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Development of an Analytical Method for the Determination of β_2-Agonist Residues in Animal Tissues by High-Performance Liquid Chromatography with On-line Electrogenerated $[Cu(HIO_6)_2]^{5-}$-Luminol Chemiluminescence Detection

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A novel method was developed for the simultaneous determination of β_2-agonist residues such as terbutaline, salbutamol, and clenbuterol by high-performance liquid chromatography (HPLC) coupled with chemiluminescence (CL) detection. The procedure was based on the enhancement effect of β_2-agonists on the CL reaction between luminol and the complex of trivalent copper and periodate ($[Cu(HIO_6)_2]^{5-}$), which was on-line electrogenerated by constant current electrolysis. The HPLC separation used a Nucleosil RP-C_{18} column (250 mm × 4.6 mm i.d., 5 μm; pore size, 100 Å) with a mobile phase consisting of 90% acetonitrile and 10% aqueous ammonium acetate (20 mmol L^{-1}, pH 4.0) at a flow rate of 1.0 mL min^{-1}. The effects of several parameters on the HPLC resolution and CL emission were studied systematically. Liver samples were hydrolyzed with β-glucuronidase followed by a solid-phase extraction procedure using Waters OasisMCX cartridges. Under optimum conditions, the limits of detection at a signal-to-noise ratio of 3 ranged from 0.007 to 0.01 ng g^{-1} and the limits of quantification at a signal-to-noise ratio of 10 ranged from 0.023 to 0.033 ng g^{-1} for three β_2-agonists. The relative standard deviations (RSDs) of intra- and interday precision were below 4.5%. The average recoveries for β_2-agonists (spiked at the levels of 0.05−5.0 ng g^{-1}) in pig liver ranged from 84 to 110%, and the RSDs of the quantitative results were from 1.6 to 7.2%. The proposed method was successfully applied to the determination of β_2-agonist residues in pig liver samples.

KEYWORDS: β_2-Agonists; chemiluminescence detector; on-line electrogenerated $[Cu(HIO_6)_2]^{5-}$; high-performance liquid chromatography; pig liver

INTRODUCTION

The β_2-agonists, such as terbutaline (TB), salbutamol (SB), and clenbuterol (CB) (see **Figure 1**), are used in human and veterinary medicine for the treatment of pulmonary disorders. They are also extensively misused in farm animals, where high doses give rise to a preferential muscle to fat ratio, resulting in financial gain for the farmer. This abundant misuse raised serious concerns about a toxicological risk for the consumer (*1*, *2*). Because of this fact, the United States, the European Union, and most other countries forbid the use of some or all of these substances in animals raised for human consumption (*3*, *4*). However, β_2-agonists have often been found as residues in animal tissues. Several outbreaks of food poisoning were reported in China as well as other countries by the intake of meat or liver contaminated with β_2-agonist residues. Because of the great varieties of β_2-agonists, and the possibility of trace residues in edible tissue, it is necessary to develop simple, inexpensive, selective, and sensitive multiresidue screening methods for the determination of β_2-agonists.

The analytical methods applied to determine β_2-agonists in biological samples including foods of animal origin have been comprehensively reviewed (*5*, *6*). These compounds are usually extracted from the matrix into organic solvents or purified by solid-phase extraction (SPE) and analyzed by gas chromatography (GC) or high-performance liquid chromatography (HPLC). Among the GC methods, the most commonly used detection modes are mass spectrometry detections. Derivatization has an important role in the determination of β_2-agonists by GC-MS, and different derivatization procedures have been employed (*7*). Unfortunately, derivatization is a complicated and time-consuming procedure. Hence, HPLC methods seem to be good alternatives to GC-MS analysis since the derivatization step is not required. HPLC with different detection modes such as using UV (*8*), fluorescence (*9*), electrochemical (*10*), and MS (*1*, *2*, *11*, *12*) detections have been extensively employed in quantitative analysis of β_2-agonists. Of these detection modes, mass

