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Development of a capillary electrophoresis method for the screening of human urine for multiple drugs of abuse

Ahmed Alnajjar [a,*], Abubakr M. Idris [a], Marika Multzenberg [b], Bruce McCord [c]

[a] *Department of Chemistry, College of Science, King Faisal University, Hofuf 31982, Saudi Arabia*
[b] *Department of Chemistry, Ohio University, Athens, OH, USA*
[c] *International Forensics Research Institute, Florida International University, Department of Chemistry, University Park, Miami, FL, USA*

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Abstract

A new capillary electrophoresis (CE) method was developed and validated for the screening of human urine for nineteen drugs of abuse. In order to achieve sufficient separation, the electrolyte composition was modified using β-cyclodextrin (β-CD) and organic solvents. To process each sample, a sequential injection-solid-phase extraction (SI-SPE) system was constructed. Using this device, matrix clean-up, extraction, and preconcentration of analytes were performed onto a C_{18} cartridge. Optimal separation and detection were obtained using a background electrolyte consisting of 100 mM phosphate adjusted to pH 6.0, with 20 mM β-CD, 5% acetonitrile and 20% isopropanol. Electrokinetic injection was performed at 5 kV for 10 s, separation voltage was 25 kV and column temperature was set to 25 °C. The separation was carried out in a 67.0 cm × 50 μm fused-silica capillary with UV detection at 214 nm. The combination of SI-SPE and sample stacking showed significant sensitivity enhancement with limits of detection in the range of 5–30 ng ml^{-1}. A validation study showed good reproducibility of both migration time (RSD = 0.003–0.088%) and peak area (RSD = 0.54–4.8%). Overall, this automated and miniaturized SI-SPE system provides a rapid, sensitive, and robust procedure for analysis; as well as minimizes sample and solvent consumption.

Keywords: Capillary electrophoresis; Forensic analysis; Drugs of abuse; Sequential injection analysis; Solid-phase extraction

1. Introduction

Since its inception, capillary electrophoresis (CE) has been proven to have great utility in the analysis and detection of drugs of abuse due to its high efficiency, reduced sample volume and rapid analysis [1]. Unfortunately, when used in standard operating modes, CE is not sensitive enough to be applied to trace analysis due to low injection volumes and limited detection pathlengths. As a result, extensive research has been conducted in an effort to improve the sensitivity of the technique. Many approaches have been examined including the development of alternative detection techniques, such as fluorescence [2], mass spectrometry [3] and electrochemistry [4]. In addition, extended pathlength detector cells [5], such as bubble-shaped flow cells and Z-shaped flow cells have been employed. However, these approaches provide only a 3–10 fold sensitivity enhancement and this improvement is often offset by a reduction in separation efficiency.

Sample preconcentration can provide an alternative approach for sensitivity enhancement. Techniques, such as solid-phase extraction (SPE) [6–10], liquid–liquid extraction [11] and solid-phase microextraction [12,13] can be used in combination with CE to improve sensitivity. These extraction procedures can be performed through the use of sequential injection (SI) techniques [14–16]. SI, a second generation flow injection technique involves the reduction of sample and reagent volume from milliliters to microliters. In addition, the full automation of the technique enables the entire process to be rapid and precise [17,18]. Unlike FI, SI can be used for widely different chemistries simply by changing the flow programs. Many articles describing the development and application of FI techniques are available in the literature [19–24].

* Corresponding author. Fax: +96635886437.
E-mail address: anajjar@kfu.edu.sa (A. Alnajjar).

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Another approach for sensitivity enhancement in CE is field amplified sample stacking [25]. It has been shown that by combining solid-phase extraction with sample stacking, it is possible to obtain sufficiently low limits of detection (LOD) in CE to permit trace detection of drugs and their metabolites in urine [26,27].

