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Simultaneous determination of fluoxetine, citalopram, paroxetine, venlafaxine in plasma by high performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–MS/ESI)

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Abstract

Fluoxetine, citalopram, paroxetine and venlafaxine have been widely used in the treatment of depression. However, no study has been conducted to determine the four drugs simultaneously by high performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–MS/ESI).

Objective: To establish a new, rapid and sensitive HPLC–MS/ESI method for simultaneous determination and screening in human plasma of the four most commonly prescribed nontricyclic antidepressants: fluoxetine, citalopram, paroxetine and venlafaxine.

Methods: The analytes in plasma were extracted by solid-phase-extraction column after samples had been alkalinized. The HPLC separation of the analytes was performed on a MACHEREY-NAGEL C_{18} (250 mm × 4.6 mm, 5 μm, Germany) column, using water (formic acid 0.6‰, ammonium acetate: 30 mmol/l)–acetonitrile (35:65, v/v) as mobile phase, with a flow-rate of 0.85 ml/min. The compounds were ionized in the electrospray ionization (ESI) ion source of the mass spectrometer and were detected in the selected ion recording (SIR) mode.

Results: The calibration curves were linear in the 5.0–1000.0 ng/ml range for all compounds, all of them with coefficients of determination above 0.9900. The average extraction recoveries for all the four analytes were above 73.2%. The methodology recoveries were higher than 95.0%. The limits of detection (LODs) were 0.5, 0.3, 0.3 and 0.1 ng/ml for fluoxetine, citalopram, paroxetine and venlafaxine, respectively. The intra- and inter-day variation coefficients were less than 15.0%.

Conclusion: The method is accurate, sensitive and simple for routine therapeutic drug monitoring (TDM) as well as toxicologic screening, and for the study of the pharmacokinetics and metabolism of the four drugs.

Keywords: Fluoxetine; Citalopram; Paroxetine; Venlafaxine; HPLC–MS

1. Introduction

Fluoxetine, citalopram, paroxetine (selective serotonin reuptake inhibitors, SSRIs) and venlafaxine (serotonin-noradrenergic reuptake inhibitor, SNaRI [1]) have been widely used in the treatment of depression. It is reported that this new generation of antidepressants have similar efficacy and better-tolerated adverse effects [2,3] when compared with classical antidepressants, and are less likely to produce high cardiac toxicity of tricyclic antidepressant drugs [4–6] and secure drug–drug interactions of nonselective monoamine oxidase inhibitors (MAOIs) [5]. The advantages of the therapeutic profile of the four drugs [4,5,14] have led to increasing use of them in treatment of depressed patients. However, even if these new compounds have fewer undesirable side effects, they can lead to major intoxications [7–10]. Moreover, many

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treated depressed patients did not respond to their treatment and the compliance was known to be low [5], in conditions of hepatic and renal impairment, poor metabolisers of CYP450 isoenzymes and comedication with inhibitors or inducers of these isoenzymes [11,12], routine therapeutic drug monitoring (TDM) seemed to be useful [4,5,13,14], furthermore, TDM was a good solution to noncompliance. So, the development of a rapid and specific method allowing the screening and the determination of these new antidepressant drugs in biologic fluids could be of great interest either in therapeutic drug monitoring use [13,14] or in toxicologic screening in the case of suicide involving one of these compounds [15].

At present, determinations of some of these drugs have been established by the use of HPLC–UV spectrometry [16,17], or gas chromatography with nitrogen phosphorus electron capture, or mass-spectrometry [17–19] and more recently on micellar electrokinetic capillary chromatograph [20]. However, none of these methods made the quick quantification and identification of these drugs in a single run. Although method to simultaneously determine some of these drugs has been described [4,5], that was not of use since it produced too long a chromatographic run, and had low sensitivity, it appeared that no assay existed for simultaneous determination of the four drugs using HPLC–MS.

The recent trend in TDM and in quick analysis of intoxication is having been developed to find methods to determine several drugs simultaneously which are expedient, quick and of low cost. Thus we designed the method using HPLC–MS/ESI for the simultaneous determination of fluoxetine, citalopram, paroxetine and venlafaxine in human plasma (using fluvoxamine as internal standard) in order to explore the application of simultaneous determination of several drugs by HPLC–MS. The assay described here requires small mobile phase and sample volume, short chromatographic run and is sensitive, specific and fully validated.

