Forensic Toxicol (2013) 31:124–132
DOI 10.1007/s11419-012-0152-7

SHORT COMMUNICATION

Simultaneous determination of new-generation antidepressants in plasma by gas chromatography–mass spectrometry

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Received: 17 April 2012 / Accepted: 24 July 2012 / Published online: 26 September 2012

Abstract We have developed a gas chromatography–mass spectrometry (GC–MS) method for plasma for the determination of new-generation antidepressants, including olanzapine (antipsychotic used in bipolar disorder), and antidepressant selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine and its metabolite norfluoxetine, paroxetine, sertraline, venlafaxine, and mirtazapine. Sample preparation was performed by liquid–liquid extraction with tert-butyl methyl ether. Fluoxetine, norfluoxetine, sertraline, and paroxetine required subsequent derivatization with 1-(heptafluorobutyryl) imidazole (HFBI). The GC separation lasts a total of 23.76 min. Qualitative and quantitative analysis were performed using an electron-impact ionization gas chromatograph interfaced to a mass-selective detector in selected-ion monitoring mode to increase the sensitivity of the method. Method validation was performed taking into account linearity, sensitivity, selectivity, accuracy, precision, and recovery, achieving good results for all the parameters studied. Calibration curves were prepared in the range of 0.005–2 μg/ml (according to the therapeutic and toxic concentrations of each individual compound), with all correlation coefficients $R^2 > 0.99$. The limit of quantification was between 0.005 and 0.1 μg/ml, depending on the compound, whereas the limit of detection ranged from 0.0025 to 0.05 μg/ml. The method is fast and simple, allowing the identification and quantification of some of the most widely used antidepressants at therapeutic or toxic concentrations, and may be useful in routine clinical and forensic toxicology analysis.

Keywords Antidepressant SSRIs · Olanzapine · GC–MS · LLE · Plasma

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Introduction

Depression is a mental disorder that affects millions of people around the world. According to the World Health Organization (WHO), depression is the third-leading cause of loss of years of healthy life due to disability or premature death (years lived with disability; DALYs). In 2020, depression will be the second most common disease after cardiovascular diseases [1, 2].

In the past decade, drugs for the treatment of depression have ranked at the top of drug sales due to the introduction of a new generation of antidepressants. These drugs, associated with better tolerability and ease of use, have achieved immediate and overwhelming success. They include: selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine (and its metabolite norfluoxetine), paroxetine, and sertraline; serotonin–norepinephrine reuptake inhibitor (SNRIs), whose main representative is venlafaxine; and noradrenergic and serotonergic specific antidepressants (NaSSA), such as mirtazapine [3]. In addition to the antidepressant drugs that dominate the present market, this study included olanzapine (an atypical antipsychotic drug used for the treatment of schizophrenia), which is also used as a mood stabilizer in bipolar disorder to prevent relapse of manic episodes [4, 5].

All these drugs have drastically reduced side effects and low risk of overdose, factors which are the main limitations of the older generation of antidepressants. However, there are clinical studies that question the efficacy and safety of these drugs, showing a profile of toxicity that is still not well known. These studies highlight a very worrying situation, such as the association between treatment with new generation antidepressants and an increase in suicidal thoughts, especially among young people [6–10]. Therefore, the development of more efficient analytical techniques is important in clinical toxicology, where they help in monitoring therapy (because it is difficult to interpret the relationship between plasma concentration and therapeutic and side effects), and in forensic toxicology, given the correlation between depression and premature death.

Numerous studies have been published on the determination of new-generation antidepressants by gas chromatography (GC)–mass spectrometry (MS) or liquid chromatography (LC)–MS with various extraction procedures. We have searched for a GC–MS method with a liquid–liquid extraction (LLE) procedure that would allow us to perform simultaneous determination of all the antidepressants of current interest [11–15].

In this study, we have developed a method with high sensitivity and selectivity, and provides satisfactory results in terms of quantity and quality, but at the same time is of practical use for routine analysis, reducing costs and processing time. The analysis was performed by GC–MS, the technique most commonly used in forensic toxicology laboratories because of its robustness, precision, sensitivity, and cost-effectiveness [16].

