Acta Poloniae Pharmaceutica – Drug Research, Vol. 69 No. 6 pp. 1023–1029, 2012 ISSN 0001-6837
Polish Pharmaceutical Society

CAPILLARY ELECTROPHORESIS SCREENING METHOD FOR SIX TRICYCLIC ANTIDEPRESSANTS IN HUMAN SERUM

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Abstract: A capillary electrophoretic (CE) method for screening of six tricyclic antidepressants: amitriptyline, nortriptyline, imipramine, desipramine, doxepin and nordoxepin, in human serum, has been developed. The drugs were separated in a bare fused silica capillary (50 µm i.d.) using the background electrolyte: 50 mM CAPSO (pH 9.54) in methanol/water with KCl addition. For increasing the method sensitivity, the sample concentration in the capillary (sample stacking) using pressure and electrokinetic injection has been applied. The standard addition method was used for calibration of the developed analytical procedure. The precision of the identification parameter (the relative migration time) and the quantitative parameter (the relative peak area) was within the range of 0.05–1.65 and 0.73–6.7 (RSD %), respectively. The detection limits were found to be 30 ng/mL for desipramine, 62.5 ng/mL for nortriptyline, and 50 ng/mL for remaining analytes.

Keywords: capillary electrophoresis, stacking, standard addition calibration, tricyclic antidepressants, human serum

Tricyclic antidepressant drugs (TCADs) belong to the first generation of psychotropic drugs which are still used for treatment of depression and other mental disorders. These drugs are efficacious but they are also dangerous with regard to their narrow therapeutic window.

In biological material (plasma, serum, urine or hair), TCADs are mainly determined by chromatographic methods such as gas (1–3) or liquid (4–6) chromatography. For the last decade of the 20th century, capillary electrophoresis (CE) has been playing a significant role in the drug analysis field. However, generally, CE technique with the most commonly used spectrophotometric detection, shows relatively low sensitivity. It is caused by a short optical path, as well as by injection of very small sample volumes (i.e., nanoliters). Several approaches have been taken in order to solve this problem such as: modification of optical cell shapes (7), application of high sensitive detectors (laser induced fluorescence detector (8), mass spectrometry detector (9) or electrochemical detector (10)), using effective on- and off-line preconcentration procedures, as well as analyte stacking techniques in the capillary (11). The last approach of lowering the analyte detection limit is especially convenient and suitable for the capillary electrophoresis technique. Under stacking conditions, a larger sample volume (5–50% of total capillary volume) may be injected into the capillary without disrupting peaks and loosing their resolution (12). The possibility of analyte stacking and other advantages of CE technique, such as high separation potential and small sample and reagents consumption, makes CE nowadays interesting tool for drug analysis in biological samples.

Generally, there is relatively a small number of CE procedures for determination of TCADs in biological fluids employing preconcentration of these drugs in the capillary with the universal UV spectrophotometry detection (13, 14). Chen et al. (13) proposed a sensitive CE method for determination of two tricyclic antidepressants: amitriptyline and its metabolite nortriptyline in human plasma using field-amplified sample stacking (FASS). Extracts of plasma samples (1 mL) were reconstituted with water and then electrokinetically injected (3 kV, 99.9 s) into a capillary after insertion of a 6 s water plug at pressure 10 psi. Detection of the drugs was 2.0 ng/mL for both amitriptyline and nortriptyline. The sensitive stacking-based CE method for the analysis of eight tricyclic antidepressants in human