An issue in the analysis of drugs of abuse by CE is the need to modify electrolyte composition in order to separate a wide variety of acidic, basic and neutral compounds, which may be present in biological fluids. Electrolyte additives have been used to modify the mobility of analytes, altering the electro-osmotic flow (EOF) and improving solubility [25]. In particular, the development of micellar electrokinetic capillary chromatography (MECC), in which micelle forming detergents are added to the electrolyte, has greatly expanded the utility of the technique. MECC permits the simultaneous separation of ions and neutrals based on their relative affinity for the hydrophobic micellar pseudo-stationary phase. Cyclodextrins (CDs) can also be used to produce a pseudo-stationary phase with high selectivity. CDs are cylindrical compounds with a hydrophilic outer rim and a hydrophobic cavity. The rate that a solute partitions into and out of this cavity varies with its structure, polarity and size. Once a solute is in the cavity, its velocity is retarded; but when it is in the bulk phase, mobility is unaffected [28]. The varying rates of formation of the guest/host inclusion complex between the analyte and the cyclodextrin molecule lead to excellent separation selectivity [29]. Organic solvents can be added to the electrolyte to further affect the rate of inclusion complex formation [30].

The present work describes the development of a CE method for the screening of human urine for 19 drugs of abuse. The proposed method involves a combination of β-CD and organic solvents to permit the development of highly selective separations. Sample treatment was performed using a SI-SPE manifold, in which matrix clean-up and analytes extraction and preconcentration were performed onto a C_{18} cartridge. The automation and miniaturization of SI-SPE permits a rapid, robust and cost-effective procedure. Our results show the SI-SPE preconcentration process combined with sample stacking provides a sensitive method with detection levels in the low ng ml^{-1} range.

2. Materials and methods

2.1. Chemicals and reagents

Chemicals and reagents used in this work were of analytical grade and 18 MΩ deionized water was used in all experiments. 6-Acetylmorphine·HCl (6-AM), D-amphetamine·HCl, cocaethylene, cocaine, codeine, L-ephedrine·HCl, fentanyl, heroin, D,L-methadone·HCl, methamphetamine·HCl, methaqualone, morphine, 3-β-D-noscapine, oxycodone·HCl, papaverine·HCl, thebaine were purchased from Lipomed (Cambridge, MA, USA). In addition, psilocin, diphenhydramine and pheniramine were purchased from Sigma (St. Louis, MO, USA). β-CD is commercially available from TIC America (Portland, OR, USA). Organic solvents including acetone, acetonitrile, ethanol, isopropanol and methanol as well as phosphoric acid, sodium dihydrogen phosphate and sodium hydroxide were purchased from Merk (Darmstadt, Germany).

2.2. Instrumentation

2.2.1. SI-SPE manifold

The SI system used in this study is a FIAlab 3500 (Medina, WA USA). It is composed of syringe pump, multiport valve, holding coil and personal computer. See Fig. 1. The syringe has a volume of 5.0 ml. The multiport valve is chemically inert and has 10 ports. 0.03 in. i.d. teflon tubes were supplied from Upchurch Scientific, Inc. (Oak Harbor, WA, USA). These tubes were used to connect the units of the manifold and to make a holding coil with 300 cm long. The manifold was controlled by FIAlab for Windows version 5.0. The C_{18} cartridge (5 cm length, 4.6 mm i.d.) was packed at our laboratory with modified silica particles (C_{18}, 45 μm, 60°A) supplied from Supelco (Bellefone, PA, USA).

2.2.2. CE system

Separation and detection were performed using a P/ACE 5500 CE system equipped with a UV detector supplied from Beckman-Coulter (Fullerton, CA, USA). Control of the instrumentation, data acquisition and processing were performed by Chrom Perfect software version 3.5 supplied from Justice

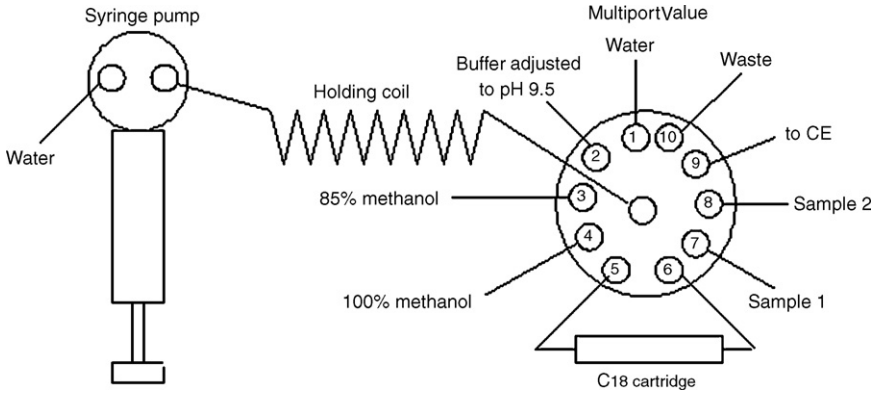

Fig. 1. SI-SPE manifold constructed for sample clean-up and preconcentration.