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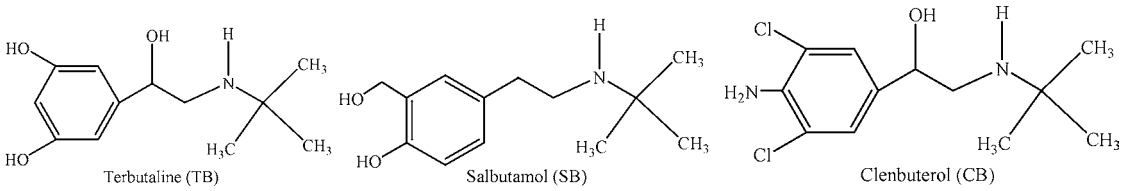

Figure 1. Chemical structures of TB, SB, and CB.

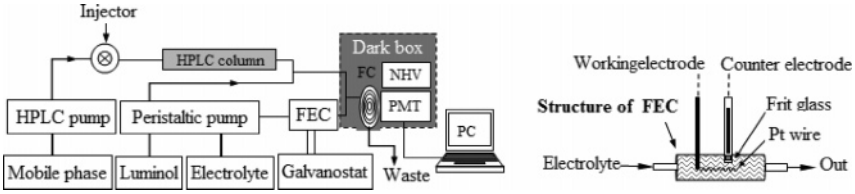

Figure 2. Schematic diagram of HPLC-CL detector of β_2-agonists: FEC, flow-through electrolytic cell; FC, flow cell; PMT, photomultiplier tube; NHV, negative high votage; and PC, computer.

spectrometry can offer high sensitivity and selectivity for the determination of β_2-agonists, but the instrumentation is expensive.

In recent years, extremely sensitive analytical technique based on chemiluminescence (CL) systems have received considerable attention. Simplicity of detection, low detection limit, and wide calibration ranges are some of the characteristics that make the method attractive. Some CL systems such as $KMnO_4$−HCHO, $[Fe(CN)_6]^{3-}$−luminol, and $[Ru(bpy)_3^{2+}]$ coupled with flow-injection analysis (FIA) and molecularly imprinted polymer technology have been developed for the determination of β_2-agonists in pharmaceutical and biological samples (*13*−*15*). Unfortunately, these methods reported can be used only for the determination of a single compound in relative simple matrices, while the determination for complex matrices, such as animal tissues, is almost impossible because similar CL reactions will lead to the interference from one to another. CL as a detection technique of HPLC is very attractive due to higher sensitivity and selectivity (*16*−*18*). The advance of CL detection has greatly catalyzed the growth and popularity of HPLC-CL applications and made trace analysis possible because of its capability of measuring picogram or femtogram quantities of compounds in the column eluate. However, to the best of our knowledge, no HPLC-CL method has been reported for the simultaneous quantitation of β_2-agonist residues in animal tissues.

Usually, most CL reactions are redox reactions. Inorganic oxidants, including H_2O_2, O_2, $KMnO_4$, $[Fe(CN)_6]^{3-}$, and Ce^{4+}, were frequently used in CL reactions; however, some inorganic oxidants such as BrO^-, ClO^-, Mn(III), Co(III), and Ag(II) cannot be used directly because they are not stable during experiment. To overcome this drawback, an on-line or in situ electrogenerated unstable reagent from a stable oxidation state of the same element in flow CL systems was developed in our laboratory (*19*, *20*).

