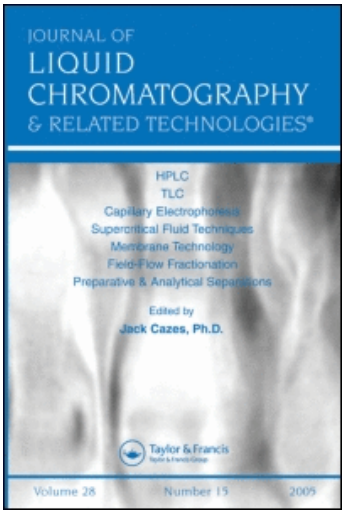

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DETERMINATION OF FLUVOXAMINE AND PAROXETINE IN HUMAN SERUM WITH HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND ULTRAVIOLET DETECTION

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ABSTRACT

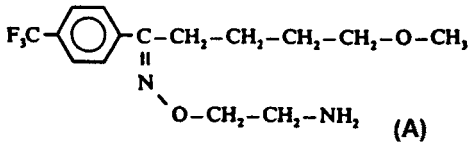
We optimized an isocratic reversed phase high performance liquid chromatography with ultraviolet detection to quantify, simultaneously, the selective serotonin reuptake inhibitors (SSRIs) fluvoxamine and paroxetine in human serum. Fluvoxamine was used as the internal standard for the determination of paroxetine, and paroxetine served as internal standard for the fluvoxamine assay. The method involved a pre-column technique for the on-line liquid-solid extraction with direct injection of serum samples and for their pre-concentration. Automation was achieved by column switching. The chromatographic separation was performed on an Ultrasep ES 100 CN-column with acetonitrile/methanol/phosphate buffer (58/19/23, v/v/v) as mobile phase. A linear relationship (r^2 >

0.99) was noted between the concentrations of fluvoxamine or paroxetine and the detector signal. The lower limits of detection in human serum of fluvoxamine and paroxetine were 5 and 2 µg/L, respectively. The accuracy of the quality control samples deviated by ± 8% with a within-day and between-day precision of less than 12.1%, and 10.5% for fluvoxamine and paroxetine, respectively. The method is presently being applied in our clinic for the routine therapeutic drug monitoring of both SSRIs.

INTRODUCTION

Fluvoxamine (5-methoxy-1-[4-(trifluoromethyl)phenyl]-1 pentanone (*E*)-O-(2-aminoethyl)oxamine; Fig. 1A) and paroxetine ((-) *trans*-4-(p-fluorophenyl)-3-[[3,4-(methylenedioxyl)-phenoxy]methyl]piperidine; Fig. 1B) are selective serotonin reuptake inhibitors (SSRIs) and are used as antidepressants. The chemical structure of fluvoxamine and paroxetine differs from that of classical tricyclic antidepressants, a feature which goes hand in hand with the SSRI's pharmacological profile. Foglia et al.[1] reported that a good response to fluvoxamine is obtained at serum concentrations between 160 to 220 µg/L. However, other investigators did not observe any relationship between serum concentration and clinical efficacy for fluvoxamine[2-4] or paroxetine.[5-6] There are few investigations on the relationship between the serum concentration of the SSRIs and clinical efficacy or the occurrence of adverse events; the number of patients is too narrow to draw definitive conclusions. For paroxetine this could be due to the lack of reliable analytical methods for therapeutic drug monitoring of this drug.

Fluvoxamine was determined by gas chromatography[7,8] or by high performance liquid chromatography with fluorescence[9-11] or ultraviolet detection.[12-15] Paroxetine concentrations in human serum were determined by gas chromatography with nitrogen-specific detection[16] or high performance liquid chromatography (HPLC) with fluorescence detection.[17] These methods are time-consuming due to lengthy sample preparation procedures and derivatization for the fluorescence detection. In 1980 De Jong[18] described a method for the analysis of fluvoxamine using on-line pre-columns that allowed direct sample injection. Recently, Härter et al.[19] presented an automated method for the on-line determination of fluvoxamine using column-switching with HPLC and ultraviolet detection. In the present paper, we describe an HPLC method for direct sample injection to quantify fluvoxamine and paroxetine in human serum.



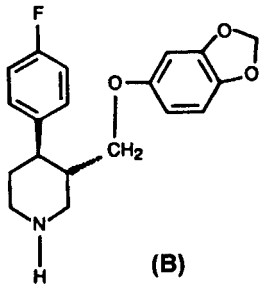


Figure 1. Chemical structures of (A) fluvoxamine and (B) paroxetine.

