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Short communication

Determination of paroxetine in plasma by high-performance liquid chromatography for bioequivalence studies

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Abstract

A high-performance liquid chromatographic method is described for the determination of paroxetine in human plasma. Dibucaine was used as the internal standard. Paroxetine was isolated by solid phase extraction using a Bond-Elut C_{18} extraction column. Separation was obtained using a reversed-phase column under isocratic conditions with fluorescence detection. The sample volume was 500 μl of plasma. The intra- and inter-assay accuracy and precision, determined as relative error and relative standard deviation, respectively, were less than 10%. The lower limit of quantitation, based on standards with acceptable relative error and relative standard deviation, was 10 ng ml^{-1}. No endogenous compounds were found to interfere. The linearity was assessed in the range 5–100 ng ml^{-1}. Stability of paroxetine during processing (autosampler) and in plasma was checked. This method proved suitable for bioequivalence studies following multiple doses in healthy volunteers. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Paroxetine, (3S-trans)-3-[(1,3-benzodioxol-5-yl-oxy)methyl]-4-(4-fluorophenyl)piperidine, is a potent 5-hydroxytryptamine uptake inhibitor currently used as an antidepressant drug. Its metabolism and pharmacokinetics have been studied extensively in man [1]. It can be determined in biological fluids by either gas chromatography [2] or HPLC with UV [3–5] or fluorescence detection [6,7]. Paroxetine has been extracted from plasma samples using both liquid–liquid extraction [4,6] and solid phase extraction (SPE) [3,7]. These previously reported methods were not automated or fully validated, and there is little information about the stability of paroxetine in plasma. The objective of the present paper was to establish a fully validated HPLC method with a quantitation limit sufficiently low to support pharmacokinetic and bioequivalence paroxetine multiple dose studies. The method reported in this paper is a simple accurate HPLC method to determine the plasma concentration of paroxetine with fluorescence detection using solid phase extraction. This method is fully validated and the limit of quantitation is 10 ng ml^{-1}. Additionally, it provides information about the stability of paroxetine both in plasma and during method processing (autosampler), which is a clear advantage for determining a large number of plasma samples for phar-

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macokinetic and bioequivalence studies in patients and healthy volunteers.

2. Experimental

2.1. Equipment

The HPLC system consisted of a Kontron Instrument comprising a 322 pump, a SFM-25 variable-wavelength fluorescence detector and a 465 autosampler equipped with computer system for acquisition and integration of data (Data System 450 MT2), supplied by Kontron Instruments S.A. (Milan, Italy).

2.2. Reagents

Paroxetine was received from SmithKline Beecham Pharmaceuticals S.A. (Madrid, Spain). Dibucaine was provided by Sigma Chemical Co. (St. Louis, USA). Acetonitrile, methanol and water for HPLC were obtained from Sharlau S.A. (Barcelona, Spain). Bond-Elut C_{18} cartridges were from Varian International (Zug, Switzerland). All other chemicals used were of analytical reagent grade.

2.3. Chromatographic conditions

The mobile phase was acetonitrile–10 m*M* phosphate buffer, pH=3.2 (45:55, v/v), delivered at a flow-rate of 1.0 ml mm^{-1}. Phosphate buffer was prepared with 1.3609 g of potassium dihydrogen phosphate and then pH was adjusted with ortho-phosphoric acid. Separation was accomplished at room temperature on a NovaPak C_{18} column (4 μm, 150×3.9 mm I.D.). The fluorescence detector was set to E_{ex}=295 nm and E_{em}=350 nm.

2.4. Plasma samples

Venous blood samples (5 ml) were withdrawn into the heparinized tubes. Blood samples were centrifuged immediately at 2500 rpm for 10 mm at 4°C and the plasma obtained was stored at −20°C until analysis.

2.5. Extraction procedure

A 0.5 ml aliquot of the sample was mixed with 5 μl of the working internal standard solution (1 mg ml^{-1}) and was applied to a 1 ml BondElut C_{18} extraction column which had been previously activated by washing successively once with 2.0 ml of methanol and once with 2.0 ml of 10 m*M* phosphate buffer, pH=3.0. The sample was passed slowly through the column under mild vacuum (100 mm Hg). The column was then washed with 2.0 ml of water and drained completely after the wash. An aliquot of 0.25 ml of acetonitrile containing perchloric acid 35% (99:1, v/v) was applied to each column. The liquid was allowed to pass through the column under gravity. A 15 μl aliquot of the eluate was injected directly into the HPLC system.

