2010

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# A new method for the analysis of $\beta_2$-agonists in human urine by pressure-assisted capillary electrochromatography coupling with electrospray ionization-mass spectrometry using a silica-based monolithic column

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## Abstract

A new pressure assisted capillary electrochromatography coupling with electrospray ionization-mass spectrometry method using a silica-based monolithic column as separation media was developed for the analysis of $\beta_2$-agonists in human urine. Experimental conditions including the mobile phase, separation voltage, assisted pressure, and sheath liquid were optimized for the analysis: mobile phase composed of 82% (v/v) ACN and 18% (v/v) 20 mmol/L ammonium acetate (pH 6.0); separation voltage 25 kV; assisted pressure 2 bar; and the sheath liquid consisting of 7.5 mmol/L acetic acid in isopropanol/water 50/50% (v/v) that was delivered at a flow rate of 3.0 μL/min. Six $\beta_2$-agonists were separated within 12.5 min with LODs (defined as S/N=3) in the range of 0.25-2.0 ng/mL. The absolute LODs of the developed method for analyzing six $\beta_2$-agonists ranged from 5.75 to 46.0 fg. Method

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repeatability of run-to-run and column-to-column was satisfactory. The recovery obtained from the analysis of spiked urine samples was between 88.2 and 106% with RSDs lower than 6.68%. The method was successfully applied to the analysis of real urine sample from volunteers.

*Keywords:* $\beta_2$-Agonists, Capillary electrochromatography; Electrospray ionization-mass spectrometry; Silica-based monolithic column

# 1 Introduction

$\beta_2$-Agonists are a class of drug normally employed for the treatment, in humans and animals, of pulmonary disorders and asthma or for the prevention of exercise-induced asthma, owing to their bronchodilator activity [1]. The compounds, especially clenbuterol, salbutamol and terbutaline, are often used as growth promoters in animal feed due to the repartitioning of carcass composition to decrease fat deposition and to increase muscle mass [2]. However, the residues of these compounds in edible tissues are potentially toxic. Several cases of $\beta_2$-agonists poisoning have been reported in recent years [3, 4]. Therefore, these compounds have been banned as growth promoters in many counties including China and European Communities [5]. The use of most $\beta_2$-agonists has been prohibited in sports by the International Olympic Committee (IOC) and World Anti-Doping Agency (WADA) [6] because stimulation and anabolic effects were observed when the intake level of $\beta_2$-agonists was higher than therapeutically indicated level.

The conventional methods for the analysis of $\beta_2$-agonists are gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass

spectrometry (LC-MS). GC-MS is a commonly used method not only for the screening and confirmation for $\beta_2$-agonists in human urine sample [7-10], but also for the multi-residual analysis in animal tissues [11, 12]. However, because $\beta_2$-agonists have high polarity and low volatility, a time-consuming, tedious and expensive derivatization is required prior to the GC-MS analysis [13]. In the last decades, liquid chromatography (LC) coupling with atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) or electrospray ionization-mass spectrometry (ESI-MS) has attracted significant attention in the analysis of $\beta_2$-agonists in human urine and multi-residual samples because no derivatization procedure was required [14-17]. As a well-known separation technique due to its simplicity, efficiency and low sample consumption, capillary electrophoresis (CE) has also been applied for the separation and determination of $\beta_2$-agonists [18, 19].

Capillary electrochromatography (CEC) coupling with mass spectrometry (MS) is an emerging microanalysis technique and a supplemental method to LC-MS. This microanalysis technique combines the excellent features of both CE and HPLC such as high separation efficiency and low sample consumption of CE as well as high selectivity and large sample loading capacity of HPLC [20, 21]. However, CEC-MS hasn't been widely accepted as a routine analytical technique due to the difficulty in instrumental operation and poor analytical repeatability, which may be attributed to following several reasons. Firstly, a longer analysis time is usually required for CEC-MS than that of CEC with ultraviolet (UV) since UV detection is generally performed in-column or on-column, whereas MS detection normally required an

additional column connected to MS interface [22]. Secondly, air bubbles are more easily formed in CEC-MS system than that of CEC-UV system, because one end of column must be inserted to the interface. To suppress bubble formation, an assisted pressure or a special column (e.g., an internally tapered column) is usually needed [23]. Thirdly, the requirement on column is more crucial for CEC-MS technique. Therefore, much effort has recently been concentrated on CEC-MS column technology [24]. However, up to now, only limited columns are available for CEC-MS compared to those for LC-MS.

In recent years, monolithic columns have attracted considerable attention and are regarded as a new generation of chromatographic separation media due to their good permeability, fast mass transfer property, high stability and easy modification [25-27]. Compared to the packed column, a significant merit of monolithic column is that the formation of bubble can be reduced or eliminated because no frits are necessary to keep the stationary phase in columns [28]. Based on different materials, monolithic columns can be classified into two categories, namely silica-based and organic polymer-based monoliths [29]. Silica-based beds have the advantages of high mechanical strength, heat stability and resistance to organic solvents [30, 31].

To the best of our knowledge, the analysis of $\beta_2$-agonists by using CEC or CEC-MS has not been reported. The goal of present work was to develop a new, simple and sensitive pressure assisted CEC-ESI-MS (pCEC-ESI-MS) method for the determination of $\beta_2$-agonists (Fig. 1). The method using self-prepared silica-based monolithic column as separation media was applied for the analysis of the real urine

sample from volunteers.