Laboratory Software (Palo Alto, CA, USA); and P/ACE 3.0 windows control software (Beckman-Coulter, Fullerton, CA, USA).

2.3. Preparation of standard solutions and samples

A standard solution containing the nineteen drugs (as addressed in Section 2.1) was prepared at a concentration of 100 μg ml^{-1} in methanol. Working mixtures of pertinent concentrations were prepared.

Human urine samples were collected from healthy drug-free volunteers. The pH of samples was adjusted to 9.5 with sodium hydroxide. Then samples were filtered through a membrane filter (0.45 μm pore size). The supernatant solution was spiked with the mixture standard solution to result a total concentration of 1 μg ml^{-1}.

2.4. Procedures

2.4.1. SI-SPE procedure

A SI manifold suitable for SPE was constructed (Fig. 1). The holding coil was placed between the out-position port in the syringe and the central port in the multiport valve. Water was linked to the in-position port in the syringe and to port-1 in the multiport valve. Buffer adjusted to pH 9.5 was linked to port-2. Methanol at concentrations of 85 and 100% (v/v) were linked to port-3 and -4, respectively. C_{18} cartridge was placed between port-5 and -6. Two standards/samples were linked to port-7 and -8. Treated samples were received in a vessel through port-9; and waste was drained through port-10.

A volume of a liquid was first loaded in the HC by aspiration and then dispensed through the appropriate port. The algorithms controlling SI-SPE procedure were then programmed and are briefly described below. To condition the cartridge, 700 μl of each of 100% (v/v) methanol, water and the buffer solution (pH 9.5) were sequentially injected into the cartridge at a flow rate of 20 μl s^{-1}. For sample introduction, 500 μl of standard/sample was injected at a flow rate of 10 μl s^{-1}. The matrix was cleaned by injecting 500 μl of water at a flow rate of 10 μl s^{-1}. Finally, standard/sample was eluted by 20 μl of 85% methanol followed by 70 μl of water. The treated sample was manually transferred to the CE system for separation and detection.

2.4.2. CE procedure

The separation was performed on a fused-silica capillary (67.0 cm length (effective length 60 cm) × 51 μm i.d.). The CE system was operated using normal polarity. Prior to each run, the capillary was washed at a pressure of 20 psi with 0.1 M sodium hydroxide for 1.0 min and water for 1.5 min. Then, the capillary was conditioned by flushing with electrolyte for 1.5 min. Samples were injected using the electrokinetic mode at a voltage of 5 kV for 10 s and a column temperature of 25 °C. Detection was carried out using a UV detector operated at 214 nm.

3. Results and discussion

3.1. CE development

The optimization of the electrophoretic separation was based on the production of acceptable peak shape, resolution and separation time. Electrophoretic factors potentially affecting these responses including electrolyte concentration, pH, β-CD concentration, organic additives as well as separation voltage were considered for optimization.

The effect of the separation voltage in the range of 10–25 kV was examined. Twenty-five kilovolts provided acceptable resolution and kept analysis times short. Electrokinetic injection mode was used for all analyses with an optimum injection set at 5 kV for 10 s.

Different concentrations of phosphate electrolyte in the range of 25–200 mM and different pH values in the range of 2.5–10.0 were examined. For concentrations up to 100 mM, the resolution increased with ionic strength. Above this concentration, resolution decreased; a phenomenon that could be attributed to the generation of higher current and Joule heating. Therefore, 100 mM was adopted as the optimum electrolyte concentration.

During pH optimization, it was found that resolution decreased at low pH. This may be due to the protonation of analytes, which are mostly basic drugs. As the pH rose above 4, the separation improved, however the overall resolution was still inadequate even at the most favorable pH of 6.0.