2.6. Drug standards

Working stock solutions of paroxetine and dibucaine were prepared in acetonitrile at a concentration of 1 mg ml^{-1} and were stored at −20°C. To test the suitability of the system, a chromatographic control was prepared by dilution of the stock solutions with acetonitrile to a final concentration of 100 ng ml^{-1} of paroxetine and 10 μg ml^{-1} of dibucaine. Plasma standards were prepared from the stock solutions using drug-free plasma from healthy volunteers (5, 10, 25, 50, 75 and 100 ng ml^{-1}). Internal standard was added as a 1 mg ml^{-1} solution in acetonitrile. Both stock solutions were prepared at the beginning of the study and were stored at −20°C for 3 months. Quality control (QC) samples were prepared in the same way at concentrations of 20, 40 and 80 ng ml^{-1}, were divided into 1 ml portions and were stored at −20°C. Six QC samples (duplicates of three concentrations) were placed at random among volunteer samples in each analytical batch.

2.7. Analytical variables

Absolute extraction recoveries of paroxetine from human plasma were estimated using standard samples at concentrations ranging from 5 to 100 ng ml^{-1} of paroxetine and a constant amount (10 μg ml^{-1}) of the internal standard by comparing the peak heights from processed plasma standard samples to those

from a calibration curve prepared from analytes in acetonitrile:perchloric acid 35% (99:1, v/v). Plasma standard samples (5, 10, 50, 75 and 100 ng ml^{-1}) were analyzed in sextuplicate (intra-assay) and triplicate (inter-assay) on three separate days during method validation. Revalidation was assessed from the duplicate standard curves made on days when volunteers' samples were analyzed. The peak height ratio of paroxetine to internal standard was plotted against the concentration of paroxetine. Linearity of standard curves, intra- and inter-assay precision and accuracy were determined from these data. The limits of detection (LOD) and quantitation (LOQ) of paroxetine were determined from the peak and the standard deviation of the noise level, S_N. The LOD and LOQ were defined as the sample concentration of paroxetine resulting in peak heights of 3 and 10 times S_N, respectively. The stability of paroxetine in the autosampler was checked after 6, 12 and 24 h at room temperature, for three different standards in plasma (20, 40 and 80 ng ml^{-1}). No statistically significant difference was observed between $t=0$ and $t=24$ h for any concentrations ($p=0.8355$).

2.8. Application

The assay was applied to pharmacokinetic studies for bioequivalence assays of paroxetine following 20 mg daily multiple doses. Blood samples were taken according to a pharmacokinetic design for bioavailability studies after multiple doses. Samples from healthy volunteers were taken immediately prior to the dose (Days 0, 10 and 11) and immediately prior to and at 2, 4, 5, 6, 12 and 18 h after drug ingestion (Day 12), and immediately prior to and at 2, 4, 5, 6, 12, 18 and 24 h after drug ingestion (Day 13). Comparison of peak height ratios from the unknown samples with those from the calibration curve permitted quantitation of the assayed samples. Concentrations of paroxetine measured in plasma samples obtained from 28 healthy volunteers given a multiple oral 20 mg dose of paroxetine (Seroxat®) from SmithKline Beecham Pharmaceuticals are shown in Fig. 1.

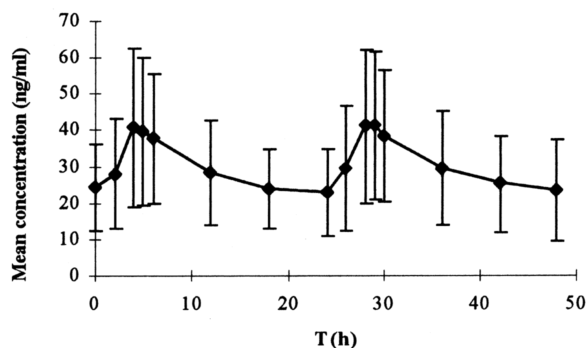

Fig. 1. Mean (±SD) plasma concentration–time profiles for paroxetine obtained from 28 healthy volunteers given a multiple oral 20 mg dose of paroxetine (Seroxat®).

3. Results and discussion

Fig. 2 illustrates a representative chromatogram of blank volunteer plasma (a), a chromatogram of blank volunteer plasma spiked with 10 μg ml^{-1} of internal standard (b), a chromatogram of blank volunteer plasma spiked with 75 ng ml^{-1} of paroxetine and 10 μg ml^{-1} of internal standard (c), a plasma sample from a volunteer at Cmin (25.9 ng ml^{-1}) (d), and a plasma sample from a volunteer at Cmax (59.3 ng ml^{-1}) 5 h after drug administration (e). Drug-free pooled human plasma yielded clean chromatograms with no significant interfering peaks. Retention times of paroxetine and dibucaine were 4.72±0.31 and 7.18±0.53 mm, respectively ($n=33$).

