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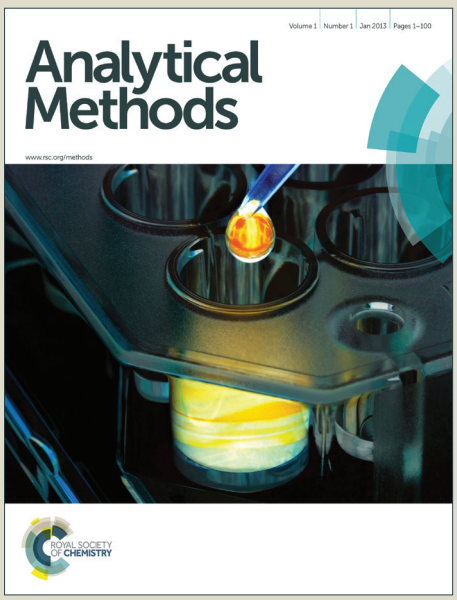

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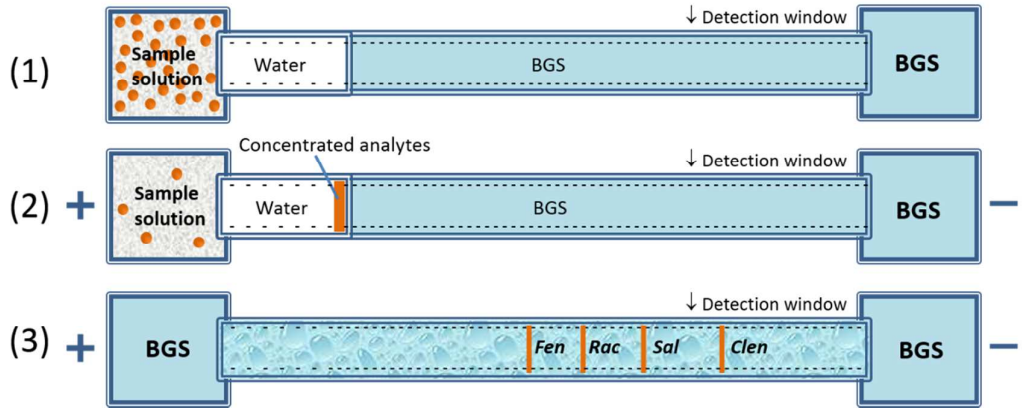

A rapid, sensitive and cost-effective CE-UV method has been developed for the simultaneous separation and sensitive determination of four beta2-agonists.

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ARTICLE

Simultaneous separation and sensitive detection of four β2-agonists in biological specimen by CE-UV with field-enhanced sample injection method

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A rapid, sensitive and cost-effective CE-UV method has been developed for the simultaneous separation and sensitive determination of clenbuterol, salbutamol, ractopamine and fenoterol in pig blood and human urine samples. Field-enhanced sample injection (FESI) as an effective online concentration technique was applied to improve the detection sensitivity of β2-agonists. Several factors, such as the concentration and pH of the background solution, sample matrix, injection time and voltage, and the length of the water plug were systematically optimized. Under the optimum conditions, the detection limits of the β2-agonists range from 2.7-9.2 nM, which were improved nearly 2000 times compared with the conventional methods by CE-UV, except for fenoterol. Eventually, the proposed method was successfully applied to the analysis of spiked blood and urine samples with good recoveries, which provides a novel method to monitor the illegal use in athletes and livestock.

