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Journal of Chromatography B, 755 (2001) 279–285

JOURNAL OF CHROMATOGRAPHY B

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Rapid determination of citalopram in human plasma by high-performance liquid chromatography

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Received 26 September 2000; received in revised form 11 January 2001; accepted 6 February 2001

Abstract

A rapid high-performance liquid chromatographic method for the quantitation of citalopram in human plasma is presented. The sample preparation involved liquid–liquid extraction of citalopram with hexane–isoamyl alcohol (98:2 v/v) and back-extraction of the drug to 0.02 *M* hydrochloric acid. Liquid chromatography was performed on a cyano column (45×4.6 mm, 5 μm particles), the mobile phase consisted of an acetonitrile–phosphate buffer, pH 6.0 (50:50, v/v). The run time was 2.6 min. The fluorimetric detector was set at an excitation wavelength of 236 nm and an emission wavelength of 306 nm. Verapamil was used as the internal standard. The limit of quantitation was 0.96 ng/ml using 1 ml of plasma. Within- and between-day precision expressed by relative standard deviation was less than 7% and inaccuracy did not exceed 6%. The assay was applied to the analysis of samples from a pharmacokinetic study.

Keywords: Citalopram

1. Introduction

Citalopram is an antidepressant drug, which selectively potentiates serotonin neurotransmission by inhibiting serotonin reuptake. It offers similar therapeutic efficacy and a more favourable tolerability profile than the tricyclic antidepressants [1]. Citalopram is a racemic mixture, its pharmacological effect resides mainly in the *S*-(+) enantiomer and, to a lesser degree, in the *S*-(+)-desmethylcitalopram [2]. Nevertheless, for bioequivalence studies the recent guidance [3] recommends measurement of the racemate using an achiral assay.

Several gas chromatographic or gas chromatographic–mass spectrometric [4,5] and high-performance liquid chromatographic (HPLC) [6–14] methods have been described for determination of citalopram in plasma. The limit of quantitation (LOQ) of HPLC assays ranges between 0.8 and 8 ng/ml. Sample preparation is performed mainly by liquid–liquid extraction [5–9,11], some methods employ solid-phase extraction [12–14] or column switching [10]. Citalopram is detected by fluorimetric [6,7,10,12,13] or spectrophotometric [8,9,11,14] detection.

The need for more effective use of analytical resources have awakened an interest in fast analysis in recent years [15]. High-speed chromatography requires short columns; they should be packed with small particles for high efficiency separations, but for

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00121-9

many applications moderate efficiencies obtained with 5 μm particles are sufficient. The columns used for separation of citalopram from its metabolites and endogenous interfering peaks are 10–30 cm long and the analyses times range between 7 and 35 min. The aim of this study was to develop a high-speed HPLC method for citalopram determination in plasma fast enough to analyse several hundreds samples from a pharmacokinetic study in 1 working day.

2. Experimental

2.1. Chemicals

Citalopram hydrobromide and *N*-desmethylcitalopram hydrogenoxalate were obtained from Léčiva (Prague, Czech Republic). Verapamil hydrochloride was purchased in the local pharmacy in the form of injections (2.5 mg/ml in water; Aliud Pharma, Laichingen, Germany). Hexane (for spectroscopy, Uvasol) and potassium dihydrogenphosphate were manufactured by Merck (Darmstadt, Germany). All other reagents and chemicals were of either HPLC or analytical grade. Isoamyl alcohol was a product of Fluka (Buchs, Switzerland). Acetonitrile was manufactured by Riedel-de Haën (Seelze, Germany). Sodium hydroxide, hydrochloric and orthophosphoric acid were purchased from Lachema (Brno, Czech Republic).

2.2. Apparatus

All HPLC instruments were obtained from Thermo Separation Products (Riviera Beach, FL, USA). The system consisted of a membrane degasser, a ConstaMetric 4100 pump, an AS 3000 automatic sample injector, an FL2000 fluorimetric detector and a datastation with PC1000 software, version 2.5. The separation was performed on a 45×4.6 mm I.D. column filled with Ultrasphere Cyano stationary phase, particle size 5 μm (Beckman, Berkeley, CA, USA).

The mobile phase consisted of acetonitrile–30 m*M* potassium dihydrogenphosphate buffer (50:50, v/v), pH of the buffer was adjusted to 6.0 with potassium hydroxide. The flow-rate was 2 ml/min at 35°C. The fluorimetric detector settings were as follows: excitation wavelength 236 nm, emission wavelength 306 nm and time constant 2 s.

