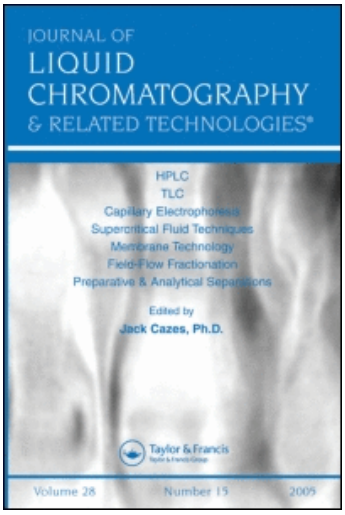

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SOLID-PHASE EXTRACTION FOR THE DETERMINATION OF TRICYCLIC ANTIDEPRESSANTS IN SERUM USING A NOVEL POLYMERIC EXTRACTION SORBENT

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ABSTRACT

A fast and easy solid-phase extraction method was developed for the determination of amitriptyline, doxepin and their metabolites (nortriptyline and nordoxepin) in porcine serum matrix by high performance liquid chromatography. The spiked serum sample was pretreated with 2% phosphoric acid followed by a simple and rugged SPE procedure using OasisTM HLB extraction cartridges.

The SPE method requires only one mL of simple solvent throughout the entire SPE process. High and reproducible recoveries were obtained even though the cartridges ran dry. For five replicate analyses, the average recoveries of parent tricyclic antidepressants and their metabolites were all greater than 94%, and the RSDs were all less than 4.0%.

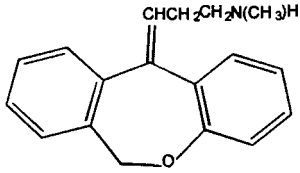
INTRODUCTION

Over the past 40 years, tricyclic antidepressants (TCAs) have been widely used to treat patients suffering from depression. They inhibit the reuptake of the neurotransmitters serotonin and norepinephrine in the central nervous system, and they are among the most widely prescribed drugs in current medicine. The monitoring of their concentrations in biological fluids such as plasma, serum or urine can be important in determining clinical efficacy, predicting side effects, and monitoring compliance.[1-2] The quantification of these drugs is also useful in emergency settings.[3] Moreover, TCAs are also used to treat enuresis, migrainous headaches, and obsessive-compulsive disorders.[4]

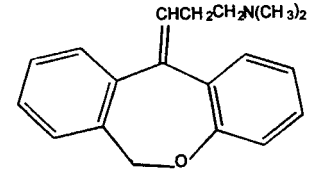
Many analytical methods have been applied to the analysis of TCAs. These include gas chromatography,[5-7] liquid chromatography,[8-18] and immunoassay.[19-21] As shown in Figure 1, the structures of TCAs are very similar; this might exhibit mutual interference in any immunoassay format which lacks antibody specificity. Liquid chromatography is the predominant method used for the measurements of antidepressants. A number of HPLC methods for determining TCAs and their metabolites have been developed and reviewed.[15-16] As pointed out by Wong,[15] the attributes for using HPLC are as follows. First, it is cost-effective due to the low reagent cost for extraction solvent and mobile phase. Second, it allows the possibility of simultaneous assays of multi-drugs and metabolites. Third, the routine use of LC always presents minor technical problems; experience and simple procedure enhance its application. The modes of HPLC include ion-paring, normal phase, and reversed phase chromatography. Among them, the reversed phase chromatography is the dominant method for the analysis of TCAs.

For the sample preparation prior to the HPLC analysis of TCAs in biological fluids, either liquid-liquid extraction[8,10-12,14-17] or solid-phase extraction[9,13,15,16,18] is used. The purpose of the sample preparation is to extract analytes of interest from the sample matrix in a high concentrated form prior to HPLC analysis.

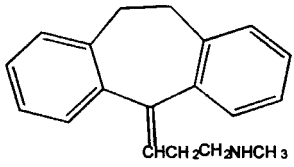
The trends of sample preparation have been shifted from liquid-liquid extraction to solid-phase extraction. In the most recent survey conducted by LC-GC, 40% of respondents use SPE.[22] Reversed phase sorbents, such as C_8 and C_{18}, are the most widely used packing materials. However, a troublesome feature of the reversed phase SPE is its irreproducibility. Also SPE is not easy to use. Many authors have reported that reversed phase SPE cartridges should not run dry before loading sample solution.[23-25] If accidentally the cartridges



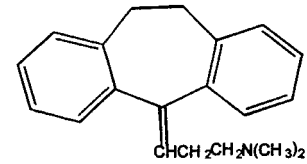
Nordoxepin



Doxepin



Nortriptyline



Amitriptyline

Secondary Amines Tertiary Amines

Figure 1. Structures of tricyclic antidepressants. They have very similar structures with two phenyl rings and one seven member ring-structure. Nordoxepin and nortriptyline are secondary amines; doxepin and amitriptyline are tertiary amines. Nordoxepin is a metabolite of doxepin, nortriptyline is a metabolite of amitriptyline. All of them are basic compounds with pKa values around 10.

run dry, the consequences are low and variable recoveries. This tedious and time consuming process is the major drawback for SPE. Therefore, it is desirable to have an easy SPE procedure which can provide excellent recovery and reproducibility.

