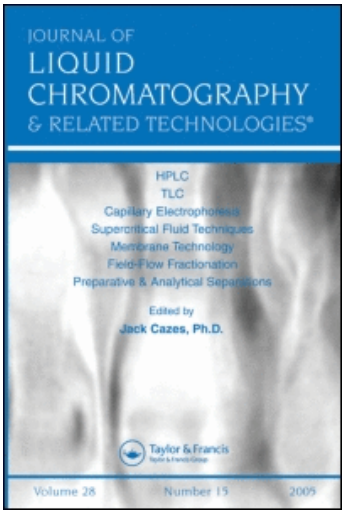

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Madej, Katarzyna , Kochana, Jolanta and Woźniakiewicz, Michał(2007) 'Study of SPE Conditions for CE Determination of Tricyclic Antidepressants in Body Fluids', Journal of Liquid Chromatography & Related Technologies, 30: 2, 185 — 198
To link to this Article: DOI: 10.1080/10826070601063526
URL: http://dx.doi.org/10.1080/10826070601063526

Journal of Liquid Chromatography & Related Technologies®, 30: 185–198, 2007

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070601063526

Study of SPE Conditions for CE Determination of Tricyclic Antidepressants in Body Fluids

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Abstract: The optimal solid-phase extraction conditions for six tricyclic antidepressants present in body fluids have been studied. The compounds were separated and detected by capillary electrophoresis with UV spectrophotometry. In the examinations, three sorbents, five pretreatments of body fluids, and four extraction procedures were taken into account. On the basis of preliminary experiments, the best starting conditions were chosen for further optimization of such factors as sorbent mass and volumes of conditioning, washing, and eluent reagents. In the optimized conditions, the average extraction recoveries and their repeatability for all drugs present in whole blood and serum have been evaluated.

Keywords: Tricyclic antidepressants, Solid-phase extraction, Capillary electrophoresis, Whole blood, Serum, Urine

INTRODUCTION

Body fluids, such as whole blood, serum, and urine, are among the most important materials for routine clinical and forensic toxicological analyses. These fluids possess complex biological matrices, which usually complicates analytical measurements. These matrices become still more complex during storage of biological samples, which is especially often in forensic analysis. There are a small number of analytical techniques, e.g., immunoassays,

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which do not require any preparation, or need only small modification[1] of biological fluids before analysis. In some cases, medicines present in urine or serum at relatively high concentrations, capillary electrophoresis with direct injection of diluted body fluid sample may be successfully used.[2] However, in most analytical methods appropriate preparation of biological material is necessary.

Liquid-liquid extraction (LLE) and solid-phase extraction (SPE) are of special interest in the analyses of drugs in biological samples. Generally, the SPE technique is used when one selective drug or one group of drugs analysis should be performed. Some variations of the SPE technique, such as classical,[3] microextraction,[4] disc format,[5] and, lately, molecularly imprinted polymer solid-phase extraction,[6] have been described in literature and applied to drug analysis. Besides clean and selective drug extracts, other advantages of the SPE technique may be mentioned: small amounts of reagents consumption, many factors for optimization, and possibility automation.

Tricyclic antidepressants (TCAD) belong to the classic psychotropic drugs, which are still frequently prescribed for treatment of depression and other mental disorders. These drugs are characterized by efficacy, but they are also dangerous due to their narrow therapeutic window. Thus, a number of drug related deaths caused by overdosing of TCAD are reported.

SPE conditions were studied for the HPLC assay of five antidepressant drugs: trazodone, doxepin, desipramine, maprotiline, and imipramine.[7] Recoveries of these drugs were examined using sorbents, such as: C^{18}, C_8, C_2, cyclohexyl (CH), cyanopropyl, phenyl Bond Elut, and copolymer HLB. As optimum conditions for the extraction of tested drugs in plasma samples, CH cartridges were chosen. The HPLC-UV method with an effective extraction disc pretreatment of serum samples was developed for therapeutic drug monitoring of 22 commonly prescribed psychotropic drugs and some of their metabolites.[8] A comparative study of SPE conditions using two kinds of extraction columns, Chem Elut and Bond Elut Certyfy, has been performed for simultaneous determination of nine antidepressants in whole blood by capillary GC-NPD.[9] However, generally, the number of papers concerning SPE methods for isolation of tricyclic antidepressants from body fluids, especially from whole blood samples, is rather limited.