Experimental

Materials and methods

Chemical reagents and standards

Fluoxetine, fluoxetine-D6, paroxetine-D6, and olanzapine were from Cerilliant (TX, USA). Paroxetine, norfluoxetine, mirtazapine, and 1-phenyl-2,3,4,5-tetrahydro-(1H)-3-benzozepine-7,8-diolhydrochloride (SKF) were from Sigma-Aldrich (Steinheim, Germany). Sertraline was from Laboratorios Dr Esteve (Barcelona, Spain). Venlafaxine was from Normon (Madrid, Spain). Methanol and HPLC-grade water (milliQ) were from Merck (Darmstadt, Germany). Toluene and 1-(heptafluorobutyryl) imidazole (HFBI), used as derivatizing agent, were from Sigma-Aldrich.

Tris A buffer was prepared by dissolving 1.21 g of Tris (hydroxymethyl) aminomethane buffer (Scharlau, Australia) in 100 ml of deionized water to give a 0.1 M solution of pH 10. A magnetic vortex was used to accelerate the dissolution; pH adjustment was achieved by adding HCl or NaOH solution.

Preparation of solutions

Primary stock solutions were prepared in methanol at a concentration of 1 mg/ml for each individual compound and stored at about 0 °C. Only olanzapine was conserved in an acetonitrile solution. Stock solutions in methanol at 100 μg/ml were made for preparing the deuterated internal standards.

Working solutions were prepared by dilution from stock solutions with methanol (acetonitrile for olanzapine), obtaining concentrations of 1, 10, and 100 μg/ml for fluoxetine, norfluoxetine, and venlafaxine, and concentrations of 0.5, 1, and 10 μg/ml for mirtazapine, sertraline, paroxetine, and olanzapine, depending on the therapeutic range of each compound. Working solutions at 10 μg/ml were prepared for the internal standards (IS). All these solutions were stored in vials protected from light at 2 °C.

Biological matrix

In this work, plasma from unidentified subjects was used as a blank matrix; this plasma was provided by the Centro de Transfusión de Galicia (Blood Transfusion Center of Galicia, Spain). At the start of every sample preparation, plasma was centrifuged at 4000 rpm for 10 min to remove further interferences.

Gas chromatography–mass spectrometry

An HP 6890 CG from Hewlett–Packard (Little Falls, DF, USA) was used, equipped with an HP 7683B autoinjector from Agilent and connected to an HP 5973 inert mass selective detector from Agilent (Las Rozas, Spain). Chromatographic separation was achieved with an HP-5MS capillary column (crosslinked 5 % phenylmethylsiloxane, 30 m × 250 μm i.d., 0.5 μm film thickness). The initial temperature of the column was 100 °C for 1 min, then ramped progressively at 30 °C/min up to 200 °C, held constant for 2 min, and then ramped again at 7 °C/min (more slowly to ensure the best separation of analyzed compounds) up to 280 °C and held for 6 min. After that, the temperature was increased to 290 °C for 4 min to clean the column. The total chromatographic separation time was 23.76 min, and the total run time was 27.76 min.

Identification of compounds

The identification of compounds (studying the information of the existing libraries [17]), was performed using the full-scan acquisition mode, which allowed the analysis of the

total ion chromatogram (TIC), extrapolating retention times and characteristic ions.

Retention times were as follows: norfluoxetine (11.89 min), fluoxetine (12.75 min), venlafaxine (13.46 min), mirtazapine (15.79 min), SKF (16.41 min), sertraline (19.06 min), paroxetine (21.18 min), olanzapine (22.44 min). Quantifier and qualifier ions used for each analyte were selected based on their abundance and *m/z* values. The ions selected were as follows: norfluoxetine (**117**, 330,226), fluoxetine (**117**, 104,344), fluoxetine-D6 (**123**, 110,350), venlafaxine (**58**, 134,119), mirtazapine (**95**, 167,180), SKF (**86**, 165,99), sertraline (**274**, 501,503), paroxetine (**526**, 388,266), paroxetine-D6 (**531**, 394,272), olanzapine (**242**, 229,213). Because of their reproducibility and lack of interference, high mass ions were selected when possible. This was not possible when there were ions in common with those of the deuterated internal standard. In fact, the substitution of a hydrogen atom with a deuterium influences the molecular weight of the substance but not the physicochemical properties. This causes the retention times of the analyte and IS to be the same; for this reason, SKF was used to replace olanzapine-D3 as the IS. With olanzapine-D3 as the IS, all major ions were in common with the analyte and those of olanzapine that could be analytically useful had an abundance so limited that they could not be detected at the low concentrations used.

