Simultaneous Determination of Viloxazine, Venlafaxine, Imipramine, Desipramine, Sertraline, and Amoxapine in Whole Blood: Comparison of Two Extraction/Cleanup Procedures for Capillary Gas Chromatography with Nitrogen-Phosphorus Detection

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Abstract

A comparative study for the simultaneous gas chromatographic (GC) resolution and detection of the six antidepressants viloxazine, venlafaxine, imipramine, desipramine, sertraline, and amoxapine in whole blood at concentration levels of 100–2000 ng/mL was developed. Two extraction/cleanup analytical procedures were compared regarding their recovery, precision, sensitivity and matrix purification efficiency. The first procedure consists of the employment of Chem Elut columns (diatomaceous earth) and is based on the principle of liquid–solid absorption extraction that is closely related to conventional liquid–liquid extraction. The second focuses on the use of Bond Elut Certify columns and a mixed SPE, reversed-phase and cation-exchange sorbent, more recently developed for the market. Each procedure required 2.0 mL of whole blood extraction and injection into a capillary GC equipped with a nitrogen-phosphorus detector. Mepivacaine was used as the extraction standard (surrogate), and prazepam was used as the chromatographic standard. No interferences were found, and the time for the chromatographic analysis was 16 min for one sample. Recoveries of the compounds using Chem Elut columns at 500 ng/mL were in the range of 28–74% with intra-assay and interassay precisions of less than 7% and 19%, respectively. Limits of detection (LOD) and quantitation (LOQ) ranged from 39 to 153 ng/mL and from 128 to 504 ng/mL, respectively. Recoveries of the compounds using Bond Elut Certify columns at 500 ng/mL were in the range of 64–86% with intra-assay and interassay precisions of less than 4% and 10%, respectively. LODs and LOQs ranged from 21 to 100 ng/mL and from 70 to 330 ng/mL, respectively. An excellent linearity was observed with both procedures from the LOQs up to 2000 ng/mL. The use of the reversed-phase and cation-exchange sorbent Bond Elut Certify showed advantages compared with Chem Elut columns for the screening of these antidepressants such as higher recoveries, cleaner extracts, better sensitivity, better precision, and less solvent consumption and disposal.

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Introduction

In systematic toxicological analysis (STA) one of the main purposes is screening analysis. Antidepressants make up an important class of drugs in forensic and clinical cases. These drugs are widely used for the treatment of a variety of depressive states and other psychiatric disorders. In addition, antidepressants are among the most commonly encountered causes of self-poisoning.

The general scheme for determining drugs in a biological matrix is usually divided into two stages: sample pretreatment and isolation followed by the actual determination of the drugs. Solid-phase extraction (SPE) is a powerful technique for the pretreatment of biological samples for clinical and toxicological drug analysis. SPE offers several advantages over traditional liquid–liquid extraction (LLE) such as higher selectivity, cleaner extracts, more reproducible results, and the prevention of emulsion formation (1). However, the major applications of SPE have been limited to plasma, serum, and urine (2–7). Until now, only a few publications have described SPE methods for whole blood (8–14). However, in practice, whole blood is the sample encountered most frequently in forensic cases.

Capillary gas chromatography with nitrogen-phosphorus detection (GC–NPD) has proven to be a powerful tool in the area of underivatized drug analysis. NPD is the most convenient technique for screening toxicological analysis because of its outstanding sensitivity for the detection of traces of drugs and negligible interference from non-nitrogenous compounds, both endogenous and exogenous (15).

This method allowed the simultaneous determination of six antidepressants belonging to the main classes of these drugs: tricyclic antidepressants (TCAs) and selective serotonin reuptake inhibitors (SSRIs). We compared two extraction/cleanup analytical procedures: antidepressants were submitted in

parallel for SPE with Chem Elut and Bond-Elut Certify columns without derivatization. Mepivacaine and prazepam were used as the extraction standard and the chromatographic standard, respectively.

The chemical structures of the six antidepressants studied in this work, viloxazine, venlafaxine, imipramine, desipramine, sertraline, and amoxapine, and the compounds used in this work to control the whole analytical procedure, mepivacaine and prazepam, are presented in Figures 1 and 2.

This paper presents a rapid and sensitive method that allows the determination of blood therapeutic and toxic concentrations of the antidepressants viloxazine, venlafaxine, imipramine, desipramine, sertraline, and amoxapine as part of STA comparing Chem Elut and Bond-Elut Certify columns and GC–NPD analysis without derivatization.

