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An online field-amplification sample stacking method for the determination of β_2-agonists in human urine by CE-ESI/MS

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ABSTRACT

Field amplified sample stacking (FASS) was combined with a simple, rapid, sensitive CE-ESI–MS method to achieve the on-line enrichment and simultaneous determination of Clenbuterol (CLE), Salbutamol (SAL), Terbutaline (TER) and Formoterol (FOR). Samples were diluted in deionized water, and electrokinetic injection (10 kV × 50 s) was employed to carry out FASS. With FASS, the four β_2-agonists had simultaneously baseline enhancement as much as 319, 332, 297 and 115 fold, respectively. Consequently, satisfactory LODs ($S/N=3$) of 0.08, 0.1, 0.1 and 0.5 ng/mL for CLE, SAL, TER and FOR were obtained. The separation of the four analytes was performed at 22 kV in ammonium acetate/ammonia (20 mmol/L, pH 9.0), using 7.5 mmol/L acetic acid in isopropanol/water 50/50% (v/v) as sheath liquid. In addition, an excellent linear response was obtained with RSD less than 1.3% for migration times and less than 6.7% for peak areas ($n=5$). The recoveries of spiked urine samples were in the range of 82.7–101% with RSD lower than 9.8%. The proposed method has been applied to analyze human urine samples successfully.

1. Introduction

β_2-Agonists (structures see Fig. 1) is a class of drugs commonly used in the clinical treatment of pulmonary disorders and asthma owing to their bronchodilator activity [1]. Moreover, these drugs have been used as stimulants in sports for their stimulation on the central nervous and protein assimilation. Thus β_2-agonists have been placed on the list of banned drugs by the International Olympic Committee (IOC) and World Anti-Doping Agency (WADA) [2]. In addition, β_2-agonists, especially clenbuterol, are often employed as growth promoters in animal feed due to repartitioning of carcass composition to decreased fat deposition [3], which has caused some β_2-agonists poisoning cases in recent years. Therefore, a sensitive and time-saving method to determine β_2-agonists in biological specimen is urgent needed for the misuse monitor and control.

It was reported that β_2-agonists excretory present in urine in trace amounts (less than ng/mL level) [4], thus sufficiently sensitive and selective analytical methods for the determination of β_2-agonists were expected. Besides the most commonly used methods for analysis of β_2-agonists such as GC and LC combined with MS [5–12], ELISA [13,14], CE [15,16] has been utilized gradually. CE is a well-known microanalysis technique for its simplicity, high efficiency, rapid analysis and low sample consumption. Nevertheless, the restricted injection volumes in CE method unavoidably cause relative lower sensitivity, and consequently limit the widespread applications. To overcome this limitation, more sensitive detectors (e.g. MS) and/or the on-line stacking methods have been developed for coupling with CE [17,18].

With a higher universal specificity, MS is an apposite detection for CE, which has the advantage of analyte identification. Combining the rapid and highly efficient separations of CE with the selective mass identification and low sensitivity of MS, CE-MS [19] is a desirable coupling technique. To achieve the coupling of CE and MS, a sheath-flow was introduced to mix with the CE buffer and provide the potential to the CE separation.

Besides the combination with MS detector, on-line sample stacking methods is also an efficient approach to enhance the sensitivity. Field-amplified sample stacking (FASS), as the simplest on-line sample stacking techniques, is based on the mismatch conductivities between the sample solution and background electrolyte (BGE) [20]. FASS only requires higher conductivity of BGE than the sample. Analytes slow down and are compressed into a narrow sample bands when they are penetrated into the BGE region under a strong electric field. The pre-concentration is thereby achieved with this meaning of stacking on the edge of

