Journal of Pharmaceutical and Biomedical Analysis 70 (2012) 557–562

Contents lists available at SciVerse ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Short communication

A fully validated method for the simultaneous determination of 11 antidepressant drugs in whole blood by gas chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 15 February 2012
Received in revised form 3 May 2012
Accepted 6 May 2012
Available online 15 May 2012

Keywords:
Antidepressants
Gas chromatography
Mass spectrometry
Whole blood

ABSTRACT

Antidepressant drugs are widely used for the treatment of depression and other psychiatric disorders and as a result they are involved in numerous clinical and forensic cases. The aim of this study was the development, optimization and validation of a simple, specific and sensitive GC/MS method for the simultaneous determination of 11 antidepressant drugs and 4 of their metabolites (amitriptyline, citalopram, clomipramine, fluoxetine, fluvoxamine, maprotiline, desmethyl-maprotiline, mirtazapine, desmethyl-mirtazapine, nortriptyline, paroxetine, sertraline, desmethyl-sertraline, venlafaxine and desmethyl-venlafaxine) in whole blood. The combination of solid-phase extraction with derivatization using heptafluorobutyric anhydride efficiently reduced matrix effect and improved sensitivity of the method. In this assay, protriptyline was used as internal standard. Absolute recovery values for all analytes were ranged from 79.2 to 102.6%. LODs and LOQs were found to be between 0.30–1.50 μg/L and 1.00–5.00 μg/L, respectively. The calibration curves were linear ($R^2 \geq 0.990$) within the range of 5.00–1000 μg/L for all analytes. Accuracy expressed as the % E_r was found to be between −12.3 and 12.2%. Precision expressed as the % RSD was found to be less than 11.7% for all antidepressants. The developed method proved to be suitable for routine work and it was used to successfully analyze more than 2500 clinical and forensic blood samples.

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1. Introduction

Antidepressant (ATD) drugs are widely used in clinical practice for the treatment of different types of depression and other psychiatric disorders like phobias and anxiety [1–3]. These drugs are frequently prescribed in numerous combinations, leading to more possible drug–drug interactions, while dosage is largely based on trial-and-error [4,5]. Involvement of ATD drugs is common in many forensic cases such as driving under the influence of drugs, cases of violent crime, cases of drug-facilitated sexual assault and other cases of sudden or violent deaths [6,7]. Therapeutic drug monitoring (TDM) of ATDs is of critical importance in situations where patients under treatment do not respond as expected, in cases of drug interactions with co-medication either with inhibitors or inducers of CYP450, non-compliance, and in patients at risk, such as elderly, poor metabolisers or with liver impairment [4]. In such cases, monitoring of ATDs is of a great importance for therapy optimization and dose adjustment, considering also the inter-individual variability of drug metabolism [8].

Several chromatographic methods have been developed for the determination of ATDs in biological fluids separately or with their metabolites [9,10] and in combinations [1–4,6,7,11–15]. All the above assays are mainly based on gas chromatography (GC) [1,4,9,12–15] or liquid chromatography (LC) [2,3,5–7,10,11]. These already published methods are used either for only screening purposes or suffer from disadvantages like that not all common ATDs are included in the same method, too long chromatographic runs or low sensitivity. The aim of our study was the development, optimization and validation of a simple, sensitive and specific method, based on GC/MS for the simultaneous identification and quantification of the 11 most commonly prescribed ATDs and 4 active metabolites of them (amitriptyline, citalopram, clomipramine, fluoxetine, fluvoxamine, maprotiline, desmethyl-maprotiline, mirtazapine, desmethyl-mirtazapine, nortriptyline, paroxetine, sertraline, desmethyl-sertraline, venlafaxine and desmethyl-venlafaxine) in whole blood samples, that are normally available in forensic cases. This method was successfully applied in routine framework of our laboratory and proved to be useful for the toxicological analysis during the investigation of different clinical and forensic cases.