Since the last decade of the previous century, capillary electrophoresis (CE) has become a powerful analytical technique in the pharmaceutical industry and also in other fields of drug determination such as clinical[10] or forensic[11] toxicological analysis. There are a number papers dedicated to drug analysis and there are also some elaborated excellent CE procedures, which have been developed only on standard drug solutions.

We tried to apply the CE separation conditions[12] for analysis of forensic samples (whole blood and urine) containing the compounds from the TCAD group commonly used in our laboratory, employing the liquid-liquid extraction method. The obtained extracts were not suitable for further analysis by the CE method.[12] We also applied a re-extraction step for sample

cleanup, but the loss of the examined drugs was rather considerable. This fact encouraged us to study optimal conditions for solid-phase extraction of six TCADs: doxepin, nordoxepin, amitriptyline, nortriptyline, imipramine, and desipramine present in three kinds of body fluids: whole blood, serum, and urine, which were then analyzed by capillary electrophoresis. During our examinations, a considerable influence of blood and urine matrix variability on the effectiveness of extraction has been observed. In order to find a universal extraction procedure for blood samples (regardless of the degree of sample decomposition), we have also made an appropriate modification of sample pretreatment.

EXPERIMENTAL

Apparatus and Accessories

A Prince 550 air thermostated capillary electrophoresis system (Prince Technologies, Emmen, Holland) with Lambda 1010 spectrophotometer (Bischoff, Leonberg, Germany) was used. The separations were conducted at 30 kV in a bare fused silica capillary of 50 μm ID and 100 cm long (66 cm to the detector).

A Vac Elut Manifold (Varian, USA) for SPE extractions was used. Three kinds of columns (Varian, USA): Bond Elut® C_{18} (100 and 500 mg), Bond Elut® TCA (100 mg), abselut™ Nexus (30 mg) were examined.

Reagents

Acetonitrile and methanol of HPLC grade were purchased from Merck (Germany).

CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid) was from Sigma-Aldrich (Germany).

2-propanol, 25% ammonia, chloroform, 85% phosphoric acid, ice acetic acid, ethyl acetate, sodium acetate, potassium dihydrophosphate, sodium hydrophosphate, sodium hydroxide, potassium hydrocarbonate, sodium hydrocarbonate, and diethylamine (DEA) were of analytical grade and supplied by POCh (Polish Chemical Reagents, Poland).

The separation buffer consisted of 50 mM CAPSO in methanol/water (30:70, *w*/*v*) with addition of 5 M NaOH to pH 9.55.

Examined Drugs and Materials

Six tricyclic antidepressants: amitriptyline, nortriptyline, doxepin, nordoxepin, imipramine, and desipramine were involved in the examinations.

Trimipramine was used as the internal standard (IS) to control volume repeatability of injected solutions.

The standards of drugs were purchased from Sigma-Aldrich (St. Louis, USA) and Polish pharmaceutical companies.

Drug free: serum and whole blood (control fluids) were taken from the blood bank in Kraków, Poland.

Drug free urine originated from a volunteer with no history being medicated.

Preparation of Samples and Drug Mixture Solutions

In order to achieve final concentrations of 20 μg or 80 μg mL^{-1} of each drug in a standard mixture, solutions were prepared by mixing six 0.1% methanolic standard drugs having suitable dilutions with deionized water.

A control body fluid (1 mL) was spiked with aqueous standard drug mixtures (2 or 0.5 μg of each drug in a mixture) and submitted to SPE extraction, according to the appropriate method. The obtained eluate was evaporated to dryness under the stream of nitrogen at 40°C. The remains were dissolved in 25 μL of a solution of 80 μg mL^{-1} trimipramine in 0.01% phosphoric acid/methanol (1:1, *v*/*v*).

Preparation of Body Fluids Before Application on a Column

Each examined body fluid containing the drug was prepared before extraction in three ways from among the following procedures: 1. diluting with deionized water (1:1, *v*/*v*), 2. adding 2 mL of deionized water and 1 mL of 1 M $NaHCO_3$, 3. acidation with 20 μL of 85% H_3PO_4, 4. sonication (15 min), then addition of 6 mL phosphate buffer pH 6.0, and centrifugation of supernatant, 5. precipitation with 2 mL of acetonitrile, centrifugation of supernatant, and then the supernatant was evaporated to dryness under nitrogen at 40°C and reconstituted in 6 mL of phosphate buffer (pH 6.0). After the appropriate preparation, the examined material was subjected to the extraction procedures given below.