## 2 Materials and methods

### *2.1. Chemicals and reagents*

Clenbuterol, terbutaline, salbutamol, formoterol, procaterol, and salmeterol were purchased from the Chinese Institute of Biological Products Control (Beijing, China). Fused-silica capillary of 100 μm id and 375 μm od was obtained from Yongnian Optic Fiber Plant (Hebei, China). Tetramethoxysilane (TMOS), methyltrimethoxysilane (MTMS), and PEG ($M_r$=10 000) were supplied by Alfa Aesar (Tianjin, China). Urea was donated by Cxbio Biotechnology (Shanghai, China). Salbutamol sulfate tablets were provided by Pingguang Pharmaceuticals (Jiangsu, China)

Acetonitrile, methanol and isopropanol (HPLC grade) were obtained from Sinopharm Chemical Reagents (Shanghai, China). Acetic acid glacial, ammonium acetate, and ammonium hydroxide were analytical reagent grade and purchased from Sinopharm Chemical Reagents. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

### *2.2. Column preparation*

Silica-based monolithic columns were prepared according to the procedure as previously described [32]. Briefly, a rehydroxylation process was performed to maximize the number of silanol groups on the silica surface before preparation of monolithic columns. The capillary was flushed with water, 1.0 mol/L sodium hydroxide, water, 0.1 mol/L hydrochloric acid, water and acetone for 30 min, 3 h, 30

min, 3 h, 30 min and 30 min in order, respectively, and then purged with nitrogen at 180 °C for 3 h prior to use. 0.44 g PEG and 0.45 g urea were dissolved in 5.0 mL acetic acid solution (10 mmol/L), and then 1.8 mL TMOS, 0.2 mL MTMS were added. The mixed solution was stirred for 45 min in ice bath. The resultant transparent sol was introduced into the pretreated capillary, and both ends were sealed with silicon rubbers. Then the polymerization was carried out at 40 °C in water bath for 20 h. The wet gel was treated for 3 h at 120 °C, and followed by a washing with water and methanol. After drying, heat-treatment was carried out at 330 °C for 25 h.

*2.3. pCEC-ESI-MS Instrumentation*

All pCEC-ESI-MS experiments were performed on an Agilent $^{3D}$CE (Agilent Technologies, Waldbronn, Germany) system coupled with an Agilent 1100 series single quadrupole mass spectrometer (Agilent Technologies). The sheath liquid was delivered by an Agilent 1100 series isocratic LC pump equipped with a 1/100 split flow (Agilent Technologies). A capillary cassette was used to facilitate thermostating of the silica-based monolithic column. Agilent CE/MSD ChemStation with pCEC-MS mode was used for the instrument control, data acquisition and data analysis.

Analysis was carried out with the self-made silica-based monolithic column (100 μm id, 66.0 cm total length). Injections were performed with electrokinetically injected at 10 kV for 5 s and injection volumes were about 23.0 nL. The column temperature was set at 25 °C inside the capillary column cassette (*ca.* 40.0 cm). The section between the pCEC instrument and the MS system (*ca*. 28.0 cm) was not thermostated. Before first use, the column was rinsed with running elution at 10 bar

assisted pressure for 30 min. And the column was flushed with running elution at 10 bar assisted pressure and 10 kV for 5 min between two runs.

MS detection was performed in the ESI positive ionization mode. The electrospray voltage was 3.5 kV for the positive mode in all experiments. MS was operated in full scan mode (m/z range from 200 to 500) and SIM scan mode ($[M+H]^+$ molecules at m/z 277 and 279 for clenbuterol, m/z 226 for terbutaline, m/z 240 for salbutamol, m/z 416 for salmeterol, m/z 345 for formoterol, and m/z 291 for procaterol) simultaneously. Nitrogen was used as the nebulizer gas. The nebulizing gas pressure, the drying gas flow rate and the drying gas temperature were set at 0.69 bar, 6.0 L/min and 150 $^{o}$C, respectively. The fragmentor voltage, step size, and gain were set at 150 V, 0.15 amu, and 1.0, respectively.

*2.4. Preparation of standard and buffer solutions*

Standard solutions were prepared by dissolving corresponding chemicals in methanol at a concentration of 1.0 mg/mL for clenbuterol, terbutaline, salbutamol, formoterol, procaterol, and salmeterol. All buffer stock solutions were prepared conventionally, and the working buffer solutions were prepared by diluting the stock solutions. The buffers were filtered through a 0.22 μm membrane filter. Standard solutions and running buffer were degassed by ultrasonication for 5 min prior to the use.

*2.5. Sample preparation*

Urine samples were collected from healthy male volunteers who took a single dose of salbutamol sulfate tablets (4.8 mg, equivalently with 4.0 mg of salbutamol) orally.

The doses were performed according to the principle of Public Health Bureau of China. Prior to the drug administration, blank urine samples of the volunteers were collected. The urine samples were collected at regular intervals after the drug administration and then stored in the refrigerator at -20 $^{o}$C. To remove the protein components and other solid particles in urine, the samples were diluted 4x with methanol, centrifuged at 4500 rpm for 10 min and filtered through a 0.22 μm membrane filter.

# 3 Results and discussion

## *3.1 Selection of mobile phase*

For CEC coupling with ESI-MS, nonvolatile running buffers are not suitable as the mobile phase due to the possibility of contaminating the ion source of ESI-MS. Therefore, volatile ammonium salts are usually selected as running buffer. To obtain the optimal separation conditions, the effect of organic solvent, concentration of running buffer, and pH of running buffer in mobile phase were investigated.

### *3.1.1 Effect of ACN concentration*

Based on our previous study, ACN was selected as solvent organic in mobile phase owing to its superior EOF promoting ability [33]. The percentage of ACN in mobile phase not only affects EOF but also influences the partition between stationary phase and mobile phase. Therefore, the effect of various ACN content (70%, 80%, 82%, 85%, v/v) in mobile phase on the separation of $\beta_2$-agonists was studied. When 70% ACN was used, low abundance and broad peaks were observed. Moreover,

compounds 2, 3 and 4, 5 were coeluted (Fig. 2A). With increasing ACN content to 80%, a base-line separation for compounds 4, 5 with a relatively high peak abundance were achieved (Fig. 2B). Compounds 2 and 3 were separated by increasing ACN to 82%, under which the highest peak abundance for all analytes were achieved (Fig. 2C). When ACN content was further increased to 85%, however, compounds 3 and 4 were not base-line separated, and the decreased abundance of analytes was observed (Fig. 2D). Therefore, 82% ACN in mobile phase was selected.