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http://dx.doi.org/10.1016/j.jpba.2012.05.007

2. Materials and methods

2.1. Chemicals and reagents

Amitriptyline, citalopram and nortriptyline were offered from Lundbeck (Copenhagen-Valby, Denmark). Clomipramine, maprotiline, and desmethyl-maprotiline were offered from Novartis (Basel, Switzerland). Fluoxetine and fluvoxamine were offered from Lilly (Windlesham, UK) and Solvay Pharma (Brussels, Belgium), respectively. Mirtazapine and desmethyl-mirtazapine were offered from Organon (Rijnberg, Netherlands). Paroxetine was offered from Glaxo Smith Kline (Brentford, UK). Sertraline hydrochloride and desmethyl-sertraline maleate were offered from Pfizer (Groton, CT, USA). Venlafaxine and desmethyl-venlafaxine were offered from Wyeth (New York, NY, USA). Internal standard (IS), protriptyline hydrochloride, was purchased from Sigma–Aldrich (St. Louis, MO, USA). All solvents were HPLC grade (methanol, isopropanol and ethyl acetate) and were purchased from Merck (Darmstadt, Germany). Heptafluorobutyric anhydride (HFBA) 99% was purchased from Fluka (Steinheim, Germany). For solid-phase extraction (SPE), Bond Elut LRC Certify (sorbent mass 130 mg, column volume 10 mL, Varian) columns were used. Human blood samples were obtained from healthy donors, after their informed consent, and were collected in test tubes containing anti-coagulant (EDTA K_3). Blood was screened before its use for the presence of drugs by GC/MS.

2.2. Calibration and quality control samples

Standard stock solutions (1.00 mg/mL) of the analytes of interest and protriptyline (IS) were prepared separately in methanol. The stock solution of IS was further diluted in methanol (1:5000, v/v) to give a working solution of 0.20 μg/mL. Working solutions of the mixture of the ATDs and their metabolites at three concentrations (0.025, 0.25 and 5.00 μg/mL) were prepared in methanol from the corresponding standard stock solutions of each compound and were used for the preparation of LOQ, calibration and quality control (QC) samples. Spiked blood samples were prepared by spiking 1.0 mL of blank blood with the appropriate volumes of the corresponding working solutions of the mixture of the ATDs. The six calibrators contained all the drugs at the following concentrations: 5.00, 10.0, 30.0, 100, 400 and 1000 μg/L and QC samples were prepared at three concentration levels (15.0, 150 and 800 μg/L).

2.3. GC/MS analysis

GC/MS analysis was performed on a Shimadzu 17A GC equipped with a Shimadzu AOC-20i autosampler system and interfaced with a Shimadzu QP 5000 mass spectrometer (Shimadzu, Kyoto, Japan). The separation of analytes was carried out using a cross-linked HP-5MS capillary column (5% phenyl-methylsilicone, 30 m × 0.25 mm i.d., 0.25 μm film thickness) supplied by Agilent Technologies (Illinois, IL, USA). Helium was used as carrier gas at a flow rate of 1.0 mL/min. The injection port and the transfer line temperatures were at 280 and 300 °C, respectively. Initial oven temperature of 100 °C was held for 1 min, followed by an increase to 300 °C at a rate of 40 °C/min with a final hold time of 4 min (total run time: 10 min). The mass spectrometer (MS) was operated at electron impact ionization mode (EI, 70 eV). Preliminary mass spectra of our HFBA derivatized compounds were obtained in SCAN mode, from *m*/*z* 50 to 600 amu with a scan rate of 0.9 scan/sec and a solvent delay of 5 min. Three characteristic ions for each analyte were selected for ion monitoring at SIM acquisition mode (dwell time of 10 ms) for their identification. The basic ion of each drug was used for the quantification of analytes. The characteristic ions and the retention times of ATDs and their metabolites are presented in Table 1. The basic ion of each substance is marked in bold. The characteristic ions of the IS (derivatized protriptyline, retention time 6.90 min) were **191** (quantification ion), 459 and 165.

Table 1
Characteristic ions (*m*/*z*) and retention times of the eleven antidepressant drugs and their metabolites.

Antidepressant drugs and metabolites	Characteristic ions (*m*/*z*)	Retention time (min)
Amitriptyline	**58**, 202, 215	6.32
Citalopram	**58**, 238, 324	6.78
Clomipramine	**58**, 85, 269	6.94
Fluoxetine-HFB	**240**, 344, 117	5.80
Fluvoxamine-HFB	**226**, 240, 258	5.76
Maprotiline-HFB	**191**, 445, 218	7.17
Desmethyl-maprotiline-HFB	**191**, 431, 202	7.08
Mirtazapine	**195**, 208, 180	6.48
Desmethyl-mirtazapine-HFB	**195**, 250, 447	6.84
Nortriptyline-HFB	**232**, 217, 204	6.53
Paroxetine-HFB	**138**, 109, 135	7.79
Sertraline-HFB	**274**, 276, 501	7.22
Desmethyl-sertraline-HFB	**274**, 276, 487	6.65
Venlafaxine-HFB	**58**, 115, 121	5.59
Desmethyl-venlafaxine-HFB	**58**, 115, 141	5.31

The basic ion of each analyte that was used for their quantification is marked in bold.