Upon selection of unique ions, the MS was run in selected-ion monitoring (SIM) mode due to the high sensitivity required with the low concentrations used. This allowed us to develop a more specific method of analysis for each compound. Several tests were performed to optimize the chromatographic separation of the peaks to obtain six time windows based on the retention time of each compound. Within the windows, the mass spectrometer identified and selected only the specified ions, thus reducing the background noise and increasing sensitivity. Some of the drugs analyzed needed a postderivatization step to increase the volatility or decrease the polarity, thereby allowing analysis by GC–MS.

Extraction procedure

To 1000 µl of plasma, 20 µl of IS was added, followed by Tris A buffer (500 µl) to obtain alkaline pH. After vigorous shaking, 5000 µl of *t*-butylmethylether was added, and the mixture was vortexed (10 min) and centrifuged at 4000 rpm for 10 min.

The organic layer was extracted and transferred to a conical tube and evaporated at 40 °C under a nitrogen stream.

For venlafaxine, mirtazapine, and olanzapine, after evaporation, the residue was redissolved with 40 µl of methanol, and 1µl was injected into the GC–MS. For fluoxetine, norfluoxetine, sertraline, and paroxetine, derivatization was necessary.

Derivatization was achieved after evaporation by adding 30 µl of HFBI and maintaining the capped tubes at 80 °C for 30 min to optimize the reaction. After cooling to ambient temperature, a simple extraction was performed by adding 500 µl of HPLC-grade water and 2000 µl of toluene. The sample was vortexed and centrifuged at 4000 rpm for 10 min, obtaining the derivatized compounds in the organic layer (the aqueous solution with the residues was eliminated). The toluene layer was then separated in a conical tube and evaporated at 40 °C under a nitrogen stream. The residue was redissolved in 40 µl of methanol, and 1 µl was injected into the GC–MS.

A chromatogram of all the analytes is shown in Fig. 1.

Method validation

Analytical validation of the method was performed by establishing selectivity, linearity, limits of detection and quantitation, intraday and interday precision and accuracy, and recovery according to FDA guidelines [18].

For evaluation of linearity, standard calibration curves were obtained for each analyte, using the same biological matrix. The sample matrix was spiked with standard solutions to obtain concentration ranges selected on the basis of therapeutic and toxic doses of the individual compound. The sensitivity of the method was determined by calculating the limit of detection (LOD) and the limit of quantification (LOQ). LOD was determined by an empirical method in which a series of plasma samples containing decreasing amounts of the analytes was analyzed. LOD was the lowest concentration that presented a signal-to-noise ratio higher than 3 for at least three diagnostic ions for each substance. The LOQ is the lowest concentration of the analyte that can be determined quantitatively with appropriate precision and accuracy.

The selectivity of the method was demonstrated by analyzing blank samples of the biological matrix that were obtained from at least six different sources. Samples were extracted and analyzed for assessment of potential interferences from endogenous substances. The apparent response at the retention times of the analytes under investigation was compared with the response of the analytes at the limit of quantitation.

