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Repetitive injection field-amplified sample stacking for cationic compounds determination

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ABSTRACT

The development of a field-amplified sample stacking technique is presented. Sensitivity enhancement in this technique was obtained by repetitive injections of a sample followed by steps of sample matrix removal through the application of counter-pressure. Under optimized conditions the background electrolyte (BGE) was composed of 80 mM H_3PO_4 while the sample matrix contained 0.5 mM H_3PO_4 and 30% (v/v) methanol. The elaborated method enabled a 4-fold effective injection of the sample (53 s, 0.5 psi). Each injection was followed by a focusing step during which the application of a voltage (2 kV) and counter-pressure (−1 psi) was performed for 0.65 min. The method was developed for the determination of six psychiatric drugs (opipramol, hydroxyzine, promazine, amitriptyline, fluoxetine, and thioridazine). The elaborated method was applied for analysis of human urine samples after a simple liquid–liquid extraction procedure. The detection limits obtained were in the range of 2.23–6.21 ng/mL.

1. Introduction

The separation of chemical compounds is one of the most intensively developed fields in analytical chemistry. Among applied techniques chromatography is the most popular and most widely used. In the last 20 years capillary electrophoresis (CE) has gained in popularity and is more often considered as a supplementary or even alternative to liquid chromatography (LC). Despite many advantages, like a shorter analysis time and high separation efficiency, CE usually provides higher detection limits in comparison to LC. This limitation can be overcome by the application of on-line preconcentration techniques.

Field-amplified sample stacking (FASS) is the oldest and the simplest of techniques whose theoretical foundations were created by Mikkers et al. [1]. The principle of this technique is based on the rapid decrease in migration velocity of the analyte on the boundary of a high electric field sample zone and a low electric field background electrolyte (BGE) zone. An interesting review on this topic was provided by Quirino and Terabe [2].

The FASS technique can be easily implemented for the elaborated separation method by a simple lowering of the sample conductivity. However, it has some limitations. The most crucial is a greatly limited volume of the sample that can be injected. The application of a sample injection longer than a few percents of the capillary length can cause current failures and analysis breakdowns. A longer injection plug can be obtained using large volume sample stacking (LVSS) techniques [3,4]. In these techniques greater sensitivity improvement can be achieved, in comparison to FASS, and it is limited by the capillary volume [5,6]. Electrokinetic injection (EKI) usually provides better signal strength enhancement. This is caused by the additional electrophoretic mechanism of the injection of the ions. EKI is used in the most powerful on-line preconcentration techniques developed so far [7,8]. However, the application of these techniques requires the optimization of many parameters. It has been also proved that hydrodynamic injection can be more repeatable than EKI [9]. Pressure-based injection modes also enable the simultaneous introduction (into the capillary) of analytes with different charge states (cations, anions, ampholytes and non-charged molecules) [10]. Thus, the development of preconcentration strategies coupled with hydrodynamic injection mode is still an important topic despite the fact that a greater sensitivity improvement can be achieved with the use of EKI [9].

The limitation of the capillary volume has been overcome by Urban et al. [11]. The application of charged β-cyclodextrines (β-CD) as a pseudostationary phase enabled the separation and double stacking of anabolic steroids using full-capillary injection. The authors have shown that the third injection, although it was possible, resulted in low separation efficiency and poor repeatability [11]. Wang et al. reported up to a fivefold whole-capillary

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injection [12]. The developed method was based on the LVSS technique followed by a sweeping of analytes coupled with the removal of the sample matrix after each injection. The elaborated method provided three orders of magnitude sensitivity enhancement.

This work presents a simple and repeatable way of sensitivity enhancement in the FASS technique. The low conductivity of samples used in the FASS technique does not enable the performance of injections longer than a few % of the whole capillary volume due to the inefficiency of stacking effect. This can result in the broadening of bands and loss of repeatability. Moreover, longer sample injection can lead to current destabilization during analysis and run collapse. The proposed repetitive injection field-amplified sample stacking (RI-FASS) technique was elaborated as a solution to this problem. The applicability of this method was shown for determination of selected psychiatric drugs in human urine samples after a simple liquid–liquid extraction clean-up step.