2.4. Sample preparation

The sample preparation procedure was performed according to Khraiwesh et al. [9]. 50 μL from the working solution (0.20 mg/L) of protriptyline was added in 1.0 mL of spiked (LOQ, calibration and QC) and routine blood samples. The final concentration of IS in all samples was 10.0 μg/L. 4 mL of phosphate buffer (pH 6.0; 0.1 M) was added during vortex-mixing. The samples were then centrifuged at 2000 rpm for 10 min. SPE columns were conditioned subsequently with methanol (2 mL) and with 0.1 M phosphate buffer pH 6.0 (2 mL). The blood samples were loaded onto the columns and slowly passed through under vacuum at a rate of approximately 1 mL/min. The columns were washed subsequently with deionized water (2 mL), acetic acid 1.0 M (1 mL) and methanol (3 mL). They were allowed to dry for 5 min under high vacuum (≥20 mmHg). The retained analytes were eluted twice by using 1.5 mL of a freshly prepared mixture of ethyl acetate: isopropanol: ammonium hydroxide (85:15:3, v/v/v). The eluents were evaporated to dryness under a gentle stream of N_2 at room temperature. The residues were acylated by 50 μL HFBA in 100 μL ethyl acetate, at 50 °C for 30 min. After evaporation, the residues were reconstituted in 50 μL ethyl acetate, and an aliquot of 1.0 μL was injected onto the GC/MS system.

3. Results and discussion

3.1. Method development

The present work describes a sensitive and selective GC/MS method for the simultaneous determination of 11 ATDs and 4 of their active metabolites in whole blood after SPE and acylation with HFBA. GC/MS was selected for this method as it is a sensitive and selective enough analytical technique and it is available at most clinical or forensic laboratories that perform everyday routine

analysis. Blood samples were preferred as they are normally available in both clinical and forensic cases.

Gas chromatographic analysis of underivatized amines is generally unsatisfactory due to adsorption and thermal decomposition of the analytes on the column [13]. Therefore, the predominant reason for derivatization of the ATDs is the improvement of the chromatography of these amines, by decreasing their polarity, and increasing the sensitivity of the method. Heptafluorobutyrylation replaces a labile hydrogen atom attached to the nitrogen-atom, for a less polar, non-labile group. Another reaction is the dehydrogenation of tertiary alcohols such as in venlafaxine and its metabolite. The reaction of 50 μL HFBA in ethyl acetate environment at 50 °C for 30 min gave the highest yield (more than 95%) for all the substances that can be derivatized. HFBA can derivatize primary and secondary amines, but not tertiary amines [13] like amitriptyline, citalopram and clomipramine. Mirtazapine cannot be derivatized also. These underivatized four compounds are volatile for GC analysis as it is clear from their chromatographic performance and their low LOQ. The total chromatographic run time of the proposed method is short (10 min) and it makes the method rapid and applicable for the investigation of both forensic and clinical cases.

SPE was chosen for this study due to its several advantages over LLE, like high selectivity, better specificity, ability to obtain clear extracts, avoidance of emulsion formation, good reproducibility, ability to automate the extraction procedure and substantial reduction in the volume of the solvents required. More specifically, Bond Elut Certify LRC SPE columns with a mixed mode sorbent that combines strong cation exchange (benzene sulfonic acid) and non-polar (C8) properties were selected as they retain the compounds by both, reverse-phase and cation exchange mechanism [9]. SPE ensured a simple and reproducible assay, and gave good sample purification and absolute recovery values for all analytes higher than 79%.

3.2. Method validation

Various parameters of the developed method such as selectivity, specificity, sensitivity, linearity, absolute recovery, accuracy and precision were evaluated according to international criteria [16,17].

