Journal of Chromatography B, 879 (2011) 2576–2582

Contents lists available at ScienceDirect

Journal of Chromatography B

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

Development and validation of an EI-GC/MS method for the determination of sertraline and its major metabolite desmethyl-sertraline in blood

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ARTICLE INFO

Article history:
Received 14 February 2011
Accepted 10 July 2011
Available online 20 July 2011

Keywords:
Sertraline
Blood
GC/MS
Solid-phase extraction
HFBA-derivatization

ABSTRACT

A sensitive and specific GC/MS method for the determination of sertraline and its main metabolite desmethyl-sertraline in whole blood has been developed, optimized and validated. Sample preparation included solid-phase extraction of both analytes and their derivatization with heptafluorobutyric anhydride (HFBA). Protriptyline was used as internal standard for the determination of both analytes. Limits of detection and quantification for both sertraline and desmethyl-sertraline were 0.30 and 1.00 μg/L, respectively. The calibration curves were linear within the dynamic range of each analyte (1.00–500.0 μg/L) with a correlation coefficient (R^2) exceeding 0.991. Extraction efficiency ranged from 90.1(±5.8)% to 95.4(±3.0)% for sertraline, and from 84.9(±8.2)% to 107.7(±4.4)% for desmethyl-sertraline. The precision for sertraline and desmethyl-sertraline was between 3.6–5.5% and 4.7–7.2%, respectively, while the accuracy was in the range of −6.67% to 2.20% and −6.33% to 2.88% for sertraline and desmethyl-sertraline, respectively. The method was applied to real blood samples obtained from patients that follow sertraline treatment and also in cases of forensic interest. The developed method can be used in routine every day analysis by clinical and forensic laboratories, for pharmacokinetic studies, for therapeutic sertraline monitoring or for the investigation of forensic cases where sertraline is involved.

1. Introduction

Sertraline [(1S,4S)-4-(3,4-dichlorophenyl)-1,2,3,4-tetrahydro-N-methyl-1-naphthylamine] is a selective serotonin reuptake inhibitor (SSRI) [1,2]. It is considered to be safe, effective within a wide therapeutic range, and with minimal and well-tolerated adverse effects due to its highly specific affinity to central serotonin uptake sites [3–5].

Sertraline is mainly used therapeutically to treat the symptoms of depression and anxiety [2,4]. It is also prescribed for the treatment of different kinds of psychiatric disorders like obsessive–compulsive, post-traumatic stress, premenstrual dysphoric disorder, panic or social phobia/anxiety [2,3,6–8].

Sertraline is completely but slowly absorbed from the gut with a time to peak plasma concentration (T_{max}) of 6–8 h, and undergoes extensive first-pass metabolism by the action of different cytochrome subtypes to form desmethyl-sertraline, an active metabolite with longer half-life. Due to its extensive metabolism and its wide distribution to tissues (Vd exceeds 20 L/kg), the plasma concentrations of sertraline are normally very low. At the therapeutic dose of 50 mg/day, the plasma concentration of sertraline does not exceed 15 μg/L and its terminal half-life in healthy volunteers was reported ranging from 22 to 32 h [3,8–10].

The widespread use of sertraline, its involvement in intenional or accidental cases of poisoning and Therapeutic Drug Monitoring (TDM) requirements indicate the necessity for the determination of sertraline in biological fluids. TDM for sertraline is of significant importance for dose adjustment and therapy optimization, considering the interindividual variability of drug metabolism [11]. A significant number of methods based on gas chromatography (GC) [12–23] or liquid chromatography (LC) [24–32] has been published for the determination of sertraline. Some of them require a lengthy, tedious and time-consuming extraction procedure combined with high cost equipment [25,28,29,32], that is not available at common clinical or forensic laboratories for everyday routine analysis, cover narrow calibration range [23–25,27,32], and are applied on plasma or serum samples [11–13,15,17,24–27,29,30,32] that normally are not available in forensic cases. Whole blood is in practice the most frequently encountered sample in forensic cases while its drug concentration can provide an estimation of the clinical response of the patient. There are also published articles concerning the determination of sertraline with or without its metabolite (desmethyl-sertraline) in whole blood [14,18,19,21–23,28,31,33,34], but most of them require complex liquid–liquid extraction (LLE) procedure [21–23,28,31,33,34] and to our knowledge, only three articles exist in the literature concern-

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doi:10.1016/j.jchromb.2011.07.015

ing the determination of both sertraline and desmethyl-sertraline in whole blood using solid-phase extraction (SPE) [14,18,19]. Furthermore, the usually low therapeutic concentrations of sertraline and desmethyl-sertraline demonstrate the need for a highly sensitive method for their determination, as the sensitivity is yet not satisfactory [18,19,22,23,31].

The aim of our study was the development, optimization and validation of a new simple, rapid, sensitive and specific method, based on GC/MS for the identification and quantification of sertraline and its major metabolite desmethyl-sertraline in whole blood, using SPE and derivatization with HFBA. Measuring of sertraline blood concentrations is of great importance in the areas of Clinical (TDM, diagnosis of poisoning) and Forensic Toxicology (suicide or driving under the influence of drugs). The developed method can be applied in routine toxicological analysis during the investigation of both clinical and forensic cases.