Extraction Procedures

1. Extraction procedure for Bond Elut® TCA (100 mg/1 mL) recommended for TCAD in plasma by Varian (column conditioning with 1 mL of methanol/0.6% diethylamine and 2 mL of aq. potassium bicarbonate/10% acetonitrile; column rinsing with 1 mL 20% acetonitrile in water and 1 mL water/methanol/acetonitrile (2:3:3, v/v/v); elution with 600 μL of 0.6% diethylamine in methanol).

2. Extraction procedure for abselut™ Nexus (30 mg/1 mL) recommended for TCAD in serum by Varian (column rinsing with 1 mL of water; elution with 1 mL of methanol).
3. Extraction procedure for BAKERBOND spe™ Octadecyl C18 (500 mg/3 mL) recommended for TCAD in urine, whole blood, and plasma by J.T. Baker (column conditioning with 2 × 3 mL of chloroform/2-propanol (9:1, v/v), 2 × 3 mL of acetonitrile, and 2 × 3 mL of water; column washing with 2 × 3 mL water; elution with 2 × 1.5 mL of chloroform/2-propanol; 9:1, *v/v*).
4. Extraction procedure for TCAD and neuroleptics in urine, whole blood, and plasma (serum) using Bond Elut Certify© columns (130 mg/10 mL) (column conditioning with 2 mL of methanol and 2 mL of 0.1 M phosphate buffer (pH 6.0); column washing with 1 mL of water and 1 mL of 20% of acetonitrile (only for urine); elution with 2 mL of 2% of ammoniated ethyl acetate).[13]

CE Measurement Procedure

In turn, new capillary was conditioned by flushing 0.5 M hydroxide (30 min), deionized water (10 min), and the separation buffer (30 min) using the pressure of 2000 mbar. During the last step 30 kV was applied.

Between measurements, the washing steps were appropriately shorter: 10, 5, and 10 minutes.

The main measurement procedure was as follows: 1. washing the capillary with the separation buffer under pressure of 2000 mbar (2 min), 2. injecting the sample in 0.1 min, using pressure of 100 mbar, 3. separating analytes at 30 kV.

The detection of the drugs was performed at 254 nm.

RESULTS AND DISCUSSION

In the first part of the examinations, the appropriate combinations of three sorbents (Bond Elut® C18, Bond Elut® TCA, and abselut™ Nexus), five ways of sample pretreatment (see "Experimental" 1–5), and four extraction procedures (see "Experimental" 1–4) were tested for whole blood, serum, and urine samples (Tables 1–3). Thus, suitable electropherograms of the examined fluid extracts and appropriate diluted standard drug mixtures were compared. The average extraction recoveries for six TCADs were calculated, and purity of the obtained extracts was observed for each tested combination (Tables 1–3).

On the basis of the above mentioned experiments, the most promising extraction conditions were chosen, i.e., the sorbent Bond Elut® C18 and the extraction procedure 3 (Tables 1–3). Among all tested sorbents, octadecyl

Table 1. Extraction of six TCAD from body fluids using Bond Elut® C18 columns

Examined fluid	Sample pretreatment	Extraction procedure[a]	Average extraction recovery for six TCAD (%)
Whole blood	Sonication	III	31
		IV	52
	Precipitation with acetonitrile	III	—[b]
		IV	17
	Addition of 1M $NaHCO_3$	III	72
		IV	—[b]
Serum	Dilution with water (1:1, *v*/*v*)	III	84
		IV	—[b]
	Addition of 1M $NaHCO_3$	III	65
		IV	22
	Addition of 85 % H_3PO_4	III	—[b]
		IV	—[b]
Urine	Sonification	III	65
		IV	26
	Precipitation with acetonitrile	III	90
		IV	—[b]
	Addition of 1M $NaHCO_3$	III	46
		IV	51

[a]III - extraction procedure for BAKERBOND spe™ Octadecyl C18 (500 mg/3 mL) recommended for TCAD in urine, whole blood and plasma by J.T. Baker IV - extraction procedure for TCAD and neuroleptics in urine, whole blood and plasma (serum) using Bond Elut Certify© columns (130 mg/10 mL).[12] (for more details see "Experimental").