EXPERIMENTAL

Chemicals and Instrumentation

Fluvoxamine maleate (FGB 87B16A) and paroxetine hydrochloride (BRL 29060) reference standards were provided by Duphar Pharma (Hannover, Germany) and Beecham Pharmaceuticals (Sussex, England), respectively. Acetonitrile, water, methanol (J.T. Baker B.V., Deventer, Holland), and n-heptane (Rathburn, Walkerburn, Scotland) were all of HPLC-grade. All other chemicals were of analytical grade and obtained from Merck (Darmstadt, Germany).

The HPLC system was equipped with two HPLC pumps, a solvent degasser unit SDU 2003 (Bischoff, Leonberg, Germany), and a Waters 717 Autosampler equipped with a cooling module (Millipore-Waters, Eschborn, Germany). The ultraviolet detector SPD-10 A (Shimadzu, Duisburg, Germany) was set at 215 nm. The detector signals were analyzed by Hyperdata Chromsoft computer software with a model 1605 serial chromatography signal interface (Bischoff). The six-port switching valve, Rheodyne model 7010,

operated on an electronic actuator model 732 (both by Bischoff). The cartridge clean-up column (25 x 4 mm ID) filled with Lichrospher RP-18 ADS (25 µm particle size, Merck) was placed between pump B and the switching valve. The cartridge guard column (10 x 4 mm ID) and the analytical column (250 x 4.6 mm ID) contained Ultrasep ES 100 CN (4 µm particle size, Bischoff).

Standard Preparation and Sample Acquisition

Blood from healthy donors was collected into 500 mL bottles (without additives) and stored for 3 h at 20°C; serum was obtained by centrifugation at 2000 g for 15 min. The supernatant (serum) were combined and used immediately for the preparation of standards. The calibration range was 20-1600 µg/L for fluvoxamine and 10-1000 µg/L for paroxetine. Quality control samples contained 120 or 600 µg fluvoxamine/L serum or 40 or 300 µg paroxetine/L serum. The serum standards were prepared by adding the appropriate amount of fluvoxamine or paroxetine stock solutions in water to human serum. The quality control samples were run with each series of analysis. Patients' blood samples were drawn into 10 mL Venoject (Terumo, Leuven, Belgium) blood collecting tubes sealed with brown stoppers and without additives. After 30 min the serum was obtained by centrifugation of the patients' blood samples at 1800 g for 10 min at 4°C. Blood samples from patients on monotherapy with drugs which were used as a co-medication with the SSRIs were collected and prepared accordingly. Fluvoxamine was used as the internal standard for paroxetine and paroxetine as the internal standard for the fluvoxamine determination; 50 µL aqueous standard solution (6 µg/L fluvoxamine or 12 µg/L paroxetine) was added to 950 µL of the patients' serum sample; a second aliquot of 1 mL of the serum was stored without this additive to test putative interferences. The aqueous stock solutions, as well as serum standards, quality controls, and serum samples of patients were kept at -20°C.

On-Line Liquid-Solid Extraction and Chromatography

The mobile phase (solvent A) for the analytical chromatography consisted of 58:19:23 (v/v/v) of acetonitrile:methanol:0.05 M phosphate buffer (pH 6.8). Water:methanol 95:5 (v/v) was used as the mobile phase for the sample clean-up solvent (solvent B). Solvents A and B were filtered through a 0.45 µm Nylon filter (Millipore, Eschborn, Germany). The chromatography was conducted at two different modes, mode A for sample application and mode B for sample analysis (Fig. 2). The chromatographic system was set at mode A (sample application) and 100 µl of the serum sample was injected onto the