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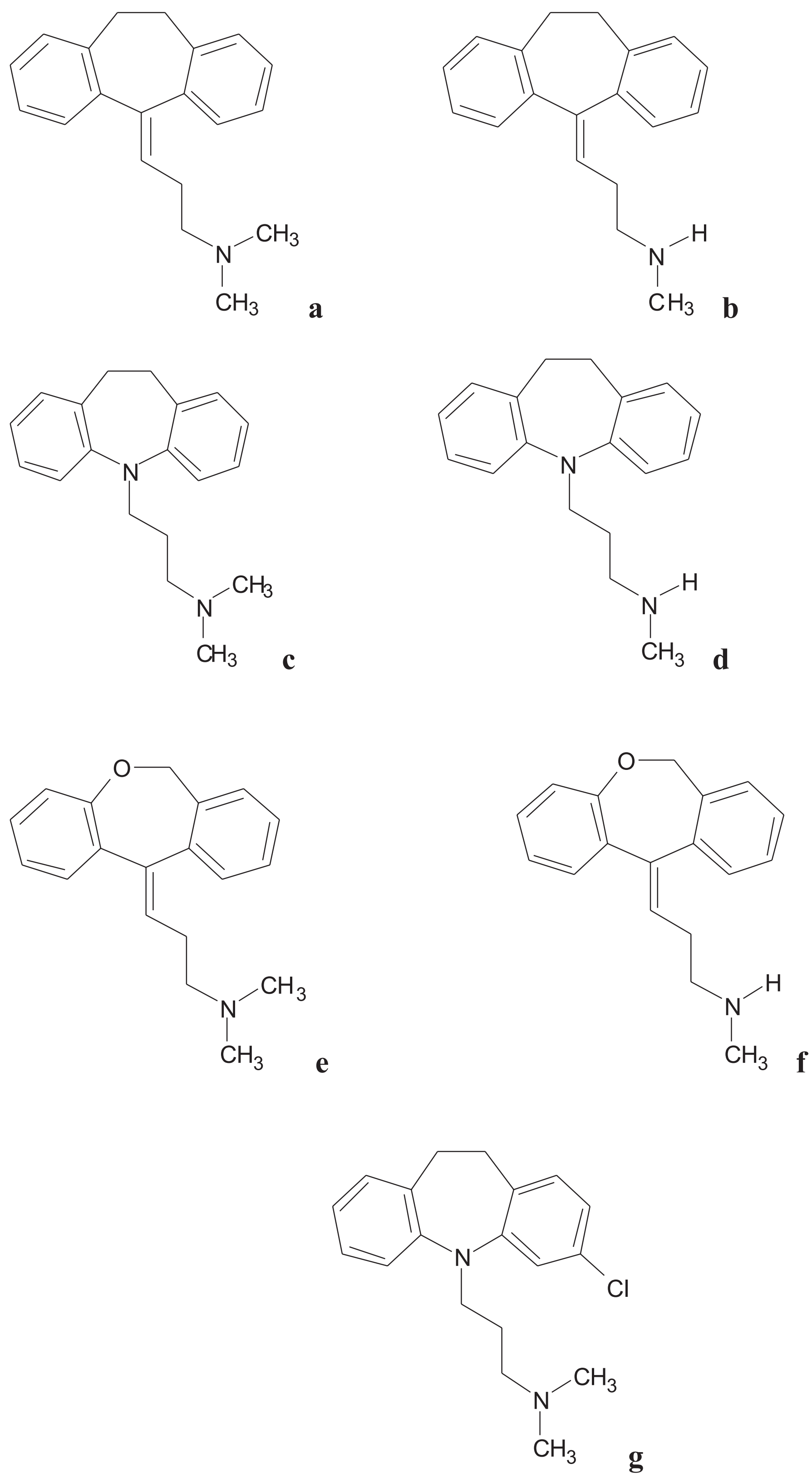

Figure 1. Chemical structures of the examined tricyclic antidepressant drugs: **a**. amitriptyline (Ami), **b**. nortriptyline (Nort), **c**. imipramine (Imi), **d**. desipramine (Des), **e**. doxepin (Dox), **f**. nordoxepin (Nordox) and **g**. clomipramine (IS)

serum has also been described (14). Stacking was caused by use of acetonitrile in the presence of sodium chloride as a sample medium. For analysis, 400 µL of spiked serum sample was mixed with acetonitrile (600 µL) and sodium chloride, and then centrifuged. The supernatant was introduced in the CE system. Using a bubble-shaped capillary, the limits of detection were in the order of 5 ng/nL.

This paper focuses on development of a simple CE method for screening analysis of six tricyclic antidepressants: amitriptyline, nortriptyline, imipramine, desipramine, doxepin, and nordoxepin (Fig. 1) in human serum. For experimental conditions, the capillary zone electrophoresis (CZE) technique was chosen and the sample was stacked by injecting it into the capillary by hydrodynamic or electrokinetic mode, in an organic (acetonitrile) or water-organic (0.01% H_3PO_4-methanol) medium. In order to find the optimal composition of the background electrolyte, a 3^2 factorial design was used. A three point standard addition method was applied for calibration of the proposed analytical procedure. This approach was found to be effective for simultaneous reduction of sample volume and time consumption.