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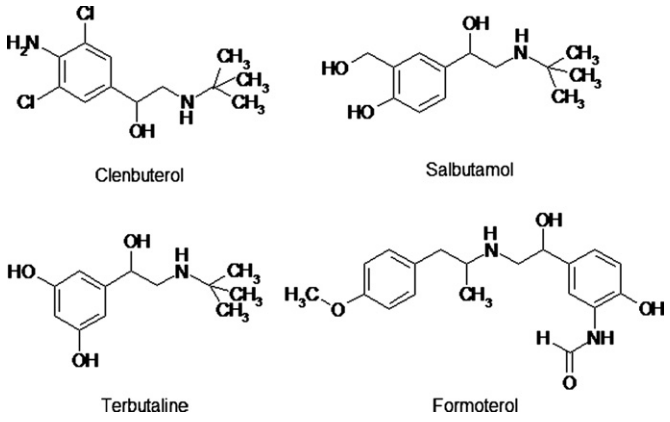

Fig. 1. Chemical structures of four β_2-agonsits.

two solutions. FASS has been applied to illicit drugs analysis in recent years. G. Manetto [21] has employed FASS to the analysis of opiate drugs in hair. Combining the advantages of MS detection and FASS to improve the sensitivity, the determination of trace analytes in biological samples becomes possible. The work presented here developed CE-ESI–MS method coupled with FASS technique for the analysis of β_2-agonists. Four β_2-agonists were satisfactorily separated within 6 min with the LODs ($S/N=3$) in the ng/mL concentration range, which were obviously lower compared with conventional injection. Moreover, the proposed method has been successfully applied to determination of β_2-agonists in the urine samples from volunteers.

2. Materials and methods

2.1. Chemicals and reagents

Clenbuterol, salbutamol, terbutaline, and formoterol were purchased from the Chinese Institute of Biological Products Control (Beijing, China). Salbutamol sulfate tablets were provided by Pingguang Pharmaceuticals (Jiangsu, China).

Methanol, acetonitrile and isopropanol (HPLC grade) were purchased from Sinopharm Chemical Reagents (Shanghai, China). Acetic acid glacial, ammonium acetate, and ammonium hydroxide were analytical reagent grade and obtained from Sinopharm Chemical Reagents. All the water used was deionized water purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

2.2. CE-ESI–MS instrumentation

All CE-ESI–MS experiments were performed on an Agilent ^{3D}CE system coupled with an Agilent 1100 series single quadrupole mass spectrometer (1100 series LC/MSD; Agilent Technologies, Waldbronn, Germany). The sheath liquid was delivered at 1/100 split flow by an LC pump (1100 series, Agilent Technologies). The instrument control, data acquisition, and data analysis was performed in Agilent CE/MSD ChemStation with EC-MS mode.

MS detection was conducted in the ESI positive ionization mode with 3.5 kV of electrospray voltage in all experiments. Full scan mode (m/z range from 200 to 400) and SIM scan mode was operated simultaneously with 150 V of fragment voltage, 0.15 amu of step size and 1.0 of gain. Nitrogen was employed as nebulizer gas and drying gas. With 0.69 bar of nebulizing gas, the drying gas (150 °C) flowed at a rate of 6.0 L/mL. The sheath liquid was consisted of methanol–water (50:50, v/v) in presence of 7.5 mM acetic acid and pumped at a rate of 3.0 μL/min.

2.3. Preparation of standard and buffer solution

All standard stock solutions were prepared by dissolving corresponding chemicals in deionized water at a concentration of 1.0 mg/mL for clenbuterol, salbutamol, terbutaline and formoterol. All buffer stock solutions were prepared conventionally, and the working buffer solutions were prepared by diluting the stock solutions. All buffers were passed through a 0.22 μm membrane filter before used. Standard solutions and running buffer were degassed by ultrasonication for 5 min before used.

2.4. Sample preparation

After blank urine sample collection, the healthy male volunteer was administered a single dose of salbutamol sulfate tablets (4.8 mg) orally. The doses were administered according to the principle of Public Health Bureau of China. Urine samples were regularly collected from volunteers and stored at −20 ℃. To remove the protein components and solid particles, all urine samples were mixed with methanol (1:1, V/V), followed by centrifugation at 4500 rpm for 10 min, and passed through a 0.22 μm membrane filter.

