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Dialysis–solid-phase extraction combined on-line with non-aqueous capillary electrophoresis for improved detectability of tricyclic antidepressants in biological samples

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Abstract

Dialysis–solid-phase extraction (SPE) sample pretreatment is combined on-line with non-aqueous capillary electrophoresis for the determination of tricyclic antidepressants in urine and serum. After clean-up and enrichment, the water is removed from the sample matrix and the analytes are eluted from the cartridge by means of an organic solvent. Next, the eluate is transported to the capillary and the injection is performed electrokinetically. This injection, which does not suffer from an adverse sample matrix effect because of the SPE step, results in further analyte concentration. The detection limits are in the 0.02–0.1 μg/ml range and the day-to-day repeatabilities are between 2.5 and 9.5%, which is quite satisfactory.

Keywords: Dialysis; Sample handling; Extraction methods; Non-aqueous capillary electrophoresis; Antidepressants

1. Introduction

Capillary electrophoresis (CE) is a technique that is highly suitable for the determination of charged compounds, with the analysis of compounds in biological fluids being a typical field of application. However, when biological fluids are injected directly into a CE capillary, problems such as clogging of the capillary by particulate matter, irreversible binding of proteins and peak distortion can occur because of the high salt concentration. Proper sample preparation prior to a CE analysis is the preferred way to solve these problems.

In previous papers, solid-phase extraction (SPE) [1–4] or dialysis–SPE [5,6] sample preparation was coupled at-line or on-line with CE, via a laboratory-made interface. The at-line SPE–CE system was successfully used to determine positively [1] and negatively [2,3] charged and amphoteric [4] drugs in urine and serum. The methods featuring dialysis–SPE–CE which is a truly on-line set-up, have the advantage of using less sample, and were used for negatively charged [5] and amphoteric [6] drugs in the same matrices.

One main difference between the SPE–CE studies on negative and amphoteric as against positive analytes (tricyclic antidepressants; TCADs) was that, in the latter case, a non-aqueous buffer had to be used. Non-aqueous CE has been presented multiple

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times over recent years. Quite a number of papers were devoted to the theoretical aspects [7,8], as well as to the improved or other separations [9]. However, the separations shown in previous papers were performed using sample stock solutions into organic solvents. This is because the main problem using non-aqueous CE is the samples may only contain small amounts of water and even little amounts of salts. Because of this, the use of non-aqueous CE is not straightforward. In the present paper, the ease of use of dialysis–SPE for sample preparation on-line coupled with non-aqueous CE will be studied for the first time. To show that the method is generic, an existing published non-aqueous CE method (published only for stock solutions) will be used for the analysis of the determination of TCADs in urine and serum matrices.

Although the same approach will be used as in-line SPE–non-aqueous CE, the present procedure is more complicated because of the on-line character of dialysis–SPE–CE. One critical step is the removal of the bulk of the water from the SPE cartridge without losing the analytes. Another important difference is that with the SPE–CE system, desorption from the SPE cartridge was performed using methanol, which was subsequently mixed with acetonitrile–acetic acid and injected hydrodynamically into the CE capillary. This cannot easily be mimicked in the on-line set-up: if a plug of a non-conducting solvent, like pure methanol in this case, is injected into the CE, no separation can be performed. Therefore, electrokinetic injection has to be used as an alternative, to prevent the entrance of the methanol plug. The system modifications required to ensure that no methanol will enter the capillary will be discussed below.

Electrokinetic injection usually is not one's first choice as an injection method, because a change of sample matrix, especially a change of conductivity, results in changing amounts of analyte being introduced into the CE capillary. This especially creates problems when biological samples are injected. However, in this study, an automated on-line sample preparation procedure effects the removal of the proteins, salts and water before injection of the analytes into the CE. As a result, the analyte-containing matrix that is introduced has a constant composition, viz. that of the eluent buffer. The overall result is the injection of a relatively large amount of analytes, which are contained in a very narrow injection plug. Consequently, the initial peak width is very small.