Precision and accuracy were determined by interday and intraday assays. Interday precision and accuracy were evaluated by six determinations per concentration on different days. Intraday precision and accuracy were determined at three concentrations, low, medium, and high, by preparing and analyzing five replicates for each level on the same day. Precision, expressed as the coefficient of variation (CV) of the measured values, was calculated as (standard deviation/mean) × 100. It is expected to be less than 15 % at all concentrations, except at the LOQ for

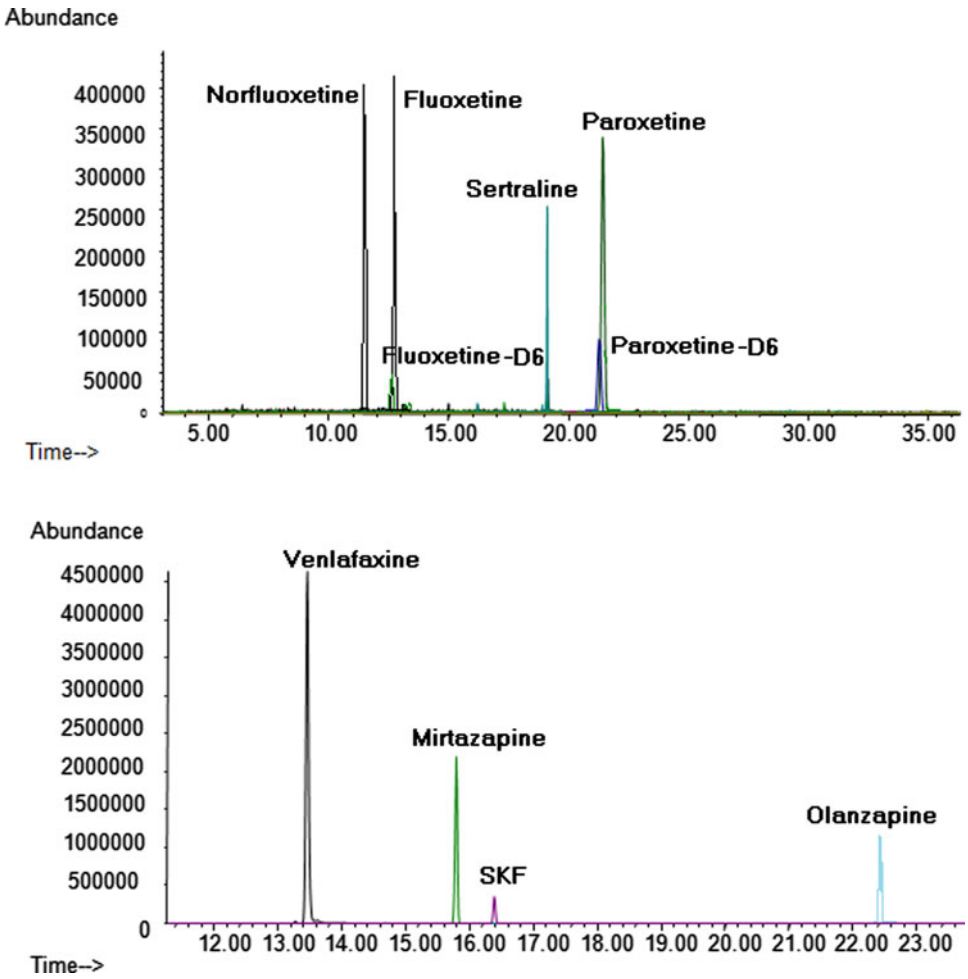

Fig. 1 Chromatogram of all the analytes (concentrations in middle of calibration range: 1.2 μg/ml for fluoxetine, norfluoxetine, and venlafaxine; 0.2 μg/ml for mirtazapine, sertraline, and paroxetine; 0.1 μg/ml for olanzapine)

which 20 % is acceptable. In the same way, accuracy was evaluated using the mean relative error (MRE), which had to be less than 15 % of the theoretical values at each concentration level except for the LOQ, for which 20 % is acceptable.

Recovery or extraction efficiency (%) for the analyte was determined at low and high concentration levels (five replications per concentration). Calculations were performed by comparing the areas of the peaks after extraction of the samples with the internal standard and the drug, with those obtained containing only the IS and subsequently spiked with the drug at the same concentration.

Results and discussion

Sample preparation

Sample preparation is one of the most important steps in the majority of analytical procedures to determine constituents in samples with complex matrices. An ideal sample preparation technique should be simple, inexpensive, efficient, selective, and compatible with various analytical techniques. It should give as high a recovery as possible, use the minimum amount of solvent, and be environmentally friendly.