2.5. Capillary precondition

Analysis was conducted in a bare fused-silica capillary (50 μm i.d × 70 cm, Yongnian Optical Fiber Factory, Hebei, China). New capillaries were pre-treated by sequentially rinsing for 30 min with deionized water, 1.0 M NaOH, deionized water, 1.0 M HCl, deionized water, respectively. Between runs the capillary was flushed for 5 min with deionized water and 6 min with operating electrolyte.

All experimental solutions were prepared in doubly distilled water and passed through a polypropylene filter (0.22 μm) prior to use.

3. Results and discussion

3.1. Optimization of CE parameters

3.1.1. Effect of buffer acidity and concentration

Since nonvolatile BGEs are likely to contaminate the ion source of ESI–MS, they are not suitable for CE coupling with ESI–MS. Moreover, BGE components may suppress the response of analytes due to ionization competition with analytes. Ammonium acetate and ammonia, therefore, were introduced as buffer components because of high volatility, low ionic strength and less competition.

pH of buffer has influence on the magnitude of the EOF as well as the ionization of each individual analyte, thereby affecting on the separation and response. In this work, the effect of varying the pH (7.0–10.0) was investigated. It was observed that the separation efficiency increased in the pH range of 7.0–9.0. While pH=10.0, separation of peaks 2 and 3 became poor (Fig. 2). Therefore, pH=9.0 was considered as the suitable acidity to achieve the baseline separation and the best signal intensity.

At pH=9.0, the influence of the buffer concentration was also studied in this experiment. A series of buffer solution was prepared with different concentrations (10–40 mmol/L). As expected, the separation was improved with the increasing buffer concentration. Nevertheless, higher concentration led to longer retention time, broadened peaks and lower signal intensity

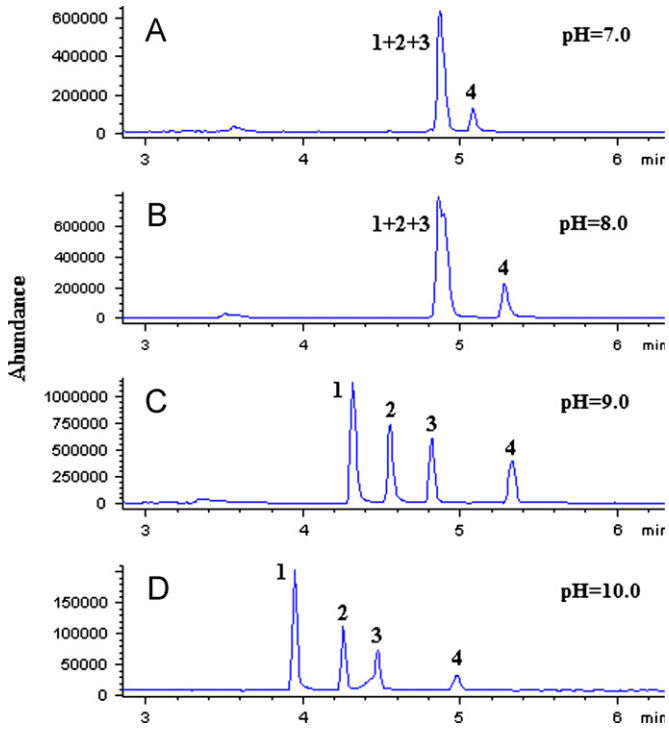

Fig. 2. Effect of the pH of buffer on the separation of four β_2-agonsits. Experimental conditions: Column: 70 cm × 50 μm of uncoated fused-silica capillary; running buffer, 20 mmol/L ammonium acetate (pH 7.0–10.0); separation voltage, 22 kV; sheath liquid, 50/50% (v/v) isopropanol/water containing 7.5 mmol/L acetic acid with a flow rate of 3.0 μL/min; injection, electrokinetic injection with 10 kV and 5 s; flow of drying gas, 6.0 L/min; temperature of drying gas, 150 °C; nebulizing gas pressure, 0.69 bar. Peak identification: (1) clenbuterol, (2) salbutamol, (3) terbutaline, (4) formoterol.

because of the influence of Joule heating. Concentration was consequently compromised on 20 mmol/L for further work.