2. Experimental

2.1. Chemicals and samples

Acetonitrile, boric acid, methanol and phosphoric acid were obtained from J.T. Baker (Deventer, The Netherlands). *n*-Decanoic acid, sodium dihydrogenphosphate monohydrate and disodium hydrogenphosphate 12-hydrate were from Merck (Darmstadt, Germany) and acetic acid and sodium acetate from Riedel-de Haën (Seelze, Germany). Water was demineralized and distilled before use. In all cases, chemicals of the best available quality were used.

Urine was collected from five healthy volunteers on 3 subsequent days. The samples were pooled and divided into 100-ml portions and frozen at −18°C. Bovine serum of untreated animals was purchased from Sigma; it was divided into 10-ml portions and frozen at −18°C. The biological samples were stored for a maximum period of 3 months.

2.2. Methods

The set-up of the dialysis–SPE–CE system (Fig. 1) and the instruments used were described in detail in Refs. [5,6]. The experimental parameters are summarized in Table 1. During optimization, the protocol elaborated in one of our earlier papers [6] was followed as far as was possible. Items that received special attention in the present study are marked by an asterisk in Table 1.

3. Results and discussion

In the present paper, parameters such as CE buffer selectivity, back-pressure optimization and SPE–CE interfacing were studied first. Next, the dialysis procedure was optimized, and, finally, the analysis of biological samples was studied.

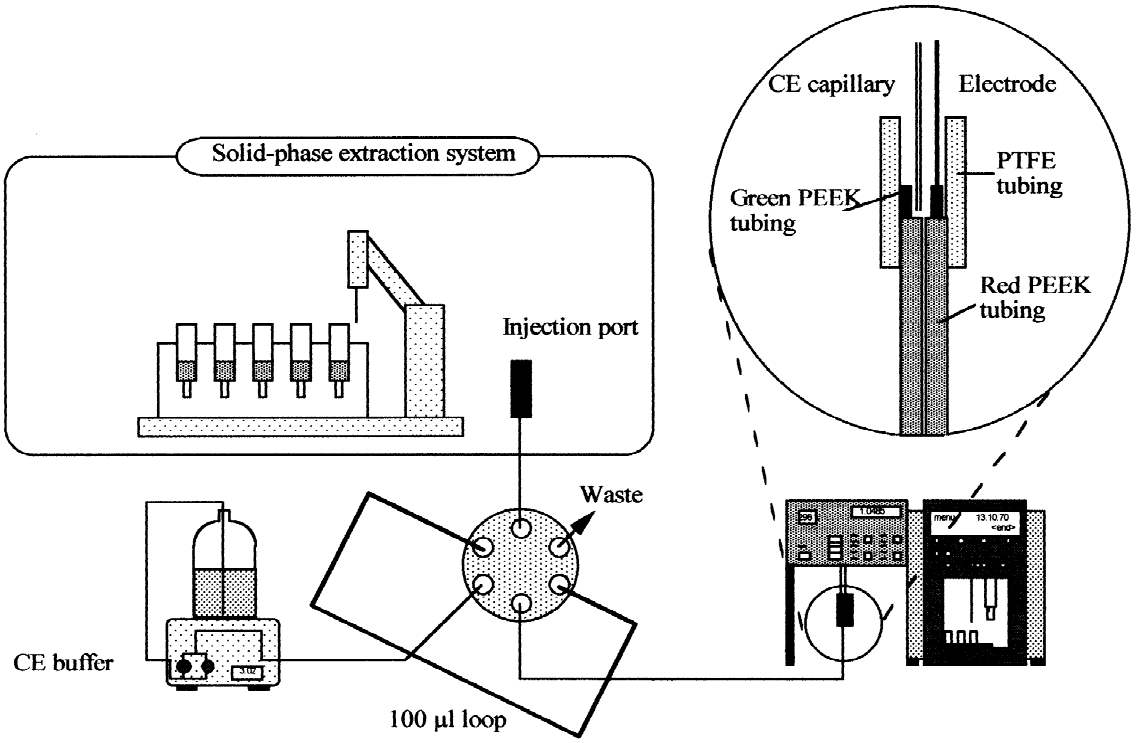

Fig. 1. At-line SPE–CE configuration. For more details, see text. PEEK, polyether ether ketone.

