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Validation of non-aqueous capillary electrophoresis for simultaneous determination of four tricyclic antidepressants in pharmaceutical formulations and plasma samples

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Abstract

We present the validation of a method using non-aqueous capillary electrophoresis (NACE) for quantitative analysis of four tricyclic antidepressants (TADs) in pharmaceutical formulations and plasma. The method presented high resolution allowing the separation of the TADs in 4.3 min at optimized conditions: 50 mM ammonium acetate, 1 M acetic acid in acetonitrile, capillary with 48 cm in length, 40 cm to the detector, and voltage of 30 kV. Acceptable precision (relative standard deviation R.S.D.14.1% from plasma samples) and linearity were achieved using the internal standard (IS) method. The limits of quantification determined for plasma, after liquid–liquid extraction (LLE), were between 30 and 50 ng ml^{-1}. These values are beyond the plasmatic therapeutic concentration. Our results were found comparable or better than those described in the literature for high performance liquid chromatography (HPLC)-based methods.

Keywords: Imipramine; Amitriptyline; Desipramine; Nortriptyline

1. Introduction

Imipramine (IMI), amitriptyline (AMI), and their respective demethyled metabolites desipramine (DES) and nortriptyline (NOR) are active principles of four psychiatric drugs widely used in the treatment of depressive disorders [1]. These compounds are secondary and tertiary amines with a common structure formed by two aromatic rings fused with an seven-atom cycle (Fig. 1). Because of their structure, this class of compounds is generally named tricyclic antidepressants (TADs).

Patients treated with identical dosages of these TADs may present large difference in the plasmatic drug concentration what may imply in suboptimal or even no therapeutic effect [2–4]. Such variability requires an individualized monitoring of the treatment, which is called, in clinical praxis, therapeutic drug monitoring (TDM) [3]. The applicability of TDM depends on the availability of rapid, sensitive, and reliable methods for drug determination.

The main technique used in TAD analysis is high performance liquid chromatography (HPLC), which requires specific stationary phases besides the use of relatively large amounts of organic solvents [5–7]. Capillary electrophoresis (CE) is a complementary analytical technique to HPLC. It presents, as main advantages, low reagent and sample consumption, high separation efficiency, reduced analysis time, and others [8,9]. Despite these advantages, CE remains less used in industrial and clinical routine analysis. As a matter of fact, it might be attributed to the relative limited number of validated quantitative analytical methods in CE.

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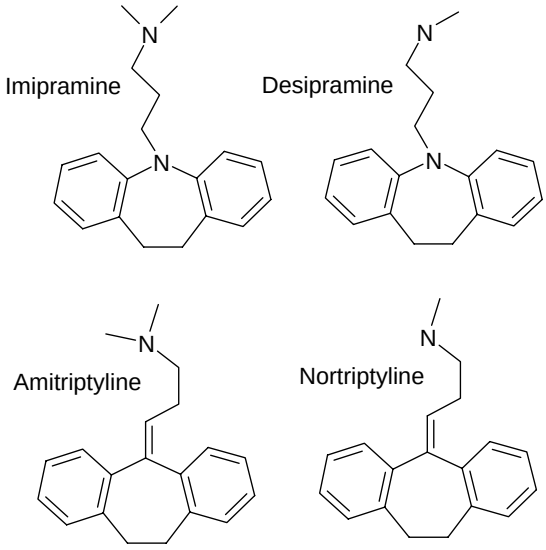

Fig. 1. Chemical structures of the TADs studied in this work.

The analysis of highly hydrophobic and structurally similar compounds by CE often requires the use of special additives like surfactants or complexing agents in order to improve the separation selectivity [10–13]. Non-aqueous capillary electrophoresis (NACE) [14–18] has emerged as an alternative to obtain high-resolution separations of hydrophobic drugs without using the above-mentioned additives. In recent years, authors have reported the separation of TADs by NACE [14,15]. Salomon et al. [19] published a work describing the separation of seven TADs using CE. Although full resolution of the analytes was achieved when methanol was added to the buffer (3-[cyclohexylamino]-2-hydroxy-1-propanesulphonic acid), the method was not fully exploited regarding quantitative analysis. Veraart et al. reported a NACE method for the determination of IMI, AMI, DES, and NOR from urine and serum after at-line solid-phase extraction (SPE) [20]. These authors emphasized the need to remove water and salt from samples to successfully separate the analytes by NACE. In another paper, the same group presented the implementation of a on-line dialysis step before the SPE in order to increase the method sensitivity [21].