3.1.2. Effect of separation voltage

Separation voltage on the separation of β_2-agonists was also investigated from 18 to 25 kV in the above chosen buffer (Fig. 3). Generally, higher the separation voltage, shorter analysis time was needed and sharper pack shapes was obtained. However, excessive voltages were not suitable due to Joule heating and bubble formation. Considering resolution, analysis time and system stability, a voltage of 22 kV was chosen for separation.

3.2. Selection of sheath liquid parameters

For coupling CE with ESI–MS by sheath liquid interface, the choice of sheath liquid was essential to enhance the ionization efficiency and make the electrospray stable. Based on our previous study [22], isopropanol/water which was mostly used as sheath liquid systems in CE/CEC-ESI–MS, usually showed higher ionization efficiency than methanol/water. Furthermore, low amounts of volatile reagents, such as formic acid or acetic acid, help to stabilize the spray and increase the ionization efficiency of analytes in the positive mode [23]. In this study, different concentrations of formic and acetic acid in isopropanol/water (50/50%, v/v) were studied, respectively. The better signal intensity and separation efficiency were obtained when 7.5 mmol/L acetic acid presented in sheath liquid. In addition, the ratio of isopropanol/water (40/60%, 50/50%, 60/40%, v/v) and the flow rate of sheath liquid (from 2.0 to 4.0 μL/min) were also investigated. When isopropanol/water (50/50%, v/v) containing 7.5 mmol/L acetate acid was pumped at a flow rate of 3.0 μL/min, the best signal response of all analytes was achieved.

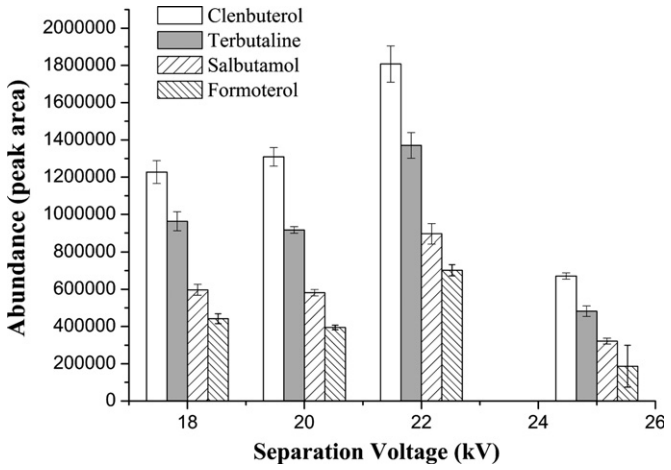

Fig. 3. Effect of separation voltage on separation of four β_2-agonsits. Experimental conditions: running buffer, 20 mmol/L ammonium acetate (pH 9.0). Other experimental conditions were the same as in Fig. 2.

The experiment showed that the drying gas velocity and temperature did not affect on the mobility or the separation efficiency apparently. To achieve the optimal detection sensitivity in the CE-ESI–MS analysis, a higher temperature was necessary. The flow rate of drying gas (150 °C) was set at 6 L/min to maintain a positive pressure in the spray chamber and avoid unfiltered air flowing into the spray chamber and vacuum system.

3.3. Field-amplified sample stacking

A pre-concentration approach could help to achieve the trace analysis of β_2-agonists in biological sample. FASS, as mentioned above, is an appropriate method applied in CE-ESI–MS for on-line sample stacking. FASS is theoretically based on the different conductivities between the sample solution and the running buffer. Therefore, the sample matrix and the injection parameters were investigated systematically.