*3.1.2 Effect of running buffer pH*

The dissociation equilibria of silanol groups on silica-based monoliths are pH-dependent. Thus, the EOF is mainly influenced by the pH of the running buffer. On the other hand, because $\beta_2$-agonists are nitrogen-containing compounds (Fig. 1), their protonation degree also depends on the pH of running buffer. Therefore, the pH of running buffer influences the EOF as well as the electromigration of the analytes. Considering the silica gel was unstable when pH is lower than 3.0 or higher than 8.0, the effect of varying the pH over the range 4.0-7.0 on separation was investigated with maintaining previously optimized 82% v/v ACN. The obtained results indicated that the best separation efficiency was achieved at pH 6.0. At pH 4.0, only four peaks were observed for the six targeted compounds because the peaks 2, 3 and 4, 5 were overlapped.

*3.1.3 Effect of running buffer concentration*

By keeping 82% ACN in mobile phase and pH of running buffer at 6.0, a series of mobile phases were prepared with different concentration of running buffer (15, 20,

25 and 30 mmol/L ammonium acetate). Experimental results demonstrated that low running buffer concentration resulted in better signals but with broadened peaks (Fig. 3A). With increasing the concentration of running buffer, the abundance of compounds gradually decreased because of the ion suppression in ESI-MS. On the other hand, high concentration of running buffer also caused poor separation efficiency and low signal abundance for the $\beta_2$-agonists (Fig. 3B-3D), in addition to producing large electric current in separation column. A concentration of 20 mmol/L ammonium acetate was found to be the optimal running buffer.

### *3.2 Selection of separation conditions*

#### *3.2.1 Effect of separation voltage*

The effect of separation voltage on the separation of $\beta_2$-agonists was investigated from 19 to 28 kV with 82% ACN/20 mmol/L ammonium acetate at pH 6.0 as mobile phase. In CEC, the EOF instead of a mechanical pump drives the mobile phase towards the detection end of the column. It is known that EOF is proportional to the separation voltage and that the change of separation voltage may be practically conducted to control the EOF in the CEC analysis. At a separation voltage of 19 kV, the analysis time was exceeded 18.0 min. With increasing the separation voltage to 28 kV, the analysis time was shortened to 11.0 min. The obtained results also indicated that the peaks were broadened considerably because of diffusion at low separation voltage, although good separation was obtained for all compounds under this condition. High separation voltage would produce large electric current in separation column. Thus, air bubbles might be easily formed with high separation voltage due to

high Joule heating. Considering the separation resolution, analysis time and system stability, a voltage of 25 kV was found to be optimal for the separation.

### *3.2.2 Effect of assisted pressure*

To suppress or eliminate bubbles that might be produced by Joule heating in CEC-ESI-MS analysis, an assisted pressure was usually added to inlet end of column. A pressure was applied to the capillary column along with an electric field in CEC-ESI-MS. The separation efficiency would be affected when EOF and assisted pressure instead of pure EOF drive the mobile phase through the column. Fig. 4 demonstrated that assisted pressure effect on the separation of $\beta_2$-agonists. It can be concluded that gradually decreased separation efficiency and broadened peaks were obtained with increasing assisted pressure from 2 to 6 bar. Also, a decreased peak abundance of compounds were obtained when increasing the assisted pressure. Therefore, assisted pressure of 2 bar was selected in this experiment.

## *3.3 Selection of sheath liquid*

For coupling CEC with ESI-MS by sheath liquid interface, it is well known that the choice of sheath liquid has significant effects on ionization efficiency and spray stability. Isopropanol/water and methanol/water were mostly used sheath liquid systems in CE/CEC-ESI-MS. Based on our previous study [34], isopropanol/water showed higher ionization efficiency than methanol/water. Furthermore, addition of low amounts of volatile acids (mostly formic or acetic acid) in sheath liquid could help to stabilize the spray and CEC current as well as to increase the ionization efficiency of analytes in the positive ion mode [35]. To study sheath liquid effect on

240 separation efficiency and signal intensity, different amount of formic and acetic acid
241 were added to the sheath liquid composed by isopropanol/water (50/50%, v/v). The
242 obtained results demonstrated that 7.5 mmol/L acetic acid in sheath liquid could
243 provide better signal intensity and separation efficiency. Subsequently, the ratio of
244 isopropanol/water and the flow rate of sheath liquid were investigated.

245 *3.3.1 Effect of the content ratio of isopropanol/water*

246 To investigate the content ratio of isopropanol/water effect on separation efficiency
247 and signal intensity, three different ratios of isopropanol/water (40/60%, 50/50%,
248 60/40%, v/v) were prepared by keeping a constant content of acetic acid in sheath
249 liquid (7.5 mmol/L). The results showed that the signal intensity was improved about
250 30% by increasing proportion of isopropanol/water from 40/60% to 50/50% (v/v).
251 However, a decreased separation efficiency and signal stability were observed when
252 increasing isopropanol/water to 60/40% (v/v). Therefore, the isopropanol/water
253 50/50% v/v containing 7.5 mmol/L acetic acid was considered as the optimized sheath
254 liquid because both reproducible spray and the best peak response were achieved for
255 all analytes under the condition.

256 *3.3.2 Effect of sheath liquid flow*

257 The effect of the sheath liquid flow rate on the ESI sensitivity was investigated in
258 the range of 2.0-4.0 μL/min. In general, the sheath liquid flow rate had significant
259 impact on both the separation and signal intensity. At higher flow rate (4.0 μL/min), a
260 slightly reduced analysis time was observed, which might be owing to increasing
261 pressure at the end of column. On the other hand, the electrospray and current in

column became unstable under low flow rate of sheath liquid (2.0 μL/min). The effect of sheath liquid flow rate on signals intensities was studied and presented in Fig. 5. Due to a dilution effect of high flow rate, gradually decreased signal intensities of analytes were observed when increasing the flow rate from 3.0 to 4.0 μL/min. However, decreased signal intensities were also obtained with reducing the flow rate from 3.0 to 2.0 μL/min, likely due to instability of the source spray at low flow rates. Therefore, a sheath liquid flow rate of 3.0 μL/min was selected.