3.1. CE buffer and SPE cartridge

A non-aqueous buffer containing 1 *M* acetic acid and 25 m*M* ammonium acetate in acetonitrile was selected for the CE separation. This buffer was successfully used in the literature for the CE separation of standard solutions of TCADs [10]. In a later study [1], it was shown that hydrodynamic injection of standard solutions in acetonitrile containing 1 *M* acetic acid yields nicely resolved analyte peaks, but that injection of aqueous standard solutions gives broad peaks. In the quoted paper it was also demonstrated that methanol has to be used to elute the analytes from the SPE cartridge. Unfortunately, the presence of a small plug of methanol in the CE capillary will cause the current to drop to zero, because it is a non-conducting solvent. As an alternative approach, we therefore used electrokinetic injections, in combination with a buffer flow from the detection towards the injection side of the capillary, with the conditions being selected in such a way that the analytes can, but methanol cannot, enter the capillary.

As regards the 20×2 mm I.D. SPE cartridge (Table 1), C_2-bonded silica was used as sorbent, because longer alkyl chains will result in too strong interaction with the analytes.

3.2. Back-pressure optimization

During injection, there will be a small electroosmotic flow towards the outlet of the capillary. This will cause the presence of a small plug of methanol in the capillary, which has a low resistance and causes the current to decrease. In order to prevent this, pressure should be applied at the far end of the capillary, preferably by using a somewhat higher buffer level there.

Sample introduction was performed by adding 1 ml of aqueous sample containing 10 μg/ml of each TCAD, to the SPE cartridge. Next, elution was effected with 1 ml of methanol at a flow of 0.2

Table 1
Experimental conditions used for TCADs

Parameter	Comment
Samples	
Urine	None
Serum, free analyte concentration	None
Serum, total analyte concentration	Add 1 part of PRR[a] to 9 parts of serum*
Dialysis	
Dialysis block	Donor phase, 100 μl; acceptor phase, 170 μl
Membrane	Cellulose acetate, molecular mass cut-off, 15,000
Sample injection	0.5 ml prior to start
Acceptor solution	10 m*M* phosphate buffer, pH 7.0
Acceptor solution flow-rate	0.5 ml/min
Dialysis time urine	12 min*
serum	12 min*
SPE	
Cartridge	20×2 I.D. mm cartridge packed with 5 μm C_2-bonded silica (Hewlett-Packard)
Desorption solvent	Methanol
Desorption buffer flow-rate	0.1 ml/min*
Wash solution	Acetonitrile–water (20:80, v/v)
Wash solution flow-rate	1 ml/min
Wash time	1.0 min
SPE–CE interfacing	
Interface	Laboratory-made interface
CE buffer flow-rate	0.2 ml/min
Δt[b]	0.02 min*
t_{inj}[c]	7 min*
CE	
Capillary	Bare silica, 106 cm (effective length 40 cm) 180 μm I.D.×350 μm O.D.
Capillary rinsing	1000 mbar for 0.5 min
Injection voltage	−4 kV*
Injection time	7 min
CE buffer	1.0 *M* acetic acid, 25 m*M* ammonium acetate in acetonitrile
UV detection	214 nm (Model 759A, Applied Biosystems)
Separation temperature	30°C
Separation voltage	−30 kV
Analysis time	15 min

*Discussed in text.
[a] PRR, protein releasing reagent (1 *M* hydrochloric acid and 25% glycerol in water).
[b] Time between start of elution and analytes passing tip of CE capillary.
[c] Time unit interval in which analytes pass tip of CE capillary.

ml/min. Injection into the CE capillary was performed using a voltage of −3 kV for 5 min, which was applied immediately upon starting the elution. Finally, the 15-min separation was performed using −30 kV, and with 1.0 *M* acetic acid, 25 m*M* ammonium acetate in acetonitrile as CE buffer. The dependence of the amount of analyte detected, expressed as the peak area, on the height difference of the buffer levels, is shown in Fig. 2. With differences of 10 mm or less, no peaks were observed because methanol then entered the CE capil-