3.3.1. Effect of sample matrix

Ions can be slowed down and stacked at the boundary when they electrophoretic migrate through a low conductivity solution into a high conductivity solution [23]. The sample is desired to be dissolved in water or a solution with lower conductivity, rather than in the running buffer. Generally, adding organic modifier to the sample matrix is helpful to reduce conductivity. In this study, several kinds of organic modifier were added respectively to the sample matrix to test the stacking effect. Unfortunately, Signal intensity had only changed slightly.

Samples diluted in deionized water and buffer solution were injected to compare the stack effect. The result showed in Fig. 4 indicated that the signal intensities of four β_2-agonists were simultaneously enhanced greatly in deionized water.

3.3.2. Effect of the injection parameters

The injection condition is the essential factor for FASS when electrokinetic injection was required. Using deionized water as the sample matrix, the influence of injection voltage (from 8 to12 kV) was studied (Fig. 5). It was indicated that the signal intensity reached the maximum at 10 kV of injection voltage.

The effect of injection time was subsequently investigated (Fig. 6). The peak heights of four β_2-agonists were found increasing gradually in the range of 30–50 s at 10 kV. Nevertheless, the

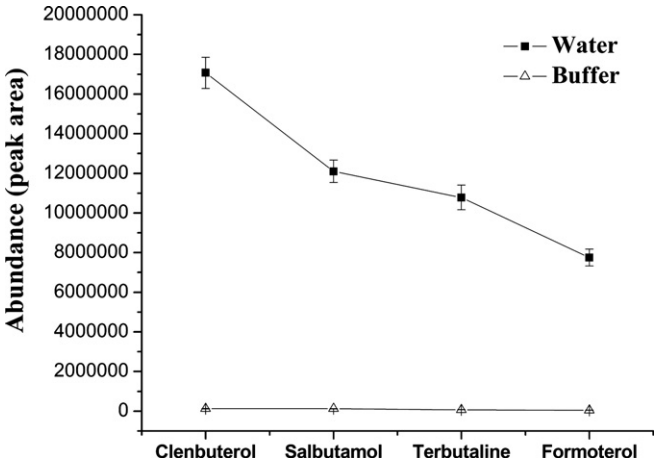

Fig. 4. Effect of sample matrix on the stacking effect. Experimental conditions: sample matrix, (a) deionized water, (b) running buffer. Other experimental conditions and peak identification were same as in Fig. 3.

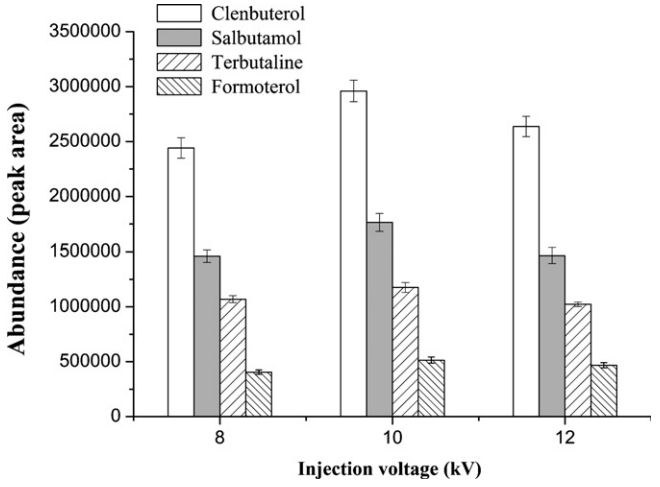

Fig. 5. Effect of injection voltage on the concentration. Experimental conditions: inject voltage, 8–12 kV; inject time, 5 s. Other experimental conditions and peak identification were same as in Fig. 3.