1. Introduction

Clenbuterol (Clen), salbutamol (Sal), ractopamine (Rac) and fenoterol (Fen) (their structures showed in Fig. 1) are members of β2 adrenergic agonists, which have been used by sufferers of breathing disorders as a decongestant and bronchodilator.[1] People with chronic breathing disorders such as asthma used drugs like β2 adrenergic agonists to make breathing easier.[2] The β2-agonist also causes an increase in aerobic capacity, central nervous system stimulation, blood pressure, and oxygen transportation.[3] As it is, the athletes used β2-agonist as a performance-enhancing drug which has been prohibited by the World Anti-Doping Agency since 2004. In addition, the β2-agonist has also been used as produced-animal feed because it can speed up muscle building and fat burning. However, after people have eaten these produced-animals for a long time, the β2-agonists would be accumulated in human body which could cause vomiting, palpitations, tachycardia, and myocardial infarction, serious enough to cause death.[4] In fact, any kinds of β2-agonists were rigorously prohibited from using as growth promoter for produced-animals in China and European Union. Only trace ractopamine can be permitted in pork or beef in the USA, Japan, Australia and Canada, usually less than 30 ppb5. Hence, there is an increasing need to develop simple, accurate and quantitative techniques to monitor the β2-agonists in biological specimen either for human urine or animal blood.

To date, different analytical methods have been reported forβ2-agonist determination. Conventional chromatographic methods connected with the mass detector have been widely applied in confirmation and precise quantitation, including CE-MS[6,7], HPLC-MS[8-10] and GC-MS.[11,12] ELC-FIA[13], CE-ECL[14] and ELISA[15] also have been utilized gradually. Although the determination of β2-agonist by these methods is promising, most of them have been associated with several shortcomings, such as complicate pre-purification procedures, expensive equipment and costly reagents, which finally limit their wider applications.[16] Therefore, more conveniently operated, cost-and-time effective methods are still highly required to be developed for the analysis of β2-agonists.

CE-UV has been considered as a fast, efficient and cost-effective analytical separation method, and it has already become a powerful analytical technique in pharmaceutical and biomedical aspects. Unfortunately, due to the small loaded sample volume and the narrow optical path length, the sensitivity is relatively low when utilizing an online UV detector. The online sample pre-concentration is an efficient approach to enhance the sensitivity for trace analysis such as field-enhanced sample injection (FESI),[17,18] field amplified sample stacking (FASS)[19] and large-volume sample stacking (LVSS).[20]

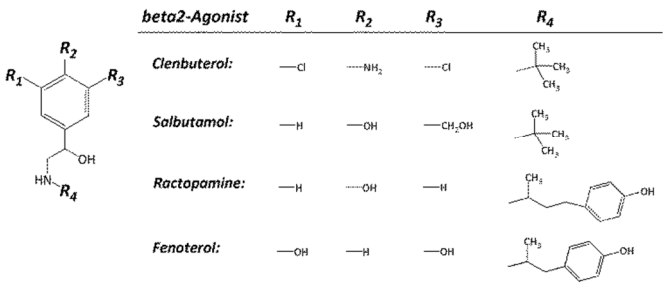

Fig.1 Chemical structures of four β2-agonists.

As shown in Fig. 2, FESI is based on the fact that each given analyte presents a different velocity either in the high-conductivity background electrolytes (BGS) zone or in the low-conductivity sample zone (water). Due to presence of a

preinjected short plug of low conductivity (diluted buffer or water) at the capillary inlet, the analyte moves rapidly from the sample vial into the capillary at a higher electric field. Once they reach the high conductivity BGS, their velocity slows down significantly where a fast attenuation of the electric field strength occurs. Thus it will result in a stacking process at the boundary between sample matrix and BGS.[21] In this work, we developed a method to separate and detect four kinds of β2-agonists rapidly and conveniently with the advantages of low detection limit, fast analysis time and wide linear range.

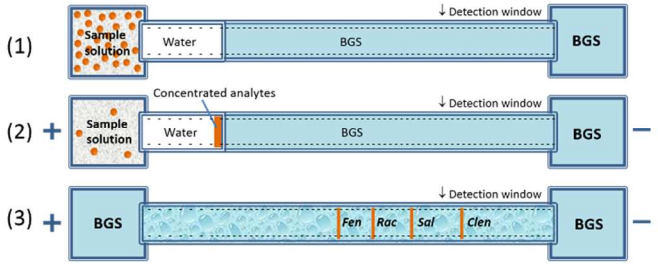

Fig.2 Schematic diagram of a preconcentration mechanism with FESI. (1) Pressurized injection of water plug; (2) Field-enhanced sample injection; (3) Separation after injection.