2.3. Standards

Stock solutions of citalopram hydrobromide were made by dissolving approximately 9 mg in 25 ml of methanol (factor 0.80037 for conversion to the free base). Separate solutions were prepared for the calibration standards and quality control samples. Further solutions were obtained by serial dilutions of stock solutions with methanol. These solutions were added to drug-free plasma in volumes not exceeding 1% of the plasma volume.

The solution containing 6.25 ng/ml of the verapamil hydrochloride was prepared by serial dilution of the injection solution with water. A 10-μl volume of this solution was added to 1 ml of the plasma as the internal standard.

The methanolic solutions were stored at −18°C, while aqueous ones were kept at +4°C. All solutions were protected from light.

2.4. Preparation of the sample

The samples were stored in the freezer at −18°C and allowed to thaw at room temperature before processing. A 10-μl volume of the internal standard solution (62.5 ng of verapamil hydrochloride) was added to 1 ml of plasma and the tube was briefly shaken. A 100-μl volume of 1 *M* NaOH was added and the tube was shaken again. Then the mixture was vortex-mixed with 4 ml of hexane–isoamyl alcohol (98:2, v/v) for 120 s at 2000 rpm. The tube was centrifuged for 3 min at 2600 *g* and the upper organic phase was transferred to another tube. A 100-μl volume of 0.02 *M* HCl was added and the tube was vortex-mixed again (2 min, 2000 rpm). The phases were separated by centrifugation for 3 min at 2600 *g* and approximately 80 μl of the lower aqueous phase was transferred to the polypropylene autosampler vial. A 25-μl volume of the 0.1 *M* KH_2PO_4 (pH 7.0 adjusted with NaOH) was added to neutralize the acid, the mixture was briefly shaken and 30 μl was injected into the chromatographic system.

2.5. Calibration curves

The calibration curve was constructed in the range 0.955–81.60 ng/ml to encompass the expected concentrations in measured samples. The calibration curves were obtained by weighted linear regression (weighting factor $1/y^2$): the ratio of citalopram peak area to verapamil peak area was plotted vs. the ratio of citalopram concentration to that of internal standard in ng/ml. The suitability of the calibration model was confirmed by back-calculating the concentrations of the calibration standards.

2.6. Limit of quantitation

LOQ was defined as the lowest concentration at which the precision expressed by relative standard deviation (RSD) is better than 20% and accuracy expressed by relative difference of the measured and true value is also lower than 20%. Six identical samples were analysed for the determination of LOQ.

2.7. Application to plasma samples

Citalopram (40 mg single dose) was orally administered to 26 healthy volunteers. Venous blood samples were collected in heparinized tubes before and at 0.75, 1.5, 2.25, 3, 4, 5, 6.5, 9, 14, 24, 36, 60, 84, 108 and 132 h after drug administration. Within 30 min after blood collection, blood plasma was separated by centrifuging (1500 *g*) for 12 min. The samples were stored at −18°C until analysis.

3. Results and discussion

3.1. Chromatography

Citalopram can be separated from its metabolites, matrix constituents and internal standard both on octadecylsilica (ODS) and cyanopropylsilica (CN) stationary phases. The separation of citalopram and *N*-desmethylcitalopram can be easily accomplished at pH≥6. At these pH values, the column efficiency was poor on the ODS column and thus the CN stationary phase was selected. The percentage of acetonitrile in the mobile phase was selected to minimize the analytical time while maintaining baseline resolution of citalopram–desmethylcitalopram and citalopram–verapamil peak pairs (R_s>2). The column efficiency calculated on citalopram peak was approximately 2000 theoretical plates which corresponds to 45 000 plates/m. This relatively low value was influenced by several factors: the high flow-rate 2 ml/min, extracolumn peak broadening which is significant for the column with dimensions 45×4.6 mm and the time constant 2 s. The flow-rate and column dimensions were selected to minimize the analysis time, the time constant was set to higher value in order to lower baseline noise and thus increase limit of detection.

It must be emphasized that the conditions selected for minimal analysis time are not identical to conditions for maximum column efficiency. However, the criterion for good separation is based on resolution and analysis time only, not on the column efficiency.

