation. *Clin. Chem.* 28, 284–290 (1982).

6. Remp, D. G., Uric acid (uricase). *Stand. Methods Clin. Chem.* 6, 1–11 (1970).

7. Cornbleet, P. J., and Gochman, N., Incorrect least-squares regression coefficients in method-comparison analysis. *Clin. Chem.* 25, 432–438 (1979).

8. Technicon Instruments Corp., Uric acid (uricase), method No. SG4-0054FJ9, Product Labeling, Volume III, 27-3 to 27-11 (1979).

9. Musser, W. A., and Ortigoza, P., Automated determination of uric acid by the hydroxylamine method. *Tech. Bull. Regist. Med. Technol.* 30, 21 (1966).

10. Robertson, E. A., Sliva, C. A., and Young, D. S., Values for uric acid in patient's sera as measured with SMAC (phosphotungstate) and AutoAnalyzer (uricase). *Clin. Chem.* 24, 173–174 (1978). Letter.

Determination of Fluoxetine and Norfluoxetine in Plasma by Gas Chromatography with Electron-Capture Detection

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This gas-chromatographic method for assay of fluoxetine and norfluoxetine in human plasma involves extraction of the drugs and use of a ^{63}Ni electron-capture detector. The linear range of detection is 25 to 800 μg/L for each drug. Overall precision (CV) in the concentration range of 10 to 100 μg/L for both drugs was approximately 10%. Accuracy (relative error) in the same concentration range was approximately +10%. None of the commonly prescribed antidepressants or tranquilizers that we tested interfere with the assay.

Additional Keyphrases: *drug assay* · *antidepressants*

Fluoxetine hydrochloride, or dl-N-methyl-3-phenyl-3-[(α-,α-,α-trifluoro-p-tolyl)oxy]propylamine hydrochloride, a specific neuronal inhibitor of serotonin reuptake, is currently undergoing clinical trials as an antidepressant drug (1). After oral dosing, the drug is well absorbed and rapidly metabolized to its desmethyl metabolite, norfluoxetine, which has selectivity similar to and pharmacological activity equivalent to fluoxetine (2). There is a therapeutic effect when 60 mg of fluoxetine hydrochloride is administered daily as a single dose. With this regimen, steady-state concentrations in plasma range from 103 to 282 μg of fluoxetine and 47 to 181 μg of norfluoxetine per liter. Lemberger et al. (1) previously reported minimal anticholinergic or other side effects at a dose as large as 90 mg.

We describe a sensitive, specific, and precise procedure for determining fluoxetine and norfluoxetine in plasma, in which we use a gas chromatograph with an electron-capture detector.

Materials and Methods

Instrumentation. We used a gas chromatograph equipped with a ^{63}Ni electron-capture detector (Model 5710A; Hewlett-Packard, Avondale, PA 19311) and an automatic sample injector (Hewlett-Packard, Model 7671A). The siliconized looped glass column, 1.2 m by 3 mm (i.d.), was packed with 3% SP 2100 on Supelcoport, 80/100 mesh (Supelco, Inc., Bellefonte, PA 16823).

Reagents. All reagents used were AR grade. The solvents butyl chloride, methanol, benzene, and hexane were distilled in glass (Burdick and Jackson Labs., Muskegon, MI 49442). Pentafluoropropionic anhydride (Pierce Chemical Co., Rockford, IL 61105) was used as purchased.

Solutions used were hydrochloric acid, 10 and 200 mmol/L, and carbonate buffer, pH 9.85, prepared from equal parts of saturated solutions of sodium carbonate (2.7 mol/L) and sodium bicarbonate (1.2 mol/L).

Standards. Fluoxetine hydrochloride, its mono-N-demethylated metabolite (norfluoxetine), and 4,4-bis(p-fluorophenyl)-N-ethylbutylamine hydrochloride (the internal standard) were obtained from Lilly Research Laboratories, Indianapolis, IN 46285. We prepared 10 mg/L standard solutions of fluoxetine and norfluoxetine by dissolving 1.12 or 1.13 mg of their respective hydrochlorides in 100 mL of 10 mmol/L HCl. For the 1 mg/L internal standard solution, we dissolved 1.00 mg of the internal standard in 1 L of 10 mmol/L HCl.

Plasma standards were prepared to contain 0, 20, 40, 60, 80, and 100 μg of both fluoxetine and norfluoxetine per liter.

Chromatographic conditions. The column was conditioned for 1 h at room temperature with helium flowing, then heated at 100 °C for 1 h followed by 250 °C for at least 24 h. The argon/methane carrier gas (95/5 by vol) flow was at 40 mL/min. An oxytrap (Applied Science, State College, PA 16801) was used to remove trace quantities of oxygen in the carrier gas. The injector temperature was 250 °C, the detector temperature 300 °C, and the column temperature 190 °C.