Experimental

Materials

All chemicals (Merck, Darmstadt, Germany) and solvents (Scharlau, Barcelona, Spain) were of analytical grade. The tested drugs were obtained from commercial suppliers and were of pharmaceutical quality. Borate buffer (pH 9.0) and dichloromethane/isopropanol (85:15) were used for the diatomaceous earth (Chem Elut) procedure. Phosphate buffer (0.1M, pH 6.0), 0.01M acetic acid, acetone/dichloromethane (1:1), and dichloromethane/isopropanol/ammonia (78:14:8) were used for the mixed-mode bonded silica (Bond Elut Certify) procedure. Individual stock solutions (1 mg/mL) were prepared by dissolving the appropriate amount of each drug in methanol. These stock solutions were stored in glass tubes at 4°C. The extraction standard (surrogate) solution was prepared by diluting the stock solution of mepivacaine with deionized water to 16 µg/mL. The chromatographic standard solution was prepared by diluting the stock solution of prazepam with methanol to 5 µg/mL.

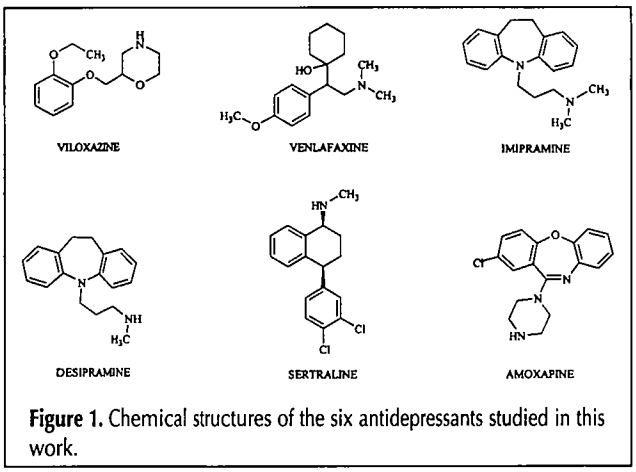

Figure 1. Chemical structures of the six antidepressants studied in this work.

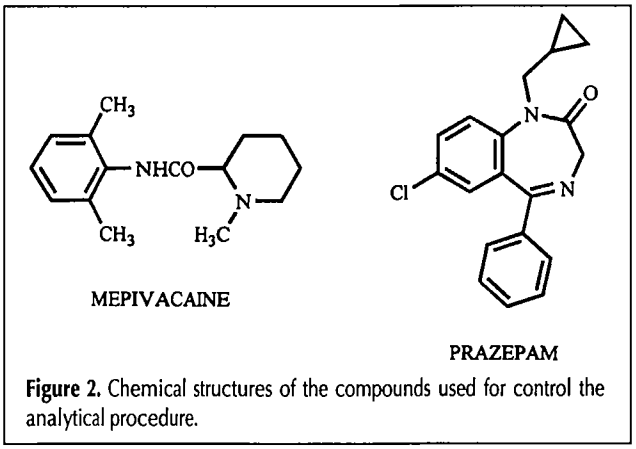

Figure 2. Chemical structures of the compounds used for control the analytical procedure.

Chem Elut CE 1010 columns (10 mL of column reservoir volume) and Bond Elut Certify columns (130 mg of sorbent mass, 3 mL of column reservoir volume) were both supplied by Varian Sample Preparation Products (Harbor City, CA).

A pool of citrated human whole blood samples was obtained from Hospital 12 de Octubre (Madrid, Spain) and verified to be drug free. No interferences were found for the studied compounds and the samples were kept frozen at –20°C until used. The blood was spiked with appropriate drugs. The concentration of each drug in spiked samples was 0.1, 0.5, and 2 µg/mL, respectively.

Instrumentation

A VAC-ELUT SPS 24 vacuum manifold system for the manual mixed-mode bonded silica SPE was purchased from Varian. A P-Selecta sonication bath and a P-Selecta Centronic S centrifuge were both obtained from Selecta (Barcelona, Spain).