2. Experiment

2.1. Equipments and reagents

A system of HPLC (Waters 2690, American)–MS with a Micro mass ZQ mass spectrometer (Wythenshawe, Manchester, UK) with mass-selective detector equipped with an electrospray ionization (ESI) ion source was used. COMPAQ Deskpro Workstation and MassLynx^{TM} 3.5 software were utilized. Auto Science® AP-01P Vacuum Pump (Automatic Science Instrument Co., Ltd., TianJin) and Waters Oasis^{TM} Extraction Cartridges (HLB1cc, LOT NO: WO195J2) (Waters Corporation, Milford, MA, USA) were utilized.

Fluoxetine (>99.8%), fluvoxamine (>99.8%) and citalopram (>99.8%) were purchased from Sigma (Steinheim, Germany), paroxetine (>99.73%) and venlafaxine (>99.0%) were generously donated by Huahai Pharmaceutic Co., Ltd. (Zhe-Jiang, P.R. China).

HPLC grade reagents (methanol, acetonitrile, formic acid and ammonium acetate) were obtained from Tedia Company Inc. (Fairfield, USA). Other AR grade reagents (acetic acid, sodium hydroxide) were obtained from Chemical Reagent Factory of Hunan (Changsha, Hunan, China). Control human plasma was obtained from the Blood Center of Shanghai (Shanghai, China) or from the volunteers.

2.2. Standard solutions

The primary stock solutions of fluoxetine (375.7 μg/ml), citalopram (1.02 mg/ml), paroxetine (1.89 mg/ml), venlafaxine (1.732 mg/ml) and fluvoxamine (117.2 μg/ml, I.S.) were prepared by dissolving appropriate amount of pure substance in methanol. Working solutions were obtained by diluting the stock solutions with distilled water. All the standard solutions were stored at −20 °C.

Routine daily calibration curves were prepared in drug-free serum. Appropriate volumes of working solutions and drug-free human plasma were added to each test tube. Final concentrations were 5, 10, 30, 70,200, 400 and 1000 ng/ml. Quality control samples that were run in each assay, were prepared in the same way, and final concentrations were 10, 70 and 400 ng/ml.

2.3. Chromatographic conditions

The analytes were separated on a MACHEREY-NAGEL C_{18} (4.6 mm × 250 mm, 5 μm, Germany) column with column temperature 40 °C. The mobile phase was water (formic acid: 0.6‰, NH_4Ac: 30 mmol/l)–acetonitrile (35:65) and was filtered using 0.45 μm filters in a Millipore solvent filtration apparatus and was never recirculated. The flow-rate was 0.85 ml/min, and the postcolumn splitting ratio was 3:1.

2.4. MS/ESI detection conditions

The compounds were ionized in the positive electrospray ionization ion source (ESI^+) of the mass-spectrometer. Selected ion recording (SIR) mode was used for quantitation by the protonated molecular ions of each analyte. The detection conditions were as follows: capillary voltage, 3.00 kV; cone voltage, 18.68 V for fluoxetine, 36.51 V for citalopram, 37.97 V for paroxetine, 19.66 V for venlafaxine and 21.61 V for fluvoxamine (I.S.); extractor voltage. 3.54 V for fluoxetine, 1.59 V for citalopram, 3.05 V for paroxetine, 2.81 V for venlafaxine, and 1.83 V for fluvoxamine (I.S.); source temperature, 100 °C; desolvation temperature, 225 °C; cone gas flow, 100 l/h, desolvation gas flow, 300 l/h.

2.5. Sample preparation

AUTO SCIENCE® AP-01P VACUUM PUMP (Automatic Science Instrument Co., Ltd., TianJin) was

used. WATERS OASIS™ EXTRACTION CARTRIDGES (HLB1cc, LOT NO: WO195J2) (Waters Corporation, Milford, MA, USA) were preconditioned by two aspirations with 2 ml methanol, and two with 2 ml distilled water. The sample (0.5 ml) was spiked with 50 μl of internal standard 501.8 ng/ml and 100 μl of sodium hydroxide 0.1 mol/l. The mixture was shaken for 1 min, then added to each column and aspirated slowly. The columns were washed with distilled water containing 5% methanol and air-dried for about 3 min. After any adherent liquid was cleaned from the outlet tube of the column, the analytes were eluted with 2 ml 2% HAc-methanol. The flow-rate was manually maintained at ≤1 ml/min. The eluent was evaporated under a stream of nitrogen at 40 °C. The residue was reconstituted in 100 μl mobile phase. Twenty microliter-solution was injected for analysis through auto sampling injector.