Procedure. All the glassware was siliconized according to the procedure of Fenimore et al. (*3*). Transfer 1.0 mL of plasma (blank, standards, or patient's sample) into a 20-mL disposable centrifuge tube (Corning Glass Works, Corning, NY 14830) and add 0.5 mL of the 1 mg/L internal standard solution. Alkalinize the plasma with 2.0 mL of carbonate buffer and vortex-mix. Add 11 mL of butyl chloride and shake by hand for 3 min. Centrifuge for 5 min and transfer 10 mL of the organic (top) phase to another 20-mL disposable centrifuge tube. Add 5 mL of 0.2 mol/L HCl, shake for 3 min, and centrifuge for 2 min. Aspirate the butyl chloride phase and make the aqueous phase basic with 2 mL of carbonate buffer. Then mix with another 6 mL of butyl chloride and separate the phases as before. Transfer 5 mL of the butyl chloride to a second centrifuge tube. Add one drop of 0.3 mol/L methanolic HCl, vortex-mix, and let stand for 5 min. Evaporate the butyl chloride under N₂ at 40 °C on the N-Evap (Organomation Associates, Inc., Northborough, MA 01532). Dissolve the residue in 0.2 mL of dry benzene, and react the compounds with 50 μL of pentafluoropropionic anhydride in a Temp-Bloc heater (Lab Line Instruments, Melrose Park, IL 60660) at 90 °C for 30 min. Cool the reaction mixture to room temperature and evaporate under N₂. Dissolve the residue in 0.7 mL of hexane and inject 3 μL of this solution onto the chromatographic column for analysis.

Perform a least-squares analysis of the calibration curves for concentration vs peak height ratios of fluoxetine and norfluoxetine to the internal standard. Determine the concentration of fluoxetine and norfluoxetine in the sample by comparing their peak height ratios with the least-squares line.

Results

Figure 1 shows representative chromatograms of the extracts of a human plasma blank, a standard, and a patient's sample. The blank showed no significant peaks that would interfere with the quantitation of fluoxetine and norfluoxetine. Gas chromatographic–mass spectrometric analyses of plasma extracts, when compared with spectra of the known
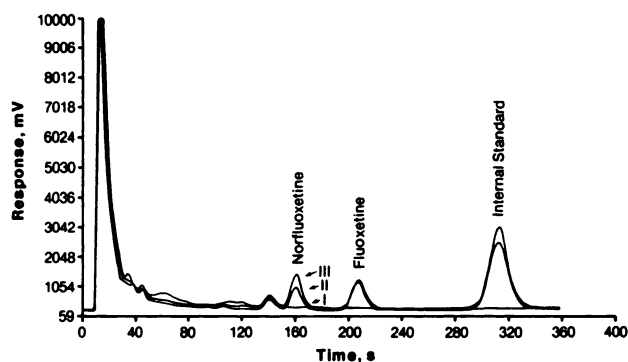

Fig. 1. Gas chromatograms of (*I*) a plasma blank with internal standard; (*II*) plasma standard containing 40 μg of both fluoxetine and norfluoxetine per liter; and (*III*) a patient's plasma after administration of 60 mg of fluoxetine hydrochloride

compounds, confirmed the presence of the monopentafluoropropionate derivatives of fluoxetine (*m/e* 294, 117, 149, 143), norfluoxetine (*m/e* 117, 280, 143, 163), and the internal standard (*m/e* 340, 436, 416, 203), at their respective retention times. Plasma calibration curves containing 20, 40, 60, 80, and 100 μg of both fluoxetine and norfluoxetine per liter, assayed on three separate days, gave a slope of 0.0050 (SD 0.0002), a y-intercept of 0.0130 (SD 0.0055), and a correlation coefficient of 0.995 (SD 0.001) for fluoxetine; and a slope of 0.0061 (SD 0.0002), a y-intercept of −0.0262 (SD 0.0041), and a correlation coefficient of 0.994 (SD 0.003) for norfluoxetine. The average analytical recovery of fluoxetine from plasma is 76% (9% CV); of norfluoxetine, 88% (7% CV). The accuracy and precision of the method is +11% (RE) and 11% (CV) for fluoxetine; +6% (RE) and 9% (CV) for norfluoxetine.

The following antidepressants or tranquilizer drugs at therapeutic concentrations did not interfere in the assay: nortriptyline, imipramine, desimipramine, amitriptyline, doxepin, diazepam, chlordiazepoxide, prochlorperazine, and chlorpromazine.