The chromatographic analysis of the extracts was performed on a Hewlett-Packard (HP) (Avondale, PA) model 5890 series II GC equipped with an NPD and linked to an HP 3396A integrator. A 25-m × 0.20-mm i.d. fused-silica capillary column coated with cross-linked methylsilicone (0.11-µm film thickness) was employed. The carrier gas was helium (Air Liquid, Madrid, Spain) delivered at a column head pressure of 195 kPa. Injection port and detector temperatures were 280°C and 300°C, respectively. The splitting ratio was 1:20. The column temperature was initially held at 180°C for 1 min and then increased to 300°C at 10°C/min. The final temperature was held for 3 min. The total chromatographic time, including 2 min of equilibration time, was 18 min. Insert liners silanized with dimethyldichlorosilane/toluene (5:100) and packed with Supelco silanized glass wool (Supelco Park, Bellefonte, PA) were used.

Samples

Human whole blood (100 mL) spiked with the appropriate drugs at three concentration levels 0.1, 0.5, and 2 µg/mL, was sonicated in a sonic bath for 15 min at room temperature. The spiked blood samples were extracted in parallel by solid-phase extraction using Chem Elut and Bond Elut Certify columns.

Extraction procedure using Chem Elut columns

The extractions were performed using a procedure based on that described by Breiter et al. (16) and Logan et al. (17) that has been optimized for general use in this laboratory for screening basic drugs. To each aliquot (2 mL), 7.0 mL of borate buffer (pH 9.0) and 100 µL of a mepivacaine aqueous solution of 16 µg/mL as extraction standard (surrogate) were added,

vortex mixed for 5 min, and loaded onto the Chem Elut columns. After 5 min, elution was carried out by adding two aliquots of 10 mL of dichloromethane/isopropanol (85:15). Eluates were evaporated under a nitrogen stream. The extraction residues of blood were reconstituted with 200 µL of methanolic solution of chromatographic standard (5 µg/mL), and 2 µL was injected for GC analysis.

Extraction procedure using Bond Elut Certify columns

The extraction was performed on a VAC-ELUT SPS 24 vacuum manifold system assembled with Bond Elut Certify columns. Extractions were performed using a procedure based on that described in the Bond Elut Certify instruction manual (5) and by Chen et al. (6,14), which has been optimized for general toxicological screening use in this laboratory. To each 2.5-mL aliquot, 7.5 mL of phosphate buffer (pH 6.0), and 125 µL of a mepivacaine aqueous solution of 16 µg/mL as extraction standard (surrogate) was added, sonicated for 5 min, and centrifuged at 4000 rpm for 10 min, then 8 mL of the supernatant, equivalent to 2 mL of whole blood, was used for further extraction (12).

The columns were preconditioned with 1 mL methanol, followed by 1 mL 0.1M phosphate buffer (pH 6.0) under light vacuum (approximately 2 in. Hg) to avoid the columns becoming dry before the application of the sample. Then the samples of pretreated whole blood were applied onto the columns and drawn through completely at a flow rate of approximately 1.5 mL/min. The columns were washed with 2 mL deionized water. The columns were acidified by passing through 0.5 mL of 0.01M acetic acid. Then the columns were dried under full vacuum (15 in. Hg) for 4 min. Methanol (60 µL) was added, and the columns were dried under full vacuum for 1 min.

After the column outlets were wiped with tissue, the labelled evaporation tubes recently rinsed with methanol, in order to avoid retention of polar drugs in the walls of the glass tubes, were placed into the manifold basin. To each column, first 3.5 mL of acetone/dichloromethane (1:1) was added, and after 3 mL of dichloromethane/isopropanol/ammonia (78:14:8). The eluents were pulled through completely at flow rates of 0.8 mL/min and 0.5 mL/min, respectively, and the combined eluates were evaporated under a nitrogen stream. The extraction residues of blood were reconstituted with 200 µL of methanolic solution of chromatographic standard (5 µg/mL), and 2 µL was injected for GC analysis.

Validation of the methods

Calibration curves were prepared with standard solutions of each antidepressant. The concentrations were 0.5, 1, 5, 15, and 30 µg/mL and the concentration of prazepam (chromatographic standard) was fixed at 5 µg/mL. The peak-area ratio (peak area antidepressants to that of prazepam) was measured, and the calibration curves were generated from least-squares linear regression. The regression lines were used to calculate the absolute recoveries (n = 6) of individual antidepressants from spiked blood at three concentration levels.

The intra-assay precision was assessed at three concentration levels by the extraction and analysis on the same day using six spiked blood samples for each level. The interassay precision was assessed by analyzing a set of nine spiked blood samples at a concentration of 0.5 µg/mL on two different days.

