CAPILLARY ELECTROPHORESIS AS A VERIFICATION TOOL FOR IMMUNOCHEMICAL DRUG SCREENING

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† Dedicated to Bretislav Smysl in memoriam

Background: The aim of this work was to develop a simple capillary electrophoretic method as the verification and confirmation tool in the screening analysis for amphetamines, opiates, benzodiazepines and cocaine and their metabolites for toxicological applications.

Methods: 50 mM phosphate Tris pH 2.0 with 30 % (v/v) of methanol was used as a background electrolyte that enabled fast separation of drugs and their metabolites in saliva and urine. Verification of the data from the electrophoretic method was done by High Performance Thin Layer Chromatography (HPTLC) and the immunochemical screening test QuikScreen.

Results: The experimental conditions of the Capillary Electrophoresis (CE) were partially optimized (mainly the influence of concentration and types of additives, e.g. cyclodextrines, organic solvents) and validated; the method was used for analysing samples from drug abusers.

Conclusions: The non-instrumental, immunoassay tests could only confirm qualitative addictions and are mainly employed when the emergency detection of drugs is needed. For quantitative analysis and verification of obtained results the confirmation step is strongly recommended. The simple screening capillary zone electrophoresis method allows recognition of the most abused drugs. The agreement of the results from CE, HPTLC and QuikScreen test was more than 95 %.

INTRODUCTION

These days the misuse of addictive drugs has become very widespread especially in certain professions and groups of people such as the military, police, professional drivers, managers, teachers and students. Therefore a new broader range of drug screening has been researched and developed¹. These screenings are usually done at drop-in centres, police and certified laboratories using immunochemical one-step tests^{2,3}. These simple tests determine common drugs in small sample volumes (saliva, urine, plasma, serum, etc.) and can be applied directly, anywhere outside the laboratory. However positive results of immunochemical screening of abused drugs in biofluids must be verified by another independent and sophisticated analytical method. Hyphenation techniques that include extraction and sample concentration (L-L extraction, SPE, SPME); high performance separation (HPLC, GC) and mass spectrometric detection are commonly used for detection and identification of drugs and their metabolites^{4–9}.

Capillary electrophoresis (CE) can be an alternative efficient technique which is characterized by high separation efficiency and selectivity, high sample throughput,

small sample consumption, resistance, simple instrumentation, etc.^{10–13}. All CE modes employ the same instrumentation; samples can be easily analyzed subsequently as differencing separation mechanisms. Generally, CE is a very promising tool for clinical analysis at the present time^{14–19} and it could be a simple and less time-consuming alternative technique to HPLC and GC which are also based on different separation mechanisms than CE. The selectivity of CE can be utilized for separation of complicated mixtures of target analytes (e.g. abused drugs, diagnostic markers, etc.) from matrix without multistep sample preparation. Moreover CE is a highly effective technique for chiral separations of drugs using many types of chiral electrolyte additives (cyclodextrins, crown-ethers, chiral surfactants, macrocyclic antibiotics, glycopeptides, etc.) which give CE a very large potential for studying the metabolism of chiral and non-chiral drugs^{20–22}.

A number of studies on toxicological screening and monitoring of abused drugs by CE techniques have been published over the past ten years^{23–26}. These contributions discuss problems with the sample preparation of drugs in biofluids (such as urine, blood, plasma, serum) for CE separation and the ability of CE methods to screen various types of abused drugs. Unfortunately the majority of

Table 1. Method validation.

Compound	Repeatability of migration times		Tested linear calibration range ($\mu\text{g/mL}$)	Limit of detection ($\mu\text{g/mL}$)
	Intraday, RSD (%)	Interday, RSD (%)		
AMP (1)	0.2	7.3	2.0–100.0	1.5
MET (2)	0.3	8.1	2.0–100.0	1.6
cocaine (3)	0.5	8.3	5.0–100.0	2.1
codeine (4)	0.8	11.7	5.0–50.0	2.4
MOR (5)	1.2	11.8	5.0–50.0	2.5
ETM (6)	1.2	12.3	5.0–50.0	2.5
MAM (7)	1.3	11.5	5.0–50.0	2.8
DAM (8)	1.3	13.2	5.0–50.0	2.8
7-AF (9)	0.8	11.7	5.0–100.0	1.6
diazepam (10)	0.7	12.5	5.0–100.0	1.4
BLN (11)	0.9	10.8	5.0–100.0	1.6
FNAP (12)	0.9	11.4	5.0–100.0	1.8
ACBP (13)	1.0	12.9	5.0–100.0	1.9

Intraday precision for 3 days; average RSD values for intraday precision are shown; linear calibrations were made with 6 points (each with 5 repetitions).