2. Experimental

2.1. Instrumentation

All experiments were performed on a PA 800 plus CE system (Beckman Instruments, Fullerton, CA, USA) equipped with a diode-array detector. Analytical wavelength was set at 200 nm. Analyses were carried out using uncoated fused-silica capillaries (50 μm × 60 cm, 50 cm to detection window; Beckman) thermostated at 25 °C. The pH values were measured using a Crison GLP-21pH meter (Barcelona, Spain). The pKa values of the analytes were calculated using ACD/ChemSketch (version 12.01) software (Advanced Chemistry Development, Inc., Toronto, ON, Canada). Injection plug length was calculated using CE Expert software (Beckman). pH values in Section 3.2 were calculated using PeakMaster 5.3 Complex software [13].

2.2. Reagents and solutions

All solvents and reagents were of analytical grade. Solvents used in experiments were sodium hydroxide 0.1 M (Beckman Coulter, Brea, USA), sodium hydroxide (Sigma-Aldrich, St. Louis, MO, USA) phosphoric acid 85% (Sigma-Aldrich), methanol (Baker, Analyzed LC–MS reagent, NJ, USA), dichloromethane (Sigma-Aldrich) and redistilled water (Millipore, Mili Q Direct 16). The analyzed drugs (opipramol, hydroxyzine, promazine, amitriptyline, fluoxetine, thioridazine and oxazepam) were purchased from Sigma-Aldrich. Drug stock solutions were prepared in pure methanol to a concentration of 1 mg/ml. The calculated pKa values were as follows: 3.45 and 7.45 for opipramol, 2.10 and 6.62 for hydroxyzine, 9.43 for promazine, 9.15 for amitriptyline, 10.05 for fluoxetine, and 9.84 for thioridazine.

2.3. General electrophoresis procedure

Optimal composition of BGE for both separation and preconcentration was found to be 80 mM H_3PO_4. The sample matrix was composed of 30% methanol (v/v) and 1 mM H_3PO_4.

Before each analysis, capillaries were rinsed with 0.1 M NaOH (0.5 min), water (1 min) and BGE (1.5 min) at 20 psi. Samples were injected for 53 s at 0.5 psi followed by a short BGE plug (12 s, 0.5 psi). Next, the stacking step with simultaneous sample matrix removal was performed for 0.65 min (2 kV, −1.0 psi). Under optimized conditions, injections and stacking were repeated four times in total. In the end, high voltage (30 kV) was applied for separation of the compounds.

2.4. Sample preparation

The extraction procedure applied in the presented assay was a modified method reported by Rabanes et al. [14]. Urine samples were collected from healthy volunteers and stored frozen (−20 °C). Before CE analysis, the samples were defrosted and centrifuged at room temperature (7378g for 10 min) to remove visible sediments. Each of the urine supernatants (3 ml) was spiked with 15 μL of a drug mixture that contained 6.0–200.0 μg/mL of each analyte and 10 μL of I.S. (200 μg/mL). This was equivalent to 0.03–1.0 μg/mL final concentration in an enriched sample. 2 M NaOH was added to the urine sample until the pH was 12 to render the analytes electrically neutral. The solution was extracted 3 times with 1 mL of dichloromethane. The organic extract was recovered by centrifugation for 15 min at 3000 rpm. Next, 1.2 mL of the pooled extract was collected in a clean tube and evaporated at 25 °C to dryness in an argon stream. Finally, the residue was dissolved in a matrix solution buffer, centrifuged and analyzed.