For further optimization of separation, the effect of different concentrations of β-CD (5–20 mM) in the CE electrolyte was investigated. The resolution between most analytes increased with the concentration of β-CD and therefore 20 mM was adopted as the optimum. These experiments also demonstrated that the addition of organic solvents to the electrolyte containing β-CD improved the separation. Various amounts of acetonitrile, acetone and isopropanol were added to the electrolyte individually and in mixtures. Typical electropherograms showing the effect of the addition of organic solvents to the electrolyte on separation are depicted in Fig. 2. Although the best results were obtained at the highest concentrations of these solvents, concentrations above 25% reduced the solubility of β-CD and the separations became reproducible. A mixture of 5% acetonitrile and 20% isopropanol provided the optimal results.

Ultimately, the optimum CE conditions for the separation were determined to be 100 mM phosphate, pH 6, 20 mM β-CDs, 5% acetonitrile and 20% isopropanol with electric field strength of 0.373 kV cm^{-1}. Fig. 3 shows typical electropherogram resulting from the SI-SPE and CE separation of a mixture standard solution contained 1 μg ml^{-1} of each of the examined drugs using these conditions.

3.2. SI-SPE development

Experimental parameters affecting the adopted SI-SPE procedure were next optimized. These conditions included concentration and volume of solvents and standard/sample as well