Just recently a paper was published by Peri-Okonny et al. [22] reporting the simultaneous determination of seven TADs and two bronchodilator drugs using a NACE–mass spectrometry method. The authors optimized the composition and apparent pH of the buffer and compared the separation performance with reversed-phase gradient and isocratic HPLC. Baseline resolution was achieved in about 30 min and no method validation was presented.

In this work, we report the separation optimization and the validation [23–25] of a NACE method for simultaneous determination of IMI, AMI, DES, and NOR in two different applications. TADs were extracted from plasma samples after liquid–liquid extraction (LLE) and pharmaceutical formulation were analyzed regarding purity and specifications.

2. Experimental

2.1. Chemicals

All chemicals and solvents were of analytical grade. Methanol, acetonitrile, acetic acid, and isoamyl alcohol were purchased from Merck (Darmstadt, Germany). Ammonium acetate, NaCl, NaOH, and hexane were purchased from Mallinckrodt Baker (Xalostoc, Mexico). TAD standards were purchased as hydrochloride salts from different suppliers as follows: IMI and DES from Ciba Geigy (São Paulo, Brazil), AMI from Sigma, (St. Louis, MO, USA), NOR from Sandoz (São Paulo, Brazil) and clomipramine (3-chloro-10,11-dihydro-*N*,*N*-dimethyl-5H-dibenz[b,f]azepine-5-propanamine), used as internal standard (IS), from Squibb.

Antidepressant tablets were purchased from local drugstores and are produced by Brazilian branch of Novartis (TOFRANIL® and PAMELOR®), and Prodome (TRYPTANOL®). Plasma samples used for method development were supplied by Hospital das Clínicas de Ribeirão Preto, University of São Paulo, Brazil.

2.2. Instrumentation and CE procedures

CE experiments were carried out in a HP ^{3D}CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a diode-array detector. Analytes were monitored at 214 nm with a bandwidth of 16 nm. CE was performed in normal mode, by applying a 30 kV positive voltage, using a fused silica capillary with 50 μm i.d. Samples were introduced hydrodynamically using 50 mbar. Best separation was achieved with acetonitrile solution of ammonium acetate 50 mM and acetic acid 1 M as non-aqueous separation medium. The capillary was washed with 0.1 M NaOH and demonized water for 10 min at the beginning of each day. Before every injection a column conditioning was performed with 0.1 M NaOH (1.5 min), water (2.0 min), and run buffer (2.5 min).

2.3. Solutions and sample preparation

Stock solutions of TADs (2 mg ml^{-1}) and internal standard (1 mg ml^{-1}) were prepared in methanol; adequate aliquots were diluted in order to generate the work calibration standards.

Sampling procedure for pharmaceutical formulations was carried as follows. The tablets were finely powdered and homogenized in a mortar and an amount corresponding to the average tablet weight was dissolved in methanol. The solution was filtered through a PTFE hydrophobic membrane with 0.45 μm pore diameter (Millipore, Bedford, USA).

Blank human plasma (plasma from patients not exposed to any drug for at least 72 h) was fortified with standard

solutions of AMI, IMI, NOR, DES, and the internal standard and homogenized in a vortexer. The extraction procedure was performed as reported by Queiroz et al. [26]. Briefly, 200 μl of a solution containing 1.5 M NaOH and 1 mg ml^{-1} NaCl and 5 ml of 99:1 (v/v) hexane/isoamyl alcohol were added to 1 ml plasma sample. The mixture was stirred for 30 min in a shaker (100 oscillations/min) and centrifuged at 750 × *g* for 5 min. An amount of 4 ml from the organic phase was then collected and evaporated under a gentle nitrogen flow at room temperature. The solute extract was dissolved in 20 μl of methanol for CE analysis.