3. Results and discussion

3.1. Mechanism of preconcentration in a repetitive injection field-amplified sample stacking technique

The scheme of the preconcentration mechanism is presented in Fig. 1. At the beginning a low conductivity sample was introduced into the capillary, preliminarily filled with the BGE (Fig. 1A). The sample injection was followed by a buffer plug (Fig. 1A). Next, a high voltage with counter-pressure was applied (Fig. 1B). Due to the applied voltage the analytes migrated towards the capillary outlet, stacking on the sample/BGE boundary. Simultaneously, under the presence of counter-pressure, the low conductivity sample matrix was removed from the capillary. This stacking/removing step was conducted until the current value reached 95% of the current obtained when the capillary is filled only with the BGE (Fig. 1C). The sample and BGE injection followed by stacking/removing steps was repeated (Fig. 1D, F). In the end, a high voltage was applied and separation was performed (Fig. 1G).

There are a few crucial factors influencing the sensitivity enhancement in the presented technique, such as the sample

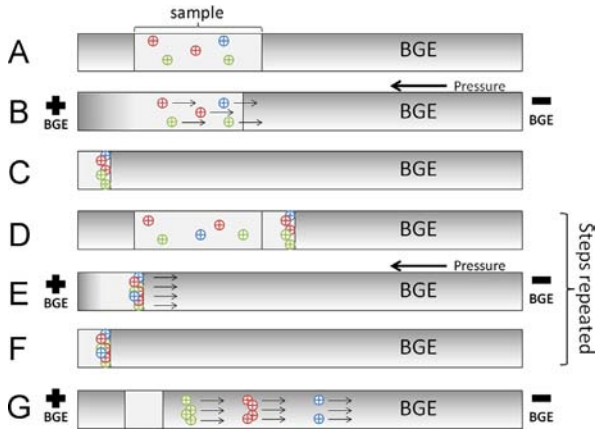

Fig. 1. Scheme presenting mechanism of the repetitive injection field-amplified sample stacking technique. (A) cationic analytes in low conductivity matrix were introduced into the capillary followed by short BGE plug. (B) Application of voltage induced cations to migrate toward the cathode and stacking on the sample/BGE boundary. Simultaneous counter-pressure removed the sample matrix from the capillary. (C) pressure and voltage were stopped when current value reached ~95% of current value when capillary was filled only with BGE. (D, E, F) Injection of sample and short BGE plug and stacking/matrix removal steps were repeated. (G) In the end high voltage was applied and the separation occurred.

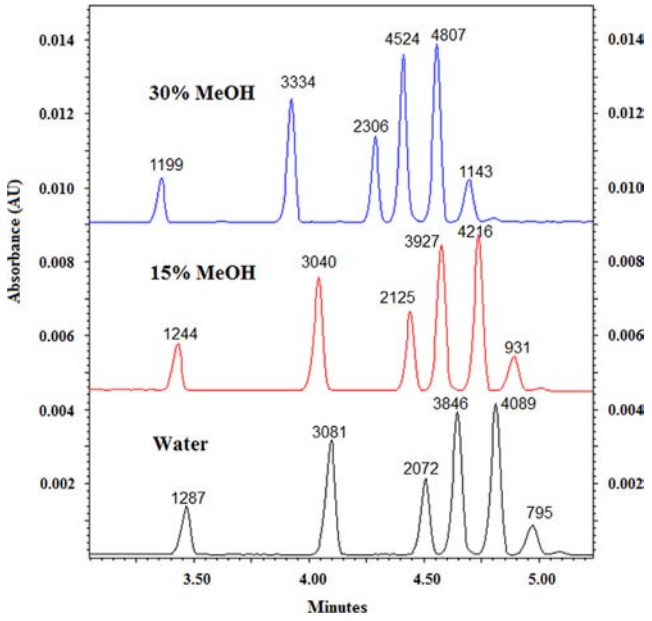

Fig. 2. Influence of methanol (MeOH) addition to sample matrix. Conditions: BGE, 80 mM H_3PO_4; sample, 0.5 mM H_3PO_4 in (black) water, (red) 15% (v/v) MeOH, and (blue) 30% (v/v) MeOH; capillary, 50 μm × 60 cm; voltage, 30 kV; injection, three times 53 s (0.5 psi) followed by BGE (12 s, 1.0 psi); stacking/matrix removal conditions, 2 kV, −1 psi, 0.65 min; analytical wavelength, 200 nm. Peaks identification (from left): opipramol, hydroxyzine, promazine, amitriptyline, fluoxetine, and thioridazine. Numbers above peaks determine their height. (For interpretation of the reference to color in this figure, the reader is referred to the web version of this article).