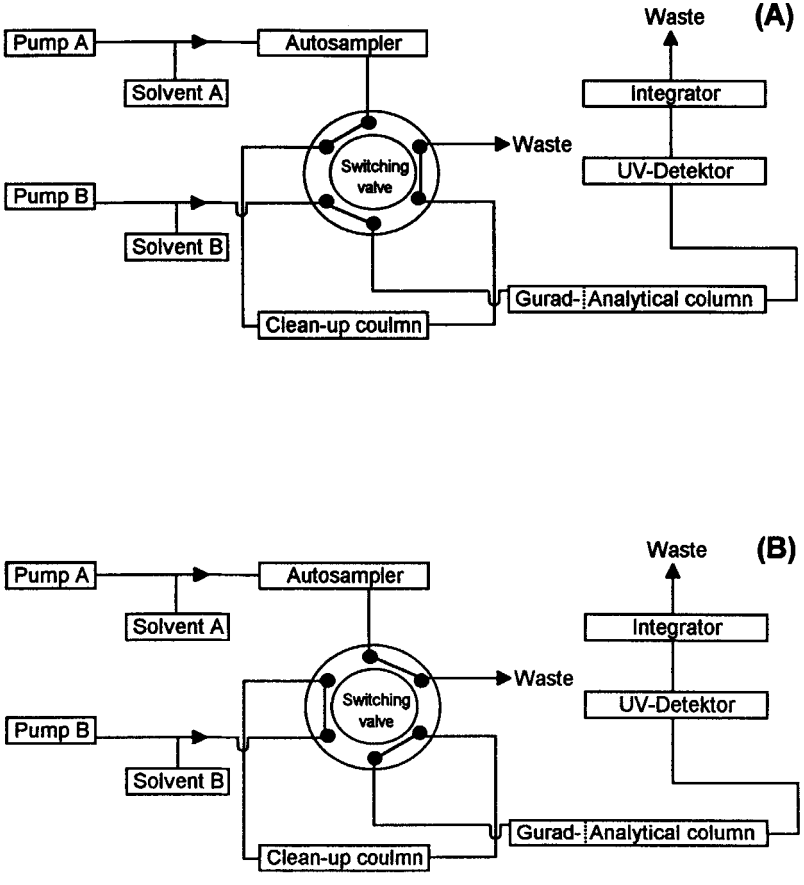


Figure 2. Instrumental arrangement with a six-port switching valve for the direct sample injection for high-performance liquid chromatography with integrated on-line liquid-solid analysis of fluvoxamine and paroxetine by column switching: mode (A) for sample injection and mode B for sample analysis (according to Härter et al. 1992).

clean-up column. The clean-up column was flushed with solvent B for 10 min at a flow rate of 1.5 mL/min. The drugs absorbed onto the clean-up column were then washed onto the analytical column and separated chromatographically with solvent A at a flow rate of 1.5 mL/min by switching the six-port valve to mode B (sample analysis). After 2.5 min the clean-up column was disconnected from the analytical column by back-positioning to

mode A (sample application) and the clean-up column was re-equilibrated with solvent B for 12.5 min for the next sample application. The chromatographic separation was achieved within 15 min.

Liquid-Liquid Extraction

Alternatively, to the on-line liquid-solid extraction, we used the liquid-liquid extraction procedure for weekly routine drug monitoring. This was carried out as follows: 2.0 mL water and 2.0 mL 2 M sodium hydroxide were added to the respective 0.5 mL serum standard, quality controls, or patient sample in 15 mL screw-capped borosilicate glass tubes, and the mixture was vortexed for 10 s. After the addition of 5.0 mL water-saturated n-heptane/isoamylalcohol (99:1) the mixture was gently shaken for 20 min; it was then centrifuged for 10 min at 2800 g and 4°C and the upper layer was aspirated. This procedure was repeated and the two extracts combined. The solvent was evaporated under vacuum to dryness, the residue dissolved in 500 μL of solvent A, and 100 μL of the latter mixture was analyzed following the same procedure for the on-line analysis of serum samples as described above.

Calculation, Recovery, and Interferences

The calibration was performed by linear regression of the peak height ratios of fluvoxamine to the internal standard (paroxetine) and of the peak height ratios of paroxetine to the internal standard (fluvoxamine) versus the respective standard concentration. The best fit was obtained with a weighing factor of $1/\text{concentration}^2$.

The recoveries of the extraction procedures were calculated by comparing the slope of the regression of peak heights obtained by direct injection of the aqueous standards onto the analytical column to those after liquid-solid or liquid-liquid extraction of the serum standards.

Interferences from endogenous serum constituents were accounted for by analysing serum from healthy medication-free subjects. During method validation, serum samples of patients treated with fluvoxamine or paroxetine and with antidepressants, benzodiazepines or neuroleptics were determined twice, namely with and without internal standard. Interferences were then checked by comparing the location of the peaks from co-medicated drugs with that of the respective internal standard (fluvoxamine or paroxetine). Serum samples of patients on monotherapy with other drugs were analysed following

the same protocol to tested putative interferences from the mother compound and that of the metabolites. The retention time of the mother compounds were determined with serum standards of the antidepressants, benzodiazepines or neuroleptics; the retention times of the metabolites with serum samples of patients on monotherapy with that drug.