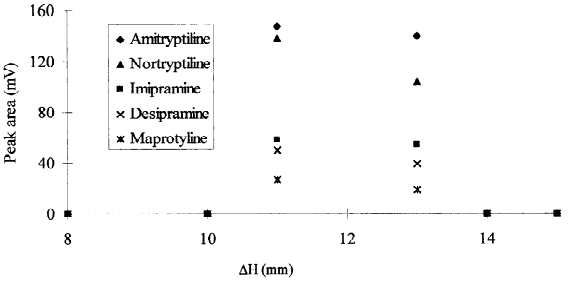

Fig. 2. Dependence of analyte response (peak areas) on height difference of the two buffer vials.

lary, making separation impossible. On the other hand, when using a height difference of 14 mm or more, no peaks showed up either, because the flow of the CE buffer from the detection to the injection side was higher than the speed of the analytes towards the CE capillary outlet; in other words, the analytes could not enter the capillary. Good, and mutually rather similar, results were obtained for height differences of 11–13 mm, and an intermediate value of 12 mm was selected for all further work.

3.3. SPE–CE interfacing

With regard to interfacing, there are two main parameters that have to be optimized: (i) the injection window, i.e. Δt and t_{inj} in the protocol of Table 1 and (ii) the flow-rates used during desorption.

In earlier studies [5,6], a long injection time of the desorption solvent resulted in band broadening. Under the present conditions this effect is absent, because the desorption solvent, methanol, does not enter the capillary anyway. Consequently, Δt was set at 0.02 min or, in other words, the voltage was applied almost immediately after starting the desorption of the TCADs from the SPE cartridge, to avoid loss of analytes.

As regards the effect of the flow-rate of methanol on the desorption of the TCADs, the cartridges were loaded with 1 ml of a solution containing 10 μg/ml of each TCAD and were, next, desorbed with 2 ml of methanol using flow-rates varying from 0.1 to 2.0 ml/min. Over the range tested, increasing the flow resulted in a steadily decreasing amount of analyte desorbed per unit of volume. In addition, the amount of TCADs desorbed from the cartridge was more reproducible when using low flow-rates. Because of these two observations, a low flow of 0.1 ml/min was selected for desorption. Since, on the other hand, the amount of analyte desorbed per time unit was higher at a high flow, a flow-rate of 2.0 ml/min was selected for cartridge regeneration.

To study the minimum volume of methanol necessary to remove all TCADs from the cartridge, a test sample containing 10 μg/ml of each TCAD was injected onto the SPE cartridge and, next, eluted using varying amounts of methanol (0.1–1.0 ml), at a flow of 0.1 ml/min. The injection time into the CE system was 10 min in all cases. Complete desorption was found when using 0.7 ml of methanol, and an injection time, t_{inj}, of 7 min and the low flow-rate were used for desorption in all further experiments.

In conclusion, even when using a low flow-rate during desorption, 0.7 ml of methanol is still required to desorb all TCADs; this is quite a lot more than the 0.2 ml typical of earlier studies. The slow desorption is probably caused by the strong interaction of the TCADs with free silanol groups with the surface of the C_2-bonded silica. In principle, the interaction can be reduced by adding an excess of amines to the desorption solvent. Unfortunately, this approach cannot be used here, because the amines will also interact with the free silanol groups on the CE capillary wall. This will cause the electroosmotic flow to fluctuate, and the migration times to become non-reproducible. Because of this, pure methanol was used for desorption, and a 7-min desorption time was accepted. As will be shown below, the adverse effect of analyte dilution was made up for by urine, an injection technique, which results in analyte (re)concentration.

3.4. Injection voltage

The injection voltage applied for 7 min after the desorption has been started should be optimized to increase the sensitivity of the procedure, without causing additional band broadening as is described in the protocol of Ref. [6].

To study this aspect, 1 ml of standard solution (1 μg/ml of each TCAD) was applied to the SPE cartridge. Upon desorption with methanol and flushing of the analytes to the CE system, electrokinetic injection was performed for 7 min using voltages ranging from 1 to 14 kV. Unfortunately, no clear-cut picture emerged. For the rapid migrating TCADs, amitryptiline and imipramine, the peak width was found to decrease about twofold when the voltage was doubled; or in other words, increasing the amount injected resulted in broader peaks with the same peak height. Essentially, no effect on peak width was observed for the slow migrating nortryptiline and desipramine, while peak width was increased with an increasing voltage for the slowest migrating TCAD, maprotyline. Most probably, the explanation is that the stacking effect is more

efficient for slower compounds as compared with faster migration compounds. In all further experiments, a compromise voltage of −4 kV was used.