Trivalent copper has generally been considered to be an uncommon oxidation state and cannot be used directly in the CL reaction because it is not stable in aqueous solution. Previous work has shown that the complex of trivalent copper and periodate, $[Cu(HIO_6)_2]^{5-}$, could be on-line electrogenerated on the near surface of platinum electrode by constant current oxidizing $Cu(NO_3)_2$ in KIO_4−KOH medium using a new flow-through electrolytic cell (FEC) (*21*). The $[Cu(HIO_6)_2]^{5-}$ concentration could be readily adjusted on-line over a wide concentration range with a change in the electrolytic current. In this paper, we found that the chemical reaction between nascent $[Cu(HIO_6)_2]^{5-}$ and luminol in alkaline medium was accompanied with the weak emission of light and the β_2-agonists including TB, SB, and CB, which had strong enhancing effects on this weak CL intensity. The CL emission signal was proportional to the β_2-agonist concentrations. On the basis of this enhancement effect, a new, selective, sensitive, and inexpensive HPLC-CL method for the determination of β_2-agonist residues was developed. The proposed method was successfully applied to the determination of β_2-agonist residues in pig liver samples.

EXPERIMENTAL PROCEDURES

Material and Reagents. Acetonitrile was of HPLC grade. All of the reagents were of analytical reagent grade unless specified otherwise, and deionized and doubly distilled water was used throughout.

Luminol was obtained from Kangpei Technology Co. (Xi'an, China). TB, CB, and SB were purchased from Sigma Aldrich (Poole, Dorset, United Kingdom), while mabuterol, cibuterol, tulobuterol, pirbuterol, and clenproperol were obtained from Chinese Pharmaceutical and Biological Test Institute (Bingjing, China). β-Glucuronidase was purchased from Sigma Chemical Co. (St. Louis, MO). Other reagents were purchased from Xi'an Chemical Reagent Factory (Xi'an, China). Oasis MCX (3 mL, 60 mg) SPE columns were obtained from Waters (Milford, MA).

Stock solutions (100 μg mL^{-1}) of β_2-agonists were prepared in methanol and stored at 4 °C in the dark. A 1×10^{-2} mol L^{-1} stock solution of luminol was prepared in 0.1 mol L^{-1} KOH. A 0.01 mol L^{-1} amount of $Cu(NO_3)_2$ was prepared by dissolving 1.21 g of $Cu(NO_3)_2 \cdot 3H_2O$ in 500 mL of 0.08 mol L^{-1} KIO_4, and then, 22.4 g of KOH was added to the mixture of 0.01 mol L^{-1} $Cu(NO_3)_2$ and 0.08 mol L^{-1} KIO_4. The electrolyte [0.01 mol L^{-1} $Cu(NO_3)_2$−0.08 mol L^{-1} KIO_4−0.8 mol L^{-1} KOH] was stirred until it became transparent. The cathodic chamber was filled with 1.0 mol L^{-1} KOH solution. The HPLC mobile phases were prepared fresh daily, filtered through a 0.22 μm membrane filter (Xinya Co., Shanghai), and then degassed prior to use.

Apparatus. The experimental setup for the HPLC-CL was shown in **Figure 2**. HPLC was a LC-6A (Shimadzu, Tokyo, Japan) liquid chromatography equipped with a Rheodyne 7725i syringe-loading sample injector valve (20 μL loop, Cotati, CA) and a Nucleosil C_{18} column (i.d., 250 mm × 4.6 mm; particle size, 5 nm; pore size, 100 Å; Macherey-Nagel, Düren, Germany). The mobile phase was the mixture of 90% acetonitrile and 10% aqueous ammonium acetate (20 mM, pH 4.0), and the flow rate was 1.0 mL min^{-1}. After passing through the HPLC system, the mobile phase was mixed at a mixing tee with luminol solution and then merged just prior to reaching (a flat spiral-coiled colorless glass tube, i.d. = 1.0 mm, total diameter of the flow cell (FC) = 2.4 cm, without gaps between loops, V = 78.5 μL) with the stream of $[Cu(HIO_6)_2]^{5-}$ electrogenerated from the FEC (V = 0.147 mL). The FC was placed close to the window of the photomultiplier tube (PMT). The FEC was made of plexiglas (30.0 mm × 20.0 mm × 25.0 mm) in which cathodic and anodic chambers were separated from