*3.4 Validation of the method*

Figure 6 shows the total ion electrochromatograms in SIM mode (A) and the extracted ion chromatograms of $[M+H]^+$ ions (B) of the six $\beta_2$-agonists standard mixtures obtained under the optimal conditions. To validate the method, a series of standard mixture solutions of the $\beta_2$-agonists with concentrations from 0.25 to 10000 ng/mL were prepared. The standard analyses were performed on pCEC-ESI-MS with optimized conditions. Ion electrochromatogram in SIM scan mode was applied to the quantitative analysis. Ions with m/z 227 and 229 were selected as characteristic ions of clenbuterol because the molecule contained two Cl atoms (Fig. 1).

The calibration curves of six $\beta_2$-agonists were presented in Table 1. The calibration curves exhibited good linearity with correlation coefficients ($R^2$) in the range of 0.9951-0.9987. The LODs of proposed method for these $\beta_2$-agonists were lower than 1.0 ng/mL except procaterol (2.0 ng/mL). The absolute LODs of the method for six $\beta_2$-agonists were 5.75-46.0 fg because the injection volume was about 23.0 nL. The low detection limits might allow the new pCEC-ESI-MS method to be used as an

284 alternative to the well-established LC-MS method (the typical LOD of the LC-MS
285 method for analyzing β2-agonists is in the range of 0.1-10 ng/mL) [5, 15, 17].

286 The method precision was examined in following experiments. Firstly, run-to-run
287 repeatability was investigated by analyzing five injections (interval 1 h every time) of
288 the standard mixture solution at the concentration of 600 ng/mL for all analytes. The
289 statistical results of retention times and peak areas were listed in Table 2. The
290 obtained data indicated that RSDs of retention times were in the range of 0.46 and
291 0.87%, and the RSDs of the peak areas were in the range of 2.4 and 3.9%. In order to
292 study the batch-to-batch column repeatability, three columns from different batches
293 were examined (Table 2). The inter-columns RSDs of retention times were found
294 lower than 1.8%, and the inter-columns RSDs of peak areas were lower than 6.2%.

295 *3.5 Analysis of real urine sample*

296 Urine samples collected from three volunteers were analyzed. The collection and
297 pretreatment of urine samples were performed according to the procedures described
298 in section 2.5. Fig. 7A shows the electrochromatogram of blank urine samples. No
299 interference was observed for the analysis of $\beta_2$-agonists in urine matrix. In the urine
300 samples collected from the normal or healthy human, no $\beta_2$-agonists were detected. In
301 order to investigate the method accuracy, precision and applicability, spiked urine
302 were analyzed. Standard mixture solutions of six $\beta_2$-agonists at two different
303 concentrations (2000 and 200 ng/mL) were spiked to urine samples that were
304 subsequently analyzed by using the developed method. Methanol was used to remove
305 the proteins in urine before adding the standard $\beta_2$-agonists solution. By comparing

306 the peak areas, the recoveries of the six $\beta_2$-agonists were determined in the range of
307 88.2 and 106% with RSDs of lower than 6.68% (Table 3). For the further
308 investigation of method applicability, human urine samples were collected from three
309 volunteers who had orally taken salbutamol sulfate tablets. A typical
310 electrochromatogram from the analysis of the 4-h urine sample collected from one of
311 the volunteers was illustrated in Fig. 7B. The peak of salbutamol was clearly detected
312 in the urine sample. Fig. 7C showed the mass spectrum of the peak in Fig. 7B for the
313 confirmation of salbutamol detection. The RSD of urine samples from three different
314 volunteers was investigated. The obtained data showed that the RSDs of retention
315 time and peak intensity were 1.6%, and 14.6%, respectively. The high RSD of peak
316 area reflected the difference behaviors of salbutamol sulfate tablets among individual
317 volunteers.

318 The developed method might be applicable for the analysis of real biological
319 sample, in term of sensitivity. To achieve anabolic-like effects for increasing the ratio
320 of muscle to fat, a higher dosage of $\beta_2$-agonists (usually 5 to 10 times than that used
321 for therapeutic treatment of bronchial diseases) was often used not only by athletes in
322 sporting competition, but also in animals as growth promoters. Therefore, the
323 sensitivity of the developed method should be higher than that required by WADA.

## 324 4 Conclusions

325 A method for the analysis of six $\beta_2$-agonists by pCEC-ESI-MS using silica-based
326 monolithic column was demonstrated for the first time. The experimental results
327 showed that the developed method was simple, robust and sensitive. Compared with

the conventional LC-MS methods, the pCEC-ESI-MS approach not only provided a comparable detection limits ranging from 0.25 to 2.0 ng/mL, but also possessed several potential advantages including the fast and highly efficient separation with small injection amount. The absolute LODs of the method ranged from 5.75 to 46.0 fg with SIM scan mode. This method was also considered to be an environment-friendly technique due to the consumption of micro-amount organic solvent. The method was successfully applied for the analysis of real urine samples collected from the volunteers who had been orally dosed with salbutamol sulfate tablets.

**Acknowledgements**

The authors are grateful for the National Nature Sciences Foundation of China (20675016, 20735002), the Key Science and Technique Cultivation Fund of College Innovation Project, Ministry of Education of China (708056), the Key Program of Science and Technology Department of Fujian Province, China (2007I0020), the Key Special Purpose Foundation of Physical Education Bureau of Fujian Province (HX2005-74) and the Plan of the University New Century Outstanding Talent of Fujian Province (HX2006-101).

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## Figure Legends

**Fig. 1.** Chemical structures of six $\beta_2$-agonsits.

**Fig. 2.** Total ion electrochromatograms in SIM mode showing the effect of different ratios of ACN/buffer (A, 70/30%; B, 80/20%; C, 82/18% and D, 85/15%; v/v) on the separation of six $\beta_2$-agonsits. Experimental conditions: mobile phase, various rations of ACN/20 mmol/L ammonium acetate (pH 6.0); separation voltage, 25 kV; assisted pressure, 2 bar; 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; column, 66 cm length self-prepared silica monolithic column with 100 μm id×375 μm od; flow of drying gas, 6.0 L/min; temperature of drying gas, 150 $^{o}$C; nebulizing gas pressure, 0.69 bar. Peak identification: (1) clenbuterol, (2) terbutaline, (3) salbutamol, (4) salmeterol, (5) formoterol, (6) procaterol.