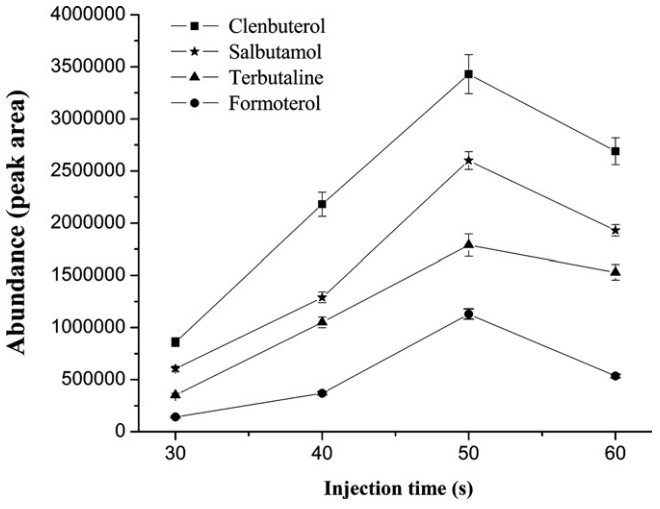

Fig. 6. Effect of injection time on the concentration. Experimental conditions: inject time, 30–60 s; inject voltage, 10 kV. Other experimental conditions and peak identification were same as in Fig. 3.

resolution got poorer when the injection time exceeded 50 s. The reason might be that the high electric field of sample solution zone could produce the partially Joule heat, which would generate the bubble and peak-broaden. Hence, 10 kV × 50 s was eventually selected as the optimum injection parameters.

Electrophorograms at the optimum pre-concentration injection and conventional injection were compared in Fig. 7(A and B). With the conventional injection, sample was dissolved in running buffer and injected at 10 kV for 5 s. The concentration factor could be calculated by multiplying the peak height ratios with the concentration dilution factors [24]. Compared with the conventional injection, the stacking efficiency of β_2-agonists with pre-concentration method could be improved 319, 332, 297, 125 fold.

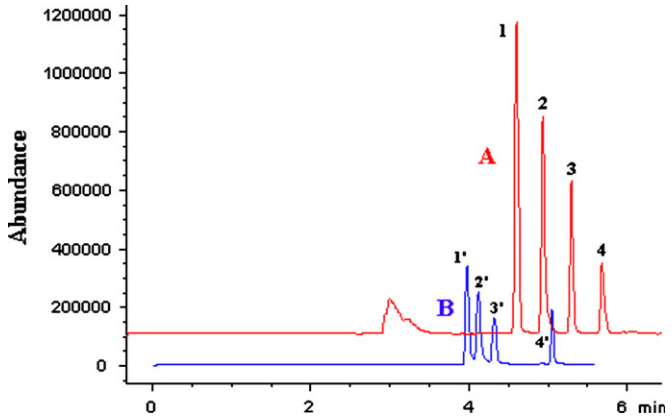

Fig. 7. Comparison electropherograms between without concentration (B) and after concentration (A). Experimental conditions: Capillary: 70 cm × 50 μm; separation voltage: 22 kV; buffer solution: 20 mmol/L ammonium acetate (pH 9.0); sheath liquid, 50/50% (v/v) isopropanol/water containing 7.5 mmol/L acetic acid with a flow rate of 3.0 μL/min; flow of drying gas: 6.0 L/min; temperature of drying gas: 150 °C; nebulizing gas pressure: 0.69 bar. (A) diluent solution: ultrapure water; sample injection: 10 kV × 50 s; the concentration of four β_2-agonist are 0.1 μg/mL. (B) diluent solution: buffer; sample injection 10 kV × 5 s; the concentration of four β_2-agonist are 10 μg/mL. Peak identification: (1) CLE, (2) SAL, (3) TER, (4) FOR.

Table 1
Regression equations, linearity, the detection limits and repeatability of CE-ESI–MS for the analysis four β_2-agonists[a].