The limits of detection and quantitation were determined as the lowest concentration giving a response of 3 times and 10 times, respectively, the average of the baseline noise defined from six control samples.

The linearity of the method for each compound was checked by preparing six replicates of the calibration curves at three different concentrations, ranging from 100 to 2000 ng/mL, by addition of known amounts of each drug to human whole blood.

Results and Discussion

General considerations regarding extraction procedures

The principle of SPE using Chem Elut columns (diatomaceous earth) is closely related to conventional LLE. It involves the absorption of the aqueous phase on the diatomaceous earth, a porous material which acts as a support for the aqueous phase. This provides a large surface area for partition into an eluting solvent, which flows through the immobilized specimen under gravity, eluting the analytes of interest (16). Theoretically superior recoveries are expected due to the continuous process of elution compared to LLE. Other advantages are the elimination of centrifugation, aspiration, and filtration steps and the prevention of emulsion formation. However, large volumes of hazardous organic solvents are required. For STA purposes, where acidic, neutral, and basic substances may be present, this type of SPE must be carried out with at least two columns: one for the acidic and neutral substances and one for basic and neutral substances (1). In our case, pH 9.0 was chosen to test antidepressants in Chem Elut columns because dissociation constants for these compounds are higher than 8 (18,19) and pH values near the isoelectric point, where the number of molecules without net charge (zwitterionic form) is maximum, are the most suitable for LLE as well as for SPE with nonpolar sorbents.

Chemically modified silica with either hydrophobic groups or with ion exchange groups can only bind one type of substance. Mixed-mode bonded silica (Bond Elut Certify) can retain at a suitable pH acidic and neutral substances by hydrophobic interactions with the alquil chains (octylsilane, n-C_8) and the basic substances by interaction with the cation exchange groups (benzenesulphonylpropylsilane) using one column. Sample pH is adjusted to 6.0 with the purpose of minimizing the number of compounds that could be coextracted with the target analytes by a non-polar retention mechanism. The studied antidepressants are in the ionic form at pH 6.0, and the octyl chain is able to retain these analytes by nonpolar interactions with aliphatic terminations. Simultaneously, a cationic exchange retention mechanism acts at this pH because all the target analytes are positively charged, both mechanisms increase the process selectivity, as only positively charged analytes will interact by electrostatic attraction with the negatively charged benzenesulfonic group. The octyl chain is said to supply superior selectivity compared to the most widely used reversed-phase, octadecylsilane, in the field of medium polarity molecules. The slight rise in polarity of the octyl chain, compared to the

octadecyl chain, allows many contaminants that would probably be retained on the C_{18} sorbent to break through on the C_8 sorbent. Dichloromethane/isopropanol/ammonia disrupts the ionic exchange interactions, the main interactions, and also the hydrophobic interactions, secondary interactions, through the neutralization of the antidepressants amino groups that are otherwise positively charged in acidic pH.

In the field of toxicology, blood specimens available for analysis are sometimes very small. Problems arise if a full-drug toxicological screening is required and all drugs of interest must be