2. Materials and Methods

2.1 Reagents

Clenbuterol hydrochloride, salbutamol, ractopamine hydrochloride and fenoterol hydrochloride were purchased from Sigma. All the water used was the commercial purified water Wahaha (Hangzhou, China). All the chemicals and reagents are of analytical grade and used without further purification.

2.2 CE apparatus

All experiments were performed with a P/ACE MDQ instrument coupled with a diode-array UV detector (Beckman Coulter, USA). Instrument control and data acquisition were carried out using 32Karat software (Version 8.0). A 50.2 cm (40.0 cm to detector) uncoated fused-silica capillary tubing with 50 μm i.d and 364 μm o.d was obtained from Polymicro Technologies (USA).

2.3 CE procedure

0.30 M stock solutions were prepared by dissolving Clen, Sal, Rac and Fen standards in water and stored in the refrigerator at 4 ℃. They were diluted with water to the desired concentration before using. Prior to experiment, new capillaries were pre-treated by sequentially rinsing with 1.0 M HCl for 10 min, then with water for 10 min, and finally running buffer for 15min. Between each run, the capillary was flushed with running buffer for 3 min so as to maintain an active and reproducible inner surface. The voltage applied in the separation was +20 kV. The capillary was held at 25 ℃ and the wavelength of the UV detector was maintained at 200 nm. Injection was performed electrokinetically after a short water plug in FESI. The injection voltage, injection time and the length of water plug were studied and optimized in this work. All solutions were filtered through a 0.22 μm pore polytetrafluoroethylene membrane filter before injection to capillary. All measurements were carried out at least three times.

2.4 Preparation of sample

The whole blood samples of pigs were collected from a slaughterhouses in Lanzhou, China, followed by centrifugation at 8000 rpm for 10 min, then collected the supernatant sample and diluted 10-fold with water. In order to keep a lower ionic strength of sample matrix, no anticoagulant was added into samples. The urine samples were collected from one of authors and the preparation procedure is similar to that of blood samples. The as-prepared samples should be tested within 24 h. All experiments were performed in compliance with the relevant laws and institutional guidelines, and were approved by the College of chemistry and molecular engineering of Peking University.

3 Results and discussion

3.1 The effect of pH of buffer

At the very beginning, the separation condition was conventionally investigated in capillary zone electrophoresis (CZE). Since the background electrolytes, including the pH and concentration, have significant impact on efficiency, resolution and sensitivity of FESI-CE separation, the effect of running buffer was first investigated. The four mixed β2-agonists samples were introduced into the capillary at a low pressure of 0.5 psi for 5 s in the optimization experiment. As shown in Fig.1, these four β2-agonists possess a common β-hydroxyamino group on the side chain of aniline (Clen) or phenol (Sal, Rac and Fen), but are differentiated from each other by varied substituents on the aryl moiety. The more phenolic hydroxyl groups would lead to more negative charge of the compound, and the alcoholic hydroxyl group like R_3 in Sal is relatively weak. Obviously, the molecular weight is also a factor that cannot be neglected. So of the four β2-agonists Clen runs fastest in capillary, followed by Sal, Rac and Fen.