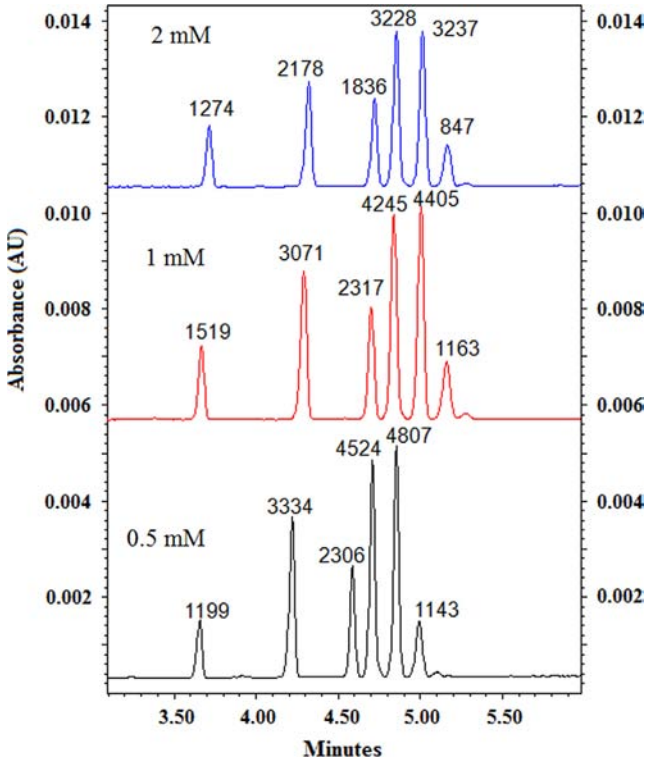

Fig. 3. Influence of phosphoric acid addition to sample matrix. Conditions: sample, (black) 0.5 mM H_3PO_4, (red) 1 mM H_3PO_4, and (blue) 2 mM H_3PO_4 in 30% (v/v) MeOH. Other conditions are the same as in Fig. 2. Peaks identification (from left): opipramol, hydroxyzine, promazine, amitriptyline, fluoxetine, and thioridazine. Numbers above peaks determine their height. (For interpretation of the reference to color in this figure, the reader is referred to the web version of this article).

and BGE composition (ionic strength difference, pH value and conductivity), the voltage and pressure values used for the stacking step, the number of performed injections, the sample injection plug length and to some extent the short BGE plug length injected after the sample.

3.2. Sample matrix composition

The difference in the conductivity of the sample and BGE is one the most important factors affecting stacking efficiency [2,15]. Samples diluted in pure water were used at the beginning of the study. The addition of an organic solvent and phosphoric acid was taken into consideration during the optimization of the method. The presence of an organic solvent in the sample matrix lowers the conductivity of this medium, enlarging the electric resistance difference between the sample and BGE. Moreover, it can also accelerate the migration of analytes during the stacking steps, preventing the loss of analytes by removing them with the sample matrix. In Fig. 2 the effect of methanol content in the sample matrix is presented. As can be expected, the amplification of the signal strength increases with the addition of methanol. More than 30% (v/v) of methanol in the sample provided an even greater sensitivity enhancement. However, it also caused current failures and an addition of 30% (v/v) methanol was considered as the optimal.