RESULTS

The average recoveries of on-line liquid-solid and liquid-liquid extraction over the entire calibration range were 93% and 89% for fluvoxamine and 88% and 83% for paroxetine, respectively. The retention times of the mother compound or metabolites of drugs commonly used as add-on therapy and considered as possible interferences are given in Table 1. Occasionally, the analysis of serum sample from patients on monotherapy showed more than one peak in the chromatogram. These peaks corresponded to the spectrum of peaks of an administered drug and its metabolites.

The specificity of the assay was also monitored by checking the chromatograms of drug-free human serum for interfering peaks from endogenous components (Fig. 3). As Table 1 and Fig. 3 imply, there were no interferences from co-medicated drugs or endogenous serum components at the respective retention times of fluvoxamine and paroxetine. Typical chromatograms of serum samples from patients receiving 200 mg fluvoxamine per day (C) and 30 mg paroxetine per day (D) are shown in Fig. 3.

With an injection volume of 100 μL at a signal-to-noise ratio of 3, the detection limits were 5 and 2 μg/L for fluvoxamine and paroxetine, respectively. Sensitivity was improved by a factor of two, when the injection volume was increased to 300 μL. The calibration curves were linear for fluvoxamine (range: 20 to 1600 μg/L; r^2=0.9965 ± 0.0049) and paroxetine (range: 10 to 1000 μg/L; r^2=0.9960 ± 0.0026). The within-day precision determined with concentrations of 120 μg and 600 μg of fluvoxamine/L serum and 40 and 300 μg paroxetine/L serum (each n=10) was 7.5% and 2.1% for fluvoxamine and 7.6% and 3.0% for paroxetine.

The between-day coefficients of variation for the entire concentration range and the quality control samples were within 12.1% and 10.5% for fluvoxamine and paroxetine, respectively (Table 2). The accuracy of the standards and quality control samples deviated within ± 8% at the various concentrations of both SSRIs.

Table 1

Retention Times of Drugs (Mother Compound and Metabolites) Tested for Possible Interferences in the Determination of Serum Fluvoxamine and Paroxetine Concentrations by HPLC

Drug	Retention Time (Min)	
	Mother Compound[a]	Metabolites[b]
Alprazolam	n.o.*	n.o.*
Amitriptyline	8.3	11.7
Carbamazepine	n.o.*	n.o.*
Chlorproxthixene	4.7	8.4; 9.5
Clozapine	5.7	n.o.*
Diazepam	n.o.*	n.o.*
Doxepin	7.1	12.9
Flupentixol	n.o*	n.o*
Fluvoxamine	6.4	
Haloperidol	n.o*	n.o*
Imipramine	8.6	11.8
Levomepromazine	5.9	11.9
Lorazepam	n.o*	n.o*
Maprotiline	13.9	8.2
Melperone	9.1	n.o*
Mianserin	n.o*	9.2
Paroxetine	10.1	
Perazine	n.o*	n.o*
Promethazine	n.o*	n.o*
Thioridazine	8.8	7.7; 8.2; 8.6; 12.3

* n.o., not observed in the chromatogram

[a] The retention time of the mother compound was determined from serum standards.

[b] The retention time of the metabolites was determined from serum samples of patients on monotherapy with this drug.

Using the on-line liquid-solid extraction the sample extraction as well chromatographic analysis was accomplished within 25 min; approximately 100 samples could be determined within two days. The cost of one serum sample analysis, calculated according to Müller et al.,[20] with on-line liquid-solid or liquid-liquid extraction amounted to 31 or 41 US $, respectively.