Compound	Regression Equation[b]	R	Linear range (ng/mL)	Detection limit (ng/mL)
CLE	$y=16988x+621314$	0.9959	1.0–1 000	0.08
SAL	$y=11888x+383352$	0.9979	1.0–1 000	0.1
TER	$y=10555x+213369$	0.9986	1.0–1 000	0.1
FOR	$y=5845.7x+45742$	0.9995	1.0–1 000	0.5

[a] Experimental conditions were the same as in Fig. 7 (A).
[b] y: peak area of MS detection (counts); x: concentration of analytes (ng/mL).

Table 2
Repeatabilities of run-to-run ($n=5$) and day-to-day ($n=3$) of four β_2-agonists[a].

Compound	Run-to-run (RSD, %)		Day-to-day (RSD, %)	
	Retention time	Peak area	Retention time	Peak area
CLE	0.6	4.1	0.8	4.5
SAL	0.6	3.2	0.8	7.9
TER	0.6	4.1	0.7	5.7
FOR	1.3	6.7	0.9	7.9

[a] Experimental conditions were the same as in in Fig. 7(A).

3.4. Validation of the method

The linearity of the method was determined by constructing a calibration curve with different concentrations of the four β_2-agonists. A series of standard mixture solutions of β_2-agonists with concentrations from 1.00 to 1 000.00 ng/mL were prepared. All studies were performed on CE-ESI–MS with optimized conditions for three replicate measurements and the results were listed in Table 1.

The calibration curves exhibited a good linearity with correlation coefficients (R) in the range of 0.9959–0.9995. The LOD (defined as $S/N=3$) ranged from 0.08 to 0.5 ng/mL, indicating that the analytical assay was sensitive to determine β_2-agonists in biological sample. Practical repeatability was obtained for all analytes with RSD values ($n=5$) better than 1.3% for migration times and for less than 6.7% peak areas, which indicating good precision of this method (Table 2).

Considering the effect of matrix and ion suppression during the quantitative method based on CE-MS [25–27], the matrix effect (ME) was studied by comparing the response of analytes spiked in the blank urine sample (A) with the response of analytes in standard solution (B). Urine sample spiked with 4 concentration levels were analyzed under the optimized conditions. The ME (%) was defined as A/B to evaluate the ME. As the results in Table, the MEs of four analytes were ranging from 80.1 to 101.3, indicating that the results were not adversely affected by matrix effect with the proposed method (Table 3).

To verify the reliability of the method, recovery experiments were also performed. Under optimized conditions, two different concentrations of four β_2-agonists mix standard solutions were added into blank urine sample. As shown at Table 4, the recoveries of four β_2-agonists were in the range of 82.7%–101% with the RSD less than 9.8%.

Table 3
Matrix effect of method[a].

Compound	Added concentration (ng/mL)	ME (%)	R^2
CEL	1000	80.8	0.9917
	750	91.1	0.9923
	100	98.0	0.9937
	75	96.4	0.9945
SAL	1000	80.8	0.9918
	750	89.8	0.9923
	100	97.8	0.9925
	75	87.9	0.9933
TER	1000	80.7	0.9935
	750	90.9	0.9938
	100	96.1	0.9939
	75	89.5	0.9956
FOR	1000	98.5	0.9995
	750	101.3	0.9979
	100	96.3	0.9987
	75	96.4	0.9987

[a] Experimental conditions were the same as in Fig. 7(A).

Table 4
Recoveries of spiked urine sample[a].

Compound	Added (μg/mL)	Found (μg/mL)	Recovery (%)	RSD (%) ($n=3$)
CEL	0.75	0.68	90.7	6.3
	0.075	0.071	94.7	2.7
SAL	0.75	0.67	89.3	4.2
	0.075	0.062	82.7	2.0
TER	0.75	0.68	90.7	9.8
	0.075	0.065	86.7	1.3
FOR	0.75	0.76	101	5.3
	0.075	0.072	96.0	6.0

[a] Experimental conditions were the same as in Fig. 7(A).