The addition of phosphoric acid to the sample was also optimized in view of the efficiency of the separation. Its presence in the sample matrix has a double effect. First of all it enables longer sample plugs to be injected without current failures. The second effect is better ionization of analytes which improves their stacking. The effect of different concentrations of phosphoric acid in the sample matrix is shown in Fig. 3. The addition of acid to a concentration of 0.5 mM (pH = 3.34) enabled longer sample plugs (53 s, 0.5 psi) to be injected. Without acid, current failures occurred, which led to analysis breakdowns. An increase in H_3PO_4 content in the sample reservoir to 1 mM (pH = 3.06) improved the opipramol signal height to about 25% which can be explained by greater positive charge on the molecule under these conditions ($pKa_1 = 3.45$). On the other hand a decrease in the intensities of the signals for hydroxyzine, amitriptyline and fluoxetine can be noted. This can be explained by the fact that the addition of acid reduces the difference in conductivity between the sample and BGE. That is why a higher content of acid causes a general signal decrease in this case (2 mM H_3PO_4, pH = 2.80). Nevertheless, the reduction of signal strength for hydroxyzine, amitriptyline and fluoxetine is below 10% and in the case of other analytes a slight height improvement is observed. Thus we considered the presence of 1 mM phosphoric acid in the sample matrix as the optimal.

3.3. Stacking step parameters

Stacking with the simultaneous removal of the sample matrix is a crucial step in the elaborated technique. The duration of the applied voltage and pressure are among the factors influencing this process.

The difference in electric potentials used directly induces the migration of analytes and their stacking on the sample/BGE boundary. This relationship is presented in Fig. 4. The greatest signal enhancement was obtained after the utilization of 2 kV during the stacking steps. A lower voltage was not sufficient for effective stacking. Moreover, a higher voltage caused a faster migration of analytes towards the cathode, which prevented them from being pushed out of the capillary according to the presence of counter-pressure. The application of 2.5 kV did not improve sensitivity. It can be noted that a broadening of peaks occurred. This was the result of the destacking of analytes and their further

migration into the BGE. We found the voltage value of 2 kV to be the optimal.

Negative pressure applied during the stacking step was used to remove low sample matrix conductivity. It was essential to eliminate most of the injected sample matrix without the loss of analytes. The process was conducted until the current reached 95% of the current value when the capillary was filled only with the BGE. On the one hand, short stacking steps with relatively high pressure caused the intensity of the signals to drop due to the partial removal of analytes. On the other hand, longer processes with relatively low pressure resulted in the loss of separation efficiency due to the destacking of analytes. Optimal parameters for the stacking steps (sample matrix removal step) were −1 psi pressure applied for 0.65 min.

We also found it necessary to introduce into the capillary a short buffer plug after each sample injection. This prevented the loss of analytes during the sample matrix removal process. Without this step a loss of intensity of the signals was observed; 10 s (0.5 psi) BGE injections were sufficient for this aim. However, we extended the injection time to 12 s to improve the repeatability of the stacking/sample matrix removal steps (2 kV, −1 psi, 0.65 min).