[b]Low extraction recoveries and/or strong interferences with matrix.

bonded (C_{18}) proved to be the best one with regard to efficiency and universality, for extraction of TCAD from all examined body fluids. Using C_{18} columns with the combination of extraction 3 the average recoveries and repeatabilities for all examined drugs were higher than in the case of two other sorbents tested. Nexus columns were also shown to be universal for two kinds of examined fluids - serum and urine (Table 2), but repeatability of extraction efficiency was rather poor and strongly depended on the variability of column packing.

The optimal sample preparation before extraction, appeared to be different for each body fluid. Serum sample was prepared by simple dilution with deionized water (1:1, *v*/*v*); whole blood required addition of 1 M sodium hydrocarbonate, and urine was precipitated with acetonitrile. Addition of aqueous hydrocarbonate caused disruption of red blood cells, releasing from them analytes and salted out proteins, which could block the extraction column. However, further experiments showed that the treatment

Table 2. Extraction of six TCAD from body fluids using Bond Elut® TCA columns

Examined fluid	Sample pretreatment	Extraction procedure[a]	Average extraction recovery for six TCAD (%)
Whole blood	Sonification	I	29
		IV	35
	Precipitation with acetonitrile	I	—[b]
		IV	41
	Dilution with water and addition of 1M $NaHCO_3$	I	—[b]
		IV	—[b]
Serum	Dilution with water (1:1, v/v)	I	50
		IV	—[b]
	Dilution with phosphate buffer (pH = 6)	I	74
		IV	—[b]
	Dilution with water and addition of 1M $NaHCO_3$	I	—[b]
		IV	—[b]
Urine	Sonification	I	—[b]
		IV	46
	Precipitation with acetonitrile	I	—[b]
		IV	42
	Dilution with water and addition of 1M $NaHCO_3$	I	—[b]
		IV	—[b]

[a]I - extraction procedure for Bond Elut® TCA (100 mg/1 mL) recommended for TCAD in plasma by Varian IV - extraction procedure for TCAD and neuroleptics in urine, whole blood and plasma (serum) using Bond Elut Certify© columns (130 mg/10 mL)[12] (for more details see "Experimental").

[b]Low extraction recoveries and/or strong interferences with matrix.

mentioned above was not sufficient to hemolize fresh blood, thus sonication as an additional step had to be used.

For further optimization of the extraction conditions (the sorbent Bond Elut® C18, extraction procedure 3), whole blood was selected, as this material is of special importance in forensic toxicological analysis. During the optimization process, the mass sorbent, volumes of conditioning and washing reagents, and volume of eluent were taken into account. The mass sorbent was examined on two levels:100 and 500 mg, and the following volumes of conditioning and washing reagents were used: 1.5, 3, 6, and 8 mL (Table 4). From these experiments, the optimal extraction parameters were determined: mass sorbent - 100 mg, volume - 3 mL for each conditioning reagent (1. chloroform/2-propanol; 9:1, *v*/*v*, 2. acetonitrile and 3. water), and volume - 3 mL of washing reagent (water). Then, the reduction of eluent (chloroform/2-propanol; 9:1, *v*/*v*) volume to 2 mL was achieved without loss of extraction efficiency.

Table 3. Extraction of six TCAD from body fluids using abselut™ Nexus columns

Examined fluid	Sample pretreatment	Extraction procedure[a]	Average extraction recovery for six TCAD (%)
Whole blood	Dilution with water and addition of 1M $NaHCO_3$	II[c]	7
		II[d]	—[b]
		III[d]	51
Serum	Addition of 85 % H_3PO_4	II[c]	27
		II[d]	91
		III[d]	89
Urine	Precipitation with acetonitrile	II[c]	24
		II[d]	—[b]
		III[d]	100

[a]II - extraction procedure for abselut™ Nexus (30 mg/1 mL) recommended for TCAD in serum by Varian, III - extraction procedure for BAKERBOND spe™ Octadecyl C18 (500 mg/3 mL) recommended for TCAD in urine, whole blood and plasma by J.T. Baker (for more details see "Experimental").

[b]Low extraction recoveries and strong interferences with matrix.

[c]Fast elution.

[d]Slow elution.