**Fig. 3.** Total ion electrochromatograms in SIM mode showing the effect of running buffer concentration on the separation of six $\beta_2$-agonsits. Mobile phase was composed by 82% ACN with 18% various concentration of running buffer (A, 15 mmol/L; B, 20 mmol/L; C, 25 mmol/L and D, 30 mmol/L). Other experimental conditions and peak identification were same as in Fig. 2.

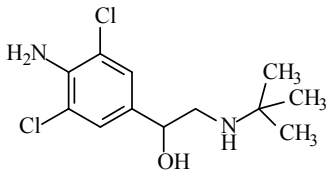
**Fig. 4.** Total ion electrochromatograms in SIM mode showing the effect of assisted pressure on the separation of six $\beta_2$-agonsits. Assisted pressure was (A) 2.0 bar, (B) 4.0 bar, and (C) 6.0 bar. Experimental conditions: mobile phase, 82/18% v/v ACN/buffer, other experimental conditions and peak identification were same as in Fig. 2.

**Fig. 5.** Effect of sheath liquid flow rate on signal intensities of the analytes. Experimental conditions: mobile phase, 82/18% v/v ACN/buffer, other experimental conditions were same as in Fig. 2. (1) clenbuterol, (2) terbutaline, (3) salbutamol, (4) salmeterol, (5) formoterol, (6) procaterol.

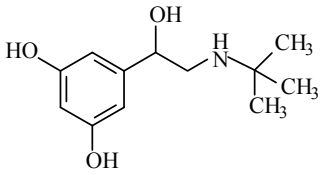
**Fig. 6.** Total ion electrochromatograms in SIM mode (A) and extracted ion chromatogram of the $[M+H]^+$ ions of (1) clenbuterol, (2) terbutaline, (3) salbutamol, (4) salmeterol, (5) formoterol, and (6) procaterol, respectively (B). Experimental conditions: mobile phase, 82/18%, v/v ACN/20 mmol/L ammonium acetate (pH 6.0); separation voltage, 25 kV; assisted pressure, 2 bar; sheath liquid, 50/50% 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; column, 66 cm length self-prepared silica monolithic column with 100 μm id×375 μm od; flow of drying gas, 6.0 L/min; temperature of drying gas, 150 $^{o}$C; nebulizing gas pressure, 0.69 bar.

**Fig. 7** Total ion electrochromatograms in SIM mode of real urine samples collected from the volunteer who took salbutamol sulfate tablets. (A) Blank urine, (B) the urine from volunteer, (C) the full-scan mass spectrum of peak 1. Experimental conditions were same as in Fig. 6.