In this paper, we report a fast and easy SPE method for high and reproducible recoveries of amitriptyline, doxepin, and their metabolites (nordoxepin and nortriptyline) in porcine serum matrices by an improved

HPLC assay. The HPLC method utilizes a Symmetry® C_{18} reversed phase column and a simple mobile phase. Oasis™ HLB extraction cartridges are used to extract the analytes from the serum matrix. The sorbents retain analytes even when the cartridges run dry.

EXPERIMENTAL

Reagents and Materials

The nordoxepin hydrochloride was from Alltech (Deerfield, IL); doxepin hydrochloride, nortriptyline hydrochloride, and amitriptyline hydrochloride were from Sigma Chemical Company (St. Louis, MO). Acetonitrile, methanol, and phosphoric acid were HPLC grade and were from J. T. Baker (Phillipsburg, NJ). Porcine serum was obtained from Equitech-Bio (Ingram, Texas). 1cc/30 mg OasisTM HLB extraction cartridges were obtained from Waters Corporation (Milford, MA).

Standard Solutions

A stock standard of each compound was prepared by dissolving 1.0 mg of the pure compound in 1.0 mL of water. Working solutions were prepared by diluting these stock standards in 20 mM phosphate buffer, pH 7.0/methanol (80:20).

Standard curves were prepared in 20 mM phosphate buffer, pH 7.0/methanol (80:20) over a concentration range from 0.50 to 10 μg/mL for amitriptyline, and from 0.25 to 5.0 μg/mL for doxepin and nortriptyline. Each standard solution contained 2.5 μg/mL nortriptyline as the internal standard.

Extraction Procedures

Aliquots of freshly thawed drug-free serum were spiked with drug solutions to produce the desired concentrations. Two levels of sample concentrations were prepared. At high level, the concentrations for nortriptyline and doxepin were each at 0.5 μg/mL; for amitriptyline was at 1.0 μg/mL. At low level, the concentrations for nortriptyline and doxepin were each at 0.1 μg/mL; for amitriptyline was at 0.2 μg/mL. Each level contained 0.5 μg/mL of nordoxepin as the internal standard. These spiked serum samples

were then acidified with 20 μL of phosphoric acid. The samples were vortex-mixed for five seconds and loaded onto OasisTM HLB extraction cartridges, which had been activated with 1 mL of methanol followed by 1 mL of water. After loading, the cartridges were washed with 1 mL of 5% methanol solution, which was discarded. The cartridge was eluted with 1 mL of methanol. The eluate was evaporated to dryness in a heating block at 40°C under a gentle stream of nitrogen and reconstituted with 200 μL of 20 mM phosphate buffer, pH 7.0/methanol (80:20).

HPLC Apparatus and Operating Conditions

Isocratic elution was used throughout the entire study. The HPLC system consisted of a Waters 616 LC system, a 717 plus Autosampler, and a 996 Photo Diode Array Detector. The Millennium 2010 Chromatography Manager was used for system control and data acquisition. The column used was a Waters Symmetry® C_{18} (3.9 mm x 150 mm, 5 μm particle size) preceded by a Sentry™ guard column (3.9 mm x 20 mm). The chromatography was carried out at 35°C. The mobile phase was 20 mM potassium phosphate, pH 7/methanol at 30:70 (v/v). The flow rate was 1.0 mL/min. For the concentration determination, 20 (μL each of the sample and the standard solution were injected. The effluent was monitored at 254 nm.