EXPERIMENTAL

Examined drugs and materials

Stock drug solutions of: amitriptyline, nortriptyline, imipramine, desipramine, doxepin, nordoxepin and clomipramine (IS) in methanol (1 mg/mL) were used for examination. All standard drugs were purchased from Sigma-Aldrich (St. Louis, USA). Spiking solutions were prepared by appropriate dilution of these stock solutions with water. Drug-free serum (control serum) was obtained from the blood bank in Kraków, Poland. Reference human serum (Medidrug™ TCA S, MEDICHEM, Steinenbronn, Germany) containing six examined tricyclic antidepressants was supplied by Promochem (Łomianki, Poland).

Reagents

CAPSO (3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid) (Sigma Ultra) was purchased from Sigma-Aldrich (Stainheim, Germany). Methanol (HPLC gradient grade) was supplied by Merck (Darmstadt, Germany). Sodium hydroxide and potassium chloride, both of analytical grade, were obtained from POCh (Gliwice, Poland). Deionized water was used throughout.

Apparatus

The Prince 550 air thermostated capillary electrophoresis system (Prince Technologies, Emmen, The Netherlands) was used with a Lambda 1010 spectrophotometer (Bischoff, Leonberg, Germany) as a detector. Electrophoretic separation was conducted at 30 kV in a bare fused silica capillary of 50 µm i.d and 375 µm o.d. (Polymicro Technologies, Phoenix, USA), 100 cm long (66 cm to the detector). Drugs were detected by UV-light absorption at 210 nm. The initial background electrolyte was prepared by dissolving CAPSO (50 mM) in mixture of methanol/water (3:7, v/v), adjusted to pH 9.54 with 5 M NaOH. This mixture was then modified during optimization of the experimental conditions.

Table 1. Enhancement factors of the examined tricyclic antidepressants obtained in CE analysis under the stacking conditions.

Drug	Enhancement factors[a]			
	Sample injection			
	Hydrodynamic mode		Electrokinetic mode	
	Sample medium			
	CH_3CN	0.01% H_3PO_4/MeOH	CH_3CN	0.01 % H_3PO_4/MeOH
Amitriptyline	3.9	5.4	4.1	3.7
Nortiptyline	3.3	6.0	6.0	3.4
Imipramine	3.3	5.8	4.0	3.4
Desipramine	3.2	5.9	5.3	3.8
Doxepin	3.2	4.8	3.8	3.6
Nordoxepin	4.2	6.5	4.6	3.7

[a]) – calculated as the ratio of the drug peak area obtained by sample stacking (sample injecting time was lengthened till all peaks remained baseline separated) and the drug peak area obtained by usual injection (we assumed 12 s as the injection time, which corresponded to a hydrodynamically injected sample size, equivalent to 1% of capillary volume).