2.6. Validation of the method

The extraction recoveries were determined at three concentration levels by comparing the analyte peak areas obtained from the quality control samples ($n = 5$) after extraction to those obtained from the corresponding unextracted reference standards prepared at the same concentrations. The methodology recoveries were measured as the percentage difference from theoretical according to the equation:

$$\text{Methodology recovery (\%)} = \left(\frac{\text{concentration}_{\text{measured}}}{\text{concentration}_{\text{theoretical}}}\right) 100$$

Precision assays were carried out five times using three different concentrations (Table 1) on the same day and over 5 different days.

Calibration was performed by a least-squares linear regression of the peak-area ratios of the drugs to the I.S. versus the respective standard concentration.

3. Results and discussions

3.1. HPLC–MS/ESI

The column temperature was 40 °C in order to reduce the pressure of the column and improve resolution. The post-column splitting ratio was fixed (3:1), and the best condition of MS was 0.20–0.25 ml/min, so the flow-rate was set at 0.85 ml/min.

Fluvoxamine is rarely prescribed and comedicated as an antidepressant with other drugs. It is often used to probe CYP1A2 in drug metabolism in laboratories. As is known to all, this drug is a potent inhibitor of CYP1A2, CYP2C19, CYP2D6 and a moderate inhibitor of CYP2C9. So it drastically increases concentrations and effects of many operable drugs, such as caffeine, theocin, imipramine, tacrine, clozapine, tizanidine, omeprazole, etc. Potentially hazardous drug interactions may result from inhibition of hepatic CYP enzymes' activity by fluvoxamine.

The experiment was performed in changsha city of Hunan province, and fluvoxamine has not went on the market in Hunan province yet. So the samples of fluvoxamine could not be collected in the province. The complete medicinal history of those patients had been investigated, and they had not taken fluvoxamine, either. So this drug was employed as the internal standard.

However, some patients may still take this drug as an antidepressant in some places of the world, or coadminister this drug with other SSRIs, this is just the limitations of this method. In these cases, the method is not suitable for determining these SSRIs any more.

In this case, the method can be used with some modifications, and then people can choose other substance (for example *N*-methylparoxetine) as internal standard to simultaneously determine these five drugs (fluvoxamine, fluoxetine, citalopram, paroxetine and venlafaxine) by HPLC–MS/ESI.

From the chromatograms of fluvoxamine, we can see that it behaved well, so if it is possible to collect samples of

Table 1
Mean extraction recoveries (±S.D.), methodology recoveries (±S.D.) and R.S.D.

Added drug	Concentration (ng/ml)	Mean extraction recoveries (%, $n = 5$)		Mean methodology recoveries (%, $n = 5$)		
		Mean recoveries ± S.D.	R.S.D. (%)	Found ± S.D.	Recoveries (%)	R.S.D. (%)
Fluoxetine	10	73 ± 8	10	11 ± 1	106	9
	70	88 ± 3	3	69 ± 7	99	10
	400	88 ± 3	4	392 ± 35	98	9
Citalopram	10	96 ± 6	6	10 ± 1	101	10
	70	96 ± 2	2	71 ± 6	102	8
	400	94 ± 1	1	402 ± 29	100	7
Paroxetine	10	86 ± 1	1	11 ± 1	107	9
	70	92 ± 2	2	71 ± 7	102	10
	400	91 ± 3	4	381 ± 25	95	7
Venlafaxine	10	87 ± 3	4	10 ± 1	104	10
	70	90 ± 3	3	68 ± 7	98	10
	400	95 ± 1	1	402 ± 32	101	8