Selectivity was studied by analyzing blank blood samples from six different people who were not on ATD treatment. The matrix effect was assessed and there was no interference from endogenous compounds of blood at the retention times of analytes. Representative TIC chromatograms obtained from a blank and a spiked with ATDs (at the low QC concentration) blood samples are shown in Fig. 1. *Specificity* was determined by analyzing a standard mixture (10.0 mg/L) of commonly used illicit and licit drugs and metabolites (morphine, codeine, 6-acetyl-morphine, methadone, buprenorphine, nor-buprenorphine, Δ^9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, cocaine, ecgonine methylester, benzoyloecgonine, amphetamine, methamphetamine, MDMA, ephedrine, ketamine, lidocaine, paracetamol, alprazolam, bromazepam, clobazam, chlordiazepoxide, diazepam, nordiazepam, 7-amino-flunitrazepam, phenobarbitone, buflomedil, haloperidol, clozapine, chlorpromazine, levomepromazine, olanzapine, quetiapine, piracetam, risperidone, zolpidem, ethosuxumide, lamotrigine, levetiracetam, topiramate, valproic acid) and spiked blood samples ($n = 6$) with these substances at a concentration of 1.00 mg/L. The specificity study documented that blood concentrations of the above substances do not interfere with the accurate determination of the 11 ATDs and their active metabolites. The *limits of detection* (*LOD*) and *quantification* (*LOQ*) for each ATD were subsequently determined by analysis of six spiked blood samples prepared at their respective concentrations. LOD was determined to be as the sample concentration resulting in a peak area with a signal to noise ratio ≥3 and LOQ was determined to be the lowest drug concentration that can be quantified with acceptable accuracy and precision (≤20%), which gave rise to chromatographic peak whose area was equal to 10 times the baseline noise. The LOD and LOQ for each analyte are presented in Table 2. *Linearity* was determined by the calculation of the regression lines using the method of least-squares with a weighting factor $1/x^2$ and it was expressed by the correlation coefficient (R^2). Six-point calibration curves were made in the range of 5.00–1000 μg/L for all analytes. The linear range of each compound covers the respective sub-therapeutic, therapeutic and toxic levels as well [18]. Linearity results are presented in Table 2. The *absolute recovery* of all analytes was calculated by analyzing six replicates at each of the three QC concentrations and comparing the area obtained with the areas obtained from the respective methanolic standards. The absolute recoveries were found to be higher than 79.2% for all analytes and are presented in Table 2. The *accuracy* (% E_r, the percentage of difference from theoretical concentration) and the *precision* (% RSD, the relative standard deviation) of the developed method were determined by analysis of six samples at each of the three QC levels within analytes' linear range (low, medium and high concentration). Intra-day variation of the assay was assessed by six samples of each QC concentration on the same day. Inter-day variation was assessed by a total of thirty samples at each QC concentration on five different days. Accuracy and precision results are presented in Table 3.

The developed method is fully validated and shows comparable or enhanced validation results, when compared with previously published validated methods for the determination of different ATD drugs [1–4,6,7,11,13,15]. The proposed method is more sensitive as it shows lower LOQ than others previously reported [1–3,7,13]. It also shows significant selectivity and specificity, as well as

Table 2
Linearity results, LOD, LOQ and % absolute recovery of the developed method for the determination of 11 antidepressant drugs and their metabolites in blood samples.

Analyte	% RSD of slopes ($n = 5$)	R^2	LOD (μg/L)	LOQ (μg/L)	% absolute recovery
Amitriptyline	4.1	≥0.992	0.7	2.00	90.2–96.7
Citalopram	3.6	≥0.993	0.7	2.00	88.4–95.2
Clomipramine	3.8	≥0.991	1.5	5.00	82.6–87.5
Fluoxetine	4.0	≥0.995	0.3	1.00	91.6–101.2
Fluvoxamine	4.9	≥0.991	0.7	2.00	86.4–90.3
Maprotiline	4.6	≥0.995	0.3	1.00	88.2–93.5
Desmethyl-maprotiline	4.1	≥0.991	0.3	1.00	91.4–99.4
Mirtazapine	2.8	≥0.996	0.7	2.00	89.5–94.7
Desmethyl-mirtazapine	3.5	≥0.992	0.7	2.00	84.7–90.8
Nortriptyline	4.2	≥0.991	0.3	1.00	94.8–102.6
Paroxetine	4.7	≥0.990	1.5	5.00	79.2–88.4
Sertraline	3.6	≥0.996	0.3	1.00	89.6–98.1
Desmethyl-sertraline	3.9	≥0.991	0.3	1.00	83.7–93.2
Venlafaxine	4.1	≥0.991	1.5	5.00	82.9–88.4
Desmethyl-venlafaxine	4.5	≥0.991	1.5	5.00	86.2–91.2