3.5. Dialysis

As the next step, the dialysis module was connected on-line to the SPE cartridge that now essentially is a trapping column. The composition of the acceptor phase was the same as was used in earlier studies on dialysis–SPE: an aqueous 10 m*M* phosphate buffer (pH 7) [6].

The donor compartment was filled with a standard TCAD solution (concentration, 1 μg/ml each). Dialysis times were varied over the range of 2.5–17.5 min and acceptor flow-rates of 0.5–1.5 ml/min were used. At this stage of the study, the CE–UV_{214} analysis was combined on-line with the sample pretreatment. Fig. 3 shows the result obtained for imipramine (similar results were obtained for other TCADs). It is obvious that, for short dialysis times of less than about 7.5 min, a high acceptor flow has to be recommended. On the other hand, if some of the speed of analysis that can be obtained is sacrificed, analyte recoveries are increased and the flow dependency is essentially lost. A low acceptor flow of 0.5 ml/min (dialysis time, 13 min) was used to reduce the possibility of breakthrough of the analytes.

3.6. Analysis of biological samples

Two types of biological samples were used to test the optimized on-line set-up: urine and serum. As is well known, with both sample types adequate removal of interferences present on the SPE cartridge after trace enrichment of the analytes, is the main problem. In the present, non-aqueous CE, study, the earlier solution of washing with a suitable pH-stabilized buffer/organic solvent mixture cannot be used: there is a real risk that (part of) the solutes in the buffer will precipitate when coming into contact with the desorption solvent. Fortunately, even if no pH effect was used, and a simple acetonitrile–water (20:80, v/v) mixture, a 1-ml wash step was sufficient to remove most of the interferences.

With serum samples, adding a releasing agent solved the additional problem of the protein–drug binding. To this end, a 1:9 (v/v) mixture of protein-releasing reagent (PRR; 1 *M* hydrochloric acid and 25% glycerol in water) and serum sample was used (cf. Ref. [11]). It should be noted that, after having mixed the solutions by shaking, analysis should proceed forthwith, because after about 1 h the viscosity starts to increase. The analyte recoveries obtained when using PRR were 60–70% for amitryptiline, desipramine and maprotyline, 80% for nortryptiline and 95% for imipramine. This contrasted sharply with recoveries of less than 10% obtained when no PRR was used. Fig. 4 shows that the proposed procedure (with the same washing step

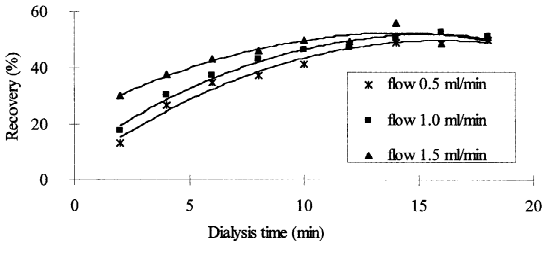

Fig. 3. Effect of dialysis time on imipramine recovery for different acceptor phase flow-rates.