2.4. Method validation

The internal standard method was used for quantitative analysis. Clomipramine was chosen since it meets most of the requirements for a good internal standard [25]. The method linearity was assessed by preparing five calibration standards in concentrations ranging from 20 to 100 μg ml^{-1} for pharmaceutical formulations and from 50 to 500 ng ml^{-1} for plasma samples. Each standard was electrophoresed in triplicates. Calibration curves were built by plotting the relative peak areas (analyte-to-IS ratio) as a function of the standard concentration.

The limit of detection (LOD) was considered as the minimum analyte concentration yielding a signal-to-noise ratio equal to three. The limit of quantification (LOQ) was adopted as the lowest analyte concentration yielding a signal 10 times greater than the noise and that could be reliably determined (relative standard deviation, R.S.D. < 15%).

The precision of the method was determined by the measure of repeatability (intraday) and intermediate precision (interday). The repeatability was assessed by the R.S.D. of replicate experiments ($n = 5$) at three different concentrations: 20, 60, 100 μg ml^{-1} for pharmaceutical formulations and from 50, 200, and 500 ng ml^{-1} for plasma samples. The intermediate precision was determined by measuring the R.S.D. of triplicate experiments carried out in different days ($n = 5$) at three different concentrations, the same as described for repeatability assays.

The method accuracy for pharmaceutical formulation determination was evaluated by recovery experiments using the standard addition technique [24]. TAD standards at three different concentrations (20, 40, and 60 μg ml^{-1}) were added to solutions of each pharmaceutical formulation containing 30 μg ml^{-1} of the TAD.

For plasma analysis, the absolute yield of the LLE was determined as follows: an amount of 100 μl of standard solution containing the four TADs was added to 1 ml blank plasma and the LLE was performed. The concentration of TADs in the extracted aliquot was compared to the corresponding standard solution. The relative yield of LLE was obtained in a similar manner, except that the IS was added to the plasma samples before de LLE procedure. All experiments were made in triplicate at three concentration levels.

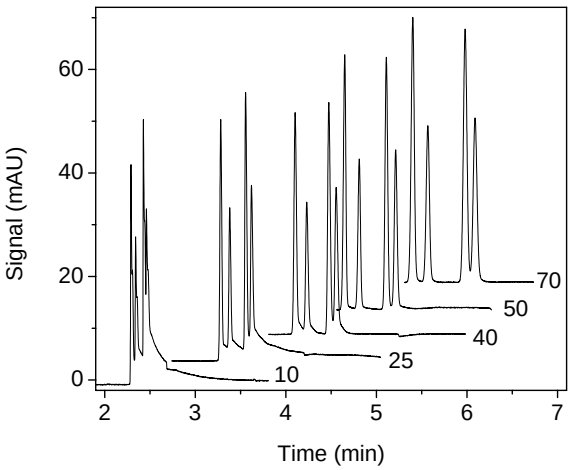

Fig. 2. Effect of electrolyte concentration on analyte separation and analysis time. Acetic acid was maintained constant at 1 M and ammonium acetate concentration was varied from 10 to 70 mM (as indicated in the graph) in acetonitrile. Peaks correspond to analytes in the following order: AMI, IMI, NOR, and DES. Fused silica capillary (48 cm; 40 cm to the detector; 50 μm i.d.); applied voltage: 25 kV; pressure-driven injection: 5 s at 50 mbar; detection at 214 nm; analyte concentration: 50 μg ml^{-1}.