AMP, amphetamine; MET, methamphetamine; MOR, morphine; ETM, ethylmorphine; MAM, 6-monoacetyl-morphine; DAM, diacetylmorphine; 7-AF, 7-aminoflunitrazepam; BLN, benzoylecgonine; FNAP, flunitrazepam; ACBP, 2-aminochlor-benzophenone

Table 2. Results from immunochemical tests and CE and HPTLC methods.

Sample No.	Immunochemical tests*				HPTLC**	CE**
	AMP	MET	OPI	BZD		
1	pos/neg	pos	neg	pos	MET, 7-AF	MET, 7-AF
2	neg	neg	pos	neg	ETM, MOR	ETM, MOR
3	pos	pos	neg	neg	MET, AMP	MET, AMP
4	neg	neg	pos	pos	MOR, 7-AF	MOR, 7-AF
5	neg	neg	neg	pos	7-AF	7-AF
6	pos	pos	pos	neg	MET, AMP, MOR	MET, AMP, MOR
7	pos/neg	pos	neg	neg	MET	MET
8	neg	neg	pos	neg	MOR	MOR
9	neg	neg	neg	pos	7-AF	7-AF
10	pos	pos	neg	neg	MET, AMP	MET, AMP
11	neg	neg	pos	pos	ETM, MOR, 7-AF	ETM, MOR, 7-AF
12	pos	pos	neg	neg	MET, AMP	MET, AMP

*AMP = amphetamines; MET = methamphetamines; OPI = opiates; BZD = benzodiazepines; pos = the test was positive; neg = the test was negative

** MET = methamphetamine; AMP = amphetamine; MOR = morphine; ETM = ethylmorphine; 7-AF = 7-aminoflunitrazepam

Fig. 1. Electropherogram of standard mixture of groups of abused drugs
BGE 50 mM phosphate-Tris buffer pH 2.0 with 30 % (v/v) of methanol.
Separation voltage 30 kV; concentration of standard mixture 10^{-4} mol.L $^{-1}$.
1: amphetamine (AMP), 2: methamphetamine (MET), 3: cocaine, 4: codeine, 5: morphine (MOR), 6: ethylmorphine (ETM), 7: 6-monoacetyl-morphine, 8: diacetylmorphine, 9: 7-aminoflunitrazepam (7-AF), 10: diazepam, 11: benzoylecgonine, 12: flunitrazepam, 13: 2-aminochlor-benzophenone

Fig. 2. Electropherograms of saliva extract from volunteer after methamphetamine dosage.
BGE 50 mM phosphate-Tris buffer pH 2.0 with 30 % (v/v) of methanol; separation voltage 30 kV.
Top electropherogram presents sample after 24 hours after dosage. Lower electropherogram corresponds to sample immediately after dosage.
1: amphetamine, 2: methamphetamine

published papers are focused on only one type of drug (e.g. opiates, amphetamines, benzodiazepines, antidepressant drugs).

The aim of this work was to develop a simple capillary electrophoretic method as the verification and confirmation tool for screening analysis of amphetamines, opiates, benzodiazepines, cocaine and their metabolites, for toxicological application. The developed method was applied to real samples of saliva and urine, which were collected from addicts in the Czech Republic. The results obtained from CE analysis were compared with the HPTLC method and with the multicomponent immunochemical test used for toxicological screenings.

MATERIALS AND METHODS

Chemicals

Phosphoric acid, tris(hydroxymethyl)aminomethane (Tris), sodium hydroxide, methanol and ethyl acetate were obtained from Sigma (St. Louis, USA). All of chemicals were analytical grade quality. Drugs and its main metabolites were kindly provided from the Institute of Forensic Medicine and Medical Law, Palacký University, Olomouc, Czech Republic. Biological samples were collected from drug addicts (narcomaniacs) in cooperation with the Drop-in Centre Olomouc. Deionised water (Elga, Bucks, England, $18 \text{ M}\Omega\cdot\text{cm}^{-1}$) was used for preparation of all solutions.