Therefore, simple, rapid, and less labor-intensive extraction techniques are needed in forensic toxicology. In this work, LLE has been employed as the extraction procedure, with the purpose of reducing the elapsed time, simplifying the method, and reducing costs while obtaining good results (results comparable to those obtained using other extraction techniques [11, 12, 14, 15, 19–21] that have some disadvantages such as being time consuming or expensive).

Tert-butyl methyl ether was used as solvent; previously it was compared with other solvents, such as diethyl ether [20], and it showed increased stability over time and a lower tendency to form toxic and explosive peroxides [22]. Several tests were performed to find the most appropriate pH; the best results were obtained with buffer Tris A (basification with 0.1 M NaOH decreased the efficiency of extraction).

The LLE technique using tert-butyl methyl ether had already been used for olanzapine [15] and in this work we have expanded the scope to the new generation of antidepressants, creating a method that includes the main

antidepressants commercially available. In the case of fluoxetine, norfluoxetine, sertraline, and paroxetine, a process of derivatization was required prior to chromatographic analysis. Other authors have used different compounds for derivatization, including *N*-methyl-*N*-(tert-butyldimethylsilyl)trifluoroacetamide, acetic anhydride, and HFBI, the latter being the most widely used [11–15].

Therefore, HFBI was used as the derivatizing agent. This compound can replace the labile hydrogen bound to a nitrogen atom and so it is able to derivatize primary and secondary amines, but not tertiary amines like mirtazapine and olanzapine. HFBI also causes the dehydration of tertiary alcohols and could therefore derivatize venlafaxine as well. In our study, this property was not put to use because venlafaxine proved to be sufficiently volatile without derivatization.

HFBI guarantees a rapid and satisfactory derivatization, obtaining more stable and volatile compounds in comparison with other derivatizing agents. Because HFBI is not an acid agent and is particularly inert, it does not cause corrosion or decomposition problems on the column. However, to eliminate excess derivatizing agent and to reduce the risk of clogging the injection needle, a further stage of extraction is necessary after derivatization. [19]. HFBI must be handled and stored in an inert atmosphere because it easily hydrolyses, is sensitive to moisture, and forms a solid precipitate when it is in contact with the air. This makes it very difficult for it to be pipetted and causes loss of effective reagent.

Validation

The developed method was fully validated. It showed significant selectivity and specificity, as well as satisfactory accuracy and precision results for all compounds. Furthermore, peak shapes and resolution were satisfactory and similar to those obtained by injecting standard solutions, as shown in Fig. 1.

The linearity of the method was evaluated by preparing calibration curves for all compounds. The range taken into account was established to include values from the therapeutic and toxic levels of each compound. Moreover, the proposed method is convenient for simultaneous analysis of the seven SSRIs in plasma samples during investigation of clinical and forensic toxicology cases. Good linearity with correlation coefficients (R^2) higher than 0.990 were obtained for all substances (Table 1).

The sensitivity of the method was determined by calculation of the LOD and the lower limit of quantitation (LLOQ). These limits are similar to those described by other authors [23].

Precision and accuracy were determined by interday and intraday assays. Good results were achieved with a CV and relative error within the limits approved by the FDA. The major variations were obtained, in general, at the lowest concentrations levels. Other authors obtained similar CV values or even higher variations at low concentrations. Paterson et al. [20] reported a CV of 36.32 % for mirtazapine and 15.34 % for sertraline at low concentration. Data are shown in Table 2.