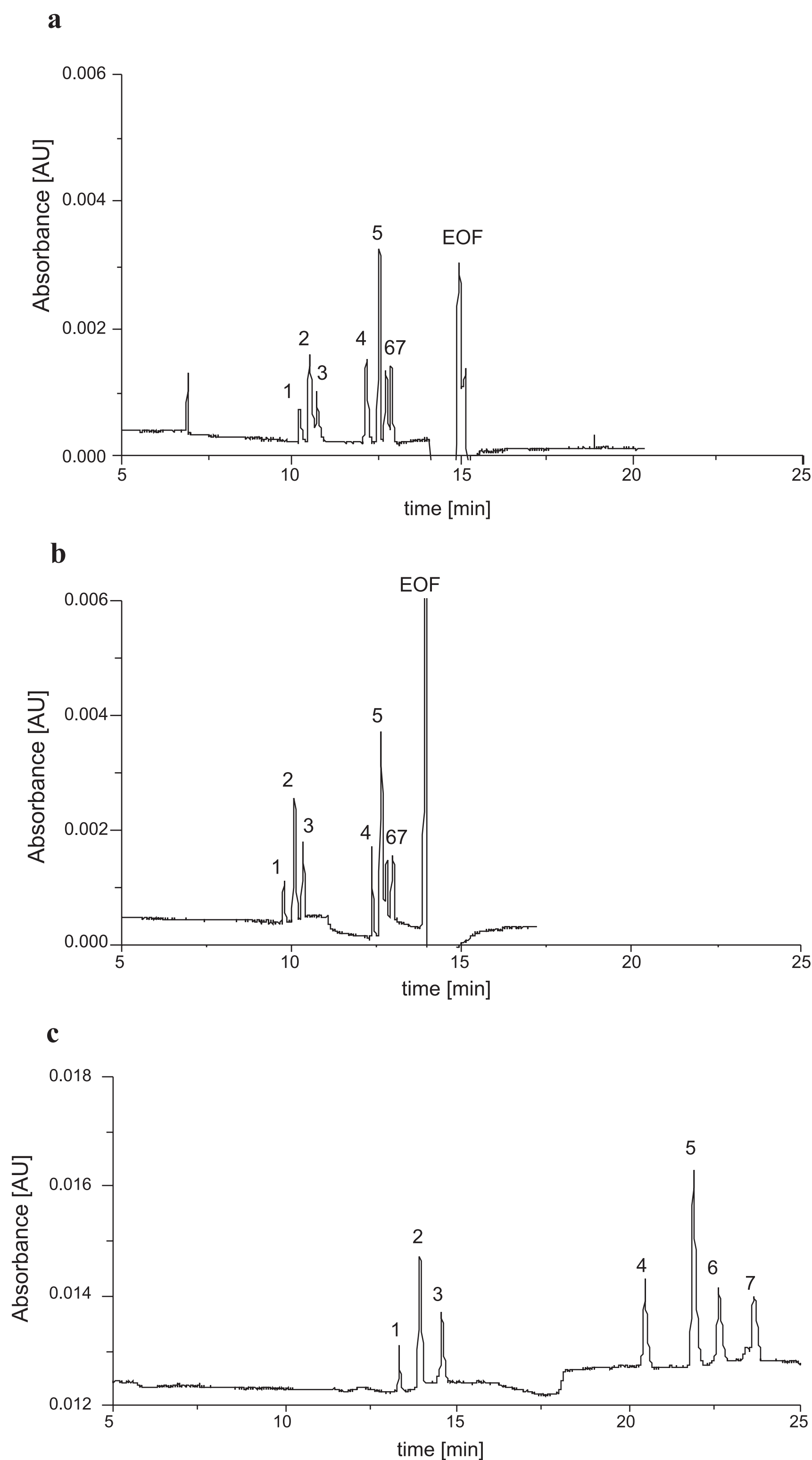

Figure 2. Optimization of separation conditions of the drugs (1 – Des, 2 – Nort, 3. – Nordox, 4 – Imi, 5 – Dox, 6 – IS, and 7 – Ami) by modification of the background electrolyte (50 mM CAPSO (pH 9.54) in methanol/water) composition: **a**. 30% (w/v) of methanol, **b**. 60% (w/v) of methanol, and **c**. 60% (w/v) of methanol and 0.143 mM of KCl. The concentrations of the drugs were as follows: 240 ng (Des), 400 ng (Dox), 500 ng (Ami, Nort, Imi and Nordox) and 2000 ng (IS). EOF – methanol

Preparation of drug standard solutions and serum samples

Drug standard solutions were prepared in acetonitrile or in a mixture of 0.01% H_3PO_4 and methanol (1:1, v/v) by adding the drug stock solutions to obtain concentration of 20 µg/mL in appropriate medium.

Lyophilized reference serum was prepared for analysis according to the procedure given by the manufacturer. After reconstitution, three (1–3) serum samples (0.5 mL) were pipetted and prepared in the following way: sample 1 remained untouched, sample 2 and 3 were spiked with standard drugs, respectively doubling and triplicating in drug concentration compared to the certificated concentrations in the reference material. For estimation of the detection limits of the analytes examined, the control serum samples were spiked with appropriate amounts of the drugs tested.

All samples were subjected to liquid-liquid extraction according to the following procedure. First, all samples were diluted with water (1:1, v/v) and spiked with the internal standard (2 µg of clomipramine). To each sample, 3 mL of 0.6 M NaOH and then 5 mL of n-hexane with isoamyl alcohol (99:1, v/v) were added and mixed. Then, the whole content was agitated for 10 min and centrifuged (4000 rpm) for 10 min. The organic layer (4 mL) was transferred successively into a 0.5 mL conical tube, and evaporated in a nitrogen stream mildly heated to 42°C. The residue was dissolved with 120 µL of the extracting mixture (n-hexane/isoamyl alcohol, 99:1; v/v) and vortexed for 0.5 min. The analytes were then re-extracted into 25 µL of 0.01% H_3PO_4. Twenty µL of the aqueous layer was mixed with 20 µL of methanol, and injected into the capillary.