3. Results and discussion

3.1. NACE method optimization

In order to optimize analyte separation the effect of background electrolyte (BGE) concentration on resolution and analysis time was investigated. A sequence of runs at different BGE concentrations is shown in Fig. 2. By raising the ammonium acetate concentration, an enhancement in resolution was observed for both pairs of analytes (AMI/IMI and NOR/DES). This effect can be assigned to the pH* (apparent pH) approaching to the analyte pK_a* values. From Henderson–Hasselbalch equation, it is easy to see that the medium pH* increases with the ammonium acetate concentration for a given acetic acid concentration. We have determined the analyte pK_a* in acetonitrile and found values were 20.60 for DES, 20.66 for NOR, 20.74 for IMI, and 20.80 for AMI. The complete resolution for the four analytes was achieved in pH* values just above 21.10 (50 mM ammonium acetate). This observation is in agreement with previous NACE studies as reported in the review by Riekkola [27].

The analysis time also increased with the BGE concentration, which is explained by the reduction of electroosmosis. It is well known that an increase in ionic strength causes a reduction of the thickness of the electric double layer. Consequently, the zeta potential decreases resulting in electroomosis suppression [28].

Since the observed resolution was not enhanced for BGE concentration above 50 mM, this value was chosen as optimal value, and therefore used for method validation. Typical separation profile at optimized conditions is shown in Fig. 3. The peak number three is the internal standard clomipramine that is totally separated of the other analytes.

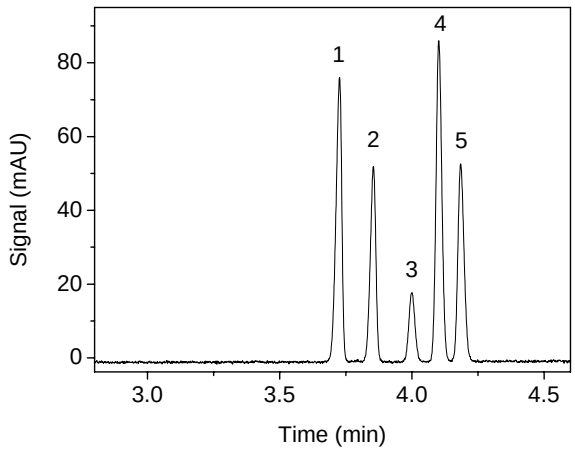

Fig. 3. Typical separation of analyte standards at the optimized conditions; (1) amitriptyline, (2) imipramine, (3) clomipramine, as internal standard, (4) nortriptyline, and (5) desipramine. Applied voltage: 30 kV; analyte concentration: 100 μg ml^{-1} each TAD and 40 μg ml^{-1} the IS. Other conditions are the same as in Fig. 2.

3.2. Method validation for pharmaceutical formulations

The suitability of CE for pharmaceutical formulation analysis has been demonstrated earlier [29–31]. The validation requires the assessment of migration time and peak area reproducibility, detector response linearity with the sample concentration, accuracy, quantification, and detection limits.

The linearity parameters related to calibration curves are presented in Table 1. The correlation coefficients obtained using the least-squares regression were satisfactory with values larger than 0.9987. The LOQ was 20 μg ml^{-1} for the four TAD studied. Limits of detection about 0.5 μg ml^{-1} were found (signal-to-noise ratio about 3) for 20 nl injections. Such values correspond to about 10^{-13} mol as typically found for CE absorbance detection systems [32]. In addition, the observed LOD are similar to the ones reported by Salomon et al. [19]. Karpinska and Starczewska [33] reported the determination of IMI and AMI in pharmaceutical formulations by reverse phase HPLC and found LOD of 0.332 and 0.443 μg ml^{-1}, respectively. Usually, the optical pathlength in a HPLC detector is about 2 mm, approximately 40-fold the capillary inner diameter. In spite of this huge difference, the LODs observed are comparable. Such results highlight one of the advantages of CE over the HPLC techniques which is the small zone dispersion effect [32].