During the examinations a significant influence of blood matrix variability on the efficiency of the extraction was observed. Considerable lowering of the analytes recoveries (to 32%) from fresh blood was noted. Thus, in order to extend the extraction method to various aged blood samples, the combination of sonication and addition of $NaHCO_3$ was applied. The addition of 0.01% DEA in 1 M sodium hydrocarbonate was also carried out to reduce adsorption

Table 4. Average extraction recovery for six TCAD at various extraction conditions, using Bond Elut® C18 columns

Volume (mL) of each conditioning[a] and washing[b] reagents	Average extraction recovery for six TCAD (%) Sorbent mass (mg)	
	100	500
8	72 ± 5	51 ± 20
6	71 ± 22	52 ± 25
3	99 ± 20	32 ± 18
1.5	78 ± 15	—[c]

[a]1. chloroform/2-propanol, 9:1 (*v*/*v*), 2. acetonitrile and 3. water.

[b]Water.

[c]The experiments at these conditions were not conducted.

of the examined drugs on glass extraction tubes.[14] The increasing average extraction recovery for six TCADs from whole blood samples using $NaHCO_3$ alone, sonication + $NaHCO_3$ and sonication + $NaHCO_3$ + DEA is shown in Figure 1.

The final optimal method for blood sample pretreatment was as follows: 1 mL of 0.01% DEA in 1 M sodium hydrocarbonate was added to 1 mL of blood sample, which was then sonicated for 15 min, and diluted with 6 mL of phosphoric buffer and centrifuged.

Under the determined conditions, four repeated extractions of six TCADs, each one present in blood samples at two concentration levels: 0.5 and 2 μg mL^{-1} were performed. The average recoveries and repeatabilities of the extractions for all drugs tested were calculated (Table 5). The electropherogram of whole blood extract was presented in Figure 2a. At a higher concentration (2 μg mL^{-1}) the average recovery for all drugs present in whole blood was 99.5 ± 6.1%. At a lower concentration (0.5 μg mL^{-1}), the average recovery for four drugs (amitriptyline, desipramine, doksepin, nordoksepin) was 71 ± 12.3%. The extraction efficiency for nortriptyline was a little lower (68.7%) and for desipramine was poor (25.5%). This phenomena may be caused by lower affinity of more polar desmethyled derivatives to nonpolar sorbent C_{18} than their parent drugs. However, for remarkably low results of extraction recovery for desipramine from whole blood should be checked by additional experiments. The effectiveness of the elaborated extraction procedure was also checked for decomposed blood, which was stored at room temperature for ten days. The results were found to be comparable with those obtained for fresh blood - the average recovery for six drugs was c.a.

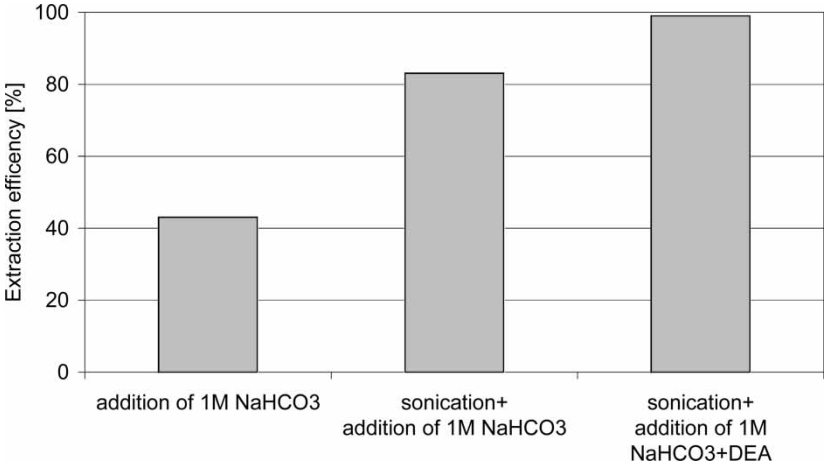

Figure 1. Influence of various pretreatments of whole blood samples on average extraction recovery of six TCADs. The recoveries have been calculated from triplicate measurements.