RESULTS AND DISCUSSIONS

Chromatographic Analysis

As shown in Figure 1, TCAs have two phenyl rings and one seven member ring-structure. On the basis of changes in the side chain, they can be divided into secondary (nordoxepin and nortriptyline) and tertiary amines (doxepin and amitriptyline). Nordoxepin is a metabolite of doxepin, nortriptyline is a metabolite of amitriptyline. They are basic compounds with pKa values around 10. They interact with the residual sites of silanol groups present on the silica based reversed phase sorbent, and cause peak tailing in HPLC and low recovery for SPE. To overcome this problem, it has been shown that the adding of competing amines, such as trimethylamine or triethylamine, is necessary in order to obtain good peak shapes for TCAs.[8,12,13,15-16] Good peak shapes are important for good quantitation, especially at low concentration. In this study, we were able to obtain good peak shapes with a very simple mobile phase, 20 mM potassium phosphate, pH 7/methanol at 30:70 (v/v), without adding any competing reagent.

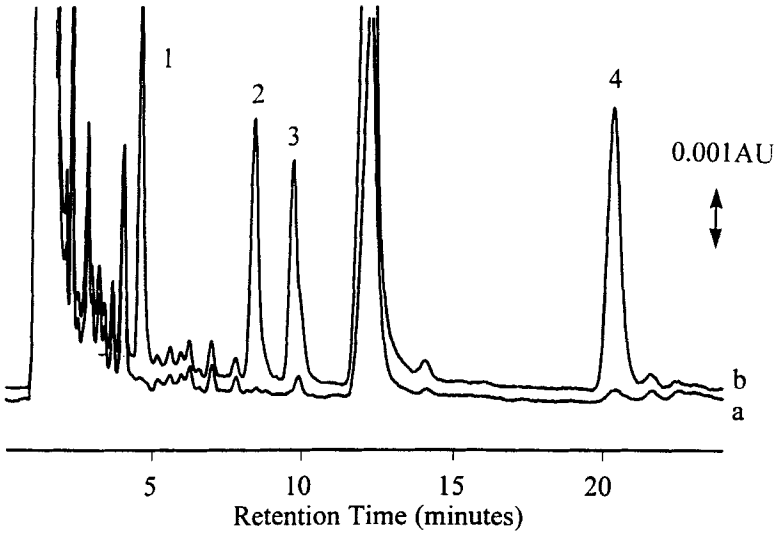


Figure 2. Representative chromatograms of a serum blank (curve a); a serum sample spiked with 1.0 (g/mL of amitriptyline, 0.5 (μ/mL each of nortriptyline and doxepin, and 0.5 (μ/mL of nordoxepin as the internal standard (curve b). Peak 1 is nordoxepin (I.S.), peak 2 is nortriptyline, peak 3 is doxepin, and peak 4 is amitriptyline. The column used was a Symmetry® C_{18} (3.0 mm x 150 mm, 5 mm particle size) preceded by a Sentry™ guard column (3.9 mm x 20 mm). The mobile phase was 20 mM phosphate, pH 7.0/methanol (30:70) at a flow rate of 1.0 mL/min, and the effluent was monitored at 254 nm.

The representative chromatograms of a serum blank and a spiked serum sample are shown in Figure 2. The serum blank is shown in curve a, and the spiked TCAs in the serum matrix, at the concentration of 1.0 μg/mL of amitriptyline and 0.5 μg/mL each of nortriptyline and doxepin, is shown in curve b. The elution sequence is nordoxepin (peak 1), nortriptyline (peak 2), doxepin (peak 3), and amitriptyline (peak 4). Here, the nordoxepin is used as the internal standard. No endogenous interferences were found to interfere with the quantitation of peaks of interest.

Calibration curves were based on peak-area ratio to the internal standard, nordoxepin. Within the concentration range described in the experimental section, linear plots were obtained for doxepin, amitriptyline, and nortriptyline. The correlation coefficients were 0.999476, 0.999888, and 0.999678 for doxepin, amitriptyline, and nortriptyline, respectively.

Table 1

Recovery of TCAs from Spiked Porcine Serum*

	Compound	Nordoxepin (I.S.)	Nortriptyline	Doxepin	Amitriptyline
High Level	Spiked Conc. (μg/mL)	0.50	0.50	0.50	1.00
	Recovery (%)	103	99.7	94.0	102
	RSD (%)	3.5	2.3	1.3	2.5
Low Level	Spiked Conc. (μg/mL)	0.50	0.10	0.10	0.20
	Recovery (%)	97.6	103	102	101
	RSD (%)	3.3	3.4	3.2	3.7

* A simple and rugged SPE method was applied to each analysis. Only one mL of simple solvent was applied to each step of the SPE procedure. Results of five replicate analyses.

Recovery of TCAs

TCAs are basic compounds and have high protein binding capacity in the range of 95%.[26] To eliminate the loss of recovery due to the binding of analytes to the albumin, it is necessary to free the protein bound drugs. In this study, we observed approximately 80% of recoveries when the sample solution was not acidified. With the addition of 20 μL of phosphoric acid into the sample solution, we were able to obtain recoveries greater than 94% for these high protein binding compounds.