The solid-phase extraction reported here is simple and rapid to carry out and does not require an evaporation step. The eluate can be injected directly into the HPLC. Several substances were tried as internal standard (buspirone, 1-(2-pyrimidinyl)piperazine, imipramine, clomipramine, trimipramine, protriptyline, maprotiline, yohimbine and fluoxetine) but none showed the desired resolution. Dibucaine shows very good resolution but it must be used at high concentration.

The specificity of the method was confirmed by the analysis of a variety of different blank plasma samples from healthy volunteers ($n=10$), none of which yielded any endogenous interference [9].

Calibration curves were generated on three different days (validation) and duplicate standard curves were generated daily to determine the sample concentrations (revalidation). Linearity of the standard curves was found in the range 5–100 ng ml^{-1} and

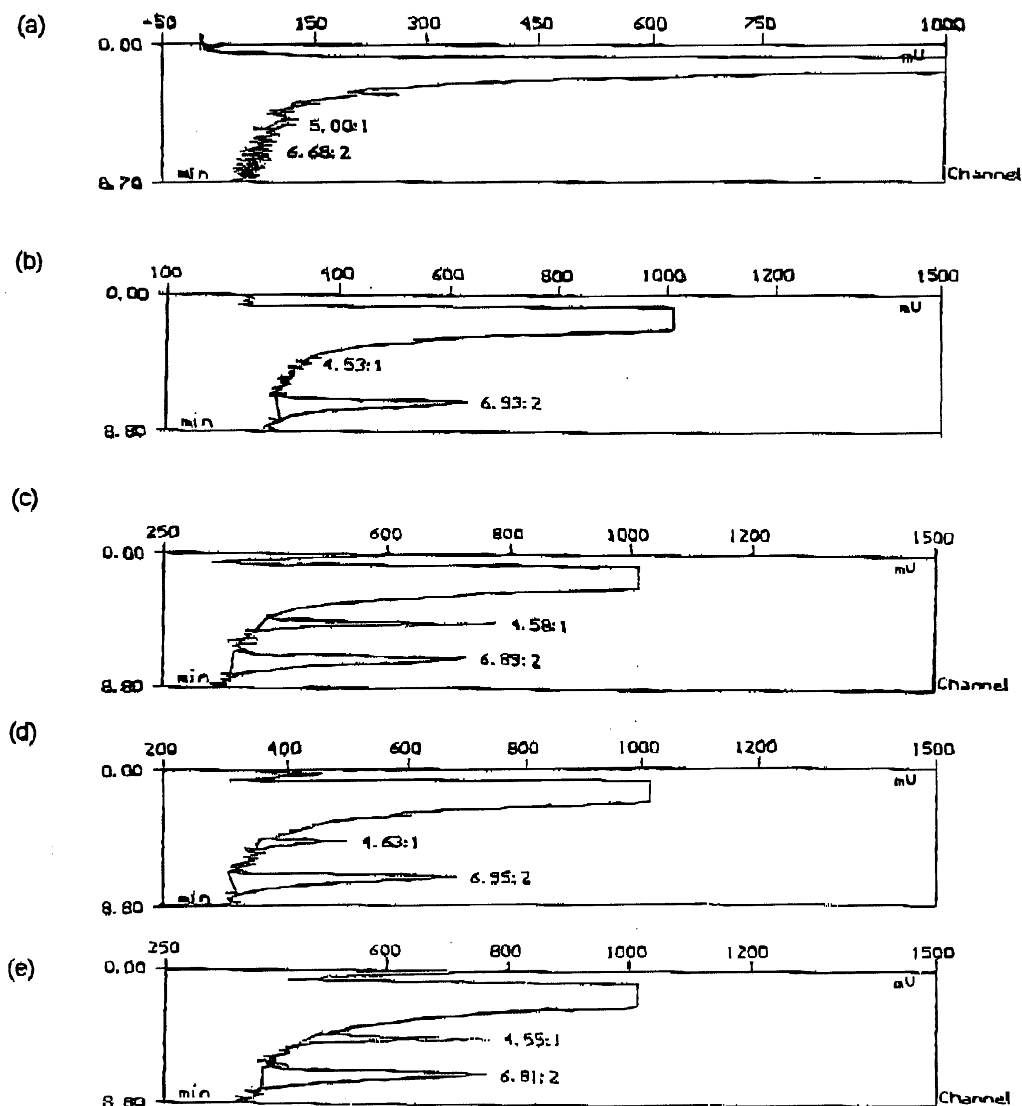

Fig. 2. (a) Chromatogram of blank volunteer plasma. (b) Chromatogram of a blank volunteer plasma spiked with 10 μg ml^{-1} of internal standard. (c) Chromatogram of blank volunteer plasma spiked with 75 ng ml^{-1} of paroxetine and 10 μg ml^{-1} of internal standard (peak 1= paroxetine, peak 2=internal standard). (d) Chromatogram of a plasma sample from a volunteer at *C*min (25.9 ng ml^{-1}). (e) Chromatogram of a plasma sample from a volunteer at *C*max (59.3 ng ml^{-1}) 5 h after drug administration.