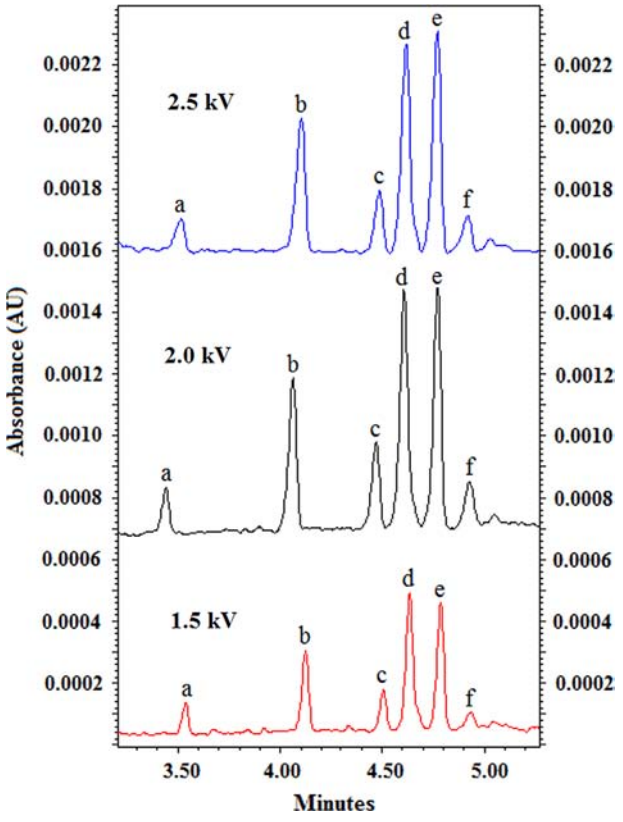

Fig. 4. Influence of value of voltage applied during the stacking steps. Conditions: sample, 0.5 mM H_3PO_4 in 30% MeOH; stacking/matrix removal step, (red) 1.5 kV, (black) 2.0 kV, and (blue) 2.5 kV, −1.0 psi, 0.65 min. Other conditions are the same as in Fig. 2 with 30% (v/v) MeOH in sample matrix. Peaks identification: a – opipramol; b – hydroxyzine; c – promazine; d – amitriptyline; e – fluoxetine; f – thioridazine. (For interpretation of the reference to color in this figure, the reader is referred to the web version of this article).

3.4. Number of sample injections

The amount of the injected sample is the most important factor influencing sensitivity enhancement. As it was discussed in Section 1, the main limitation of the FASS technique is the small sample volume that can be efficiently stacked. With the use of an optimized composition of the sample matrix (1 mM H_3PO_4 in 30% MeOH) we considered 53 s injection at 0.5 psi pressure (≈ 5% of capillary length to the detection window) as the optimal. Longer injection plugs resulted in current errors and breakdowns of analyses. It is noteworthy that current errors also occurred when a simple FASS technique (53 s at 0.5 psi; sample matrix composed of 1 mM H_3PO_4 in 30% MeOH) was used without the stacking/matrix removal step. The influence of injection modes on the effect of signal enhancement is presented in Fig. 5. The utilization of a single standard injection (5 s, 0.5 psi) resulted in low intensity signals near the limits of their detection values while a thioridazine peak was not detected. The application of a single injection FASS technique followed by the stacking/matrix removal step enabled the signal strength to be improved. However, the introduction to the capillary of about 10 times larger sample volume than in a standard injection did not provide equal signal enhancement. This was caused by a slight broadening of the peak due to the diffusion and destacking effects [16]. Every next injection improved the sensitivity enhancement effect with a slight broadening of peaks. The 5th injection did not provide a signal amplification effect. However, in every presented injection mode