3.5. Analysis of real urine sample

Under optimized conditions, the urine samples collected from three volunteers were analyzed. The collection and pretreatment of urine samples were performed according to Section 2.4. Fig. 8 showed the contrasted electrophorograms between blank urine and urine sample which was collected from volunteer after 4 h oral administration. According to the mass spectrum of peak in Fig. 8(C), the peak 1 can be identified as SAL, indicating that SAL could be successfully detected in real urine samples.

4. Concluding remarks

In present work, a sensitive and time-saving CE-FASS-MS method for analyzing β_2-agonists was demonstrated. By using the FASS method, the best detection sensitivity could be improved by 332-fold in terms of peak height and the lowest LOD can reach 0.08 ng/mL. Compared with well-known LC-MS and GC-MS method (Table 5), this microanalysis technique presented many potential advantages (e.g. fast, high efficiency, and micro-amount) and was also considered to be an environment-friendly technique due to micro-amount organic solvent consumed in analysis.

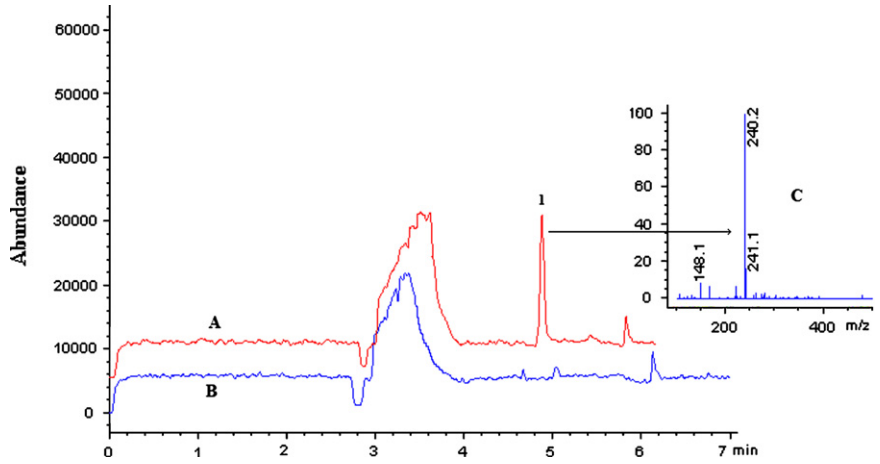

Fig. 8. Total ion electropheroograms of real samples from volunteer who took salbutamol sulfate tablets. (A) Urine sample from volunteer, (B) blank urine, (C) the mass spectra of peak 1. Other experimental conditions and peak identification were same as Fig. 7(A).

Table 5
Comparison with other reported methods.

No	LOD (ng/mL)				Method	Modification	Application to samples	Ref.
	CLE	SAL	TER	FOR				
1	0.08	0.1	0.1	0.5	CE-MS	FASS	Urine samples	**Proposed method**
2	4.4	/	/	/	GC-MS	/	Retinal tissue	**5**
3	0.5	2	/	>10	GC-MS	derivatization	Urine samples	**6**
4	1.5	20	/	20	GC-MS	derivatization	β-Adrenoceptor ligand; diuretic doping agents	**7**
7	0.5	0.5	/	/	GC-MS	Derivatization;SPE	Urine of meat-producing animals	**11**
8	1	/	/	/	LC-MS	/	Bovine urine	**12**
9	/	50	/	/	CE-AD	/	Spiked serum	**15**
10	800	2000	2000	/	NACE-MS	/	Tablet	**16**

Furthermore, the method was successfully applied to analysis SAL in urine samples. The method would promise to application in the field of pharmaceutical and biochemical analysis with the development of other techniques.

Acknowledgments

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