was confirmed statistically (*F* test for lack of fit) [10]. The correlation coefficient (r^2), the slope and the *y*-intercept for the straight lines were $0.9949\pm1.6\times10^{-3}$, $0.0107\pm9.0\times10^{-4}$ and $0.0421\pm4.4\times10^{-3}$ respectively for the validation and $0.9940\pm2.1\times10^{-3}$, $0.0122\pm1.1\times10^{-3}$ and $0.0436\pm3.3\times10^{-3}$, respectively for the revalidation. The limit of detection for paroxetine was less than 5 ng ml^{-1}, whereas the quantitative limit was 10 ng ml^{-1}. As the limit associated with reliable quantitation is the LOQ, the relative standard deviation and the relative error that are deemed acceptable may vary but usually range from 10 to 20% [8].

Precision of the assay, calculated as the relative standard deviation for intra-assay variability, was less than 10% at any concentration studied. Inter-

Table 1
Validation of analytical method

Nominal concentration added ($ng\ ml^{-1}$)	Concentration found ($ng\ ml^{-1}$)	RSD[a] (%)	Accuracy (%)
Intra-assay (n=6)			
10	10.1	6.5	101.1
25	25.1	9.1	100.2
50	49.8	4.1	99.7
75	73.7	5.0	98.4
100	100.4	3.8	100.4
Inter-assay (n=12)			
10	10.4	18.4	104.0
25	25.5	7.3	102.2
50	49.7	5.1	99.4
75	74.2	4.1	99.0
100	100.2	2.9	100.2
Revalidation (n=62)			
10	9.8	17.1	98.2
25	24.5	8.2	97.8
50	51.5	6.0	103.0
75	75.2	4.2	100.2
100	98.8	3.0	102.9

[a] RSD=Relative Standard Deviation.

assay precision ranged from 2.9% for 100 $ng\ ml^{-1}$ to 18.4% for 10 $ng\ ml^{-1}$. For revalidation, precision ranged from 3.0% for 100 $ng\ ml^{-1}$ to 17.1% for 10 $ng\ ml^{-1}$. Accuracy was within the range 90–110% in all concentrations studied.

Extraction recovery of paroxetine from human plasma was estimated using triplicate standard samples at concentrations ranging from 5 to 100 $ng\ ml^{-1}$ by comparing the peak heights from processed plasma standard samples to those from a calibration curve prepared from analytes in acetonitrile–perchloric acid 35% (99:1, v/v). In the range of calibration standards, the mean recovery of paroxetine was 88.3±7.9% in plasma and one-way ANOVA demonstrated that there were no statistically significant differences between concentrations studied. The mean recovery for internal standard at a concentration of 10 $pg\ ml^{-1}$ was 89.1% (n=6). The results of method validation and revalidation are summarized in Table 1.

The ruggedness of the method was studied from QC samples over time, under different analysts, different lots of reagents and different columns [11]. Knowledge of the stability of the drug in test material is a prerequisite for obtaining valuable data [12]. The QC results show that paroxetine was stable in plasma samples at −20°C for at least 4 months. Results from QC are shown in Table 2. All the batches met QC acceptance criteria [8] and gave values for accuracy of better than 100±10% and precision of less than 10%.

This analytical method was applied to the quanti-

Table 2
Results of quality control

Nominal concentration added ($ng\ ml^{-1}$)	Concentration found ($ng\ ml^{-1}$)	RSD[a] (%)	Accuracy (%)	n
20	19.2	9.8	96.2	68
40	37.8	8.7	94.6	68
80	75.3	6.7	94.1	68

[a] RSD=Relative Standard Deviation.

tation of plasma paroxetine concentrations in more than 1000 samples from healthy volunteers in bioequivalence studies. Extensive information about the applicability of the method was obtained from validation and revalidation data and the stability of paroxetine in plasma and during processing was established.

This paper describes a sensitive, specific, rapid and robust reversed-phase HPLC method with fluorescence detection which has proven suitable for use in pharmacokinetic measurement of paroxetine in plasma following multiple dose administration.

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