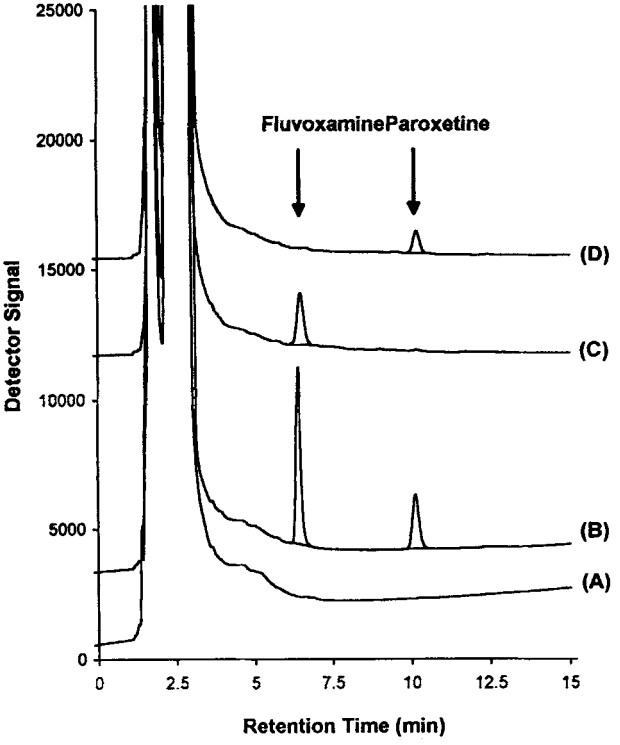


Figure 3. Chromatograms of (A) drug-free human serum, (B) serum standard containing 400 µg/L of fluvoxamine and 100 µg/L paroxetine, sera from patients receiving orally (C) 200 mg fluvoxamine or (D) 30 mg paroxetine per day. The retention times of fluvoxamine and paroxetine are indicated by the arrows and are 6.4 and 10.1 min, respectively.

DISCUSSION

In the present paper, we describe a sensitive, selective and accurate assay for the analysis of fluvoxamine and paroxetine in human serum. This is the first report on the analysis of paroxetine serum concentrations by HPLC and ultraviolet detection. The method involves on-line liquid-solid extraction by column switching for the direct injection of the serum samples, which permits sample preparation and chromatographic analysis within 25 min. A problem encountered during the analysis of serum samples by direct injection was the coating of the clean-up as well as of the analytical columns, attributable to

Table 2

Accuracy and Precision of the HPLC Determined Concentrations of Fluvoxamine and Paroxetine in Serum

Fluvoxamine			Paroxetine		
Concentration (μg/L)	Accuracy* %	CV** %	Concentration (μg/L)	Accuracy* %	CV** %
Calibration Standards					
20	1.2	5.8	10	3.9	8.3
40	0.1	7.7	20	2.6	8.3
100	2.6	5.0	40	2.0	6.0
200	0.8	10.5	100	4.6	8.3
400	6.6	3.5	400	2.8	6.0
1000	6.5	8.4	600	4.8	9.6
1600	3.5	8.6	1000	2.6	9.7
Quality Control Samples					
120	6.0	12.1	40	8.3	10.2
600	4.8	6.9	300	3.9	8.6

* The amount added was taken to be 100%.

** Inter-assay coefficient of variation.

impurities in the serum after clean-up. To maintain sensitivity it was necessary to replace the clean-up and the analytical column after approximately 250 runs. Although the frequency of changing the columns was reduced by using the new Lichrospher ADS clean-up column, recently introduced by Merck, liquid-liquid extraction of the serum samples with n-heptane/isoamylalcohol prior to analysis further improved the column life by a factor of up to three. Both extraction procedures were combined for the routine therapeutic drug monitoring of the SSRIs without changing the chromatographic equipment and conditions, owing to the internal standard technique and the fact that the recovery was similar after on-line liquid-solid and liquid-liquid extraction. This combines the advantages of both extraction procedures: direct serum sample injection and rapid sample analysis with the on-line liquid-solid extraction and cost reduction with the liquid-liquid extraction.

The determination of the SSRIs in serum from patients on co-medication posed no problems since there were no interferences. Testing the interferences of co-administered drugs by analysing the serum samples of patients on monotherapy is more reliable than analysing spiked serum samples, since not all metabolites are purchasable, and patients' serum samples certainly contain the mother compound plus the full spectrum of metabolites.

The lower calibration level of 20 µg/L fluvoxamine and 10 µg/L paroxetine proved to be sufficient. However, sensitivity is easily improved whenever necessary (for anticipated concentrations that are below the lower level of the calibration range) by increasing the injection volume; this requires only multiple injections on the clean-up column prior to the analytical chromatography.

We currently apply this method for the therapeutic drug monitoring of fluvoxamine and paroxetine to investigate the relationship between dose and serum concentration and between the drug's serum concentration and its therapeutic efficacy, as well as drug interactions during comedications.

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