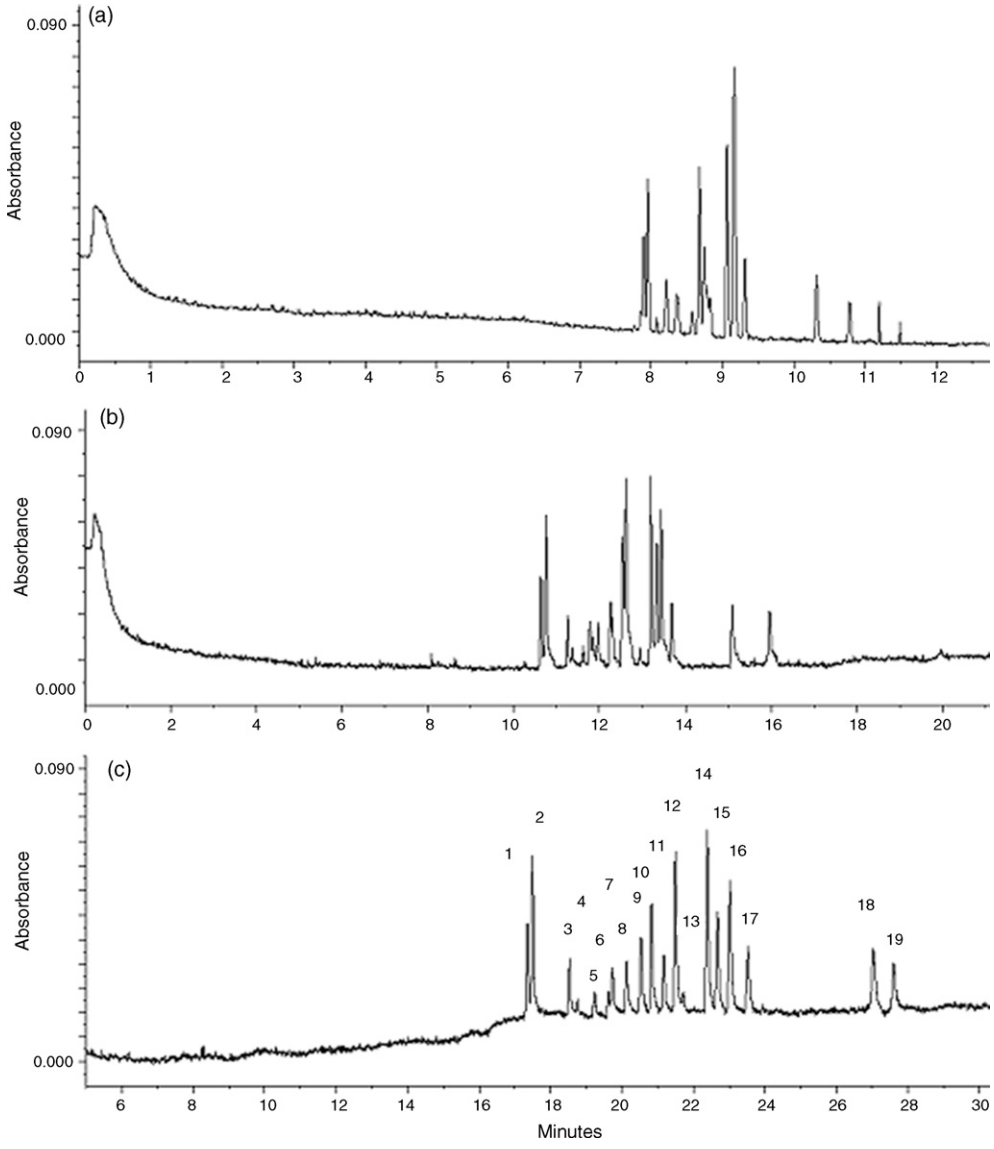

Fig. 2. Electropherograms of 19 drugs of abuse showing the effect of the addition of an organic solvent to the buffer in the concentration of 25% on the resolution and migration times: (a) acetonitrile; (b) acetone and (c) isopropanol. Conditions: buffer: 100 mM phosphate, pH 6, 20 mM β-CD. Other electrophoretic conditions as described in Section 2.4.2. Peak identification and drug concentration as in Table 1.

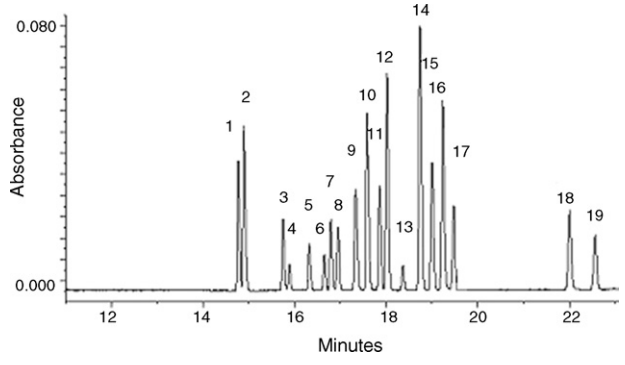

Fig. 3. Electropherogram of a standard mixture contained 1 μg ml^{-1} of each drugs of abused under the optimized conditions: 100 mM phosphate, pH 6, 20 mmol l^{-1} β-CD, 5% (v/v) acetonitrile and 20% (v/v) isopropanol. Other electrophoretic conditions as described in Section 2.4.2. Peak identification as in Table 1.

as flow rate. The method was optimized to obtain an acceptable CE baseline, good recovery, and significant preconcentration. Different flow rates ranging from 10 to 20 μl s^{-1} were tested. The use of higher flow rates speeds the procedure, though at flow rates above 20 μl s^{-1} reverse backpressure causes problems. Twenty microliters per second was found to be suitable for conditioning the cartridge and the elution of analytes. 10 μl s^{-1} was found to be suitable for matrix clean-up. Sequential flushing with 700 μl of each of 100% (v/v), water and the buffer at a flow rate of 10 μl s^{-1} was found to effectively condition and equilibrate the cartridge. For matrix clean-up, 500 μl of water was found to satisfactorily remove interferents from urine. The elution step was successfully carried out by sequential injection of 20 μl of 85% methanol and 70 μl water.

Unlike conventional procedures used in manual SPE, this SI-SPE procedure conserves both reagents and sample. The current

procedure reduces solvent volumes from the milliliter scale down to microliters. In addition, the full automation of SI technique provides a rapid, accurate and reproducible procedure that minimizes reagent handling and consumption.

3.3. Method validation

For method validation, a series of standard solutions containing each of the 19 drugs of abuse at different concentrations ranging from 0.1 to 50 μg ml^{-1} were examined under the optimum conditions. The CE method was found to be linear in the range of 0.3–6.0 μg ml^{-1} for all of the studied drugs. The calibration equation and correlation coefficient of each drug are shown in Table 1. Migration time, peak area, and their relative standard deviation values (RSD, $n = 5$) are also provided in Table 1. The results show good reproducibility of both migration time (RSD = 0.003–0.088%) and peak area (RSD = 0.54–4.8%).