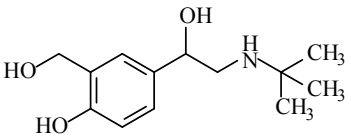
437 **Figure 1**



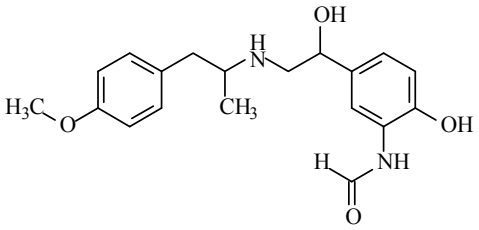
Clenbuterol



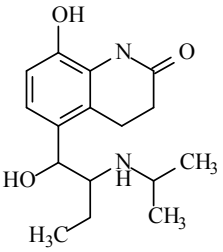
Terbutaline



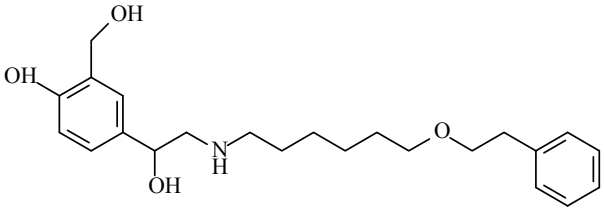
Salbutamol



Formoterol



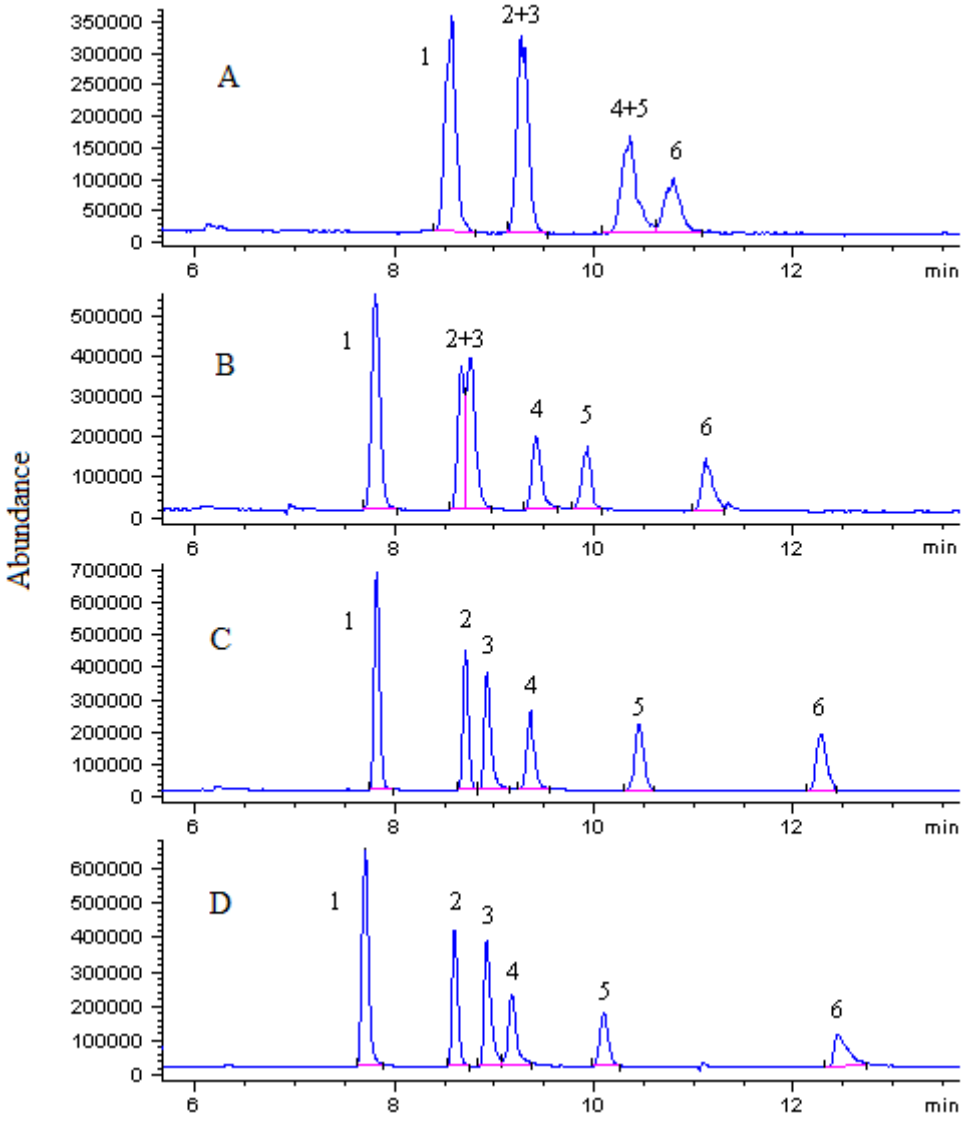
Procaterol



Salmeterol

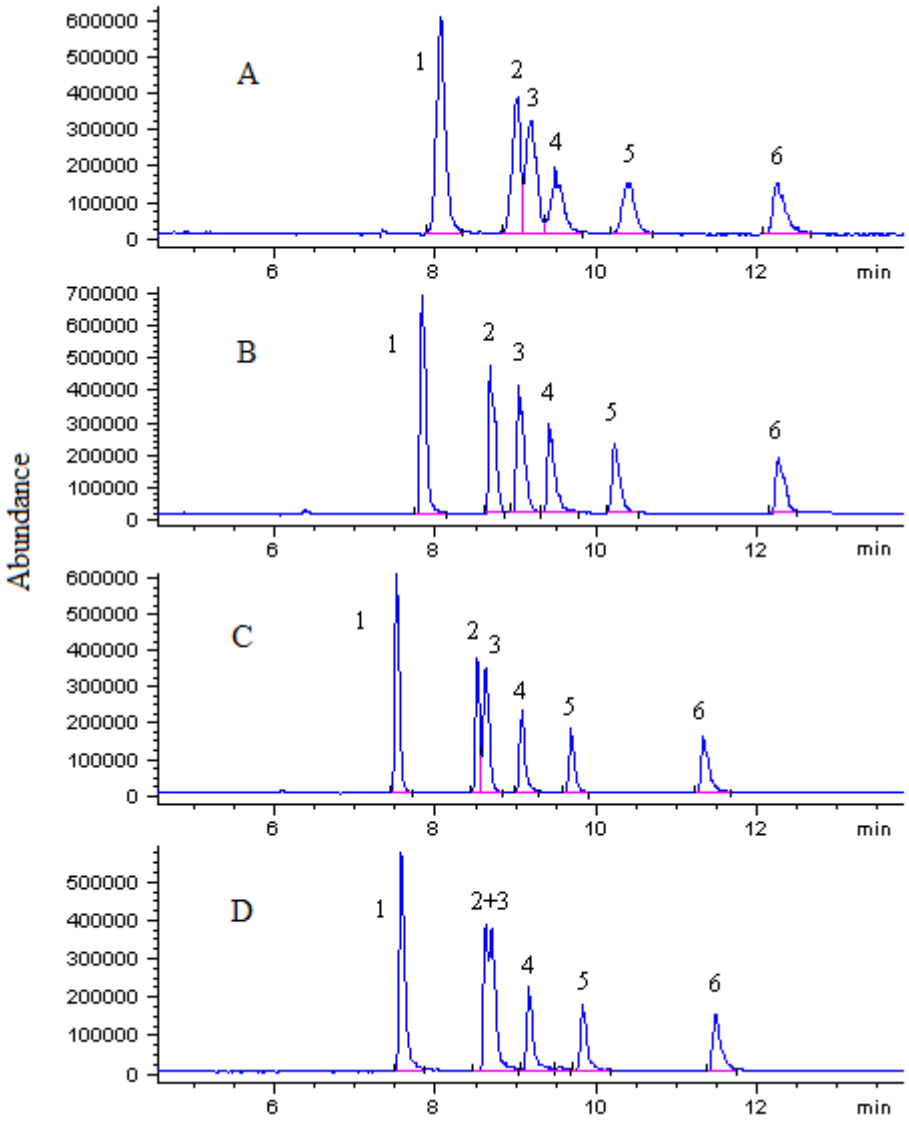
438

439 **Figure 2**



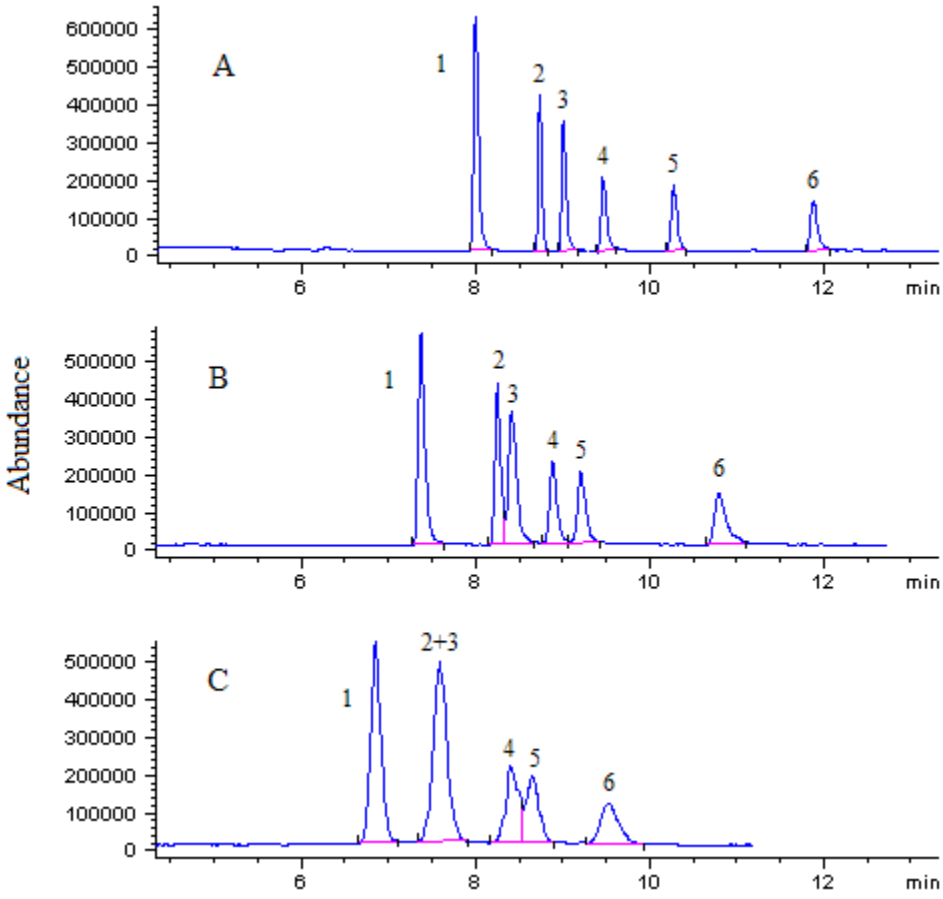

440

441 **Figure 3**



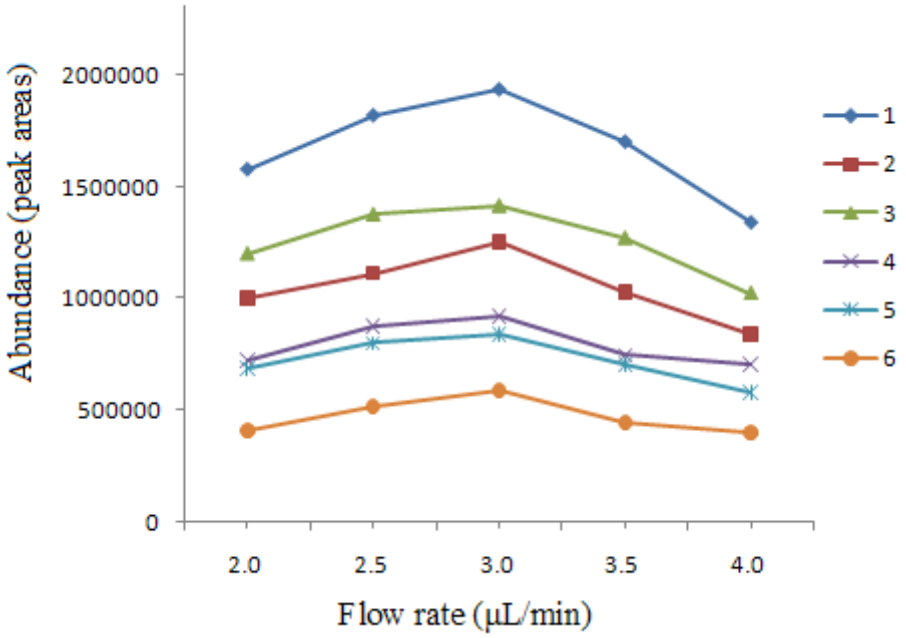

442

443 **Figure 4**



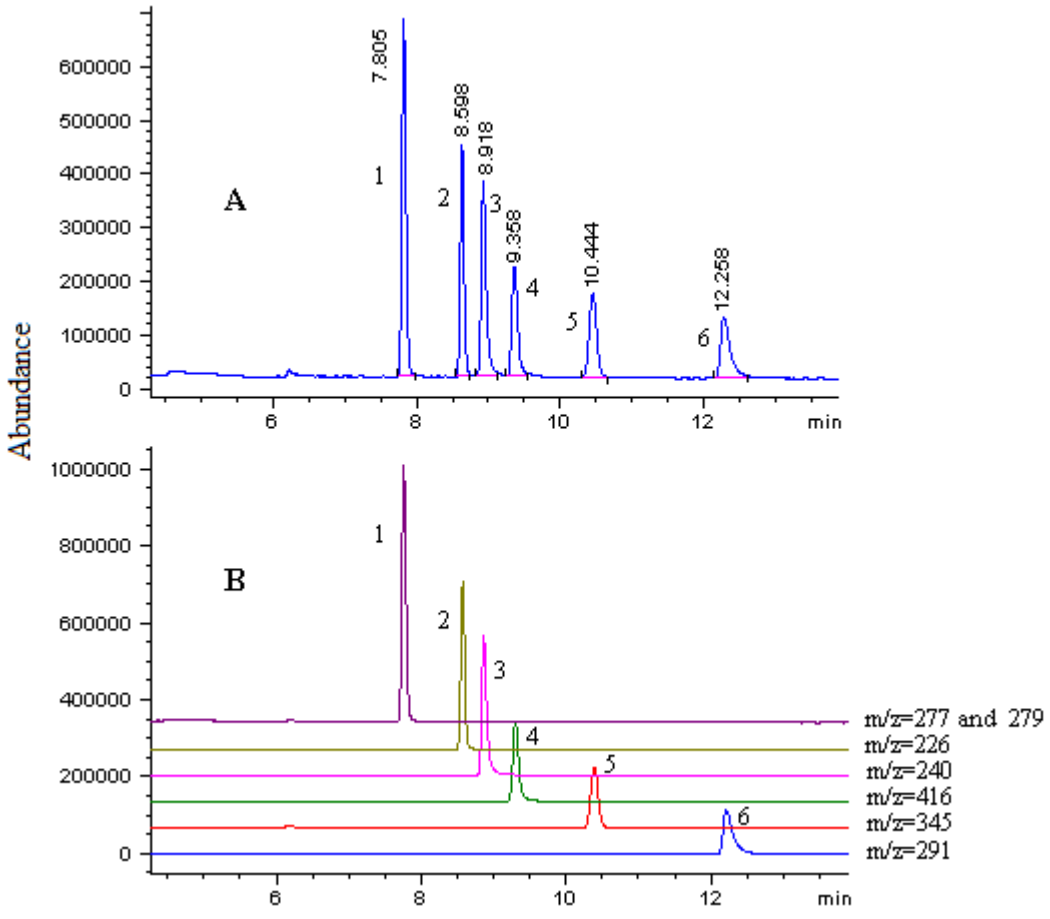

444

445 **Figure 5**



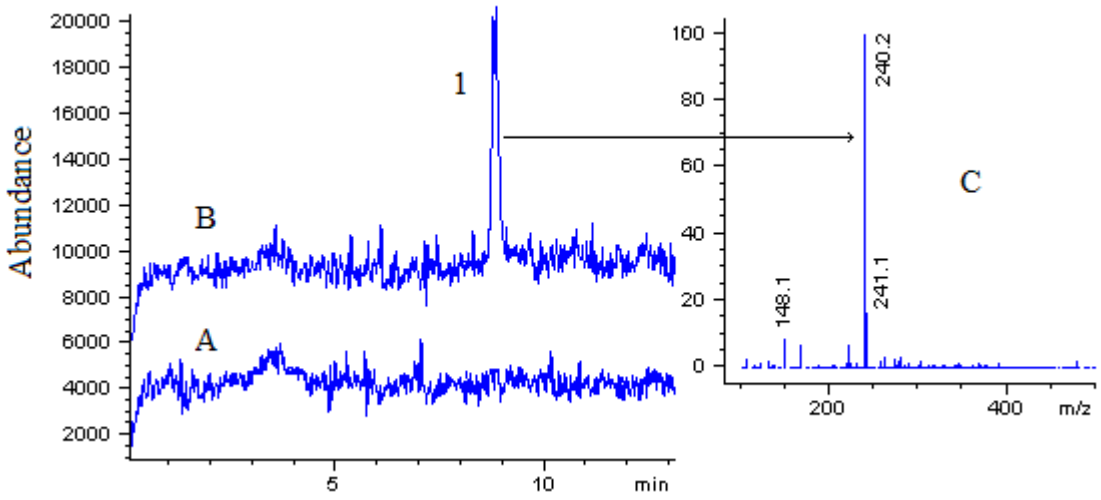

446

447 **Figure 6**




448

449 **Figure 7**

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451

452 **Table 1.** Regression equations, linearity, the detection limits and repeatability of
453 pCEC-ESI-MS for the analysis six $\beta_2$-agonists[a)].