Table 1 Linearity, limits of detection and quantification, and calibration range

Substance	LOD	LOQ	R^2	Range concentration (μg/ml)					
Norfluoxetine	0.05	0.1	0.9945	0.1	0.2	0.6	1.2	1.6	2.0
Fluoxetine	0.02	0.1	0.9954	0.1	0.2	0.6	1.2	1.6	2.0
Venlafaxine	0.01	0.1	0.9977	0.1	0.2	0.6	1.2	1.6	2.0
Mirtazapine	0.0025	0.02	0.9982	0.02	0.05	0.1	0.2	0.5	1.0
Sertraline	0.005	0.01	0.9981	0,01	0.05	0.1	0.2	0.5	1.0
Paroxetine	0.0025	0.005	0.9992	0.005	0.01	0.05	0.1	0.2	0.4
Olanzapine	0.01	0.03	0.9985	0.03	0.06	0.1	0.2	0.5	1.0

Table 2 Precision and accuracy intraday and interday and recovery of extraction

Concentration (μg/ml)	Intraday CV (%)	Interday CV (%)	Concentration (μg/ml)	Recovery (%)	CV (%)
Norfluoxetine					
0.1	16.42	15.68	0.2	100.47	5.37
1.2	3.34	7.75	2.0	110.01	3.51
2.0	0.08	1.82			
Fluoxetine					
0.1	4.03	14.54	0.2	100.04	9.73
1.2	6.91	7.98	2.0	107.03	7.69
2.0	4.29	3.30			
Venlafaxine					
0.1	8.17	10.34	0.2	91.19	12.16
1.2	2.67	4.56	2.0	74.92	1.43
2.0	6.55	3.81			
Mirtazapine					
0.02	4.66	17.60	0.05	93.93	7.8
0.1	2.18	9.76	1.0	65.41	8.28
1.0	4.99	1.22			
Sertraline					
0.01	4.78	18.57	0.01	99.39	7.9
0.2	11.34	14.55	1.0	63.10	14.089
1.0	9.78	2.69			
Paroxetine					
0.005	2.35	13.399	0.05	94.32	12.67
0.1	11.516	12.514	0.4	92.14	13.90
0.4	5.68	3.511			
Olanzapine					
0.03	19.27	18.55	0.06	96.49	19.97
0.2	11.41	5.99	1.0	96.85	5.32
1.0	9.67	1.01			