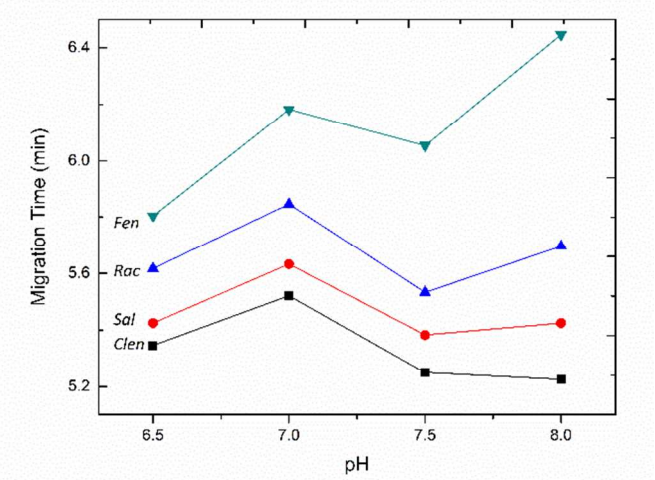

Fig.3 Effect of the pH of buffer on the separation resolution. Concentration (μM): Clen, 32; Rac, 30; Sal, 42; Fen, 26. Experimental conditions: 50 mM phosphate buffer; injection by pressure, 0.5 psi; injection time, 5s; separation voltage, +20 kV.

Fig. 3 shows the separation of the four β2-agonists under different pH in the range of 6.5–8.0 in phosphate buffer. The result indicated that the separation resolution of the four samples was improved with the increase of buffer pH from 6.5 to 8.0. Nevertheless, higher pH value would result in less buffering capacity which finally influenced the reproducibility of the migration time of the peaks. When the borate buffer was tried under the pH from 8.0 to 10.0, a system peak overlapped some of the sample peaks, which should be due to the

interaction between the β2-agonist with borate. So the best resolution was obtained under a pH of 8.0 in phosphate buffer.

3.2 The effect of the concentration of buffer

The concentration of phosphate buffer was also optimized in our study. Fig. 4 shows that various separations were performed with varying the concentration of phosphate buffer from 50 to 100 mM. Increasing ionic strength resulted in increased analysis time and the separation resolutions were better at a higher concentration. This could be explained by the fact that the loading capacity of the background electrolytes increased together with the concentration of phosphate buffer. It was found that the higher concentration would give rise to Joule heating which caused the peak broadening. Considering the peak broadening in FESI would affect the sensitivity in our study, as a result, 50 mM phosphate buffer at pH 8.0 was thus used for subsequent analyses. The results of CZE method regression analysis on calibration and the detection limits are summarized in Table.1.

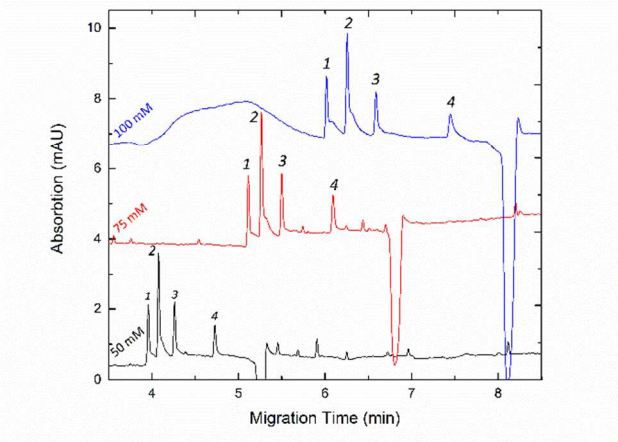

Fig.4 Effect of the concentration of buffer on the migration time and separation resolution. Experimental conditions: 50 mM pH 8.0 phosphate buffer. Peak identities: 1-Clen;2-Sal; 3-Rac; 4-Fen. Other conditions are the same as in Fig.3.

3.3 Injection parameters

FESI as an on-line concentration method was investigated to improve the detection sensitivities of the four β2-agonists. The parameters affecting the concentration efficiency of FESI, including sample matrix, injection time, injection voltage and the length of water plug were systematically investigated.