| Compound | Regression equation | $R^2$ | Linear Range (ng/mL) | Detection limit (ng/mL) |
|---|---|---|---|---|
| Clenbuterol | y = 3479.2x + 644526 | 0.9965 | 10 000-1.00 | 0.25 |
| Terbutaline | y = 3240.3x + 502004 | 0.9970 | 10 000-1.00 | 0.25 |
| Salbutamol | y = 2596.9x + 411067 | 0.9951 | 10 000-1.00 | 0.40 |
| Salmeterol | y = 1931.3x + 323660 | 0.9973 | 10 000-2.00 | 0.60 |
| Formoterol | y = 1813.1x + 304626 | 0.9967 | 10 000-2.00 | 0.60 |
| Procaterol | y = 1608.7x + 192940 | 0.9987 | 10 000-6.00 | 2.00 |

454 a) Experimental conditions were same as in Fig. 6

455 **Table 2.** Repeatabilities of run-to-run (n=5) and column-to-column (n=3) of the

456 pCEC-EDI-MS for the analysis of six $\beta_2$-agonists[a)].

| Compound | Run-to-run (RSD, %) | | Column-to-column (RSD, %) | |
|---|---|---|---|---|
| | Retention time | Peak area | Retention time | Peak area |
| Clenbuterol | 0.70 | 2.4 | 1.2 | 3.2 |