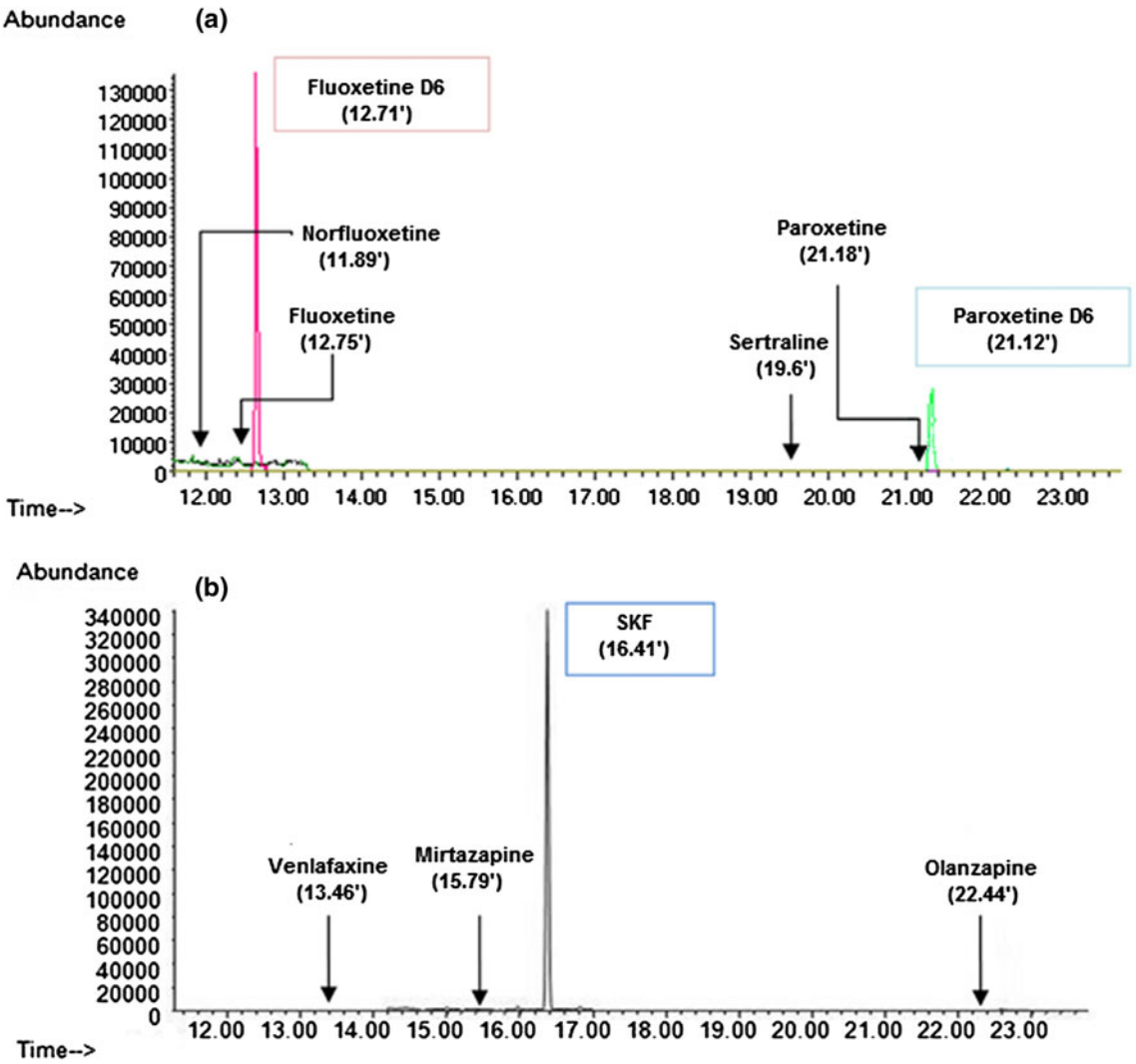

Fig. 2 Study of selectivity: **a** derivatized compounds, **b** underivatized compounds

The selectivity of the method was demonstrated by analyzing six plasma samples from different people who stated that they had not consumed antidepressant drugs. The apparent response at the retention times of the analytes under investigation was compared with the response of analytes at the limit of quantitation (Fig. 2). It was noted that there were no significant interferences.

Recovery or extraction efficiency (%) for the analytes was determined at low and high concentration levels. The data obtained demonstrates that the extraction procedure is particularly efficient, providing a recovery of 90 % for all compounds (except for the high concentrations of venlafaxine, mirtazapine, and sertraline, in which values ranged from 60 to 70 %). These data are similar to those obtained by other authors: Wille et al. [13] reported a recovery of 65 % for sertraline and 53 % for norfluoxetine at a low therapeutic level. The results are shown in Table 2.

The developed method was used to analyze 26 real plasma samples obtained from the Forensic Toxicology Service of the Institute of Forensic Sciences of Santiago de Compostela (Spain) from cases including overdose, suicide, other violent death, or death from unknown causes. Of the total 26 cases, 23 were positive for at least one of the substances analyzed. In 5 of the positive cases, the cause of death was shown to be overdose of one or more of these substances (Table 3). Other authors have published death cases related to the use of SSRIs. Compton et al. [24] published a case report of suicide involving fluoxetine, while Dahl et al. [25] reported a fatality related to a venlafaxine overdose, with similar toxic levels to those obtained in this work.