Measurement procedure

A new capillary was conditioned by flushing with 1 M NaOH (30 min), 0.5 M NaOH (5 min), deionized water (5 min) and the background electrolyte (15 min) in sequence. The measurement procedure consisted of three main steps: 1) washing the capillary with freshly prepared background electrolyte (2 min), 2) sample injection in hydrodynamic (50 mbar) or electrokinetic mode (5 kV) from 12 to 96 s, and 3) separation of analytes under 30 kV. Between measurements the capillary was purged using the following sequence of reagents: 0.5 M NaOH (2 min), water (3 min) and the background electrolyte (3 min).

RESULTS AND DISCUSSION

This study covered the most important classic antidepressants including their metabolites: amitriptyline, nortriptyline, imipramine, desipramine, doxepin and nordoxepin. Clomipramine, a drug from the same pharmaceutical group, was chosen as the internal standard.

The enhancement in the sensitivity of CE analysis was achieved by use of stacking conditions, i.e., a standard drug mixture was dissolved in a low conductivity medium (acetonitrile or mixture 0.01% H_3PO_4/methanol, 1:1, v/v) and then injected hydrodynamically or electrokinetically into the capillary. Enhancement (stacking) factors, for all examined

Table 2. Optimization of the background electrolyte (50 mM CAPSO (pH 9.54) in methanol/water with salt addition) composition.

Point No.	MeOH/water (%, w/v)	Salt cation	Salt concentration (mM)	R[a] (n = 3)
0	30	–[b]	0	7.19
1	30	Na	0.143	8.21
2	30	Na	0.072	8.17
3	30	K	0.072	7.74
4	30	K	0.143	8.17
5	60	Na	0.143	8.77
6	60	Na	0.072	8.29
7	60	K	0.072	8.64
8	60	K	0.143	9.00
9	60	–[b]	0	7.73

[a]) the global sum of peak resolutions was calculated as the median from three repeated measurements (in calculations, peak areas were taken into account). [b]) no salt was added.

drugs were calculated by dividing the drug peak areas obtained by means of sample stacking (sample injecting time was lengthened from 24 to 96 s till all peaks remained baseline separated) and by usual injection (we assumed 12 s as the initial injection time, as it corresponds to the hydrodynamically injected sample size equivalent to 1% of the capillary volume). The best stacking conditions (the highest values of enhancement factors) for the standard drug solutions were: hydrodynamic injection mode and sample medium – mixture of 0.01% H_3PO_4 and methanol (1:1, v/v) (Table 1).

In order to improve the separation efficiency of the drugs, the initial composition of the background electrolyte was optimized taking into account three factors: 1. ratio of methanol to water in the background electrolyte, 2. kind of salt cation (Na^+ or K^+) added to the electrolyte, 3. concentration of salt added to this electrolyte. Each factor was examined on two value levels and the analyses were performed according to a 3^2 factorial design. As the optimum criterion, the global sum of peaks resolutions (R) between all adjacent drugs (i–j), including internal standard were selected. A significant increase in resolution was predicted after some preliminary research, thus the injection time was prolonged to 1 min.

$$R = \Sigma R_{i\text{-}j}$$

where:

$$R_{i\text{–}j} = 2\,\frac{t_j - t_i}{w_i + w_j}$$

t_i and w_i – retention time and peak width of peak I;
t_j and w_j – retention time and peak width of peak j;
If $R_{i\text{-}j} = 1.5$ then $R_{i\text{-}j}$ was assumed to be 1.5.

At optimal conditions, the response R should be a maximum one. On the basis of the results obtained from the experiments, the following composition of the separation electrolyte was chosen as the optimum one (Table 2; experimental point N 8, response R = 9.00): 0.143 mM KCl in methanol/water (60:40; w/v). The protonation degree of the drugs was lowered by increasing the amount of methanol in the background electrolyte, but there was only a little improvement in separation of two desmethyl amine compounds (nortriptyline and nordoxepin), however, the rest of the tested drugs were not baseline separated (compare Figs. 2a and 2b). The substantial improvement of separation efficiency was achieved by addition of KCl salt into the background electrolyte. This was caused by adsorption ability of cation K^+ on the capillary surface, which resulted in reduction of the electroosmotic flow and also by increasing the electric field (15) (Fig. 2c).