2. Materials and methods

2.1. Chemicals and reagents

Sertraline hydrochloride and desmethyl-sertraline maleate were offered from Pfizer (Groton, CT, USA). Internal standard (IS), protriptyline hydrochloride, was purchased from Sigma–Aldrich (St. Louis, USA).

All solvents were HPLC grade (methanol, isopropanol, ethyl acetate, dichloromethane, and acetonitrile) and were purchased from Merck (Darmstadt, Germany).

Pentafluoropropionic anhydride (PFPA) 99%, N-methyl-N-(trimethyl-silyl) trifluoroacetamide (MSTFA), and N,O-bis (trimethylsilyl)-trifluoracetamide (BSTFA) with 1% trimethylchlorsilane (TMCS) were purchased from Sigma–Aldrich (Steinheim, Germany), heptafluorobutyric anhydride (HFBA) 99% and trifluoroacetic acid (TFAA) 99% from Fluka (Steinheim, Germany), acetic anhydride 97% from Mallinckrodt (St. Louis, USA) and pyridine 99.5% from Ferak (Berlin, Germany).

Analytical grade ammonium hydroxide (NH_4OH), glacial acetic acid (CH_3COOH), and extra pure sodium-dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) were purchased from Merck (Darmstadt, Germany).

Bond Elut LRC Certify (Sorbent Mass 130 mg, Column Volume 10 mL, Varian) and Certify II (Sorbent Mass 200 mg, Column Volume 10 mL, Varian), Abselut Nexus (Sorbent Mass 30 mg, Column Volume 10 mL, Varian) and Isolute HCX (Sorbent Mass 130 mg, Column Volume 10 mL, Biotage) SPE columns were used.

Phosphate buffer (pH 6.0, 0.1 M) was prepared by dissolving 15.6 g of $NaH_2PO_4{\cdot}2H_2O$ in 800 mL distilled water. The volume was brought to 1000 mL by adding sufficient distilled water and the pH was adjusted, with the help of a pH-meter, to 6.0 by adding NaOH (0.1 N).

Human blood samples were obtained from healthy donors, after their informed consent, and were kept in test tubes containing anti-coagulant (EDTA K_3). Blood was screened (before its use) for the presence of sertraline, desmethyl-sertraline or other drugs, by GC/MS in scan mode after basic LLE using dichloromethane:isopropanol (9:1 (v/v)).

2.2. Calibration and quality control samples

Standard stock solutions (1.0 g/L) of sertraline, desmethyl-sertraline and protriptyline (internal standard) were prepared in methanol. The stock solution of internal standard was further diluted in methanol (1:200 (v/v)) to give a 5.0 mg/L working solution. Three aqueous working solutions containing sertraline and desmethyl-sertraline (0.04, 0.5 and 5.0 mg/L) were prepared from the corresponding standard stock solutions of each compound and were used for the preparation of calibrator samples. Three other aqueous working solutions, containing sertraline and desmethyl-sertraline, at the same concentrations as above, were also prepared, from different stock solutions, and used for the preparation of quality control samples.

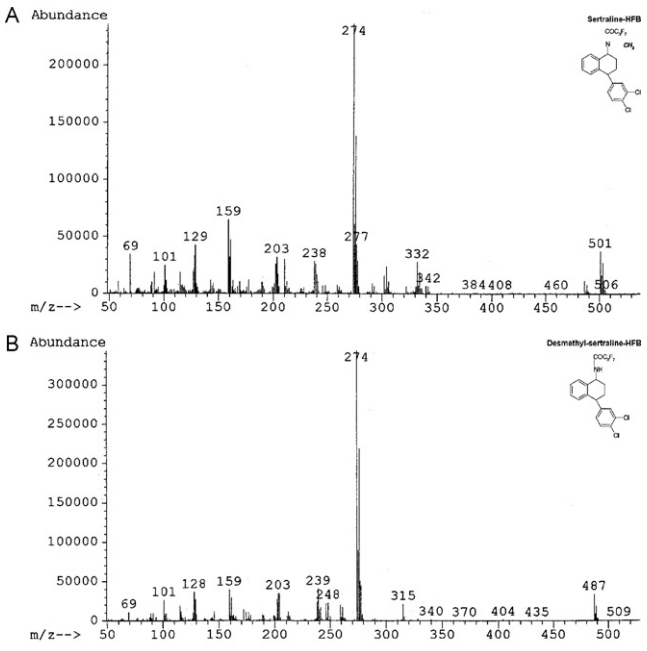

Fig. 1. Mass spectra of sertraline–HFB (A) and desmethyl-sertraline–HFB (B).

Spiked blood calibration samples were prepared by spiking 1 mL of blank blood with the appropriate volume of the corresponding working standard solution of sertraline and desmethyl-sertraline to make six calibrators containing both analytes at concentrations of 1.00, 2.00, 10.0, 50.0, 200.0 and 500.0 μg/L.