Table 1
Linearity parameters obtained in method validation

Analyte	Linearity range (μg ml^{-1})	Calibration parameters		
		Intercept	Slope	r
AMI	20–100	−0.0425	1.3976	0.9990
IMI		0.0136	0.7934	0.9987
NOR		−0.0216	1.3784	0.9995
DES		−0.0289	0.8584	0.9999

Table 2
Method repeatability and intermediate precision regarding the relative peak areas (analyte/internal standard)

Analyte	Concentration (μg ml^{-1})		
	100	60	20
Intraday R.S.D. (%) ($n = 5$)			
AMI	1.3	1.8	1.2
IMI	1.8	2.8	1.5
NOR	1.2	1.9	1.4
DES	1.7	3.7	1.6
Interday R.S.D. (%) ($n = 5$)			
AMI	4.6	4.6	3.4
IMI	3.2	3.9	3.3
NOR	5.0	3.0	5.9
DES	3.8	2.5	4.9

Relative standard deviation (%) is presented for three different concentrations.

Method precision was evaluated by means of accessing the repeatability (intraday) and intermediate precision (interday), regarding both migration time and relative peak area (analyte-to-IS ratio, Table 2). The precision assays yielded R.S.D. values ranging between 0.8 and 2.3 for migration times and between 1.2 and 3.7 for relative peak areas. For interday experiments, R.S.D. between 0.8 and 2.3, and 2.5 and 5.9 were found for migration times and relative peak areas, respectively.

Method accuracy was evaluated by recovery experiments using the standard addition technique. Mean values between 97.0 and 103.9% of recovery (R.S.D. lower than 1.7) were obtained for all TAD levels.

The validated method was employed to quantify the TADs in commercially available tablets (Fig. 4). The analysis

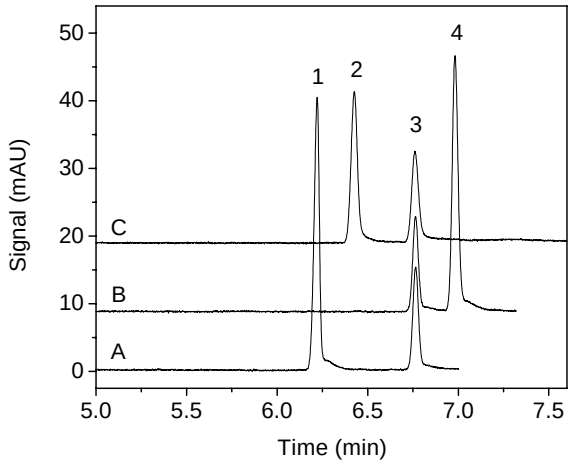

Fig. 4. Analysis of a commercial pharmaceutical formulations: (A) TRYPTANOL® (AMI), (B) PAMELOR® (NOR), (C) TOFRANIL® (IMI). (1) Amitriptyline, (2) imipramine, (3) clomipramine, as internal standard, and (4) nortriptyline. Fused silica capillary (64 cm; 55.5 cm to the detector; 50 μm i.d.). Applied voltage: 30 kV; pressure-driven injection: 5 s at 50 mbar; analyte concentration: 100 μg ml^{-1} each TAD and 40 μg ml^{-1} the IS.

Table 3
Results of TADs analysis in commercial pharmaceutical formulations

	TRYPTANOL® (AMI)	PAMELOR® (NOR)	TOFRANIL® (IMI)
Labeled claim (mg)	25	50	25
Amount found (mg)	24.56	50.20	24.93
R.S.D. ($n = 3$) (%)	0.21	0.83	0.54

results (Table 3) have shown good agreement with the labeled content, thus confirming the usefulness of NACE in routine pharmaceutical industry quality control.

3.3. Method validation for plasma samples

According to Causon, [34] an analytical method validation for biological samples must be carried out in the presence of the same biological matrix, e.g. blood plasma. Therefore, the calibration curve for method validation of plasma samples was carried out with standard solutions recovered from plasma by the LLE procedure described above. The calibration curve parameters determined are presented in Table 4. Correlation coefficients larger than 0.9992 were obtained. The achieved LOD and LOQ (Table 4) are beyond the drug plasmatic therapeutic concentration, which are 50–200 ng ml^{-1} for AMI, 75–250 ng ml^{-1} for IMI, 50–150 ng ml^{-1} for NOR, and about 145 ng ml^{-1} for DES [3].