Comparing the findings in the performed examinations with the results obtained by other authors, it may be stated that the developed method requires comparable (14) or even smaller amounts of the examined body fluid (13) (taking into account requirement of at least two samples analysis). The sample preparation method used by Galeano-Diaz et al. (14) was simpler (only enrichment of a serum sample with sodium chloride and then precipitation with acetonitrile was required) and CE measurement was faster (ca. 6 min) but not all examined drugs were completely separated and for lowering detection limits appropriate bubble shape cell (bubble capillary) was required. The detection limits in the proposed procedure are higher than those reported in (13, 14) but they are low enough to apply this method for determination of the studied drugs in human serum at therapeutic concentrations.

Table 3. Validation parameters of the developed CE method.

Drug	Repeatability of				Limit of detection [ng/mL]	Linearity correlation coefficient (r^2)	Accuracy RE [%]
	$(TM_D/TM_{IS})^a$ [RSD, %]		$(A_D/A_{IS})^b$ [RSD, %]				
	C_1^c	C_2^d	C_1^c	C_2^d			
Amitriptyline	0.24	0.05	2.31	5.38	50.0	0.999	4.0
Nortriptyline	1.04	1.74	1.03	4.32	62.5	0.980	4.0
Imipramine	0.24	0.55	1.96	6.70	50.0	0.999	4.4
Desipramine	1.11	1.90	0.76	1.19	30.0	0.975	5.1
Doxepin	0.36	0.20	0.78	4.76	50.0	0.999	4.1
Nordoxepin	1.16	1.65	0.73	1.10	50.0	0.979	4.4

[a]) – ratio of the examined drug migration time and the internal standard migration time. [b] – ratio of the examined drug peak area and the internal standard peak area. [c] – lower concentration drug level (120, 200 and 250 ng/mL for desipramine, doxepin and the rest of examined drugs, respectively). [d] – higher concentration drug level (360, 600 and 750 ng/mL for desipramine, doxepin and the rest of examined drugs, respectively).

For validation of the analytical procedure, several analytical parameters of the developed method were determined. The precision was assessed by four times repeated analyses of serum specimens containing known concentrations (two concentration levels, Table 3) of the drugs investigated. By these means, the repeatability of both qualitative (relative migration time) and quantitative (relative peak area) parameters were estimated. The detection limits (LODs) for all drugs tested in the serum were determined by estimation of the minimum concentration equivalent to five times of the background noise signal. The linearity of the method was studied for each examined drug in serum samples in the following concentration ranges: 120–360 ng/mL for desipramine, 200–600 ng/mL for doxepin and nordoxepin, and 250–750 ng/mL for amitriptyline, nortriptyline and imipramine, and the correlation coefficient (r^2) was calculated on the basis of three concentrations (points) for each drug. The accuracy of the method was calculated as the relative error RE = [(desired value – obtained value) /desired value] × 100%. The obtained values of the analytical parameters are presented in Table 3.

CONCLUSIONS

A simple and sensitive CE screening method for six tricyclic psychotropic drugs in human serum has been developed. An experimental design (3^2 factorial) was used to find optimal composition of the separation electrolyte. As the optimum criterion, the global sum of peaks resolutions (R) between all adjacent drugs (i–j), including the internal standard has been selected. In order to calibrate the proposed analytical procedure, the standard addition method was used instead of the method of the set of standards, which is commonly used in analysis of biological fluids for drug determination. The reason for choosing the standard addition method was that this method (in contrast to the latter one) was hoped to compensate the matrix effects, which could be expected in the case of analysis of biological material, especially in the case of decomposed forensic samples.

The combination of sample preconcentration in the capillary with a three point standard addition method enables analysis of relatively small volumes of (3 × 0.5 mL) serum samples, containing the examined drugs at therapeutic concentration levels. The proposed CE procedure may be an alternative for the chromatographic methods commonly used for examination of these antidepressants in clinical and forensic laboratories.

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Received: 02. 09. 2011