Quality control samples at three concentration levels (3.00, 40.0 and 400.0 μg/L) were prepared by spiking 1 mL blank blood with the appropriate volumes of the corresponding working solutions of sertraline and desmethyl-sertraline.

2.3. GC/MS analysis and apparatus

A Hewlett-Packard GC/MSD system consisting of a Model 5890 series II gas chromatograph provided with a 7673 A Autosampler, a 5970 series mass-selective detector (MSD) and Hewlett-Packard Chemstation software B.02.05 was used. The column was an HP-5MS (5% phenyl-methylsilicone, 30 m × 0.25 mm ID, and 0.25 μm film thickness) supplied by Agilent Technologies (IL, USA). The GC conditions were as follows: injection mode: splitless, injector port temperature: 280 °C; carrier gas: helium; flow rate: 1 mL/min; oven temperature rate: from 120 °C to 300 °C at a rate of 20 °C/min, initial time 1 min, final time 5 min, detector temperature: 300 °C. The MS detector had an electronic impact (EI) ionization source (ionization voltage: 70 eV) and a quadrupole analyzer. Preliminary mass spectra of our HFBA derivatized compounds were obtained in SCAN mode, from *m*/*z* 50 to 550 amu with a scan rate of 0.9 scan/s and a solvent delay of 5 min. For quantitation selective ion monitoring (SIM) mode was selected with dwell time of 10 ms. Different acquisition windows were defined taking into account the retention times and the suitable fragments of the studied compounds. The three characteristic ions from the mass spectrum of sertraline–HFB (Fig. 1A) were 274, 276 and 501, desmethyl-sertraline–HFB (Fig. 1B) were 274, 276 and 487, and that of protriptyline–HFB were 191,

189, and 459 (ions in bold were used for quantification). The retention times of sertraline–HFB, desmethyl-sertraline–HFB and protriptyline–HFB were 12.04, 11.14 and 11.38 min, respectively.

A vortex (Chiltern model MT 19) was used for the mixing of standards and samples during their preparation. A METROHM digital pH-meter with a glass combination electrode was used. An evaporating unit connected with nitrogen (Reacti-Vap PIERCE Model 18780, Rockford, IL) was used for the evaporation of all samples. Centrifugation was performed with a centrifuge (ALRESA, Spain) at a speed of 2000 rpm. A Sonicator (J.P.SELECTA®) (Abrera, Spain) was used during the preparation of the buffer.

2.4. Sample preparation

25 μL from the working solution (5.0 mg/L) of protriptyline was added in 1 mL of spiked (calibrators and quality control samples) and real blood samples as internal standard giving a final concentration of 125.0 μg/L. 4 mL of phosphate buffer 0.1 M (pH 6.0) was added and the tubes were vortex-stirred and left to stand for 1 min. The samples were then centrifuged at 2000 rpm for 10 min and the supernatants were subjected to SPE.

SPE columns were conditioned subsequently with methanol (2 mL) and with phosphate buffer 0.1 M (pH 6.0) (2 mL). The blood samples were loaded onto the columns and slowly drawn through under vacuum at a rate of approximately 1 mL/min. The columns were washed consequently with deionized water (2 mL), acetic acid 1.0 M (1 mL) and methanol (3 mL). The columns were allowed to dry for 5 min under full vacuum (≥20 mmHg). Then, the retained analytes were eluted twice by using 1.5 mL of the mixture 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 100 μL ethyl acetate and 50 μL HFBA at 50 °C for 30 min. After evaporation, the residues were reconstituted in 50 μL ethyl acetate, and an aliquot of 2 μL was injected into the GC/MSD system.

3. Results and discussion

3.1. Method development and optimization

The developed method allows the specific and sensitive determination of sertraline and desmethyl-sertraline in whole blood by GC/MS after SPE and acylation with HFBA. In this assay, protriptyline was chosen as the internal standard because it is chemically similar compound to both analytes, with an amine group that it is derivatized using HFBA, it can be extracted with the developed SPE procedure with a very good extraction efficiency [94.1(±6.3)%], and it is separated from both derivatized analytes using the optimized GC/MS parameters. Furthermore, it is an old antidepressant drug and very rare to be used therapeutically.