Table 3 Details of real cases testing positive for antidepressants

Case no.	Age (years)	Sex	Sample	Cause of death	General information and treatment	Substance
1	84	Male	Blood, urine, humour vitreous	Severe craneal trauma	Antidepressants	Venlafaxine
2	53	Female	Blood, urine, humour vitreous	Unknown	Trankimazin® (alprazolam)	Venlafaxine, BZD
3	49	Male	Blood, urine, bile, gastric contents, humour vitreous	Unknown	Previous depressive episodes (suspected suicide by drug overdose)	Venlafaxine 1.06 μg/ml, BZD, NSAIDs (ibuprofen, naproxen)
4	47	Male	Blood, urine	Suicide (hanging)	Zyprexa® (olanzapine), Tranxilium® (clorazepate), Trileptal® (oxcarbazepine)	Olanzapine, alcohol, BZD (tetrazepam, carbamazepine)
5	50	Male	Blood, urine	Acute myocardial infarction	Aremis® (sertraline)	Sertraline 0.3 μg/ml
6	23	Male	Urine, hair	– (arrested)	Consumer of cocaine and THC	Fluoxetine, cocaine, THC
7	50	Female	Urine	– (arrested)	Consumer of alcohol, BZD, fluoxetine	Fluoxetine, BZD
8	37	Male	Blood, humour vitreous	Acute pulmonary edema	Rivotril® (clonazepam), Zyprexa® (olanzapine), Vandral® (venlafaxine)	Olanzapine, venlafaxine 2.03 μg/ml, BZD
9	55	Female	Blood	Intracranial injury (fall trauma)	–	Venlafaxine
10	81	Male	Blood, urine, humour vitreous	Suicide (hanging)	Vandral® (venlafaxine), Tranxilium® (clorazepate), Tofranil® (imipramine), Elontril® (bupropion)	Venlafaxine 1.07 μg/ml, clorazepate, imipramine, desipramine
11	30	Male	Blood, humour vitreous	Unknown	Psychiatric treatment for schizophrenia	Olanzapine, alcohol phenobarbital
12	41	Female	Blood, urine, humour vitreous	Unknown	Treatment of mixed personality disorder, obesity	Venlafaxine
13	53	Female	Blood, urine, gastric contents	Drug overdose	Xeristar® (duloxetine), lormetazepam, Dormodor® (flurazepam), mirtazapine, folic acid	Mirtazapine 0.5 μg/ml, alcohol (3.05 g/l), lorazepam
14	48	Female	Blood, bile, humour vitreous	Unknown	Psychotropic drugs, antidepressants, major tranquilizers. Affected by fibromyalgia, anxious-depressive syndrome, previous breast cancer	Venlafaxine, paracetamol, caffeine, BZD
15	55	Male	Blood, urine, humour vitreous	Intracranial injury	–	Olanzapine, alcohol
16	43	Male	Blood, urine, humour vitreous, bile, gastric contents	Suspected overdose (methadone and anxiolytics)	Ex addicted to drugs	Mirtazapine, methadone, opiates, BZD, THC
17	20	Male	Blood, urine, humour vitreous	Suspected overdose	–	Olanzapine, methadone, BZD
18	44	Male	Blood	Drug overdose	Venlafaxine, clomethiazole, hydrochlorthiazide, lormetazepam, diazepam, diltiazem	Venlafaxine, clomethiazole, alcohol (2.47 g/l)
19	73	Male	Blood, urine, humour vitreous	Trauma to the pelvic blood vessels	–	Venlafaxine, ibuprofen, metamizol
20	43	Male	Blood, urine, humour vitreous, gastric contents	–	Medications for mental deficiency (admitted to mental institution)	Olanzapine, BZD

Table 3 continued

Case no.	Age (years)	Sex	Sample	Cause of death	General information and treatment	Substance
21	73	Female	Blood, humour vitreous	Ischemic heart disease	Norfenazin® (nortriptiline), Prozac® (fluoxetine)	Fluoxetine
22	56	Male	Blood, urine, humour vitreous	Suicide (hanging)	Antidepressant treatment	Venlafaxine, mirtazapine, BZD
23	42	Male	Blood, urine, humour vitreous	Suicide (suffocation)	Antidepressant treatment	Venlafaxine, mirtazapine, BZD

Conclusions

We have developed a fast and reproducible GC–MS method that allows qualitative and quantitative analysis of the most commonly used new generation of antidepressants, even when they are used at low dosages. Plasma extraction was performed by LLE using tert-butyl methyl ether, following prior alkalinization with Tris A buffer. Some of the substances studied (fluoxetine, norfluoxetine, sertraline, paroxetine) required derivatization with HFBI prior to chromatographic analysis.

The method was fully validated according to FDA guidelines. Twenty-six real plasma samples were analyzed, of which 23 were found to be positive for some of the drugs studied. The results obtained prove that the method is useful for monitoring antidepressants and identifying cases of intoxication, including those that result in death.

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