The metabolites of citalopram other than *N*-desmethylcitalopram are not commercially available. In order to develop a method for separation of citalopram from the metabolites a urine extract from a volunteer dosed with citalopram was used, because the concentrations of the metabolites in urine are higher than in plasma. The optimized separation of citalopram, *N*-desmethylcitalopram, internal standard and other, non-identified metabolite (probably didesmethylcitalopram) is shown in Fig. 1. The separation of these peaks takes only 2.6 min. An additional benefit of the fast separation is rapid column equilibration when the composition of the mobile phase is changed. The optimization of the separation proceeds very quickly and can be finished in a few hours.

The method selectivity was demonstrated on six blank plasma samples obtained from healthy volunteers: the chromatograms were found to be free of interfering peaks. The typical chromatogram of blank plasma is shown in Fig. 2 and the chromatogram of a plasma sample from a volunteer 60 h after administration of 40 mg of citalopram is shown in Fig. 3. The concentration of citalopram was 6.08 ng/ml.

It was not required to also measure levels of citalopram metabolites in this study. For bioequivalence studies, measurement of only the parent drug released from the dosage form, rather than the

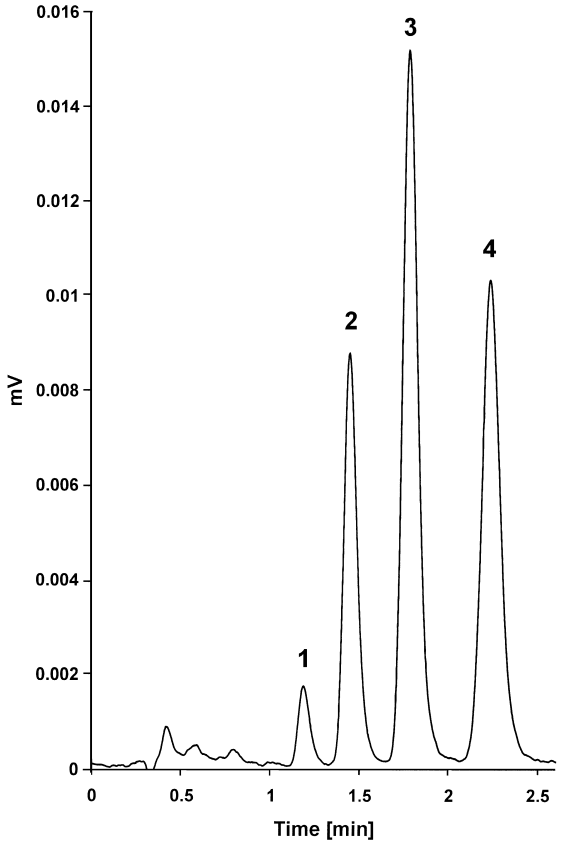

Fig. 1. Chromatogram of a mixture (urine extract obtained from a volunteer 5 days after administration of 20 mg of citalopram) of a non-identified metabolite of citalopram (1, probably didesmethylcitalopram), *N*-desmethylcitalopram (2), citalopram (3) and verapamil (4, internal standard).

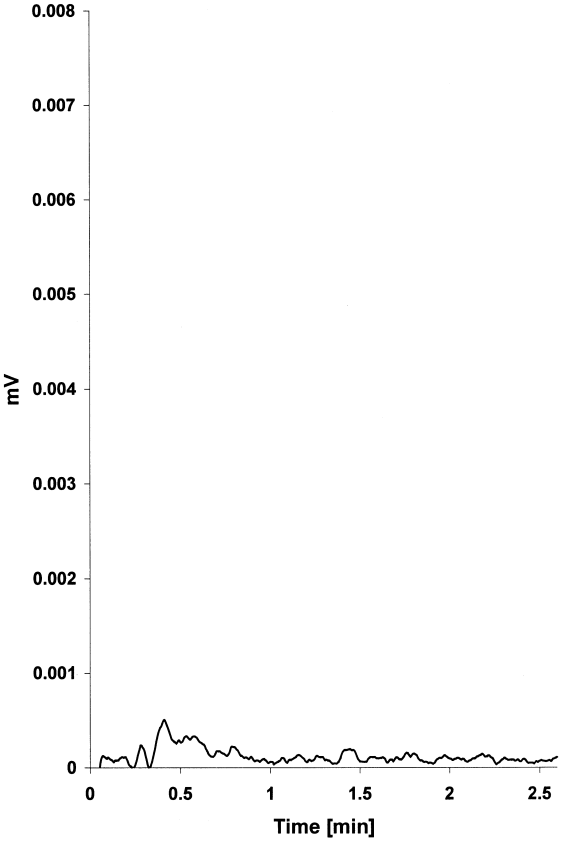

Fig. 2. Typical chromatogram of drug-free human plasma.

metabolite, is generally recommended [3]. However, from the chromatograms it is evident that the metabolites are well separated and their determination should be possible with the current assay.