GC/MS analysis of sertraline and its major metabolite desmethyl-sertraline without derivatization is not sensitive enough. Thus, derivatization by different reagents (HFBA, PFPA, TFAA, MSTFA, BSTFA with 1% TMCS, and acetic anhydride in pyridine) was tried. The derivatization procedure was optimized in order to obtain the highest sensitivity and to avoid interferences at the retention time of sertraline and desmethyl-sertraline. Very low sensitivity was observed with acetylation by acetic anhydride in pyridine as the peak areas of both derivatized analytes using this reagent were the smallest comparing with the other reagents. The highest peak areas of both derivatized sertraline and desmethyl-sertraline were achieved by silylation using MSTFA or BSTFA with 1% TMCS and by acylation using HFBA. On the other hand, during silylation (MSTFA and BSTFA with 1% TMCS) many problems were faced at the derivatization step because both reagents are very sensitive to the humidity and there was not sufficient repeatability and good linearity. Furthermore, the baseline with the both silylation reagents showed interferences at the retention time of sertraline. Moreover, peak areas for both derivatized sertraline and desmethyl-sertraline with PFPA and TFAA were lower than using HFBA and a noisy baseline was obtained when PFPA and TFAA were tested. An overall consideration of the results indicated that the acylation reagent should be the first choice for the derivatization of sertraline and desmethyl-sertraline. After perfluoroacylation, sertraline and desmethyl-sertraline were readily converted to their corresponding perfluoroacyl-derivatives which are hydrolytically stable and less sensitive to humidity compared to silylation reagents. To our knowledge, in the literature only one study refers to the determination of sertraline, but not its metabolite, in plasma after LLE and derivatization with HFBA [13], whereas there are some published studies [12,15,18] that used heptafluorobutyryl imidazole (HFBI) for the derivatization of sertraline and desmethyl-sertraline, which gives the same derivatized analytes.

Derivatization conditions, like temperature (30, **50**, 60, 90, and 110 °C), and duration of the reaction (15, **30**, 45, and 60 min) were optimized using standard solutions of the compounds. The optimal conditions are in bold. During the optimization of the derivatization temperature, the peak areas of both acylated analytes were increased until 50 °C and after that increasing the temperature resulting in decrease the peak areas of the both analytes. The duration of the reaction had no effect on the peak areas of acylated desmethyl-sertraline, while the peak areas of acylated sertraline increased significantly by increasing the duration time up to 30 min and after that time they started to decrease.

Chromatographic conditions like injector temperature (240, 260, **280**, and 300 °C), detector temperature (280, 290, **300**, and 310 °C), initial (80, 100, **120**, 140 °C) and final (285, 290, 295, and **300** °C) column temperature, as well as the column temperature rate (**20**, 30, 40, 50 °C/min) and the initial temperature time (0.0, 0.5, **1.0**, 1.5 min) were also optimized. The optimal conditions are in bold and were chosen judging by the peak areas of the derivatized analytes and according to their resolution. The best GC separation of analytes was achieved within 13 min.

Optimization of the extraction procedure was also performed. Most of the previously published methods in whole blood used complex LLE procedure [21–23,28,31,33,34] and only three articles exist in the literature concerning the determination of both sertraline and desmethyl-sertraline in whole blood using SPE [14,18,19]. Different organic solvents [diethyl ether, hexane, toluene, isooctane], mixtures of solvents [hexane:ethyl acetate (70:30 and 80:20 (v/v)), hexane:ethyl acetate:isopropanol (70:25:5 and 80:15:5 (v/v/v)), hexane:dichloromethane:ethyl acetate (2:1:1 (v/v/v))] and basic pH values (8.0, 9.0, 10.0 and 11.0) were tested for LLE during the development of the sample preparation, but LLE failed to produce sufficient extraction efficiency, clean extracts and base line free of interferences. When back extraction [26,31,33] was applied, the extraction efficiency for both analytes was found to be low (≤65%). Thus, SPE through different columns (Bond Elut LRC Certify and Certify II, Isolute HCX, and Abselut Nexus) were tested. Both Bond Elut LRC Certify II and Abselut Nexus gave low extraction efficiency (≤60%) for both analytes. The extraction efficiency for both sertraline and desmethyl-sertraline using mixed mode sorbent that combines strong cation exchange (benzene sulfonic acid) and nonpolar (C8) properties (Bond Elut LRC Certify and Isolute HCX) were reproducible, as sertraline and desmethyl-sertraline are basic (pK_a 9.5 and 9.4, respectively) with an amine function group so a cation exchange mechanism is plausible. When Isolute HCX columns tested the extracts were less clean and the extraction efficiency was constantly lower compared to Bond Elut LRC Certify. Bond Elut LRC Certify showed clear advantages over the other tested SPE columns as it ensured rapid, reproducible, simple, clean extraction and gave

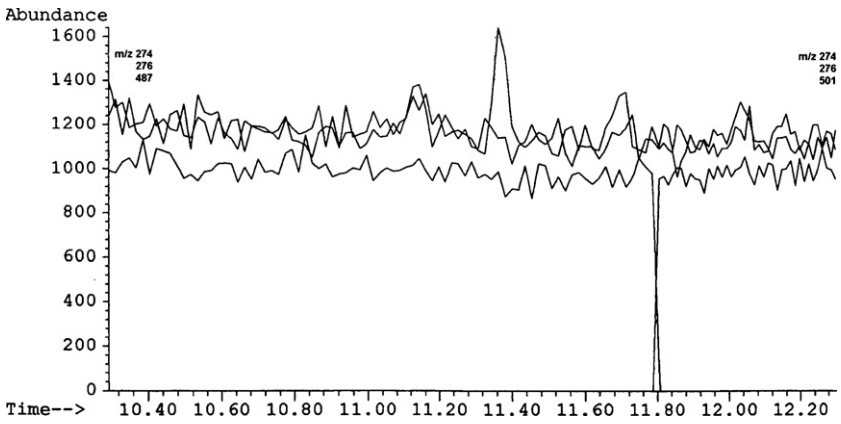

Fig. 2. SIM chromatogram of an extracted blank blood.