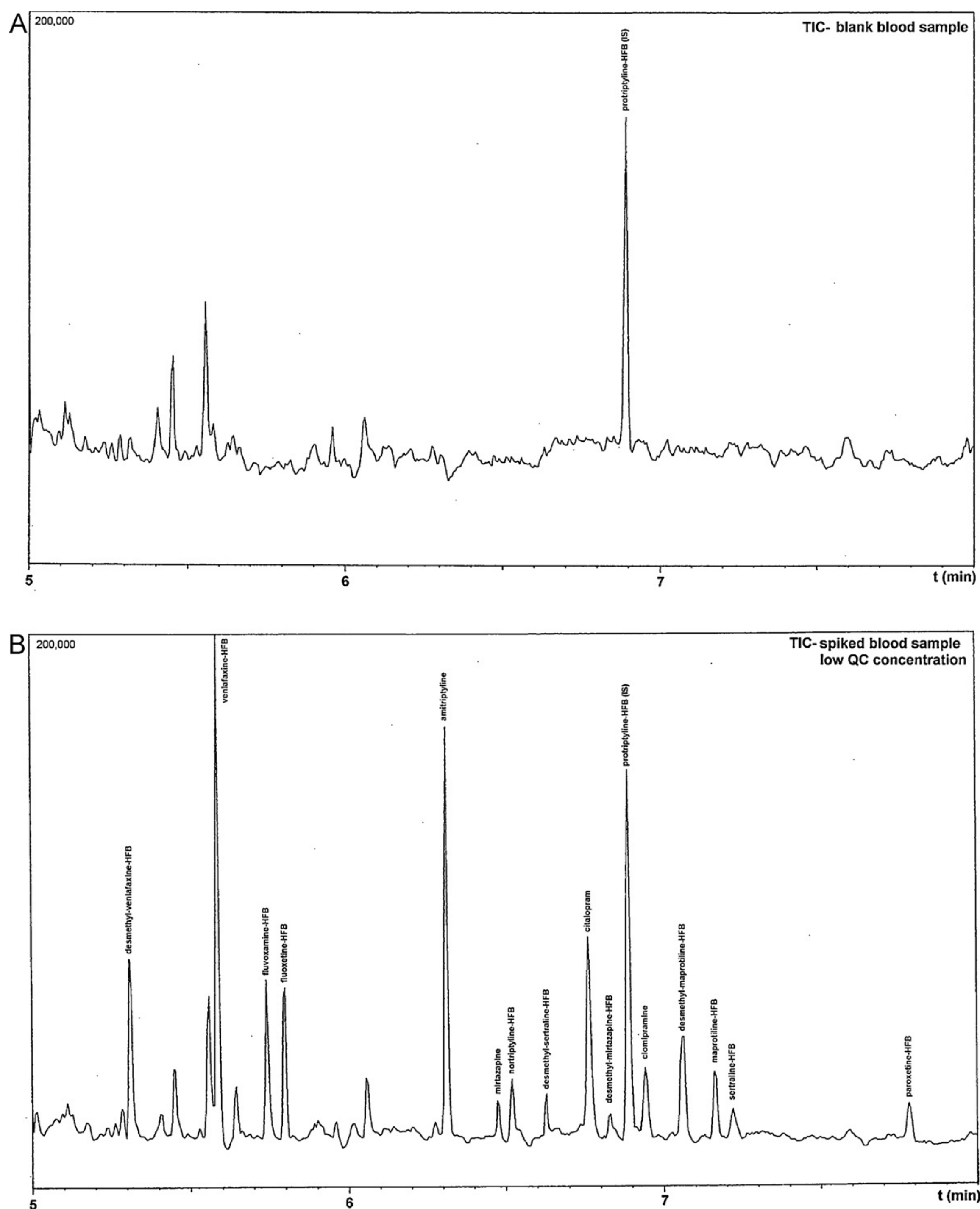

Fig. 1. (A) TIC chromatogram of a blank blood sample and (B) TIC chromatogram of a spiked blood sample with 11 antidepressant drugs and 4 of their metabolites at the low QC concentration.

satisfactory accuracy and precision results for all compounds. The method produce good sample purification and extraction efficiency of the analytes with absolute recovery values higher than others previously published [2–4,6]. Furthermore, peak shapes and resolution were satisfactory and similar to those obtained by injecting standard solutions. The linearity range (5.00–1000 μg/L) of the assay is wider than others [3,6,11,15] and it covers sub-therapeutic, therapeutic and toxic levels. Moreover, the proposed method is convenient for the simultaneous analysis of 11 ATDs and some of their metabolites in whole blood samples during the investigation of clinical and forensic toxicology cases.