3.2. Linearity and limit of quantitation

The calibration curves were linear in the studied range. The calibration curve equation is $y=bx+c$, where y represents the citalopram peak area to verapamil peak area ratio and x represents the ratio of citalopram concentration to that of internal standard. The mean equation (curve coefficients±standard deviation) of the calibration curve ($n=7$) obtained from six points was $y=0.663(\pm0.062)x+0.00041(\pm0.00090)$ (correlation coefficient $r=0.9997$).

The LOQ was 0.96 ng/ml. The precision, characterised by the RSD, was 8.7% and accuracy, defined as the deviation between the true and the measured value expressed in percents, was −7.9% at this concentration ($n=6$).

3.2.1. Intra-assay precision

Intra-assay precision of the method is illustrated in Table 1. It was estimated by assaying the quality control samples (low, medium and high concentration) six times in the same analytical run. The precision was better than 5% and the bias did not exceed 6% at all levels.

3.2.2. Inter-assay precision and accuracy

Inter-assay precision and accuracy was evaluated by processing a set of calibration and quality control

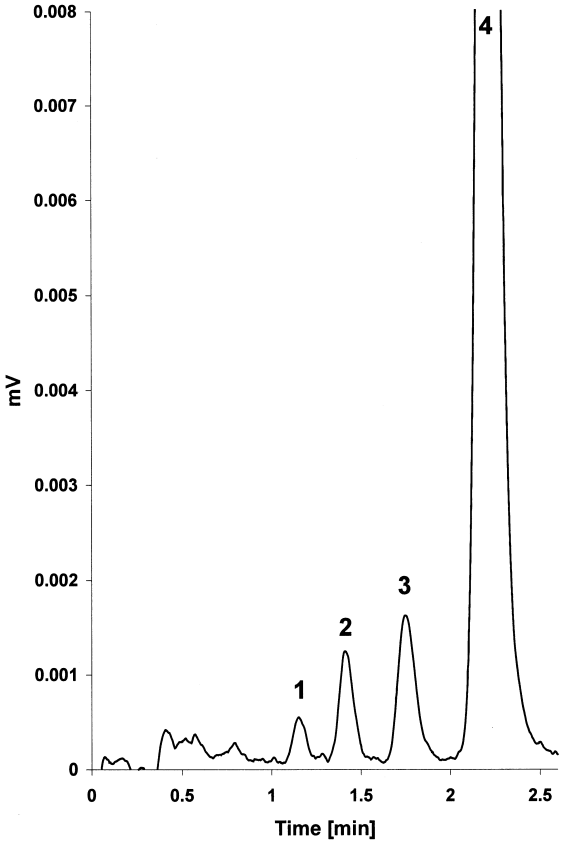

Fig. 3. Chromatogram of a plasma sample from a volunteer 60 h after administration of 40 mg of citalopram. The measured concentration of citalopram was 6.08 ng/ml.

Table 1
Intra-assay precision and accuracy

n	Concentration (ng/ml)		Bias (%)	RSD (%)
	Added	Measured		
6	1.84	1.89	2.5	4.4
6	8.89	8.78	−1.2	2.6
6	69.3	65.4	−6.0	1.5

Table 2
Inter-day precision and accuracy

n	Concentration (ng/ml)		Bias (%)	RSD (%)
	Added	Measured		
6	1.84	1.75	−4.9	7.0
6	8.89	8.82	−0.7	5.7
6	69.3	67.0	−3.3	5.3

samples (three levels analysed twice, results averaged for statistical evaluation) on six separate runs. The samples were prepared in advance and stored at −18°C. The respective data are given in Table 2. The precision was at most 7% and the bias was better than 5% at all levels.

3.2.3. Stability study

3.2.3.1. Freeze and thaw stability. Stock solutions of a low and high concentration sample were prepared. The solutions were stored at −18°C and subjected to three thaw and freeze cycles. During each cycle triplicate 1-ml aliquots were processed, analysed and the results averaged. The results are shown in Table 3. The concentrations found are well within the allowed limit ±15% of nominal concentration, indicating no significant substance loss during repeated thawing and freezing.