The results for the TCAs are summarized in Table 1. At high level, the recoveries were 99.7%, 94.0%, and 102% for nortriptyline, doxepin and amitriptyline, respectively. At low level, the recoveries were 103%, 102%, and 101% for nortriptyline, doxepin, and amitriptyline, respectively. For five replicate analyses, the RSDs were all less than 4.0%. It should be noted that these results were obtained even though the cartridges ran dry before loading the sample onto cartridges. This procedure totally eliminates the time consuming and tedious stopcock manipulation necessary with traditional SPE cartridges, while providing excellent and reproducible results.

The absolute recovery of the internal standard, nordoxepin, was determined by comparing the average peak area from five replicate analyses to the average peak area of the ten standard injections. The mean recoveries were 103% with 3.5% RSD for the high level, and 97.6% with 3.3% RSD for the low level. The ten standard injections had a RSD of 2.5%.

Comparison to Liquid-Liquid Extraction and C_{18} SPE

Extraction of TCAs is traditionally carried out by either liquid-liquid extraction or solid-phase extraction. Liquid-liquid extraction procedures are carried out at high alkaline pH with nonpolar solvents, such as n-hexane or n-heptane. Addition of isoamyl alcohol is a necessary step to prevent formation of an emulsion, which can cause low recovery. The sample solution is shaken and centrifuged. The organic layer is then carefully decanted into another test tube, evaporated to dryness, and reconstituted into a proper solvent for reversed phase HPLC analysis.[8,11] Alternatively, the organic phase is back extracted with diluted acid for subsequent HPLC analysis.[10,12]

For the liquid-liquid extraction method without the back extraction step, Amitai et al.[11] first alkalinized sample solutions to pH 10.5 with 0.1 M of sodium hydroxide. To the sample solutions, they added 8 mL of hexane/isoamyl alcohol (97/3, v/v), shook for 10 minutes, and centrifuged for another 10 minutes. The aqueous layer was frozen by placing the sample solutions in a methanol-dry ice bath. Finally, they decanted the organic phase into other test tubes, evaporated to dryness and reconstituted for HPLC analysis. They reported that the recoveries of parent TCA compounds, such as amitriptyline and doxepin, from plasma were 93% with 2% SD; the recoveries of TCA metabolites, such as nortriptyline and nordoxepin, from plasma were lower at 72% with 3% SD. Similarly, Ghahramani et al.[8] reported that the recoveries of amitriptyline, nortriptyline, and desipramine (used as an internal standard) from plasma at a concentration of 0.05 μg/mL were 90%, 87%, and 76%, respectively; the absolute recoveries, therefore, were 68.4 % and 66.1 % for amitriptyline and nortriptyline, respectively. The within-batch coefficients of variation were reported to be less than 7.4%.

For the liquid-liquid extraction method with the back extraction step, El-Yazigi et al.[12] transferred the organic phase into a tube containing 100 μL of 0.03% phosphoric acid, shook, centrifuged, and finally transferred the acid layer for HPLC analysis. They reported that the recoveries from plasma at a concentration of 0.05 μg/mL were 85.7% and 87.4% for amitriptyline and nortriptyline, respectively; at a concentration of 0.2 μg/mL, the recoveries were 70.4% and 76.5%. No absolute recoveries were reported. The within-batch

coefficients of variation were reported to be less than 9.8%.[12] Similarly, Atta-Politou et al.[10] reported that the recoveries from plasma at a concentration of 0.05 μg/mL were 93.4% with 11.8% SD and 88.7% with 7.7% SD for amitriptyline and nortriptyline, respectively; at a concentration of 0.15 μg/mL, the recoveries were 89.4% with 4.2% SD and 95.7% with 4.2% SD for amitriptyline and nortriptyline, respectively.

An alternative way to extract TCAs is solid-phase extraction method. With SPE, several steps involved in the liquid-liquid extraction are eliminated. These include shaking, centrifuging, and transferring the extracts. Therefore, sources of variability are minimized and the analysis time is also reduced significantly. In addition, SPE requires less solvent and glassware, is less costly in labor and materials, and is easier to automate compared to liquid-liquid extraction. The SPE method requires six simple steps. These steps include preconditioning cartridges, loading the sample solution, washing the cartridge, eluting the analytes, evaporating, and reconstituting into a proper solvent for HPLC analysis.