3.3. Method application

The proposed method allows the simultaneous determination of the most commonly prescribed ATDs and some of their active metabolites. The method was applied to 2517 blood samples obtained from different clinical and forensic cases during 2011, and 136 samples were found positive for one or more of the substances analyzed (5.4% of the samples), and 41 of these cases were related with suicidal attempt of the victim (30.1% of the positive cases and 1.6% of all samples). The three most frequent ATDs found were mirtazapine, citalopram and venlafaxine

Table 3
Intra- and inter-day accuracy and precision of the developed method for the determination of 11 antidepressant drugs and their metabolites in blood at three QC concentrations.

Analyte	QC concentration (μg/L)	Intra-day (n = 6)		Inter-day (n = 30)	
		Accuracy (% E_r)	Precision (% RSD)	Accuracy (% E_r)	Precision (% RSD)
Amitriptyline	15.0	4.91	7.1	7.02	8.9
	150	−5.57	3.1	5.56	6.9
	800	4.87	3.9	7.16	8.2
Citalopram	15.0	−6.44	6.2	5.50	7.1
	150	−4.05	5.8	6.55	10.1
	800	−6.94	3.8	−8.08	9.2
Clomipramine	15.0	−7.39	8.2	−5.11	11.7
	150	−10.4	9.7	3.93	10.6
	800	−2.35	4.6	−2.83	7.8
Fluoxetine	15.0	8.61	3.6	0.52	7.2
	150	−9.29	5.2	−8.72	9.0
	800	−6.81	3.0	7.04	8.6
Fluvoxamine	15.0	6.06	3.4	0.95	6.3
	150	−2.42	7.4	3.62	8.0
	800	8.19	8.7	−7.16	8.2
Maprotiline	15.0	2.19	6.1	8.50	9.2
	150	−6.69	5.4	11.1	6.0
	800	−5.87	2.0	−1.38	9.8
Desmethyl-maprotiline	15.0	0.15	5.9	10.8	10.1
	150	−8.22	5.4	−4.05	8.1
	800	−7.99	2.7	10.3	7.1
Mirtazapine	15.0	−10.1	9.8	11.4	7.0
	150	−7.06	5.6	−4.63	7.0
	800	−7.89	3.1	−11.0	9.9
Desmethyl-mirtazapine	15.0	5.09	6.2	12.2	8.4
	150	−10.7	8.5	−1.84	5.1
	800	−6.36	2.7	−0.09	7.2
Nortriptyline	15.0	−9.68	7.3	2.25	6.8
	150	8.36	9.1	−2.25	6.6
	800	−9.40	4.8	−8.50	4.5
Paroxetine	15.0	10.9	4.2	−6.57	11.6
	150	5.30	1.9	−3.66	8.4
	800	−4.90	4.0	2.69	9.5
Sertraline	15.0	4.89	6.9	−6.04	8.3
	150	6.97	5.8	−0.37	5.8
	800	6.22	4.3	8.36	9.0
Desmethyl-sertraline	15.0	3.89	6.1	−3.99	6.9
	150	−3.29	2.9	9.56	6.4
	800	−6.04	4.6	−7.19	7.0
Venlafaxine	15.0	−9.11	4.8	10.3	11.0
	150	−3.34	2.1	−11.2	9.6
	800	−0.14	8.1	−6.18	10.9
Desmethyl-venlafaxine	15.0	−12.3	6.2	8.01	10.7
	150	−0.19	3.5	−2.64	8.9
	800	−3.50	3.8	−2.61	4.6

in the 2.4, 1.9 and 0.9% of all samples (60, 48 and 23 cases), respectively. In 20 of the positive cases more than one ATDs (14.7% of the positive cases) were found during the analysis. 72 cases were found positive for other drugs or toxic substances (52.9% of the positive cases) during general screening of the relative blood samples. Alcohol was also determined in 25 of the positive cases (18.4%).

4. Conclusion

A simple, specific and sensitive analytical method was developed and validated for the simultaneous determination of the most common prescribed 11 ATDs (amitriptyline, citalopram, clomipramine, fluoxetine, fluvoxamine, maprotiline, mirtazapine, nortriptyline, paroxetine, sertraline and venlafaxine) and 4 active metabolites (desmethyl-maprotiline, desmethyl-mirtazapine, desmethyl-sertraline and desmethyl-venlafaxine) in blood by GC/MS. Sample preparation includes SPE using Bond Elut LRC Certify (mixed-mode columns) and derivatization with HFBA. This simple and sensitive procedure is suitable for screening of unknown blood samples, as well as for the determination of these ATDs and some of their active metabolites for pharmacokinetic studies, for TDM in order to adjust the dosage or in forensic toxicology investigations.

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