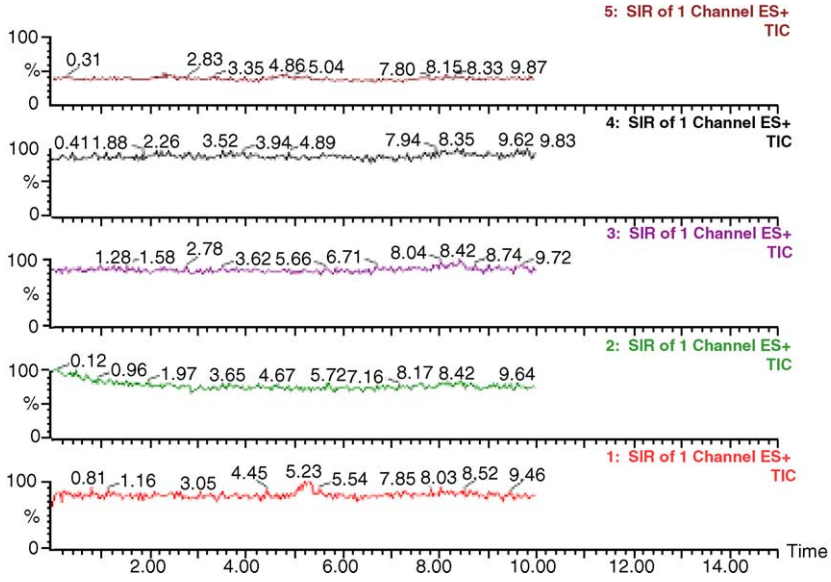

Fig. 1. Chromatograms of control human plasma. Channel 1, venlafaxine; channel 2, paroxetine; channel 3, citalopram; channel 4, fluoxetine; channel 5, fluvoxamine (I.S.).

fluvoxamine, people can choose other substance (for example *N*-methylparoxetine) as internal standard to simultaneously determine these five drugs (fluvoxamine, fluoxetine, citalopram, paroxetine and venlafaxine) by HPLC–MS/ESI.

The HPLC–MS/ESI in the SIR mode provided a highly selective method for the determination of fluoxetine, citalopram, paroxetine, venlafaxine and fluvoxamine (I.S.). The retention times of fluoxetine, citalopram, paroxetine, venlafaxine and fluvoxamine (I.S.) were approximately 8.17, 6.37, 6.87, 6.42 and 7.39 min, respectively. Compared with the published methods (the chromatographic run was as long as 13–18 min [4,5]), the chromatographic run of this method was shortened, the complete elution was obtained in less than 9.0 min. The chromatograms of control human plasma, standards in control human plasma and patient samples were shown in Figs. 1–3, respectively. The protonated molecules of the standards of ESI^+ mass spectrum (SIR) in control human plasma were identified at *m/z* 310 for $[\text{fluoxetine} + H]^+$, 325.1 for $[\text{citalopram} + H]^+$, 330.1 for $[\text{paroxetine} + H]^+$, 278.1 for $[\text{venlafaxine} + H]^+$ and 319.0 for $[\text{fluvoxamine} + H]^+$ (I.S.).

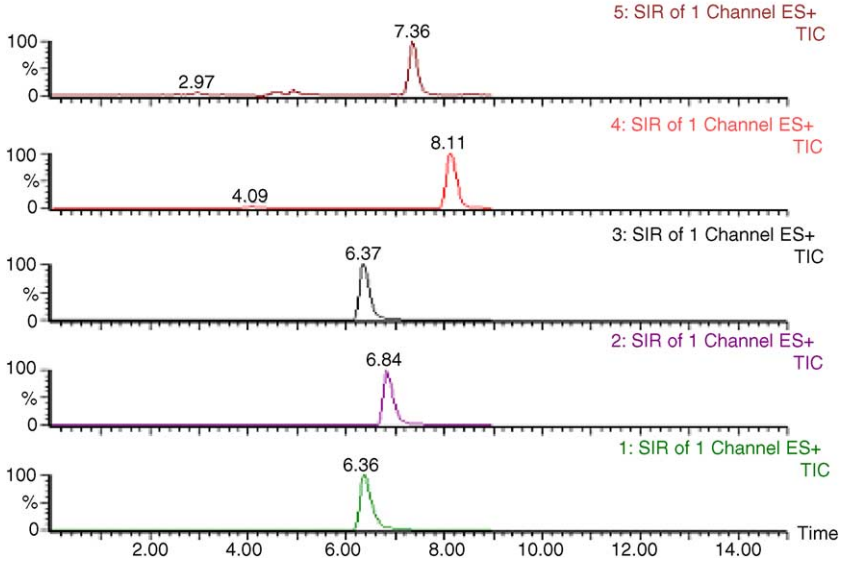

Fig. 2. Chromatograms of standards and I.S. in control human plasma. Channel 1, venlafaxine; channel 2, paroxetine; channel 3, citalopram; channel 4, fluoxetine; channel 5, fluvoxamine (I.S.). The concentrations of each compound were 83.3 ng/ml.