3.2.3.2. Processed sample stability. Two sets of samples with a low and a high concentration of citalopram were analysed and left in the autosampler at ambient temperature. The samples were analysed using a freshly prepared calibration samples 3 days later. The results are presented in Table 3. The processed samples are stable at room temperature for 3 days.

3.2.3.3. Long term stability. Two sets of samples (low and high concentration of citalopram) were stored in the freezer at −18°C for 7 weeks. The samples were then analysed using freshly prepared calibration samples. The results are within the acceptable ±15% limit of the nominal concentration (see Table 3). The samples are stable at −18°C for the studied period.

3.3. Application to biological samples

The proposed method was applied to the determination of citalopram in plasma samples from a bioequivalence study. The plasma samples were periodically collected up to 132 h after oral administration of a 40 mg single dose to 26 healthy male volunteers. Fig. 4 shows the mean plasma concentrations of citalopram. The plasma level of citalopram reached its maximum 5 h after the administration and thereafter the plasma level

Table 3
Stability of the samples

Freeze and thaw stability							
Sample concentration (ng/ml)	*n*	Cycle 1		Cycle 2		Cycle 3	
		Measured	Bias (%)	Measured	Bias (%)	Measured	Bias (%)
3.68	3	3.24	−12.0	3.50	−4.9	3.61	−2.0
69.3	3	64.1	−7.6	72.2	4.1	65.7	−5.1
Processed sample stability							
Sample		Concentration found (ng/ml)	RSD (%)	Bias (%)			
New	6	1.89	4.4	2.6			
3 days old	6	1.66	4.0	−9.8			
New	6	65.4	1.5	−5.6			
3 days old	6	67.7	0.5	−2.3			
Long-term stability							
Concentration (ng/ml)							
3.68	6	3.47	5.6	−5.8			
69.3	6	71.5	5.3	3.1			

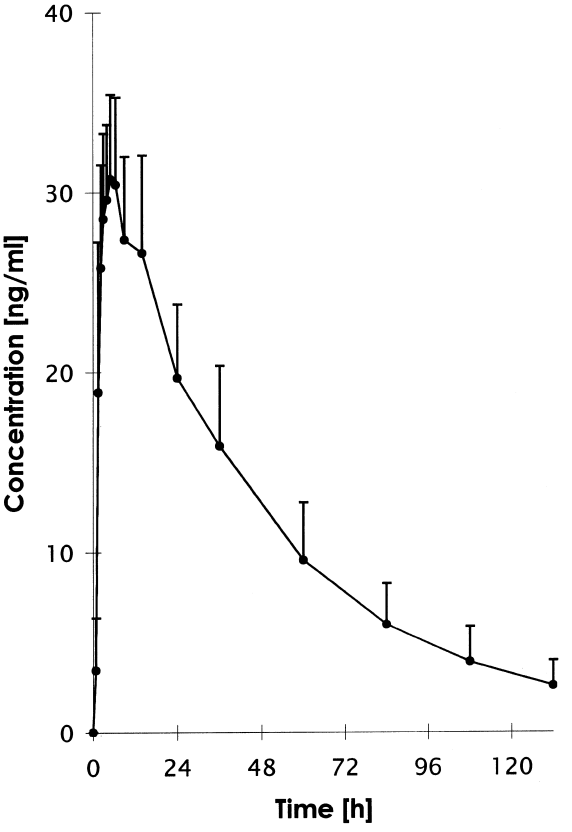

Fig. 4. Mean plasma concentrations (+SD) of citalopram after administration of a 40 mg single oral dose (26 healthy volunteers).

declined with an elimination half-time of ca. 35 h. These values agree with previously published reports [1]. The extrapolated fraction of the area under the curve (AUC) from last measurable concentration to infinity accounted only for 8% of AUC from 0 to infinity which indicates a suitability of the analytical method for pharmacokinetic studies.

4. Conclusions

The validated method allows determination of citalopram in the 0.96–82 ng/ml range. The assay is rapid, the analysis time is only 2.6 min. About 200 samples can be prepared and analysed in 1 working day. The precision and accuracy of the method are well within the limits required for bioequivalence study methods. The LOQ 0.96 ng/ml permits the use of the method for pharmacokinetic studies.

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