Apparatus and conditions

The separations were performed on the capillary electrophoresis SpectraPHORESIS 100 with fast-scanning UV/VIS detector SpectraFOCUS (Thermo Separation Products). Uncoated fused silica capillaries (CACO-Silica Tubing and Optical Fibers, Slovakia) of total and effective lengths of 75 cm and 45 cm, respectively, 75 μm I.D. x 365 μm O.D. were used for these experiments. The capillary was thermostated at 25 $^{\circ}\text{C}$, the applied voltage was +30 kV (+400 $\text{V}\cdot\text{cm}^{-1}$). Statistical evaluations were done using the data analysis programme QC Expert 2.5 (TriloByte Statistical Software, Pardubice, Czech Republic). The background electrolyte was prepared by diluting an appropriate amount of phosphoric acid in deionised water and then the solution was titrated with Tris to pH 2.0; finally methanol was added to the background electrolyte. Drugs and their metabolites were dissolved in a ten times diluted background electrolyte. Samples were injected for 0.5 s by vacuum. The capillary was washed daily before the first experiment with 0.1 $\text{mol}\cdot\text{L}^{-1}$ NaOH solution for 10 min, then with water (10 min); 0.1 $\text{mol}\cdot\text{L}^{-1}$ HCl (10 min); again with water (10 min); and finally with the working electrolyte for 15 min. The capillary was washed with BGE between individual analysing runs. All measurements were executed five times.

Comparative screening assays

The multicomponent immunochemical QuikScreenTM tests (donated by Exbio, Olomouc, Czech Republic) were used for comparison of results obtained by the CE method. The test was utilized for amphetamine (sensitivity 1.0 $\mu\text{g}/\text{mL}$), benzodiazepines (0.3 $\mu\text{g}/\text{mL}$), methamphetamine (1.0 $\mu\text{g}/\text{mL}$), and opiates/morphine (2.0 $\mu\text{g}/\text{mL}$). The procedure of the HPTLC method, used for the screening comparison, was as follows: the sample was filtrated; then alkalized with 50 % (w/w) NaOH to pH 9–10. The sample was extracted 3 times with diethylether; dried with sodium sulfate and acidified with HCl to a pH 3–4; then the extract was evaporated. The HPTLC plates Merck 60 F-254 (Merck, Darmstadt, Germany) and ethylacetate – methanol – ammonium 34:4:2 (v/v) as a mobile phase were used.

Sample preparation

The real samples of urine and saliva from addicts were extracted and prepared according to the procedure described below.

Extraction of amphetamines and benzodiazepines: The sample was adjusted with 1 M sodium hydroxide to pH 9–10 and then shaken for 3 min with ethyl acetate. The water layer was removed after the extraction. The organic layer was shaken with water and the water layer was removed again. The remaining organic layer was shaken with 0.1 M HCl for 2 minutes and the water layer was separated out and alkalized with 20% (v/v) Na_2CO_3 . The water phase was reextracted with ethyl acetate and the water layer after reextraction was removed. The organic extract was dried with anhydrous sodium sulphate and organic phase with addition of 0.1 M HCl in isopropyl alcohol was evaporated in a stream of nitrogen at 25 $^{\circ}\text{C}$.

Fig. 3. Electropherograms of urine extract from volunteer with morphine.

BGE 50 mM phosphate-Tris buffer pH 2.0 with 30 % (v/v) of methanol; separation voltage 30 kV. Top electropherogram presents the sample after 6 hours after dosage. Lower electropherogram corresponds to sample immediately after dosage.

5: morphine (MOR)

Extraction of opiates: The sample was adjusted with concentrated NH_4OH to pH 9–10 and then shaken for 3 min with a mixture of chloroform and isopropyl alcohol (4:1, v/v). The water phase was removed after extraction and the organic phase was dried with anhydrous sodium sulphate and organic phase with addition of 0.1 M HCl in isopropyl alcohol was evaporated in a stream of the nitrogen at 25 $^{\circ}\text{C}$.