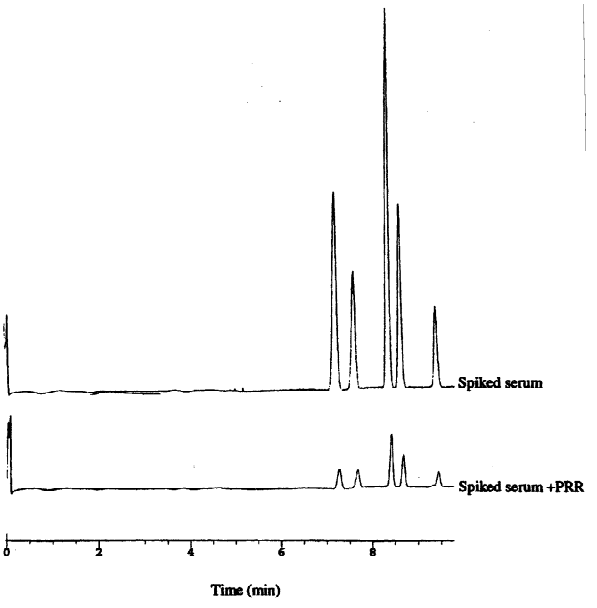

Fig. 4. Electropherogram of TCADs (spiked at 1 μg/ml each) in serum with and without the addition of PRR obtained by dialysis–SPE–CE–UV_{214}. Elution order: amitryptiline, imipramine, nortryptiline, desipramine and maprotyline. For details, see text.

as for urine), resulted in excellent electropherograms for serum samples.

Some relevant analytical performance data are summarized in Table 2. Linear calibration plots were obtained for all five TCADs in both types of biological sample. The within-day precision was 2.5–4.5% ($n=5$) for standard solutions, 2.5–5.5% ($n=5$) for urine and 4.5–9.5% ($n=5$) for serum. The values were all below the threshold of 15%, a value often used as acceptance level of an analytical method to be used for quantification of analytes in biological samples [12]. However, they may be even improved by using an internal standard. In addition, Table 2 shows that the LODs are in the 40–80 ng/ml range for urine, and the 60–100 ng/ml range for serum. This is a distinct improvement over earlier results obtained with at-line SPE–CE with hydrodynamic injection, where LOD were 30–300 ng/ml for urine, and 300–1000 ng/ml for serum. The improved detectability is largely due to the use of electrokinetic instead of hydrodynamic injection. With electrokinetic injection more analyte can be injected without creating extra band broadening; with hydrodynamic injection, restrictions are more severe because next to the analytes, solvent is also injected. The improved analyte detectability can be very gratifying, especially when one considers that the sample volumes per analysis were only 0.5 ml for both urine and serum, as against 8 ml (urine) or 1 ml (serum) in the SPE–CE study. This is an important aspect when biological fluids have to be analysed that are available in only limited amounts.

Finally, it should be added that maintenance of the automated dialysis–SPE–non-aqueous CE system was negligible during the 4 months of the present study, and that no technical or operational problems were encountered.

Table 2
Calibration data for TCADs in spiked urine and serum[a]

Compound	Linearity[b]				LOD (ng/ml)
	Slope (RSD, %)		Intercept (RSD, %)		
Urine					
Ami	0.195	(0.004)	−0.16	(0.09)	40
Imi	0.38	(0.01)	−0.1	(0.2)	80
Nor	0.181	(0.005)	−0.1	(0.1)	60
Des	0.54	(0.01)	−1.0	(0.1)	60
Map	1.07	(0.03)	0.0	(0.1)	60
Serum					
Ami	0.119	(0.002)	0.04	(0.06)	60
Imi	0.364	(0.009)	0.01	(0.12)	100
Nor	0.141	(0.003)	0.13	(0.10)	90
Des	0.358	(0.007)	0.16	(0.1)	80
Map	0.76	(0.016)	0.15	(0.1)	90

[a] Sample size: urine, 0.5 ml; serum, 0.5 ml.
[b] TCAD concentration range: LOD — 10 μg/ml, five datapoints.

4. Conclusions

Dialysis–SPE has successfully been combined on-line with non-aqueous CE with UV detection. To illustrate the practicality of the procedure, five tricyclic antidepressants were determined in serum and urine. The analytical performance data of the automated procedure were fully satisfactory, one main advantage being an over 10-fold improved analyte detectability compared with an earlier at-line SPE–CE approach. With 0.5-ml urine and serum samples, the urine detection limits were in the 40–100 ng/ml range. The successful outcome is primarily caused by the use of electrokinetic injection which is shown to provide excellent results if combined with a sample pretreatment which ensures that an extract is injected which has a constant composition, independent of the nature of the biological sample.

The present paper completes a series studies which demonstrate that at-line SPE–CE but, even more so, on-line dialysis–SPE–CE is a powerful method for the trace-level determination of cationic, amphoteric and anionic analytes (drugs) in serum and urine, and can be combined with both an aqueous or non-aqueous CE buffer.

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