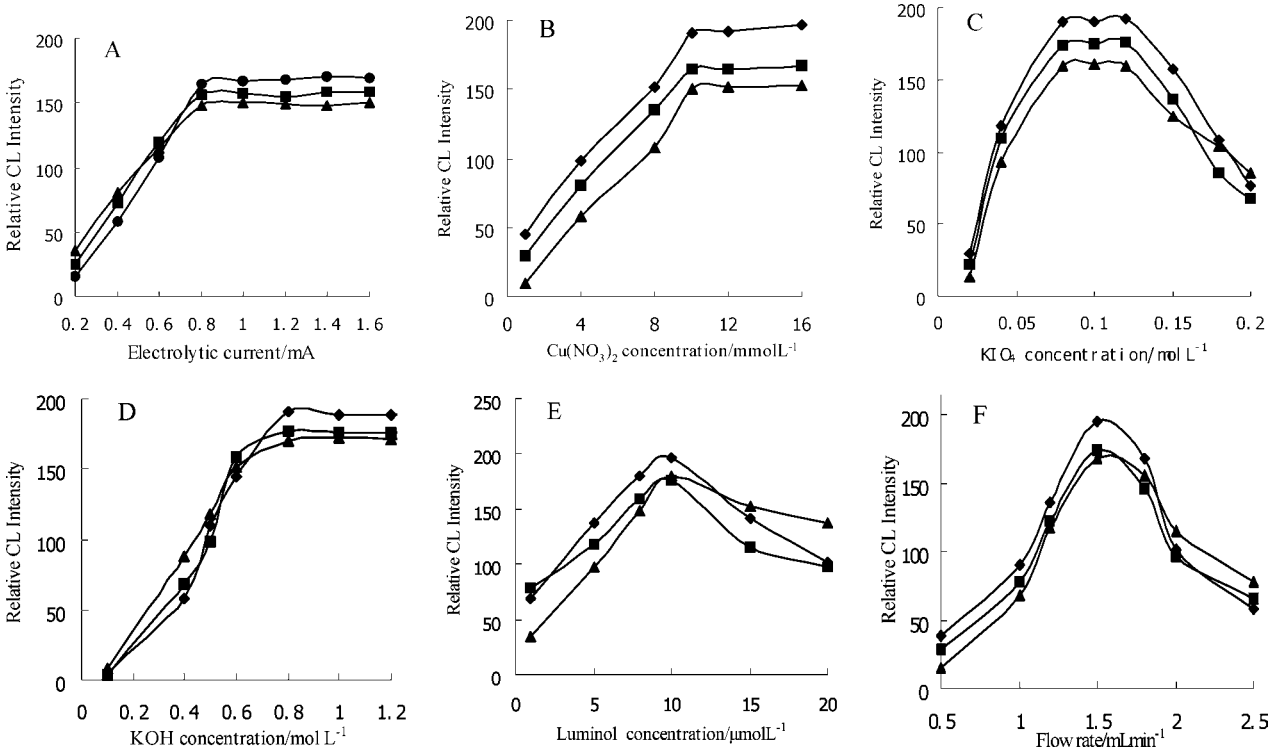

Figure 3. Effect of (**A**) electrolysis current, (**B**) $CuNO_3$, (**C**) KIO_4, (**D**) KOH, (**E**) luminol concentration, and (**F**) flow rate of electrolyte on the relative CL intensity for the HPLC-CL detecyion. β_2-Agonists: 1.0 ng mL^{-1}; TB (♦), SB (■), and CB (▲).