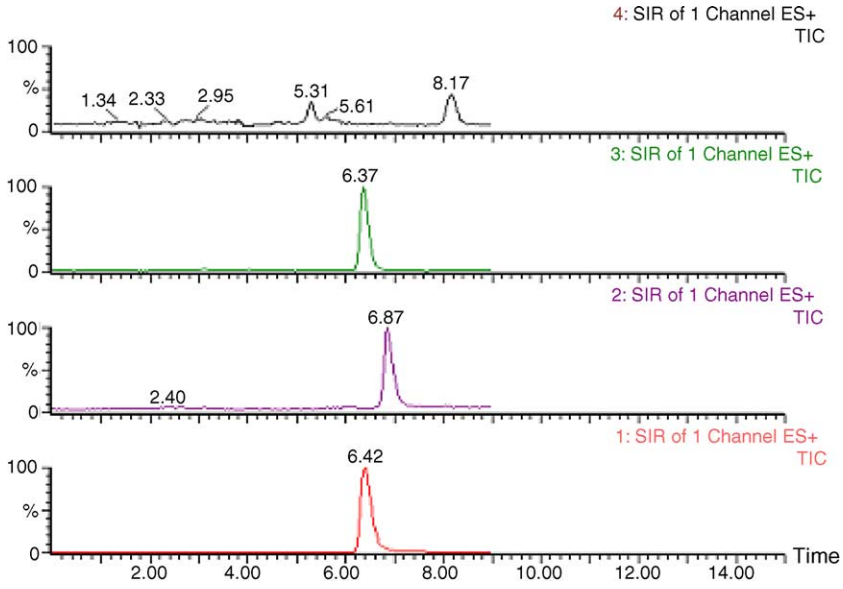

Fig. 3. Chromatograms of patient samples. Channel 1, venlafaxine, the represented patient is no. 6. Channel 2, paroxetine, the represented patient is no. 3. Channel 3, citalopram, the represented patient is no. 2. Channel 4, fluoxetine, the represented patient is no. 1.

The liquid–liquid-extraction procedure used in the published methods was relatively of low sensitivity and recovery [4,5,16–19]. Instead of that, the solid-phase-extraction procedure used in this study was relatively simple, efficient and of high sensitivity, reduced the long time of agitation in liquid–liquid extraction and the extraction recovery of this method was relatively high, allowing for analyzing samples in batches.

3.2. Linearity

The concentration range was 5.0–1000.0 ng/ml for all the four compounds. The area ratio of each analyte to I.S. was well related to the concentration. The related coefficients of fluoxetine, citalopram, paroxetine, and venlafaxine were 0.9995, 0.9964, 0.9994 and 0.9981, respectively.

The sample injection needle was washed by methanol before each injection, this could prevent sample-to-sample contamination; The carryover between injected samples was investigated: the areas of the standard solutions for each drug were very stable, and there were no impurity peaks or contamination in each chromatogram; In addition, the human-plasma negative controls were routinely used to ensure that contamination had not occurred in the extraction process; The concentrations of the quality control samples used have been showed in Section 2.2, they are 10, 70 and 400 ng/ml;The reasons for choosing these concentrations as the quality control samples were that they represented the low, middle and high concentrations of the therapeutic concentrations which can be seen from Table 2 or from the references [12,26,27]; during each extraction, two quality control samples were employed which is required in our country.

Table 2
Intra- and inter-day precision

Added drug	Concentration (ng/ml)	Intra-day precision (%, $n = 5$)			Inter-day precision (%, $n = 5$)		
		Found ± S.D.	R.S.D. (%)	RE* (%)	Found ± S.D.	R.S.D. (%)	RE* (%)
Fluoxetine	10	11 ± 1	9	10	11 ± 2	14	6
	70	69 ± 7	10	1	71 ± 8	11	2
	400	392 ± 35	9	2	390 ± 31	8	3
Citalopram	10	10 ± 1	10	1	11 ± 2	14	6
	70	71 ± 6	8	1	69 ± 7	9	2
	400	402 ± 29	7	1	403 ± 35	9	1
Paroxetine	10	11 ± 1	9	7	11 ± 1	10	5
	70	71 ± 7	10	2	71 ± 6	8	1
	400	381 ± 25	7	6	377 ± 28	7	6
Venlafaxine	10	10 ± 1	10	4	11 ± 1	11	5
	70	68 ± 7	10	3	69 ± 7	10	1
	400	402 ± 32	8	1	402 ± 35	9	1

RE*, Relative error.