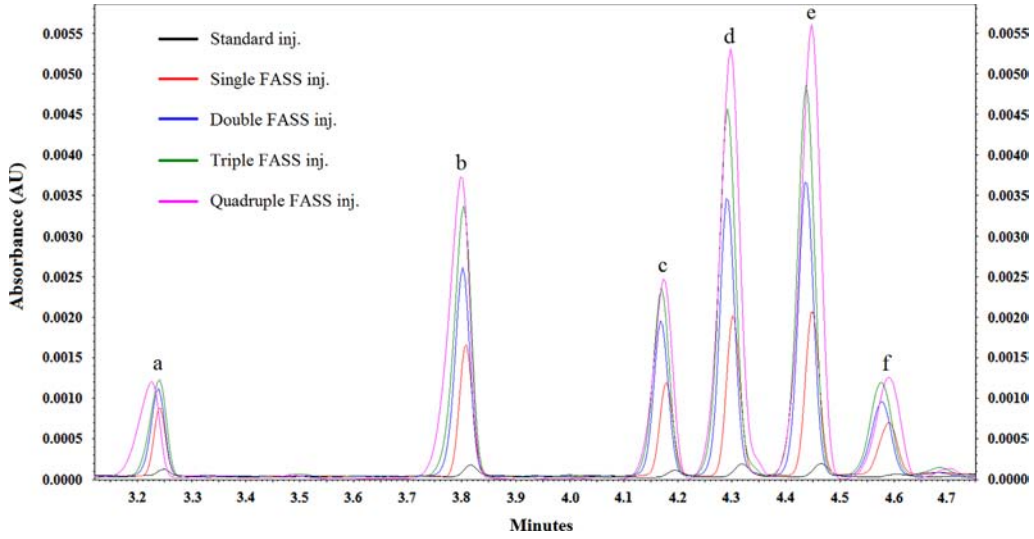

Fig. 5. Aligned electropherograms obtained by application of different injection modes. Conditions are the same as in Fig. 2 with 30% MeOH in sample matrix. Peaks identification is the same as in Fig. 4. (For interpretation of the reference to color in this figure, the reader is referred to the web version of this article).

Table 1
Designated validation parameters juxtaposition.

	Opipramol	Hydroxyzine	Promazine	Amitriptyline	Fluoxetine	Thioridazine
Sample linearity (ng/mL)	30–1000					
Slope a	1.7656	3.6891	2.597	5.3255	5.6061	1.0937
Intercept b	0.0045	0.0003	0.0173	0.0154	0.0109	0.0032
Determination coefficient R^2	0.9992	0.9996	0.9988	0.9995	0.9996	0.9989
LOD (ng/mL)	3.92	2.23	5.04	2.77	2.74	6.21
LOQ (ng/mL)	11.76	6.69	15.12	8.31	8.22	18.63
SEF[a]	12	27	29	36	35	32
N/m (× 1000)[b]	144.7	222.3	274.7	256.4	247.4	137.4
	Intra-day precision (% RSD, $n=6$)					
30 ng/mL	4.36	2.48	5.60	3.08	3.05	6.90
900 ng/mL	0.82	0.86	1.35	0.98	0.75	1.37
10 μg/mL	0.88	1.09	0.90	0.95	0.97	0.89
	Intra-day accuracy (%, $n=6$)					
30 ng/mL	101.5	99.3	98.7	99.7	99.7	102.7
900 ng/mL	99.9	99.9	100.0	100.1	100.1	100.4
10 μg/mL	99.9	99.9	100.1	99.9	100.0	99.9
	Inter-day precision (% RSD, $n=9$)					
30 ng/mL	5.11	2.54	5.74	3.22	3.49	6.73
900 ng/mL	1.05	1.14	1.39	1.13	0.88	1.51
10 μg/mL	1.03	1.21	0.99	1.10	1.07	1.05
	Inter-day accuracy (%, $n=9$)					
30 ng/mL	102.1	100.1	99.2	99.8	99.7	101.5
900 ng/mL	99.9	99.8	100.6	100.3	100.2	100.3
10 μg/mL	99.7	99.8	100	100	99.9	99.4

[a] Sensitivity enhancement factor; ratio of peak height obtained with RI-FASS technique to CZE technique with standard hydrodynamic injection (5 s, 0.5 psi) multiplied by dilution factor.
[b] Separation efficiency expressed as a number of theoretical plates per meter. Measurements were performed for analytes concentration of 80 ng/mL ($n=3$).