good sample purification and extraction yields of the analytes, so was chosen for our study. Different alkaline eluting mixtures of solvents dichloromethane:isopropanol:ammonium hydroxide (85:15:3 (v/v/v)); ethyl acetate:isopropanol:ammonium hydroxide (85:15:3 (v/v/v)); and ethyl acetate:acetonitrile:ammonium hydroxide (85:15:3 (v/v/v)) were tried. It was found that the eluting mixture of ethyl acetate:isopropanol:ammonium hydroxide (85:15:3 (v/v/v)) yielded a very good cleanup of the blood samples and gave high and reproducible extraction efficiency values.

3.2. Method validation

The combination of SPE and acylation proved to be useful for the determination of sertraline and desmethyl-sertraline blood concentrations, as no interference from endogenous and exogenous compounds was observed. Ion m/z 274 for both sertraline and desmethyl-sertraline, as well as m/z 459 for protriptyline, the internal standard, showed the desired sensitivity and were used as target ions for their determination in blood samples.

Various parameters of the method such as selectivity, specificity, sensitivity, extraction efficiency, linearity, accuracy, precision, robustness and stability were evaluated according to international criteria [35–37].

Spiked samples were assayed in replicate and the data represent the mean ± SD. SPSS (version 18) was used for the statistical analysis and the best fit was obtained with a weighting factor of $1/x^2$ for both sertraline and desmethyl-sertraline.

The lack of the interference from endogenous blood compounds (selectivity) was checked by analyzing six different blank human blood extracts with and without IS, while specificity study documented that blood concentrations of 1000 μg/L of the following drugs: amitriptyline, nortriptyline, fluoxetine, maprotiline, desmethyl-maprotiline, venlafaxine, desmethyl-venlafaxine, clomipramine, mirtazapine, desmethyl-mirtazapine, citalopram, penfloridol, clozapine, nor-clozapine, perphenazine, risperidone, thioridazine, carbamazepine, olanzapine, trifluoperazine, pipamperone, cocaine, benzoylecgonine, ecgonine methyl ester, morphine, codeine, 6-acetyl-morphine, paracetamol, methadone, lorazepam, oxazepam, bromazepam, diazepam, nordiazepam, temazepam, Δ^9-tetrahydrocannabinol, 11-nor-9-carboxy-Δ^9-tetrahydrocannabinol, 3,4-methylenedioxy amphetamine (MDA), 3,4-methylenedioxymethamphetamine (MDMA), methamphetamine, amphetamine, ephedrine, 7-amino-flunitrazepam, and alprazolam do not interfere with the determination of sertraline and desmethyl-sertraline in blood. Furthermore, no interferences were observed from the tested substances at the respective retention times of sertraline, desmethyl-sertraline and protriptyline, thus, the method was proven to be specific. The chromatogram of a blank sample is shown in Fig. 2.

Linearity was excellent with a correlation coefficient (R^2) exceeding 0.991. The linear dynamic range was 1.00–500.0 μg/L for both analytes. This range covers the sub-therapeutic, therapeutic (50–250 μg/L) and also the toxic levels (>290 μg/L) of sertraline [38]. The %RSD of the slopes during five consecutive days was found to be 4.8% and 7.6%, for sertraline and desmethyl-sertraline, respectively (Table 1).

The limits of detection (LOD) and quantification (LOQ) were subsequently determined by analysis of six spiked blood 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 it was 0.30 μg/L for both sertraline and desmethyl-sertraline. LOQ was determined to be the lowest drug concentration that can be quantified with acceptable accuracy and precision, which gave rise to chromatographic peak whose area was equal to 10 times the baseline noise. The LOQ was found to be 1.00 μg/L for both analytes. A chromatogram of a spiked sample at LOQ concentration is presented in Fig. 3.

The precision (coefficient of variation, %CV) and the accuracy (the percentage of difference from theoretical concentration, %Bias) of the developed method were evaluated at three quality control levels within analytes' linear range. Six replicates of each quality control concentration were assayed in one run for the within-day experiment (Pooled). Thirty replicates of each quality control concentration were assayed on five different days for the day-to-day experiment (ANOVA). The precision for sertraline and desmethyl-sertraline were less than 5.5% and 7.2%, respectively, whereas the accuracy was ranged from −6.67% to 2.20% and −6.33% to 2.88% for sertraline and desmethyl-sertraline, respectively (Table 1).

The extraction efficiency of both analytes at three quality control levels was estimated by comparing the area obtained from an amount of the analyte added and extracted from the biological matrix, with the areas obtained from the respective unextracted standards. The extraction efficiency values were found to be between 90.1(±5.8)% and 95.4(±3.0)% for sertraline, while they found to be between 84.9(±8.2)% and 107.7(±4.4)% for desmethyl-sertraline. A summary of the validation results of the developed method for the determination of sertraline and desmethyl-sertraline in blood is presented in Table 1.