Table 5. Average extraction recovery for six TCAD present in fresh and decomposed blood samples

	Fresh blood		Decomposed blood		
	$c_1 = 0.5$ ($\mu g\ mL^{-1}$)		$c_1 = 2.0$ ($\mu g\ mL^{-1}$)		$c_1 = 2.0$ ($\mu g\ mL^{-1}$)
Drug	Mean (%) n = 4	RSD (%)	Mean (%) n = 4	RSD (%)	Mean (%) n = 2
Amitryptyline	85.5	13.7	102.5	6.6	104.1
Doxepine	76.0	13.7	98.5	4.5	91.4
Imipramine	78.7	12.2	94.0	3.6	85.3
Nordoxepine	89.7	16.2	105.7	5.4	100.2
Nortryptyline	68.7	9.6	100.2	7.5	89.1
Despiramine	25.5	4.8	102.5	6.6	94.3

94% (Table 5 and Figure 2b). The drugs present in serum at concentration of 0.5 $\mu g\ mL^{-1}$ were extracted four times, with average recovery for all compounds $87 \pm 13.1\%$ (Table 6); the appropriate electropherogram is shown in Figure 2c. The SPE recoveries of doxepin and imipramine from spiked plasma have been higher (c.a. $95 \pm 13.7\%$) than those obtained on a CH cartridge by Bakkali et al.[7] (c.a. $83 \pm 9\%$), while we obtained a lower result ($69.5 \pm 6.1\%$) for desipramine than the authors achieved for this drug ($83.3 \pm 9\%$). The SPE disk conditions used by Frahnert et al.[8] for six tricyclic antidepressants: amitriptyline, nortriptyline, imipramine, desipramine, doxepin, and nordoxepin, tested at a concentration of 200 ng mL^{-1} in serum plasma, were c.a. $99 \pm 7.1\%$ and appeared to be better than those achieved by us for this material. Martínez et al.,[9] using SPE Bond Elut Certify columns, obtained recoveries of 69 and 62% for amitriptyline and nortriptyline from whole blood (at 500 ng mL^{-1}), respectively; which were lower than our extraction results obtained for these drugs present in this material at the same concentration (85.5 and 68.7% for amitriptyline and nortriptyline, respectively). However, as far as we know, no recoveries of imipramine, desipramine, doxepin, and nordoxepin from whole blood samples have been reported.

In the found extraction conditions, the examined TCADs were not recovered successfully from decomposed urine samples (stored for a few months in refrigerator $+5^{\circ}C$), which were precipitated with acetonitrile or pretreated in the same way as whole blood samples (Figure 3a and Figure 3b), although the drugs were extracted with relatively high efficiency (c.a. 90%) from fresh urine during initial experiments using the sorbent C_{18} (500 mg), acetonitrile precipitation, and extraction procedure 3 (Figure 3c). It should be stressed that although urine seems to be a relatively simple

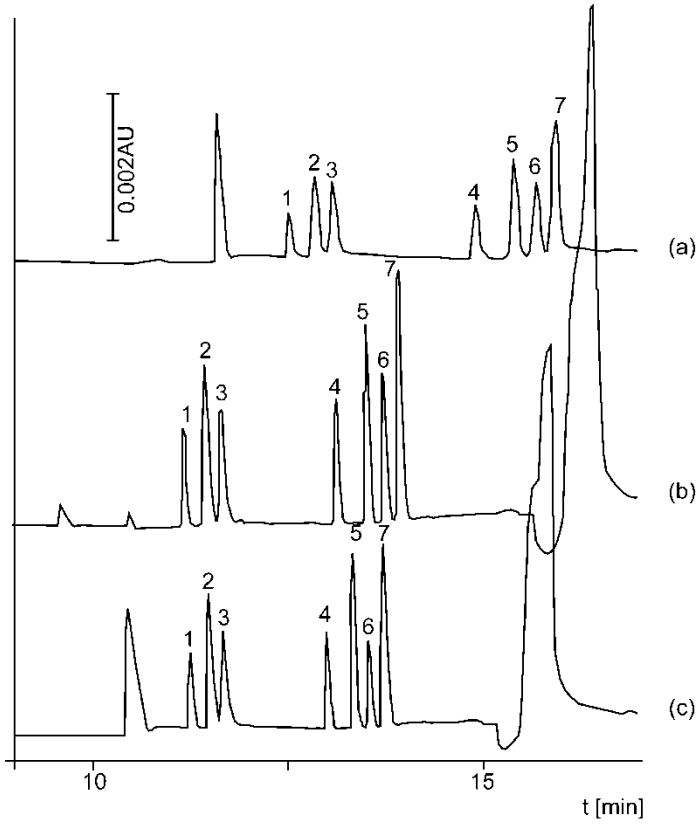

Figure 2. Electrophoretic separation of seven TCADs at the optimal extraction conditions in: (a) whole blood extract, (b) decomposed whole blood extract and (c) serum extract. 1. desipramine, 2. nortriptyline, 3. nordoxepin, 4. imipramine, 5. doxepin, 6. trimipramine (used as the internal standard to control volume repeatability of injected solutions), 7. amitriptyline.