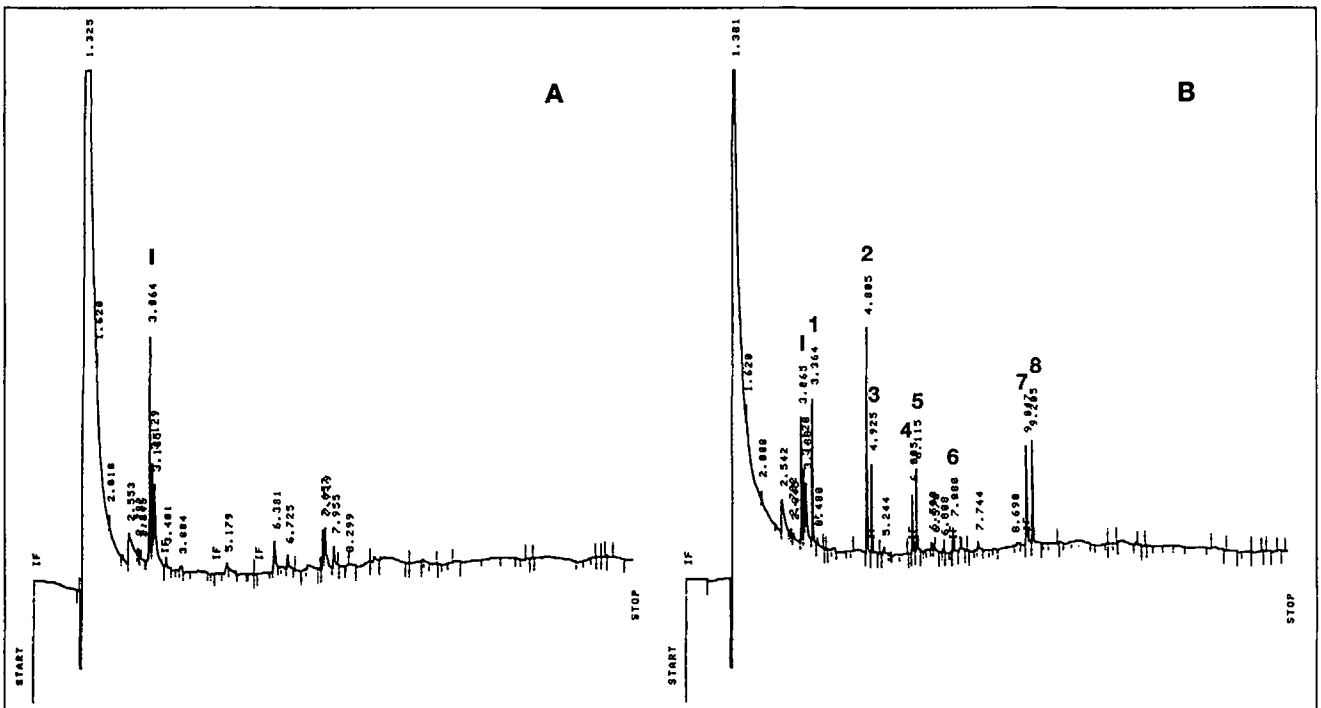

Figure 3. Representative GC–NPD chromatograms of blank human whole blood (A) and human whole blood spiked with 0.5 µg/mL of each antidepressant (B) after extraction using Chem Elut columns. Peak identification: I, caffeine; 1, viloxazine; 2, mepivacaine (extraction standard); 3, venlafaxine; 4, imipramine; 5, desipramine; 6, sertraline; 7, amoxapine; and 8, prazepam (chromatographic standard).