Robustness of the entire method was studied by changing different parameters of the procedure such as (A) injection temperature (280 → 285 °C), (B) oven temperature rate (20 → 20.6 °C/min), (C) detector temperature (300 → 295 °C), (D) extraction pH (6.0 → 6.5), (E) derivatization temperature (50 → 55 °C), and (F) ratio of the elution mixture (ethyl acetate:isopropanol:ammonium hydroxide) (85:15:3 (v/v/v) → 80:15:3 (v/v/v)). The robustness was deter-

Table 1
Validation results of the developed method for the determination of sertraline and desmethyl-sertraline in blood.

Validation parameter	Sertraline	Desmethyl-sertraline
Linearity ($N=5$)		
Range (μg/L)	1.00–500.0	1.00–500.0
Slope: mean ± SD (%RSD)	0.0398 ± 0.0019 (4.8)	0.0441 ± 0.0034 (7.6)
R^2 (range)	0.992–0.996	0.991–0.997
Recovery ($N=6$)		
Concentration used (μg/L)	3.00	3.00
Extraction efficiency (%): mean ± SD	95.4 ± 3.0	107.7 ± 4.4
Concentration used (μg/L)	40.0	40.0
Extraction efficiency (%): mean ± SD	90.1 ± 5.8	101.7 ± 6.5
Concentration used (μg/L)	400.0	400.0
Extraction efficiency (%): mean ± SD	92.7 ± 9.5	84.9 ± 8.2
Within-day variation ($N=6$)		
Theoretical value (μg/L)	3.00	3.00
Measured value (μg/L): mean ± SD	2.86 ± 0.15	2.95 ± 0.16
Precision (%CV)	4.0	4.7
Accuracy (%Bias)	−4.80	−1.67
Theoretical value (μg/L)	40.0	40.0
Measured value (μg/L): mean ± SD	40.88 ± 0.91	40.4 ± 1.6
Precision (%CV)	3.6	5.1
Accuracy (%Bias)	2.20	1.00
Theoretical value (μg/L)	400.0	400.0
Measured value (μg/L): mean ± SD	402.9 ± 3.4	411.5 ± 7.1
Precision (%CV)	5.1	4.9
Accuracy (%Bias)	0.73	2.88
Day-to-day variation ($N=30$)		
Theoretical value (μg/L)	3.00	3.00
Measured value (μg/L): mean ± SD	2.80 ± 0.12	2.81 ± 0.17
Precision (%CV)	4.0	6.0
Accuracy (%Bias)	−6.67	−6.33
Theoretical value (μg/L)	40.0	40.0
Measured value (μg/L): mean ± SD	40.4 ± 1.5	40.6 ± 2.3
Precision (%CV)	3.7	5.8
Accuracy (%Bias)	1.11	1.44
Theoretical value (μg/L)	400.0	400.0
Measured value (μg/L): mean ± SD	395 ± 21	403 ± 28
Precision (%CV)	5.5	7.2
Accuracy (%Bias)	−1.27	0.76
Limit of quantification ($N=6$)		
Theoretical value (μg/L)	1.00	1.00
Measured value (μg/L): mean ± SD	1.007 ± 0.060	1.016 ± 0.077
Precision (%CV)	6.0	7.7
Accuracy (%Bias)	0.70	1.60

mined from four replicates of spiked samples at the medium quality control level for each parameter (A–F). The mean effect of each parameter is the average difference between normal and alternative values of the same parameter. Mean effects of each parameter and standard deviations of all differences were calculated [37]. Neither a single parameter nor a combination of the ones changed, showed a significant influence on the results of the method, which was proven to be sufficiently robust, as the mean effect and standard deviation values were found to be adequately low.

Stability study was assessed by analyzing six spiked blood samples with sertraline and desmethyl-sertraline at medium quality control level at different storage conditions including 4 °C for 1 day, 7 days, and 14 days; −20 °C for 14 days, 30 days, and 90 days; three freeze/thaw cycles (−20 °C for 24 h/room temperature for 4 h), and derivatized sertraline and desmethyl-sertraline in the autosampler for over 24 h. The results were expressed as relative error (%) which was calculated by comparing the determined concentrations under specific storage conditions with that of the freshly prepared samples. Analytes were considered to be stable if the relative error % of the mean test concentration were lower than 15%. The data of stability demonstrated that sertraline was stable in blood for 30 days and desmethyl-sertraline for 14 days under −20 °C with relative errors −14.6% and −14.2%, respectively. Under 4 °C, sertraline was stable in blood for 7 days and desmethyl-sertraline for 1 day with relative errors −13.9% and −11.1%, respectively. Furthermore, both sertraline and desmethyl-sertraline were stable in blood after three freeze/thaw cycles with relative errors −4.6% and −11.6%, respectively. Finally, both sertraline–HFB and desmethyl-sertraline–HFB were also proved to be stable for over 24 h in the autosampler with relative error lower than −7.5%.