Table 6. Average extraction recovery for six TCAD from serum samples present at concentration of 0.5 μg mL^{-1}

Drug	Extraction recovery (%) n = 4	RSD (%)
Amitryptyline	105.2	18.2
Doxepine	94.4	13.1
Imipramine	95.4	14.4
Nordoxepine	88.2	15.3
Nortryptyline	71.1	11.3
Despiramine	69.5	6.1

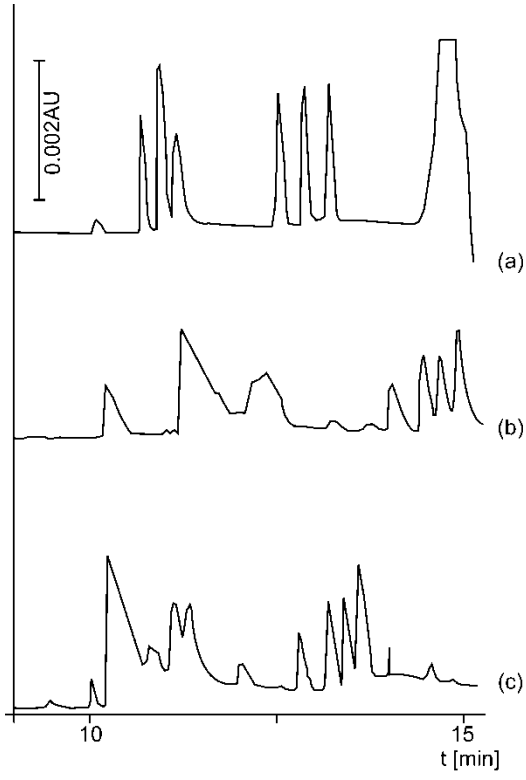

Figure 3. Electropherograms of urine extracts containing six TCADs: (a) decomposed urine after sonication and addition of $NaHCO_3$ with DEA, (b) decomposed urine after acetonitrile precipitation, (c) fresh urine after acetonitrile precipitation. The rest of the conditions are the same as in Figure 2.

material for analysis, this body fluid contains many various compounds and its matrix is considerably changeable in the course of time; it undergoes decomposition and precipitation.

CONCLUSIONS

We propose using the effective SPE procedure of five commonly used tricyclic antidepressants, present in fresh and decomposed blood at low and high level toxic concentrations, followed by capillary electrophoresis assay. We have extended this procedure to fresh whole blood samples including sonication as an additional step of extraction. The time (ten days) of blood samples kept at room temperature did not influence the extraction results. The procedure was also examined for the drugs present in serum at low toxic concentration and

has been proven to work well in this case. The addition of diethylamine to the samples before extraction also considerably improve the drug recoveries. The optimal method for extraction of the drug mixture from blood samples are as follow: Bond Elut® C18 (100 mg/1 mL) column (Varian); preparation of samples: addition of 0,01% diethylamine in 1 M sodium hydrocarbonate to 1 mL of blood sample (serum sample needs only dilution with 0.01% diethylamine in water), sonication for 15 min, dilution with 6 mL of phosphoric buffer and centrifuged; column conditioning with 3 mL of chloroform/2-propanol, 3 mL of acetonitrile, and 3 mL of water; column washing with 3 mL of water; elution with 2 mL chloroform/2-propanol.

Application of the optimized extraction conditions to decomposed urine samples, did not give expected results, most probably because of complexity and changeability (during storage) of this material matrix. This fact encourages us to study further experiments with this body fluid.

In the near feature, we would like to elaborate on a complete CE procedure for identification and determination of the examined tricyclic antidepressants present in all tested body fluids. We think that this method will be useful in our laboratory practice and in other forensic or clinical laboratories, as an alternative to the chromatographic methods.

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Received September 3, 2006
Accepted October 6, 2006
Manuscript 6933