Discussion

The pentafluoropropionyl derivatization of the secondary and primary amines not only affords high sensitivity of detection but also ensures the specificity of the method. Other halogenated derivatizing reagents were investigated, but the pentafluoropropionic anhydride produced maximum sensitivity consistent with acceptable chromatography, as well as good precision and accuracy. The reaction temperature of 90
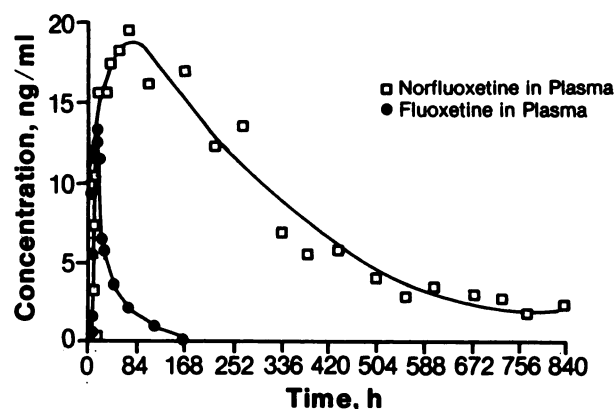

Fig. 2. Mean concentration–time profile of fluoxetine and norfluoxetine in plasma after oral administration of 30 mg of fluoxetine hydrochloride to two subjects

Table 1. Concentration of Fluoxetine and Norfluoxetine in Plasma of Six Patients Orally Administered Fluoxetine Hydrochloride [a]

Consecutive doses, mg/day [b]	Sample time after last dose, h	Concn, µg/L	
		Fluoxetine	Norfluoxetine
Patient one			
20/8, 40/2, 60/3, 40/1	0.5	120	144
60/2, 40/2	0.5	280	279
60/1, 40/1, 20/1, 0/7	0.5	100	305
Patient two			
20/7, 40/2, 60/4, 40/1	0.5	67	44
40/7	0.5	62	62
40/6, 20/2, 40/6, 20/1, 40/6, 20/1	0.5	74	90
Patient three			
20/7, 40/2, 20/1, 40/1, 60/3	8.0	107	72
60/7	8.0	212	145
60/1, 40/1, 60/2, 40/1, 60/2, 40/7, 20/4, 0/1, 20/2	7.0	118	210
Patient four			
20/7, 40/2, 60/4	8.0	91	65
60/7	8.0	201	125
60/22	6.5	282	181
Patient five			
20/8, 40/3, 60/3	1.0	56	61
60/7, 80/1	1.0	103	47
80/13, 60/7	1.0	206	172
Patient six			
20/8, 40/2, 60/5	1.0	224	89
60/2, 80/4, 60/1, 80/1	1.0	324	163
80/13, 40/8, 20/1	1.0	519	355

[a] Single analysis. [b] Quantity of dose/no. of days that dose was given (at 0800 h each day). For example, patient one received 20 mg of fluoxetine HCl for eight days, then 40 mg for two days, etc.

°C and reaction time of 30 min were necessary to obtain quantitative derivatization.

The electron-capture detection accounts for the specificity and sensitivity of the assay. As little as 200 pg of drug injected onto the column yielded a deflection that was 5% of full-scale at an instrument attenuation setting of 16.

The addition of methanolic HCl before the final evaporation and reaction steps increased the recovery of the compounds, as shown by the instrument response. The hydrochloride salts of the fluoxetine and norfluoxetine may permit better recovery of the compounds, because of less adsorption to the glassware.

The extraction of the bases at a buffered pH of 9.85 with butyl chloride gave reproducible recovery and clean separation of the phases. Negligible extraneous reactable material was co-extracted from the plasma, as shown by the low background in the chromatograms (Figure 1). A 3% SP 2250 column at 190 °C also well resolved the compounds and the internal standard, but did not adequately separate from fluoxetine a peak in the background due to the derivatizing reagent.

Plasma samples from patients administered fluoxetine hydrochloride were stored for several months at −20 °C without measurable decomposition of fluoxetine or norfluoxetine.

Because of the equivalent activity of norfluoxetine vs fluoxetine, the method was designed specifically to measure both compounds.

The sensitivity and specificity of the method made it possible to measure both fluoxetine and norfluoxetine in plasma samples from bioavailability and compliance studies. Figure 2 shows the long biological half-life, approximately 70 h, of fluoxetine and the extremely long half-life, approximately 330 h, of norfluoxetine. Table 1 shows the data obtained from several patients after oral dosing with fluoxetine for several days. Evidently the absorption, distribution, and metabolism of the drug are subject dependent.

References

1. Lemberger, L., Rowe, H., Carmichael, R., et al., Pharmacologic effects in man of a specific serotonin-reuptake inhibitor. Science 199, 436 (1978).

2. Fuller, R. W., Snoddy, H. D., Perry, K. W., et al., Importance of duration of drug action in the antagonism of p-chloroamphetamine depletion of brain serotonin-comparison of fluoxetine and chlorimipramine. Biochem. Pharmacol. 27, 193–198 (1978).

3. Fenimore, D. C., Davis, C. M., Whitford, J. H., and Harrington, C. A., Vapor phase silylation of laboratory glassware. Anal. Chem. 48, 2289–2290 (1976).