Bidlingmeyer et al.[18] used Sep-Pak® C_{18} cartridges to clean up the interferences from the serum matrix. They prewashed cartridges with 10 mL of methanol followed by 5 mL of 0.1 N NH_4OH. Then they loaded 1 mL of spiked serum, into which 2 mL of 0.1 N NH_4OH was added, onto the cartridges. These cartridges were flushed with 10 mL of 0.1 N NH_4OH, followed by 10 mL of methanol: water (50:50). The final elution was with 3 mL of 2% butylamine in methanol. The eluate was then evaporated to dryness and redissolved in 100 μL of mobile phase. The entire procedure took about fifteen minutes. The recoveries of amitriptyline and nortriptyline at a concentration of 0.1 μg /mL of serum were 97 % (with RSD of 14%) and 102 % (with RSD of 4%), respectively. These recoveries were reported after adjustment with the internal standard which was protriptyline.

SPE, first introduced in 1978, is now among the most commonly used rapid sample preparation techniques for cleaning up sample matrices and concentrating analytes prior to analysis. The most commonly used sorbents are the porous silica particles surface-bonded with C_{18} or other hydrophobic alkyl groups. The presence of silanols on the surface of packing material complicates the interaction of analytes, especially the basic compounds such as TCAs, with the sorbents. Therefore, solid-phase extraction cartridges that are packed with these sorbents are difficult to work with. It may take a long time to develop and to optimize the methods. In the above method reported by Bidlingmeyer et. al, large volumes of solvent were used, and the addition of a competing agent, butylamine, is necessary for the elution.

Table 2

Comparison of Liquid-Liquid Extraction and Solid-Phase Extracton Using Sep-Pak® C_{18} Or Oasis™ HLB Cartridges

	Percent Recovery (RSD)[a] Amitriptyline	Nortriptyline	Solvents Used	Automation	Reference
Liquid-Liquid Extraction					
Without back extraction	93 (2%)	72 (4%)	>8.0 mL	Difficult	11
	68 (<7%)	66 (<7%)	5.3 mL	"	8
With back extraction	78 (<10%)	82 (<10%)	2.3 mL	"	12
	91 (8%)	93 (6%)	6.4 mL	"	10
Solid-Phase Extraction					
Sep-Pak® C_{18}	97 (14%)	102 (4%)	40.1 mL	Easy	18
Oasis™ HLB	99 (4%)	100 (3%)	4.2 mL	"	

[a] Concentration ranges from 0.05 to 0.20 μg/mL of analyte. All recoveries were reported as absolute recovery, except for results from references 12 and 18.

Once the methods have been optimized, the cartridges must remain wet after conditioning and equilibration, in order to retain analytes from an aqueous sample matrix. These steps make the silica-based SPE sorbents time consuming, tedious, and irreproducible.

In this study, we used Oasis™ HLB cartridges to extract TCAs from serum matrix. The Oasis™ HLB sorbent is a polymeric reversed phase sorbent. This macroporous polymer [poly(divinylbenzene-co-N-vinylpyrrolidone) exhibits both hydrophilic and lipophilic characteristics. The lipophilic monomer is divinylbenzene; it provides the reversed phase properties necessary for analyte retention. The hydrophilic monomer is N-vinyl pyrrolidone; it gives the packing the necessary amount of hydrophilic property to prevent the wettability problems encountered with the traditional silica based reversed phase sorbents. These two unique and distinct characteristics are carefully balanced.

The Oasis™ HLB sorbent contains no silanol group, which simplifies the retention mechanism between sorbents and the analytes. As a result, a simple and more general SPE protocol (see experimental section) can be applied.

With only one mL of simple solvents throughout the entire SPE procedure, we were able to obtain excellent recoveries (all greater than 94%) with good reproducibility (less than 4.0% RSD).

Table 2 summarizes the above methods used for the extraction of TCAs. Clearly, the Oasis™ HLB extraction cartridge has tremendous advantages over the liquid-liquid, and traditional silica based C_{18} SPE methods. This cartridge simplifies the sample preparation procedure to an extraordinary degree. It provides fast and easy method development while giving us high and good reproducible results.

CONCLUSIONS

The assay described here provides a faster and simpler extraction method than the previously published methods, including liquid-liquid and solid-phase extraction. This method requires only one mL of simple solvent in each step of the SPE procedure. Additionally, unlike traditional reversed phase sorbents, we can let these Oasis™ HLB extraction cartridges run dry on a vacuum manifold prior to sample loading. This feature reduces errors caused by the sample preparation, and improves method ruggedness. The unique property of this sorbent makes SPE more efficient, more rugged, and less tedious than conventional SPE. This method is highly precise, easy to perform, and suitable for determining the TCAs in the biological matrices.

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