The recovery of both the SI-SPE process and the CE analysis was examined by analyzing a human urine sample spiked with a mixture of the analytes at a concentration of 1 μg ml^{-1} for all components. The recovery was calculated for each analyte and the results obtained are presented in Table 2. As shown in the table, the recoveries range from 70.4 to 98.8%. A lower recovery recorded for certain drugs (ephedrine, methadone, PCP and phenireamine) is most probably due to their loss during the SI-SPE process. It is difficult to adopt a single set of SI-SPE conditions applicable to a wide range of drugs of abuse. However, this procedure shows great utility as a screening method. The repeatability of the recovery was examined by running the same spiked human urine sample in five replicate injections. The RSD values and the results obtained are introduced in Table 2. These values did not exceed 2.5%, indicating good reproducibility of the adopted method.

Table 2
The recovery (R) and its relative standard deviation (RSD, $n = 5$) of 19 drugs of abuse in spiked human urine sample

#[a]	Drug	R, RSD (%)	LOD[a]	LOD[b]
1	Amphetamine	76.6, 1.5	26	10
2	Methamphetamine	75.8, 1.3	16	10
3	Ephedrine	74.4, 1.3	42	10
4	Psilocin	84.0, 1.2	13	7
5	Cocaine	82.4, 1.7	120	10
6	Cocaethylene	80.6, 0.8	84	10
7	Methadone	75.1, 2.5	33	10
8	PCP	76.2, 2.3	58	30
9	Pheniramine	74.6, 1.5	35	20
10	Diphenhydramine	84.7, 1.3	24	7
11	Oxycodone	98.1, 1.1	18	7
12	Thebaine	95.8, 1.4	19	7
13	Fentanyl	82.2, 1.6	47	10
14	Codeine	98.8, 1.8	15	5
15	Morphine	98.2, 1.5	28	5
16	6-AM	96.6, 1.6	21	5
17	Heroine	78.6, 1.4	29	10
18	Noscapine	81.1, 1.2	61	10
19	Papaverine	83.3, 1.1	140	10

The limits of detection (LOD, ng ml^{-1}) of direct CE; and LOD (ng ml^{-1}) using SI-SPE before CE are also introduced.
[a] Peak number.
[b] LOD using direct CE; LOD using SI-SPE prior to CE.

To determine the role of the SI-SPE process in sensitivity enhancement, a mixture of the 19 drugs was subjected directly to CE analysis. Another portion of the same standard solution was subjected to SI-SPE before CE analyses. The LOD for both electropherograms were calculated as the concentration of solute, resulting in a peak height three times the baseline noise level. The results are displayed in Table 2. The relatively low LOD for the CE analysis can be attributed to sample stacking. As shown in the table, the range of LODs was reduced from 13–140 to

Table 1
Weighed regression with correlation coefficient (r) of 19 drugs of abuse of the linearity range of 0.3–6.0 μg ml^{-1}

#[a]	Drug	Weighed regression, r	t_m, RSD (%)	Peak area, RSD (%)
1	Amphetamine	$y = 1.726x - 2.771$, 0.9998	15.4, 0.053	0.66, 4.30
2	Methamphetamine	$y = 2.881x - 4.567$, 0.9997	15.6, 0.061	1.14, 4.30
3	Ephedrine	$y = 1.069x - 1.650$, 1.0000	16.5, 0.013	0.49, 2.00
4	Psilocin	$y = 3.452x - 0.513$, 0.9999	16.6, 0.027	0.18, 4.80
5	Cocaine	$y = 0.375x - 0.852$, 0.9999	17.1, 0.051	0.27, 1.20
6	Cocaethylene	$y = 0.530x - 0.816$, 0.9998	17.5, 0.060	0.23, 2.10
7	Methadone	$y = 1.346x - 0.893$, 0.9997	17.7, 0.088	0.41, 0.54
8	PCP	$y = 0.765x - 1.121$, 0.9999	17.8, 0.028	0.37, 2.00
9	Pheniramine	$y = 1.283x - 1.595$, 0.9999	18.2, 0.052	0.92, 0.97
10	Diphenhydramine	$y = 1.853x - 2.546$, 1.0000	18.6, 0.127	1.14, 1.80
11	Oxycodone	$y = 2.418x - 1.864$, 0.9997	18.8, 0.011	0.53, 2.90
12	Thebaine	$y = 2.389x - 3.239$, 1.0000	19.0, 0.014	1.52, 3.50
13	Fentanyl	$y = 0.949x - 0.286$, 0.9997	19.4, 0.028	0.18, 0.77
14	Codeine	$y = 3.074x - 4.051$, 0.9999	19.8, 0.005	2.01, 3.90
15	Morphine	$y = 1.586x - 2.176$, 1.0000	20.1, 0.009	0.99, 4.32
16	6-AM	$y = 2.159x - 2.810$, 0.9997	20.3, 0.005	1.35, 4.61
17	Heroine	$y = 1.550x - 2.199$, 1.0000	20.7, 0.003	0.91, 2.70
18	Noscapine	$y = 0.727x - 0.088$, 0.9996	23.4, 0.021	1.31, 2.20
19	Papaverine	$y = 0.320x - 0.098$, 1.0000	24.1, 0.046	0.53, 3.70