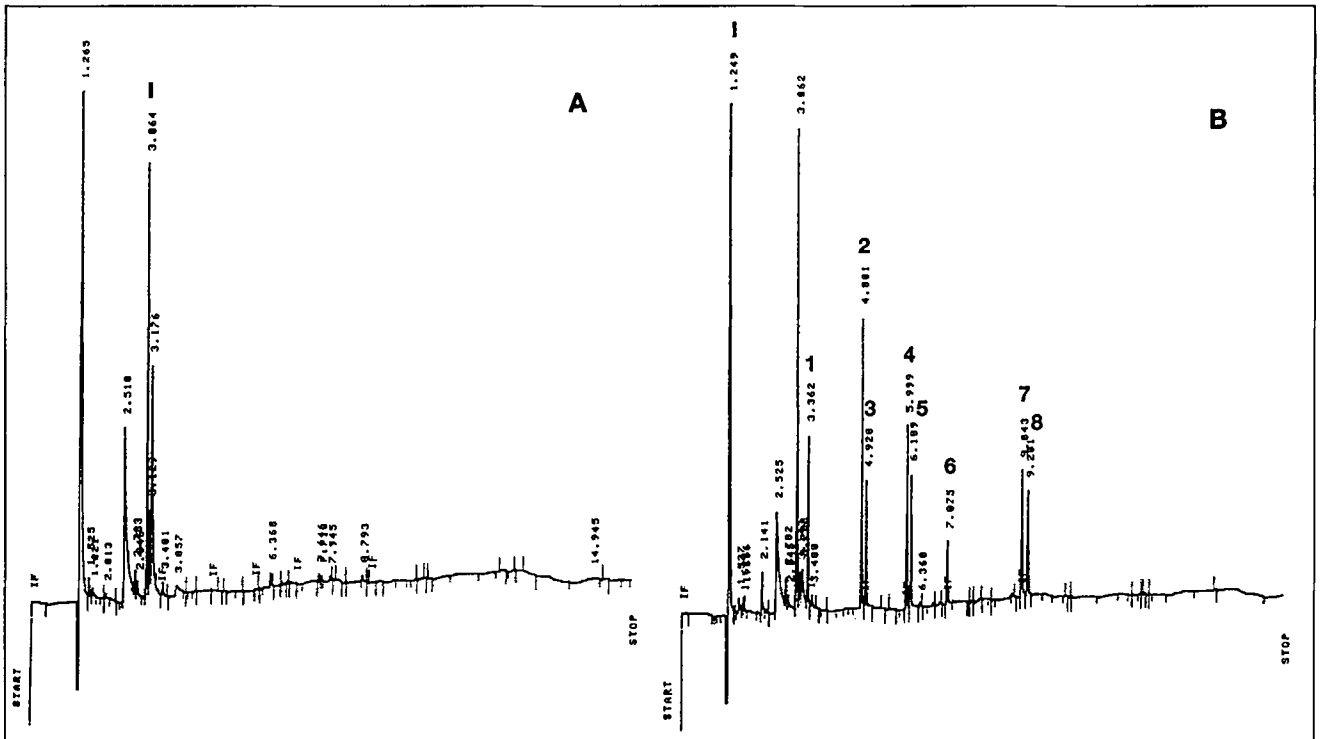

Figure 4. Representative GC–NPD chromatograms of blank human whole blood (A) and human whole blood spiked with 0.5 µg/mL of each antidepressant (B) after extraction using Bond Elut Certify columns. Peak identification: I, caffeine; 1, viloxazine; 2, mepivacaine (extraction standard); 3, venlafaxine; 4, imipramine; 5, desipramine; 6, sertraline; 7, amoxapine; and 8, prazepam (chromatographic standard).

extracted from the same aliquot of specimen. In such cases, the use of a general extraction procedure of acidic, neutral, and basic substances using one column is a great advantage.

Chromatography

Good chromatographic results were obtained from both extraction procedures. Figures 3 and 4 show representative GC–NPD chromatograms of extracted human blood for each method. Reliable separations of the six antidepressants and the chromatographic standard were obtained using the chromatographic conditions described in short chromatographic times (16 min). Excellent chromatographic behavior with good peak shapes was shown for these secondary and tertiary amine containing compounds without derivatization. A crucial point in obtaining good chromatographic behavior was found to be the injection system. Special care must be taken in silanizing the glass wool and the insert liner. Furthermore, no more than 100 injections should be performed without replacing the insert liner to prevent deterioration of the chromatographic system. Both procedures provided extracts free of chromatographic interferences in the areas corresponding to the retention time of the studied compounds. Caffeine was detected in the chromatograms because coffee is a common beverage; nevertheless, it did not interfere with the studied drugs.

Mepivacaine was added as extraction standard (surrogate) to control the extraction procedures. Mepivacaine is an uncommonly prescribed drug. According to our toxicological analytical experience, it has a very good extraction behavior (the recovery obtained for this standard was 100% with a relative standard deviation of 5%) in both extraction procedures, and it does not interfere with a large variety of abuse and prescribed drugs.

Prazepam was chosen as chromatographic standard because of its similar molecular structure and physicochemical behavior through the analytical procedure. It is not widely used or prescribed. The chromatographic standard was added after the extraction, but prior to the introduction of the extract into the GC, in order to correct for difference in the final volume of the concentrated extract and injection volumes and to check chromatographical behavior. The use of mepivacaine as the extraction standard (surrogate) and prazepam as the chromatographic standard allows us to control the whole analytical procedure during the routine performance of a screening toxicological analysis.

The temperature program utilized in this study was developed in our laboratory for general and routine use in order to obtain a good separation of a large variety of drugs during the toxicological screening. NPD is a temperature-dependent detector; its sensitivity varies with the change of temperature. In order to minimize this influence, the temperature program was utilized over a relative small range (180–300°C) and oven temperature was increased at 10°C/min, as a result, an excellent linearity was observed with both extraction procedures.

The nitrogen-containing structures of viloxazine, venlafaxine, imipramine, desipramine, sertraline, and amoxapine together with the use of SPE columns are responsible for the high sensitivity obtained using an NPD, as well as for the minimization of interferences.