RESULTS AND DISCUSSION

Separation development

The examined mixture contained five main groups of frequently misused addictive drugs (amphetamines, benzodiazepines, cannabinoids, cocaine and opiates). All of these analytes were positively charged in acidic background electrolytes. Therefore a very acidic pH of BGE was chosen. These strong acidic conditions ensure

Fig. 4. Electropherogram of urine extract from drug addict with methamphetamine (6 hours after dosage).
BGE 50 mM phosphate-Tris buffer pH 2.0 with 30 % (v/v) of methanol. Separation voltage 30 kV.
2: methamphetamine (MET)

Fig. 5. Electropherogram of urine extract from drug addict with morphine (48 hours after dosage).
BGE 50 mM phosphate-Tris buffer pH 2.0 with 30 % (v/v) of methanol. Separation voltage 30 kV.
5: morphine (MOR), 7: 6-monoacetylmorphine

protonization and moreover using low pH of the BGE and Tris⁺ ions, as coions leads to the suppression of residual electroosmotic flow. In the first experiment 50 mM phosphate Tris pH 2.0 was used for the separation of a standard mixture of drugs. The excellent separation of methamphetamine, amphetamine, cocaine, benzoyl-eckognine and both benzodiazepines from their metabolites was achieved under these conditions. However the separation of opiates and cocaine was not favourable. Therefore, the addition of native cyclodextrins and organic solvents (methanol, acetonitrile) into the background electrolyte was tested. Native cyclodextrins (α , β , γ cyclodextrin) were examined as additives for the selectivity; the addition of α -cyclodextrin gave the best baseline separation of five morphine analogues. The optimal concentration of α -cyclodextrin was 15 mM in 50 mM phosphate Tris pH 2.0. Unfortunately, separation of 7-aminoflunitrazepam and cocaine under these conditions was lost with this additive.

Furthermore, the influence of methanol and acetonitrile was studied to achieve complete separation of all drugs. Acetonitrile did not allow separating the drugs but methanol can be used. Finally, the addition of 30 % (v/v) of methanol into the BGE enabled the separation of all studied compounds (Figure 1). The identification of com-

pounds was based on their migration times and characteristic UV spectra. The proposed screening method was partially validated; repeatability, limits of detection and calibration parameters are listed in Table 1; correlation coefficients varied from 0.978 to 0.998. The precision of peak areas (RSD) did not exceed 3.5 % for intraday assays and 18.6 % for interday assays.

Screening applications

This simple CE method developed for rapid screening of abused amphetamines, opiates, benzodiazepines and cocaine with their main metabolites was applied to real samples of saliva and urine from drug addicts. The samples of urine and saliva were extracted according to the extraction procedure described in the experimental section. All analyses of real samples were carried out in 50 mM phosphate Tris pH 2.0 with 30 % (v/v) of methanol. Figure 2 presents the electropherogram of saliva extract from an addict after 24 hours of methamphetamine consuming. Saliva as a sample for monitoring drug abuse seems to be suitable because it has a relatively simple matrix (see the Figure 2) and it is easy to sample. On the other hand urine samples from volunteer (Figure 3) enable good identification of morphine and its metabolites (in this case six hours after dosage). Figures 4 (6 hours after the meth-

amphetamine dosage) and Figure 5 (48 hours after the heroin dosage) present electropherograms of extracts of urine samples from drug addicts and prove the possibility of using this method for the real analyses.

The CE method was compared with the HPTLC method and the multicomponent immunochemical screening test QuikScreen™ on a set of samples from teenage drug abusers in the region of Olomouc, Czech Republic. The results of the drug identification by these screenings are listed in Table 2. Results obtained by CE and HPTLC demonstrate 99.8 % agreement and results from CE and immunochemical analysis exhibited 95 % correspondence. These results also confirmed the current mode of drug use – the combination of several drugs in order to prolong their effects.

CONCLUSION

The simple screening capillary zone electrophoresis method that allows recognition of the most abused drugs (amphetamines, opiates, benzodiazepines and cocaine) was developed. The advantages of this CE screening toxicological method include its simplicity with relatively high selectivity for separation and identification of abused drugs, low cost and potential to become the verification tool for immunochemical screenings and the HPTLC screening of abused and/or illegal drugs (agreement of the results was more than 95%).

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