3.3.1 Effect of sample matrix. A higher ionic strength of the sample solution would lead to negative influence on concentration efficiency of stacking. The samples were desired to be dissolved in water or a solution with lower conductivity. In our study, the four samples could only be injected successfully under forward voltage when the sample matrix was weak acid solutions. It could be explained that the four samples were all positively charged in weak acid solutions. At first the dilute hydrochloric acid (pH = 4.0-6.0) was chosen as sample matrix. The injection efficiency were nearly the same under the low conductivity solution when the pH of the sample matrix was adjusted. It was probably because three of the four β2-agonists were hydrochloric salt, and the mixed solution in deionized water was weak acid. After comparing with different low concentrations of dilute hydrochloric acid and in order to convey less interfering ion into the capillary, deionized water was chosen as sample matrix.

3.3.2 Effect of water plug length. The water plug pre-injected plays a key role in FESI, which provides an enhanced electric field at the injection end of the capillary. After the samples entered the capillary through the water plug with higher electric field strength, they would slow down at the interface between water plug and background electrolytes. Taking into account that the higher electric field strength in the water plug can improve the stacking efficiency, the shorter length of the water plug was better. However, if the water plug was too short, the introduced ionic solutes would increase the conductivity of the water plug which thus decreased the efficiency of the electric injection.[17] So the length of the water plug should be short enough meanwhile it could allow a maximum amount of the solute to be injected. The pressure of the injection of water plug was kept at 0.5 psi, the injection time was investigated from 3s to 10s. Fig. 5A shows that the peak areas in 10s-injection of water plug were smaller than that in 5s-infection because the longer water plug would bring about the lower electric field strength. When the injection time was less than 5s, the peaks of the samples were broadening and overlapping which could be interpreted that the capacity of the water plug was not enough and the samples could not be focused on the interface. So the optimal injection time was 5s at the pressure of 0.5 psi which was used for subsequent analyses.

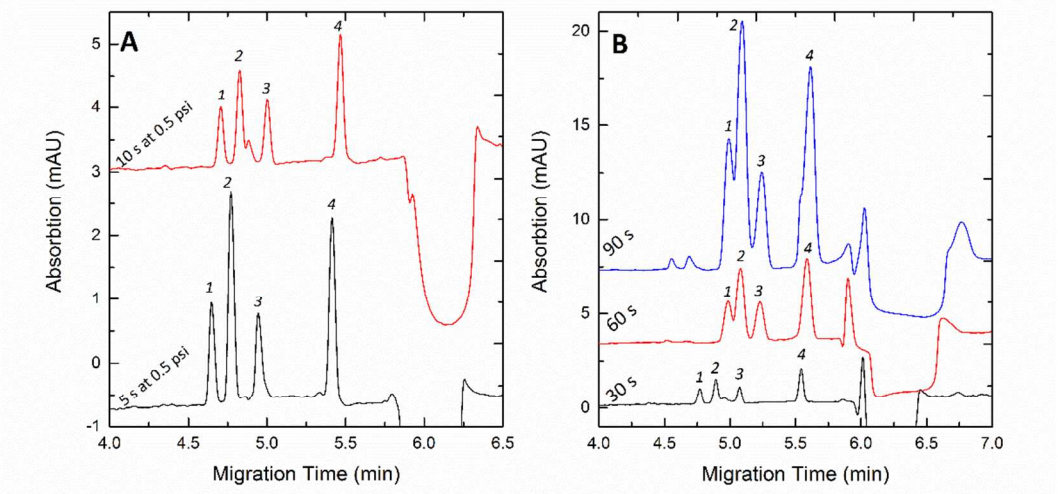

Fig.5 Effects of the water plug length (A) and the injection time (B) on stacking efficiency. Concentration (nM): Clen, 300; Rac, 300; Sal, 300; Fen, 600. Experimental conditions: 50 mM pH 8.0 phosphate buffer; injection voltage, +10 kV; separation voltage +20 kV. (A) Injection time, 30 s; (B) Water plug, 5 s at 0.5 psi. Peak identities: 1-Clen; 2-Sal; 3-Rac; 4-Fen.

Table 1 Results of regression analysis on calibration and the detection limits with CZE method.