each other by a glass frit. The working electrode was a piece of platinum (2.0 cm^2), and a similar piece of platinum was used as the counter electrode. The platinum counter electrode was inserted in a 2 cm length glass tube with an internal diameter of 2.5 mm, and the glass tube was filled with 1.0 mol L^{-1} KOH. Every day, the electrolyte in the counter compartment was changed. The constant current electrolysis was performed using a JH2C galvanostat (Shanghai Electric Instrument Plant, China). Luminol solution and electrolyte were delivered with one peristaltic pump (Qingpu Huxi Instrument and Meter Plant, China) at a rate of 1.5 mL min^{-1}. PTFE tubing (0.8 mm i.d.) was used as a connection material in the flow system. The emitted CL was collected with a PMT (operated at −800 V) of the Type IFFL-D Flow Injection Chemiluminescence Analyzer (Xi'an Remax Electronic Science-Tech Co. Ltd., Xi'an, China). The signal was recorded using an IBM-compatible computer equipped with a data acquisition interface. Data acquisition and treatment were performed with REMAX software running under Windows XP.

Sample Preparation. Pig's liver samples for analysis were purchased at local outlets. The samples were homogenized and stored at −20 °C. The frozen samples were thawed at 4 °C when analyzed. Sample treatment was performed as follows (*22*). Aliquots (5.0 g) of minced liver were homogenized with 10 mL of 20 mmol L^{-1} ammonium acetate buffer (pH 5.2) in a 20 mL polypropylene centrifuge tube using a T25 basic homogenizer (Ika Labotechnik, Staufen, Germany). Each sample was supplemented with 50 μL of β-glucuronidase and vortex mixed for 5 min and then incubated for 10 h at 37 °C. The mixtures were then centrifuged at 12000 r/min for 10 min at room temperature, and the supernatant was collected in another 20 mL polypropylene centrifuge tube. This supernatant was then purified using a Waters OasisMCX (3 mL, 60 mg) cartridge. The MCX cartridge was prewashed and activated sequentially with 2 mL of methanol and 2 mL of water. The liver extract was applied to the cartridge and washed sequentially with 2 mL of water, 0.1 mL of acetate (0.1 mol L^{-1}), and 2 mL of methanol. The β_2-agonists were eluted with 5 mL of a solution consisting of 80% chloroform, 20% isopropanol, and 5% ammonia. This solution was evaporated to dryness in a nitrogen stream at room temperature. The residue was redissolved in 1 mL of mobile phase and passed through a 0.22 μm nylon syringe filter (Xinya Co., Shanghai), and 20 μL was injected in the HPLC-CL system.

Experimental Procedure. Flow lines were inserted into the luminol solution, mobile phase, and electrolyte, respectively. The pump was started to wash the whole flow system, and electrolysis was started to generate $[Cu(HIO_6)_2]^{5-}$ until a stable baseline was recorded. The 20 μL standard solution or sample was injected into the mobile phase. This stream was merged with $[Cu(HIO_6)_2]^{5-}$ in the FC, producing CL emission. The concentration of the sample was quantified by the relative CL intensity I, $\Delta I = I_s - I_0$, where I_s is the CL intensity of β_2-agonists and I_0 is the CL intensity of the blank.

RESULTS AND DISCUSSION

Optimization of Analytical Parameters. In alkaline medium, the oxidation reaction between $[Cu(HIO_6)_2]^{5-}$ and luminol emitted weak CL. β_2-Agonists including TB, SB, and CB were observed to enhance the CL intensity of the $[Cu(HIO_6)_2]^{5-}$−luminol system. The enhanced CL intensity was proportional to the concentration of β_2-agonists. To obtain the highest β_2-agonists sensitivity, the CL reaction conditions and the HPLC mobile phase composition in the HPLC-CL system were optimized. The β_2-agonists concentration (standard solutions) used for the optimization experiments was 1.0 ng mL^{-1}.

Optimization of CL System. To obtain the maximal relative CL intensity, the design of the FEC and the components of the electrolyte, luminol, electrolytic current, and flow rate on relative CL intensity were investigated.