| Terbutaline | 0.46 | 3.8 | 1.0 | 5.4 |
| Salbutamol | 0.63 | 3.1 | 1.3 | 4.9 |
| Salmeterol | 0.72 | 3.2 | 1.6 | 4.8 |
| Formoterol | 0.68 | 3.5 | 1.5 | 3.9 |
| Procaterol | 0.87 | 3.9 | 1.8 | 6.2 |

457 a) Experimental conditions were same as in Fig. 6.

458 **Table 3.** Recoveries of spiked urine sample[a)].

| Compound | Added (ng/mL) | Found (ng/mL) | Recovery (%) | RSD (%) (n=3) |
|---|---|---|---|---|
| Clenbuterol | 2 000 | 2125 | 106 | 2.59 |
| | 200 | 205 | 102 | 3.41 |
| Terbutaline | 2 000 | 1964 | 98.2 | 3.35 |
| | 200 | 185 | 92.3 | 4.40 |
| Salbutamol | 2 000 | 1907 | 95.3 | 3.56 |
| | 200 | 181 | 90.4 | 3.85 |
| Salmeterol | 2 000 | 2029 | 101 | 3.94 |
| | 200 | 192 | 95.9 | 5.09 |
| Formoterol | 2 000 | 1890 | 94.5 | 2.94 |
| | 200 | 207 | 104 | 3.60 |
| Procaterol | 2 000 | 1836 | 91.8 | 4.78 |
| | 200 | 176 | 88.2 | 6.68 |

459 a) Experimental conditions were same as in Fig. 6.