Component	Calibration Curve[a]	Correlation coefficient	RSD (%, *n*=5)		Detection limit (μM)	Linear range (μM)
			migration time	peak area		
Clen	*y*=61.92*x*+20.8	0.9997	0.30	5.2	13	30-600
Sal	*y*=167.9*x*+168.3	0.9997	0.23	6.7	7.2	15-300
Rac	*y*=103.3*x*-124.8	0.9983	0.31	4.9	5.3	15-300
Fen	*y*=355.5*x*-244.4	0.9991	0.48	8.5	2.7	15-300

[a] *y*: peak area, mAu s; *x*: concentration of analyte, μM

Table 2. Results of regression analysis on calibration, the detection limits and enhancement factors with FESI.

Component	Calibration Curve[b]	Correlation coefficient	RSD (%, *n*=5)		Detection limit (nM)	Linear range (nM)	Enhancement factor
			migration time	peak area			
Clen	*y*=172*x*+1150	0.9969	0.41	8.2	7.2	10-300	1800
Sal	*y*=892*x*+4680	0.9984	0.28	4.5	3.6	5-150	2000
Rac	*y*=781*x*+927	0.9991	0.64	6.9	2.7	5-150	1900
Fen	*y*=768*x*+43.3	0.9997	0.53	5.3	5.3	10-300	600

[b] *y*: peak area, mAu s; *x*: concentration of analyte, nM

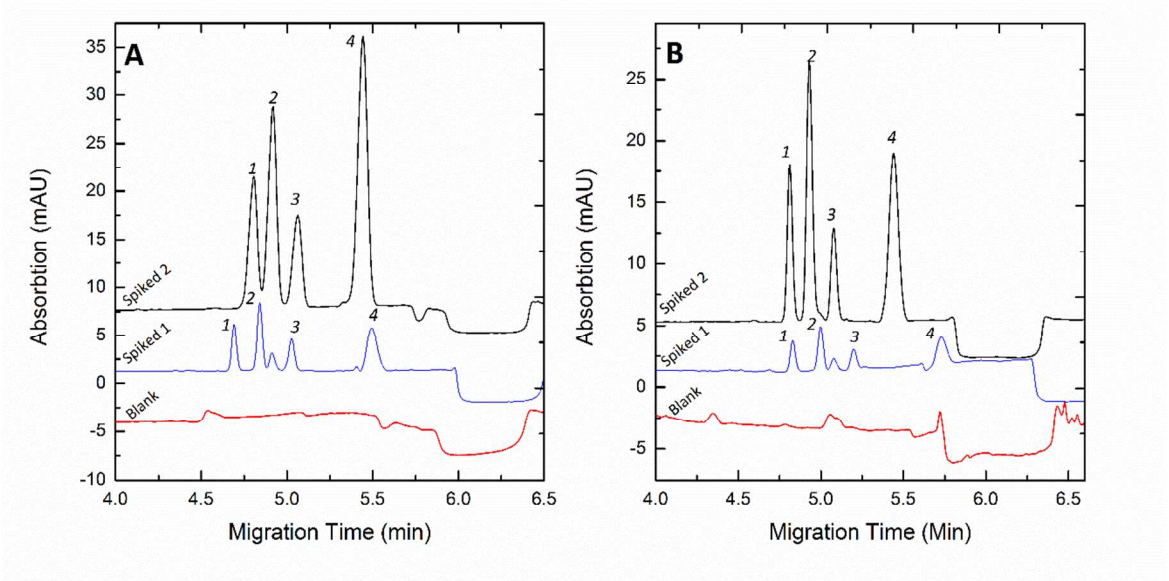

Fig.6 Electropherograms of blood (A) and urine (B) samples under optimum FESI condition. Spiked 1 concentration (nM): (A) Clen, 20; Rac,10; Sal, 10; Fen, 20. (B) Clen, 10; Rac, 5; Sal, 5; Fen, 10. Spiked 2 concentration (nM): (A) Clen, 60; Rac, 30; Sal, 30; Fen, 60. (B) Clen, 40; Rac, 20; Sal, 20; Fen, 40. Experimental conditions: 50 mM pH 8.0 phosphate buffer; water plug, 5 s at 0.5 psi; injection voltage, +10 kV; injection time, 60 s; separation voltage +20 kV. Peak identities: 1-Clen; 2-Sal; 3-Rac; 4-Fen.