The developed method is fully validated and shows comparable or enhanced validation results, when compared with previously described methods for the determination of sertraline and desmethyl-sertraline in blood. It also shows significant selectivity, specificity and robustness, as well as satisfactory accuracy and precision results. The extraction efficiency of our method is higher than other published methods using whole blood samples [18,19,28] and the calibration range covers sub therapeutic, therapeutic and toxic levels. The proposed method is highly sensitive, although some previously published methods [13,24,25,29,32] show quite low LOQ for sertraline in plasma (0.1–0.5 μg/L). These methods cover a very narrow concentration range of the drug while some of them [25,29,32] use LC/MS/MS.

The proposed GC/MS method can solve already existing analytical problems in such cases and it satisfies sensitivity requirements by using low cost elementary equipment, available at common clinical or forensic laboratories that perform everyday routine analysis. The developed method can be used for the determination of sertraline and desmethyl-sertraline in blood samples during phar-

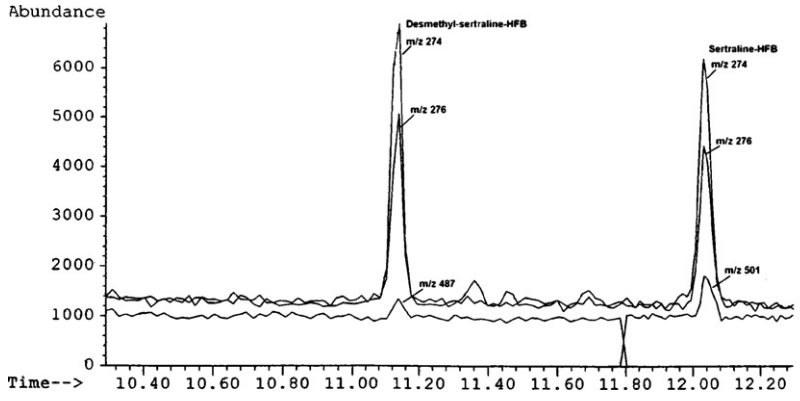

Fig. 3. SIM chromatogram of a spiked blood sample at LOQ concentration (1.00 μg/L) for both sertraline and desmethyl-sertraline (internal standard not shown).

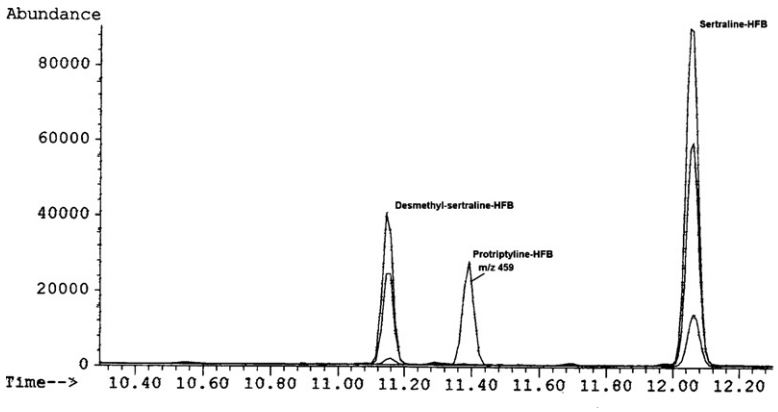

Fig. 4. SIM chromatogram of a real blood sample related to the forensic case 1 (sertraline: 182.1 μg/L and desmethyl-sertraline: 64.4 μg/L).

macokinetic studies, for therapeutic drug level monitoring when adjustment of sertraline dosage is required or for the investigation of forensic cases.

3.3. Method application to the clinical and forensic whole blood samples

The developed method was applied for qualitative and quantitative analysis of sertraline and desmethyl-sertraline in clinical and forensic cases. The determination of sertraline and desmethyl-sertraline levels was carried out on the whole blood of patients undergoing monotherapy or polypharmacy and also in cases of forensic interest. All blood samples were collected and kept until their analysis in test tubes containing anti-coagulant (EDTA K_3). None of these samples posed any problem in the determination of the analytes, and no matrix interferences were observed in any of them.

The concentrations of sertraline and desmethyl-sertraline in the blood samples were calculated according to the relative calibration curves. A representative chromatogram is shown in Fig. 4 (Forensic case 1).

3.4. Clinical cases

Case 1. A 29 years old man was taking therapeutically 100 mg sertraline once a day for more than one year. A blood sample was taken 24 h after the last drug intake and sent to our department for therapeutic drug monitoring. According to our quantitative analysis using the developed method, the blood concentrations of sertraline and desmethyl-sertraline were found to be 25.3 and 73.8 μg/L, respectively. The blood levels of sertraline and desmethyl-sertraline are in agreement with those reported in the literature [39] and were found to correspond well to the clinical response of the patient.