Method precision was measured by repeatability (intraday) and intermediate precision (interday), for relative peak area, as shown in Table 5. R.S.D. values ranging between 1.2 and 7.9 for intraday and between 4.2 and 14.1 for interday assays were found. These results are in good agreement with the method requirements according to the literature [34].

The values for absolute yield for the four TADs for every tested concentration were about 75%. Since only 80% of the organic phase is collected in the extraction procedure, the relative yield results indicate a high efficiency of the LLE method.

A typical result for the simultaneous determination of the four TDAs in plasma samples after liquid–liquid extraction is presented in Fig. 5. Our results have shown the potential of this method for application in TDM of TADs. The possibility of simultaneous determination of these drugs is of great importance since more then one TAD might be used in combination when patients show poor response to monotherapy [35].

Table 5
Method repeatability and intermediate precision regarding the relative peak areas (analyte/internal standard) for plasma samples

Analyte	Concentration (ng ml^{-1})		
	500	200	50
Intraday R.S.D. (%) ($n = 5$)			
AMI	1.4	2.0	7.9
IMI	3.8	1.9	5.6
NOR	3.2	1.2	5.5
DES	3.3	2.5	2.8
Interday R.S.D. (%) ($n = 5$)			
AMI	7.8	9.8	14.1
IMI	8.8	11.0	8.0
NOR	7.7	9.4	13.1
DES	4.2	5.9	8.5

Relative standard deviation (%) is presented for three different concentrations.

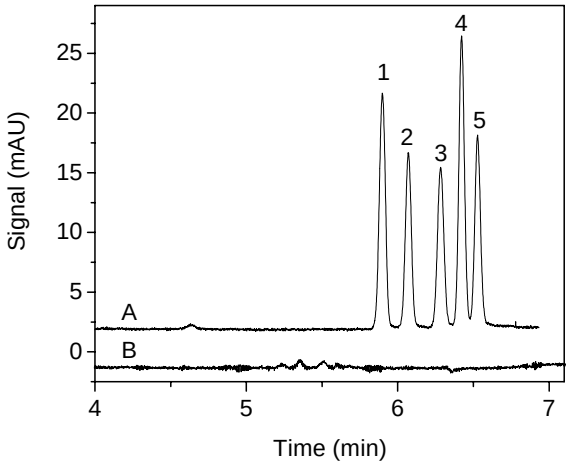

Fig. 5. Analysis of (A) analyte standards recovered from human plasma and (B) blank plasma. (1) Amitriptyline, (2) imipramine, (3) clomipramine, as internal standard, (4) nortriptyline, and (5) desipramine. Fused silica capillary (64 cm; 56 cm to the detector; 50 μm i.d.). Applied voltage: 30 kV; pressure-driven injection: 15 s at 50 mbar; analyte and IS plasmatic concentration: 500 ng ml^{-1}.

4. Conclusions

We have described a complete method validation for quantitative analysis of four TADs in pharmaceutical formulations and in human plasma samples. CE appears to be an

Table 4
Linearity parameters and limits of detection and quantification achieved in method calibration for plasma samples

Analyte	Linearity range (ng ml^{-1})	Calibration parameters			LOQ (ng ml^{-1})	LOD (ng ml^{-1})
		Intercept	Slope	r		
AMI	30–500	0.0125	0.0029	0.9994	30	20
IMI	50–500	0.0250	0.0020	0.9995	50	30
NOR	30–500	−0.0102	0.0033	0.9992	30	20
DES	50–500	−0.0162	0.0023	0.9996	50	30

ideal separation technique for TADs formulations analysis since most of the tablet composition is a neutral incipient vehicle, and therefore it will never interfere with the analyte peaks. Furthermore, silica capillaries are not susceptible to damages caused by strong adsorption of the matrix such as in HPLC columns. The limits of quantification achieved for the four TADs in plasma samples were below the plasmatic therapeutic concentration, what proves the described method to be suitable for use in clinical praxis, e.g. therapeutic drug monitoring.

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