3.3.3 Effect of injection time and injection voltage. The effect of the injection time on the enrichment of the four β2-agonists were investigated by adjusting the injection time from 30 to 90 s. As we know that a longer injection time can improve the sensitivity, but it could also cause peak boarding.[22] Fig. 5B shows that the detection sensitivity of the four samples increased with the increasing injection time and the greatest signal amplification was obtained for 90s. However, the separation became worse with the injection time decreasing. With the best peak height and shape of the four samples, 60s was chosen and applied in this experiment.

Table 3. Comparison of different detection methods with CE separation.

Method	Detection limit (nM)			
	Clen	*Sal*	*Rac*	*Fen*
CE-UV[24]	1600	2100	-	1300
CE-UV[26]	-	17000	-	-
CE-EC[27]	320	710	300	-
CE-ECL[14]	260	84	-	-
CE-ECL[28]	81	340	-	-
CE-MS[7]	0.25	0.41	-	-
This method	7.2	3.6	2.7	5.3

The effect of electrokinetic injection voltage on peak heights was studied. Actually, the injection voltage has the similar effect on the efficiency of stacking and there is a connection between the two factors which can lead to different experiment results. So we adjusted the two parameters simultaneously in order to study which would cause an additional impact of the sample staking efficiency. The peak heights were enhanced with the increasing of injection voltage from +1 to +15 kV at different injection time (30 s to 90 s). The peak broadening got serious when the electrokinetic injection voltage increasing more than +10kV. Based on an overall consideration of the two injection factors, 60 s under +10 kV injection voltage was chosen as the optimum injection time. The result of regression analysis on calibration, the detection limits and enhancement factors with FESI are summarized in

Table 2. It was observed that the detection limit with FESI-based stacking was greatly enhanced as compared with those in normal CZE. Moreover, as shown in Table 3, compared with the reported works employed the UV detector,[23-25] the detection limit for β2-agonists was 0.75-2 μg/mL (about 3-8 μM), the sensitivity is greatly improved with at least 3 orders of magnitude for these β2-agonists, even approaching to 0.1-0.5 ng/mL (about 0.5-2 nM) of the latest reported work by CE-ESI/MS which was also used field-amplification sample stacking (FASS) as an online concentration method.[7]

3.4 Applications

To evaluate the FESI techniques, the proposed methods were applied to the analysis of pig blood and human urine sample. Thanks to its high sensitivity, we could diluted the sample solutions 1000-fold with deionized water before injection in order to decrease the ionic strength of sample matrix. Typical electropherograms for the analysis of spiked samples are illustrated in Fig. 6. The quantitative results and the recoveries of this method are listed in Table 4.

Table 4. Results for the determination of the four components in blood and urine samples (n=3).

Sample	Component	Original (nM)	Added (nM)	Found (nM)	Recovery (%)
Blood	*Clen*	-	60	58	97
	Sal	-	30	34	113
	Rac	-	30	28	93
	Fen	-	60	61	102
Urine	*Clen*	-	40	42	105
	Sal	-	20	18	90
	Rac		40	44	110
	Fen		20	23	115

4 Concluding remarks

A rapid, simple and sensitive CE-FESI method has been developed for β2-agonists detection .Compared to CZE and the reported method, the proposed method has much higher stacking efficiency for the four β2-agonists with extremely low detection limit ranging from 2.7 to -7.2 nM. Results of this study show a great potential for this method to be a useful tool for the rapid and sensitive determination of β2-agonists in pig blood and human urine samples. Meanwhile, it shows that the utilization of cheap and simple CE-UV in the field of public-health risks of food and sports games is quite promising.

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Notes and references

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