Case 2. A blood sample was taken 3 days after the last drug intake from a 42 years old woman taking 100 mg sertraline daily for more than one month. According to our quantitative analysis by the developed method, the blood concentrations of sertraline and desmethyl-sertraline were found to be 12.4 and 79.2 μg/L, respectively. The results proved that sertraline and its metabolite were able to be determined in blood using the developed method 3 days after the last drug intake. This is in agreement with literature which mentions that sertraline has an elimination half-life of 22–36 h, while desmethyl-sertraline has a 3 times longer half-life (60–100 h) [38,39].

Case 3. A 29 years old man was taking 200 mg sertraline daily for more than one week. The blood sample was taken 3 h after the last drug intake and sent to our department for therapeutic drug monitoring as the patient showed symptoms such as sleepiness, nervousness, dizziness and nausea. According to our analysis the blood concentrations of sertraline and desmethyl-sertraline were 85.1 and 136.9 μg/L, respectively. The blood levels of sertraline and desmethyl-sertraline were within the therapeutic range [38] and in agreement with those reported in the literature [39]. The symptoms were considered as side effects from sertraline and a dose reduction to 100 mg daily was suggested by the clinicians.

3.5. Forensic cases

Case 1. A woman tried to commit a suicide by ingestion of Zoloft® (Sertraline Hydrochloride). Two empty boxes were found close to her and she was admitted to the hospital where gastric lavage was performed immediately during which a great amount of disintegrated tablets were removed. A blood sample was taken about 3 h after the drug ingestion and was sent to our department to quantify the sertraline. The sample was analyzed immediately. According to our quantitative analysis using the developed method, the blood concentrations of sertraline and desmethyl-sertraline were 182.1 and 64.4 μg/L, respectively. After the patient recovered, she confessed that she had ingested 20 tablets of 100 mg (one and half boxes) which corresponds to oral ingestion of 2 g of sertraline. The therapeutic range of sertraline in plasma is 50–250 μg/L [38]. The observed sertraline concentration in this case was within the therapeutic range which means that the major part of the ingested amount of sertraline was removed by the gastric lavage.

Case 2. A 39 years old man died unexpectedly in his house. Autopsy showed no pathological findings. According to our comprehensive toxicological screening protocol, sertraline, desmethyl-sertraline, mirtazapine, citalopram, venlafaxine, biperidine, diazepam, nordiazepam, levomepromazine, and chlorpromazine were found in the blood sample. The concentrations of the above drugs were within their respective therapeutic range except the observed sertraline concentration that was considered toxic. More specifically, the concentrations of sertraline and desmethyl-sertraline in the blood sample were 459.3 and 1512 μg/L, respectively. This measurement was made to a diluted blood sample (1:4 (v/v)). The toxic levels of sertraline in plasma range from 290 to 1900 μg/L [38]. During the investigation of the case there were no indications of suicide. The toxic levels of sertraline suggest that the concomitant use of the other detected drugs may resulted in a modification of the pharmacokinetics of sertraline leading this way to high blood concentrations that although toxic could not be considered as lethal. A synergistic effect between the drugs taken could not be excluded. Postmortem redistribution of sertraline was not considered as the concentrations of the other

detected drugs were within their therapeutic range. Death was clarified as sertraline poisoning.

Cases 3 and 4. A 48 years old woman and a 50 years old man were found dead in their homes without any signs of violence or needle marks on the corpses. Autopsy of the woman revealed a myocardial infarction while autopsy of the man revealed a cerebral hemorrhage. Both findings were confirmed histopathologically. Stomach content, blood and urine samples were sent to our department for toxicological analysis. The samples were analyzed. According to our comprehensive toxicological screening protocol, sertraline and desmethyl-sertraline were detected in both cases. The concentrations of sertraline and desmethyl-sertraline in the blood sample of the woman were found to be 102.9 and 149.9 μg/L, correspondingly, and that in the man's blood sample were found to be 149.1 and 293.7 μg/L, respectively. The observed sertraline concentrations in these two cases were within the therapeutic range [38], demonstrating that sertraline was not the cause of death. The manner of death for both cases was considered to be natural.

4. Conclusions

A simple, rapid, sensitive and specific method has been developed, optimized and validated for the determination of both sertraline and desmethyl-sertraline in whole blood using GC/MS. Sample preparation includes SPE using Bond Elut LRC Certify (mixed-mode columns) and derivatization with HFBA.

The presented method provides significant advantages with regard to other related methods previously published. It shows high sensitivity, has a wide concentration range and is applied to whole blood samples. Its reliability was certified by means of an exhaustive validation study.

The developed method was successfully applied to qualitative and quantitative analysis of sertraline and desmethyl-sertraline in routine analysis of actual clinical and forensic cases. The proposed method is suitable for the determination of sertraline and desmethyl-sertraline in whole blood samples for pharmacokinetic studies, for therapeutic drug level monitoring in order to adjust sertraline dosage of sertraline patients or for the investigation of forensic cases.

Acknowledgements

The authors would like to acknowledge the scientific consultation of Assoc. Prof. C. Maravelias. This study was accomplished with the valuable contribution of Assoc. Prof. G. Alevisopoulos and the research was financially supported by the Special Research Account of the University of Athens.

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