Migration time (t_m, min) and peak area; and their relative standard deviation (RSD%, $n = 5$) values are also introduced.
[a] Peak number.

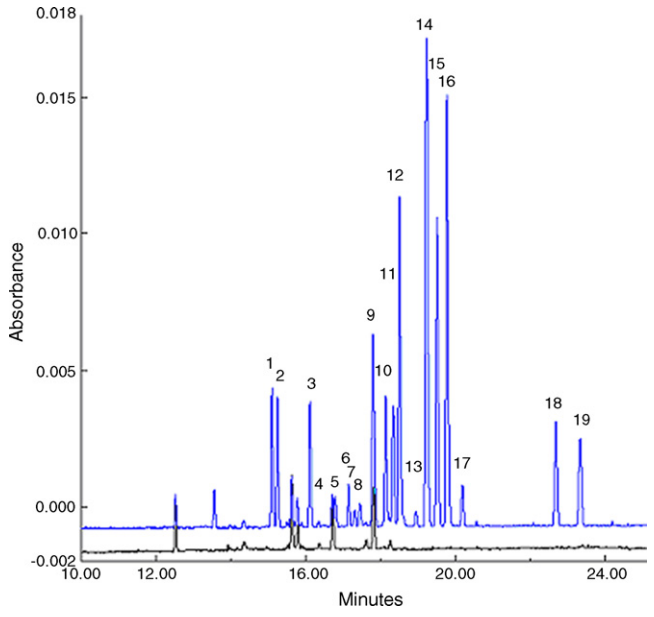

Fig. 4. Electropherograms of a urine sample spiked with 25 ng ml^{-1} of each drug (upper electropherogram); and drug-free urine sample (lower electropherogram) under the optimized conditions: 100 mM phosphate, pH 6, 20 mM β-CD, 5% (v/v) acetonitrile and 20% (v/v) isopropanol. Other electrophoretic conditions as described in Section 2.4.2. Peak identification as in Table 1.

5–30 ng ml^{-1}, when using the SI-SPE system. These levels are within the range necessary for the determination of drugs of abuse in toxicological urine samples [31].

As example, in Fig. 4, the upper electropherogram demonstrates a human urine sample spiked with 25 ng ml^{-1} of a mixture of drugs that was subjected to SI-SPE and CE. For comparative study, the lower electropherogram in Fig. 4 demonstrates a blank sample. The figures show that no significant peaks were recorded for the drug-free urine sample, except at peak 5 and 9, indicating minimal interference between the urine sample matrix and the drugs under study.

4. Conclusion

A new method for the screening of human urine sample for multiple drugs of abuse was developed using a CE technique. Sample clean-up, extraction and preconcentration were performed using SI-SPE. The automation and miniaturization capabilities of the SI-SPE procedure yield a method for drug screening that is rapid, and minimizes sample and reagent handling when compared with conventional sample treatment procedures. The combination of β-CD and organic solvents in the electrolyte composition yields high selectivity in the analysis of a wide variety of drugs of abuse. Significant sensitivity enhancement was obtained by preconcentration using the SI-SPE procedure.

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