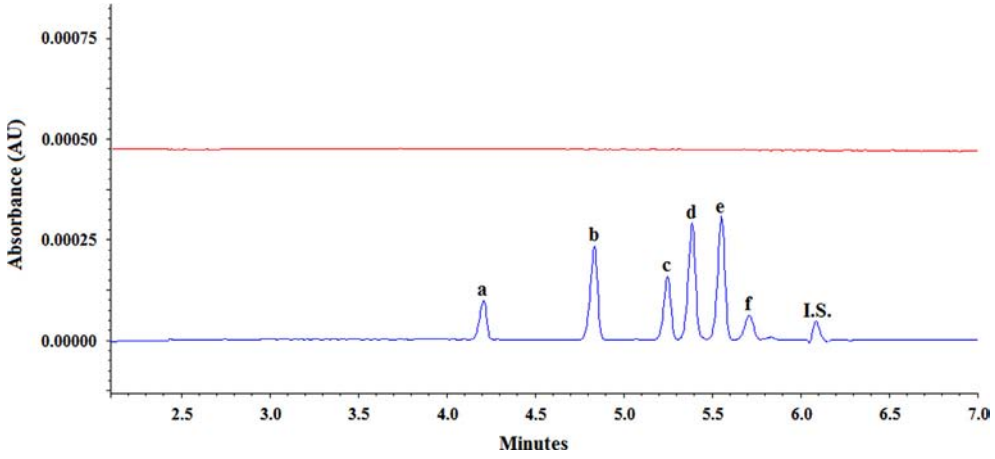

Fig. 6. Typical electropherogram of spiked (blue) and blank human urine sample (red). Extraction was performed according to the procedure described in Section 2.4. Each drug concentration was 300 ng/ml.

the resolutions were >1 and the separation efficiency was in the range of 137,400–274,700 theoretical plates per meter for quadruple injection. Thus, quadruple injection was used for quantitative analysis of biological samples.

3.5. Validation study

Under the optimum separation conditions, the calibration graphs for the six psychiatric drugs were investigated. The results are summarized in Table 1. The selectivity of the RI-FASS-CE method was confirmed on the basis of the analysis of drug-free human urine samples and extracts of urine spiked with each of the analytes and I.S. All compounds were well separated from co-extracted material under the presented electrophoretic conditions. The specificity study showed that the urine concentrations in the above substances do not interfere with the accurate determination of analytes. The peaks were symmetrical and completely resolved from one another. The limits of detection (LODs) and quantification (LOQs) for each psychiatric drug were subsequently determined by the analysis of five spiked urine samples prepared at their respective concentrations. The signal to noise (S/N) ratio equal to 3 was determined as the LOD while the LOQ corresponded to the analyte concentration at which the S/N ratio was 10. The obtained LODs and LOQs are presented in Table 1. Linearity was tested with 10 point calibration curves made in the range of 30.0–1000 ng/mL for all analytes. Satisfactory determination coefficients for the calibration curves were obtained ($R^2 > 0.998$). The linearity results are presented in Table 1.

3.6. Application

The proposed method allowed the simultaneous determination of six selected psychiatric drugs in human urine samples. As a result of small therapeutic doses of these drugs usually administered

for the treatment of mental disorders the expected concentration of these drugs in human urine samples is at the level of ng/mL. Therefore, their concentration in biological samples was reported to be below the CE detection sensitivity range when a conventional hydrodynamic injection of the sample was performed [17]. In our study concentrations of the drugs used in assay were 30, 40, 50, 80, 100, 300, 500, 700, 900 and 1000 ng/mL. The LOQs for all analytes in human urine samples were 20 ng/mL. The obtained determination coefficients (R^2) were >0.998 with acceptable RSD values. An exemplary electropherogram is presented in Fig. 6. The elaborated method may be successfully applied for monitoring the selected central nervous drugs in urine samples after their administration in therapeutic doses.

4. Conclusion

The elaborated technique enabled the performance of four effective injections of the sample under field-amplified conditions. Although the signal amplification was not linearly correlated with the injected sample volume, the developed method provided the limits of detection in a range of 2.23–6.21 ng/mL without off-line sample preconcentration. The obtained results provided a slight sensitivity improvement according to the previously reported micelle to solvent stacking technique [18]. The usefulness of the RI-FASS technique in human urine sample analysis was shown. Moreover, the preconcentration was efficient for structurally diverse amines. The presented technique can be easily implemented for methods in which FASS is used for sensitivity enhancement if the obtained results are still insufficient.

Acknowledgments

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