Table 3
Plasma concentrations in depressed patients

Patient no.	Patient gender	Drug administered	Drug dose (mg/day)	Concentration (ng/ml)	
				C_{min} [a]	C_{max} [b]
1	Female	Fluoxetine	20	41.14	–*
2	Female	Citalopram	40	170.02	290.40
3	Female	Paroxetine	20	5.58	8.15
4	Female	Paroxetine	20	–	5.95
5	Male	Venlafaxine	20	408.05	–*
6	Female	Venlafaxine	20	48.67	–*

–*, The samples had not collected because some depressant patients did not cooperate. –, The concentration was below the linear range of the paroxetine curve (5 ng/ml).
[a] The values were determined before their administration of the drugs when they had got their steady plasma-drug concentrations.
[b] The values were determined after the administration of the drugs according to the T_{max} of each drug when they had got their steady plasma-drug concentrations.

3.3. Accuracy and precision

The mean extraction recoveries (means ± S.D.), methodology recoveries (means ± S.D.), intra- and inter-day precision for the four analytes are shown in Tables 1 and 2. The average extraction recoveries for all the four analytes were above 73%. The average methodology recoveries were higher than 95%. The intra- and inter-day R.S.D. were less than 15%.

The precision assays were carried out on five continuous days before and after the analyzing of the samples. The samples were stored at −70 °C in ultra cold freezer during the time that they were not being analyzed.

3.4. Sensitivity

Five quality control plasma samples were utilized to determine the sensitivity. The limits of detection (LODs) were 0.5 ng/ml for fluoxetine, 0.3 ng/ml for citalopram, 0.3 ng/ml for paroxetine and 0.1 ng/ml for venlafaxine, respectively ($S/N = 3$).

3.5. Stability

Standard solutions of fluoxetine (0.38 mg/ml), citalopram (1.02 mg/ml), paroxetine (1.89 mg/ml) and venlafaxine (1.73 mg/ml) in methanol were stored at −20 °C for 3 months. All analytes appeared to be stable as the publications [21–25] described.

Stored at 20 °C for 24 h, the stability of fluoxetine, citalopram, paroxetine and venlafaxine in samples were as good as evidenced in previous publications [22,24,25].

3.6. Analysis of patient plasma

Plasma samples were obtained from six depressed patients. The specimen collection from human subjects was approved by the Ethical Committee of XiangYa Second Hospital of Central South University. Because some patients did not cooperate when collecting their samples, it was difficult to collect both C_{max} and C_{min} samples, so only their C_{max} samples were collected; I had been collecting samples for about 1 month, during that time at the hospital, there was only one male patient, the majority of the patients were female, and were prescribed paroxetine and venlafaxine, so their duplicate experiments (two patients) were performed for paroxetine and venlafaxine but only one each for citalopram and fluoxetine.

The administered drugs and their concentrations determined by the method are shown in Table 3. These drugs underlie an extensive metabolism with high interindividual variability, whereby cytochrome P450 (CYP) isoenzymes play a major role. Therefore, the blood concentrations are highly variable between individuals. So TDM is necessary to acquire the best treatment effect.

4. Conclusions

Compared with other methods, HPLC–MS/ESI improved the specificity and sensitivity, shortened the analytical time of the samples. The solid-phase-extraction technique simplified the preparation of the samples. The main aim of the study was to establish a HPLC–MS method that was suitable for simultaneous determination of fluoxetine, citalopram, paroxetine and venlafaxine in plasma of patients undergoing antidepressant treatment. The method described here has been found to be specific and accurate in application which is also suitable for the determination of each of the drugs studied. To the best of our knowledge, this method meets the request of the present pharmacokinetic studies of the four drugs. Thus, the method also suits for the study of pharmacokinetics and metabolism [26,27] and for the analysis of samples in batches when undertaking TDM.

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