Table I. Extraction Recovery (%), Intra-assay and Inter-assay Precision (RSD %), Linearity (r^2), and Limits of Detection (LODs) and Quantitation (LOQs) for the Six Antidepressants in Whole Blood using Chem Elut and Bond Elut Certify Columns and Analyzed by GC–NPD

Compound	Recovery (%) Mean (0.5 µg/mL) (n = 6)	Intra-assay precision RSD (%) (n = 6) (0.5 µg/mL)	Intra-assay precision RSD (%) (n = 9) (0.5 µg/mL)	Linearity r^2 (n = 6)	Limit of detection LOD (ng/mL) (n = 6)	Limit of quantitation LOQ (ng/mL) (n = 6)
Viloxazine						
Chem Elut	74	5	13	0.999	70	231
Bond Elut Certify	79	4	5	0.999	23	76
Venlafaxine						
Chem Elut	66	4	10	0.999	39	128
Bond Elut Certify	86	3	7	0.999	24	79
Imipramine						
Chem Elut	33	7	15	0.999	67	222
Bond Elut Certify	82	3	3	0.999	21	70
Desipramine						
Chem Elut	43	2	16	0.999	103	340
Bond Elut Certify	66	3	10	0.999	39	130
Sertraline						
Chem Elut	28	6	19	0.999	153	504
Bond Elut Certify	64	3	8	0.999	100	330
Amoxapine						
Chem Elut	52	4	15	0.999	62	204
Bond Elut Certify	69	2	6	0.999	42	140

Validation

Table I shows a summary of the most representative analytical parameters studied in this research. Recoveries of the compounds using Chem Elut columns at 500 ng/mL were in the range 28–74%, with intra-assay and interassay precision values (RSD) less than 7% and 19%, respectively. Recoveries of the compounds using Bond Elut Certify columns at 500 ng/mL were in the range 64–86%, with intra-assay and interassay precision values less than 4% and 10%, respectively. The use of the reversed-phase and cation exchange sorbent Bond Elut Certify represents an improvement with respect to Chem Elut columns for the screening of these antidepressants such as higher recoveries, cleaner extracts, better precision, and less solvent consumption and disposal.

The nitrogen-containing structures of viloxazine, venlafaxine, imipramine, desipramine, sertraline, and amoxapine together with the use of SPE columns are responsible for the high sensitivity obtained using an NPD, as well

as for the minimization of interferences. The respective LODs and LOQs using Chem Elut columns ranged from 39 to 153 ng/mL and from 128 to 504 ng/mL. The respective LODs and LOQs using Bond Elut certify columns ranged from 21 to 100 ng/mL and from 70 to 330 ng/mL. The use of Bond Elut Certify reversed-phase and ion-exchange sorbent resulted in better LODs and LOQs comparing with the Chem Elut columns extraction procedure.

Table II. List of Therapeutic, Toxic, and Fatal Levels for the Studied Antidepressants Published in the Literature

Antidepressant	Therapeutic level (mg/mL)	Toxic level (mg/mL)	Fatal level (mg/mL)	References
Viloxazine (plasma)	1.3	N.A.*	45	(20)
Venlafaxine (plasma)	0.07	N.A.	N.A.	(19)
Imipramine (blood)	0.09–0.126	0.1–3.2	0.85–13.1	(21)
Desipramine (blood)	0.011–0.11	0.4–1.5	3.8–16.8	(21)
Sertraline	0.03–0.21 (plasma)	N.A.	0.61 (blood)	(19)
Amoxapine (serum)	0.01–0.2	N.A.	3	(22)

* N.A.: not available.

Table II shows a list of therapeutic, toxic, and fatal antidepressant levels in blood, plasma, or serum, published in the literature. Not all of the antidepressant levels are available in blood because most of the studied drugs belong to the new generation of antidepressants introduced in the market, and nowadays there is still lack of information. The utility of the method is demonstrated comparing the literature levels with the LODs obtained in the study.

The linearity of the methods was satisfactory over the range 100 to 2000 ng/mL The calibration curves obtained using quadratic regression gave the best correlation coefficients $r^2 > 0.999$ ($n = 6$) for all tested drugs.

Case Example

Figure 5 shows the chromatograms of Chem Elut and Bond Elut Certify extracts of a blood sample obtained of a forensic autopsy. The deceased was a 67-year old man who was fatally injured in a road crash. After screening with the methods

Table III. Case Example

Extraction procedure	Blood concentrations in mg/mL	
	Venlafaxine	*O*-Desmethylvenlafaxine
Chem Elut	0.4	0.7
Bond Elut Certify	0.5	0.7

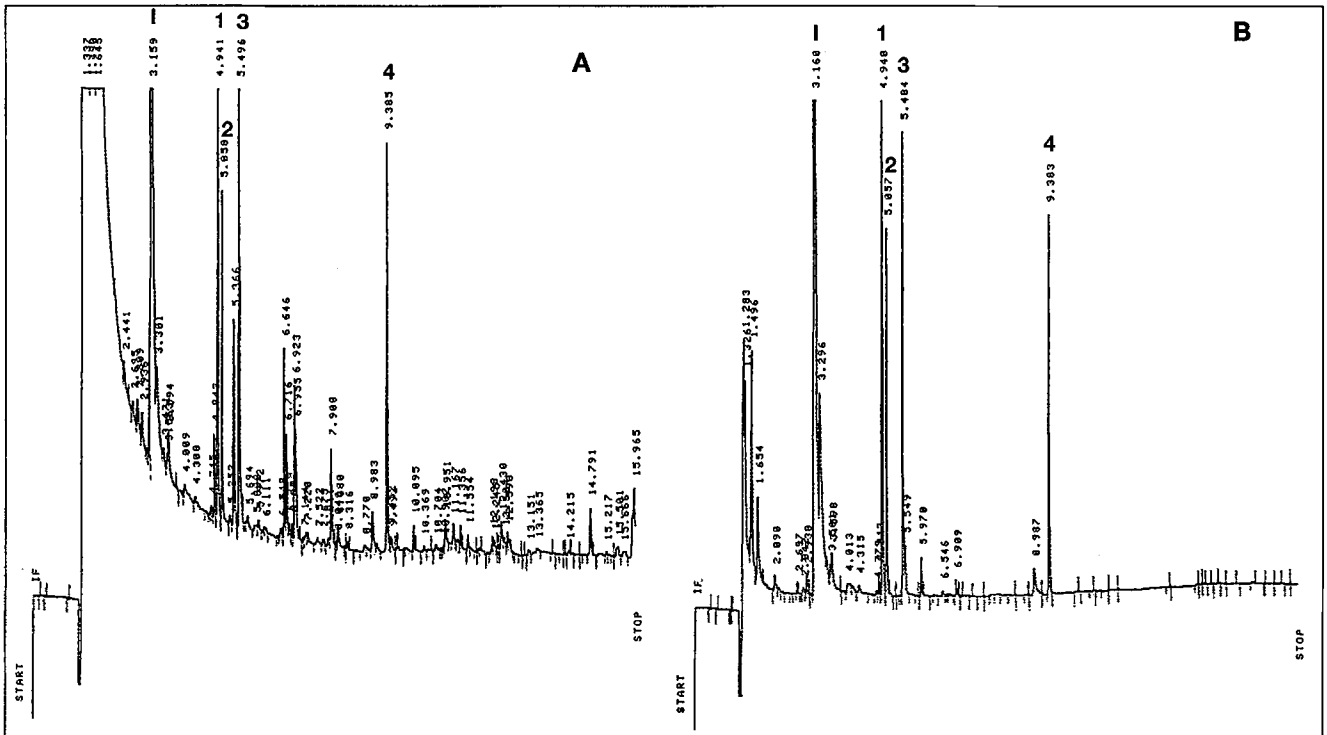

Figure 5. GC–NPD chromatograms of blood obtained at a forensic autopsy after extraction using (A) Chem Elut and (B) Bond Elut Certify columns, respectively. Peak identification: I, caffeine; 1, mepivacaine (extraction standard); 2, venlafaxine; 3, *o*-desmethylvenlafaxine; and 4, prazepam (chromatographic standard).

described here, venlafaxine and *o*-desmethylvenlafaxine (its active metabolite) at a therapeutic concentration (19) were detected and quantitated using GC–NPD and confirmed using GC–MS mode TIC. The man was driving under the influence of this prescription drug. Spanish law forbids driving under the influence of drugs with central nervous system (CNS) effects. The results using both extraction procedures are shown in Table III.

Conclusions

This research has demonstrated that the use of Bond Elut Certify reversed-phase and ion-exchange sorbent resulted in a lot of advantages besides these six antidepressant detection, such as better and more easily reproducible recoveries, cleaner extracts, and less solvent consumption and disposal, compared with the Chem Elut columns extraction procedure.

The assays described herein allowed the determination of six antidepressants in the performance of STA with satisfactory recoveries and limits of quantitation (LOQs). The good specificity of this method allows toxicological screening as well as therapeutic drug monitoring of several antidepressants available commercially.

Acknowledgments

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