In the basic design of electrochemiluminescence cell discussed by Knight and Greenway (*23*), the electrolytic FC lay directly in front of the PMT. Obviously, the electrolytic cell has an optical interference as a result of scattering, reflectance, or absorbance by the electrode, and the electrochemical reaction could influence the CL reaction. Therefore, we placed the FEC and the CL FC at different sites in the flow lines, and the optical signal was detected in the CL FC. Besides, the structure of the FEC was a vital factor for the generation of $[Cu(HIO_6)_2]^{5-}$ in this HPLC-CL flow system. The ideal FEC could offer a high and stable electrolytic efficiency for electrogenerating a suf-

Table 1. Optimum Values for the Variables Involved in the Proposed HPLC-CL System for the Determination of β_2-Agonists

mobile phase composition	90% acetonitrile:10% aqueous ammonium acetate (20 mmol L^{-1}, pH 4.0)
mobile phase flow rate (mL min^{-1})	1.0
injection volume (μL)	20
column temperature	20 °C
electrolysis current (mA)	0.8
[$Cu(NO_3)_2$] (mol L^{-1})	0.01
[KIO_4] (mol L^{-1})	0.08
[KOH] (mol L^{-1})	0.8
[luminol] (μmol L^{-1})	10 (in 0.1 mol l^{-1} KOH)
(electrolyte, luminol) flow rate (mL min^{-1})	1.5

ficiently high concentration $[Cu(HIO_6)_2]^{5-}$ for the postcolumn CL reaction. When the counter electrode and working electrode were in the same stream [$Cu(NO_3)_2$−KIO_4−KOH], Cu(II) was reduced to form Cu deposited on the counter electrode, which influenced the electrolytic reaction to generate $[Cu(HIO_6)_2]^{5-}$. So, we selected a glass frit as the separating material between the two electrode chambers. Furthermore, the potentials dispersed on the working electrode should be sufficient to give a high and stable electrolytic efficiency for generating $[Cu(HIO_6)_2]^{5-}$. On the basis of consideration of the effects of the relative locations of the two electrodes in the electrolytic cell and the area of the counter electrode, the results showed that only when the two electrodes faced each other and the area of the working electrode was the same as that of the counter electrode, the electrolytic cell satisfies our aims. In addition to the points considered above, the volume of the electrolytic flow-through cell is also an important factor for using the cell in this CL flow system since it can affect the online concentration of $[Cu(HIO_6)_2]^{5-}$. To obtain a smaller volume of the cell limited by the instruments that were used to make the cell, a 2.5 mm diameter of the chamber of the working electrode was adopted. Considering all of the points discussed above, the structure of the FC as shown in **Figure 2** was the optimum and it was used throughout subsequent experimental work.

For on-line generating $[Cu(HIO_6)_2]^{5-}$ by electrochemically oxidizing $Cu(NO_3)_2$ in KIO_4−KOH medium, both the galvanostatic method and the potentiostatic method were selected for this purpose, respectively. However, the results showed that the former presented the best analytical performance and it was selected as the method for on-line generating $[Cu(HIO_6)_2]^{5-}$. In this HPLC-CL flow system, the electrolysis current could control the concentration of electrogenerated $[Cu(HIO_6)_2]^{5-}$, which affected the relative CL intensity. Therefore, the dependence of the relative CL intensity on the electrolysis current was investigated. The results are shown in **Figure 3A**. It can be seen that the relative CL intensity of β_2-agonists increases with increasing electrolysis current from 0 to 0.8 mA because the concentration of electrogenerated $[Cu(HIO_6)_2]^{5-}$ increases. For electrolysis currents higher than 0.8 mA, the relative CL intensity of β_2-agonists almost remained constant, probably due to the excess of $[Cu(HIO_6)_2]^{5-}$ or the decline of the current efficiency. So, the electrolytic current of 0.8 mA was selected as the optimum for the subsequent studies.

The components of the electrolyte not only affect the effective concentration of the nascent spices $[Cu(HIO_6)_2]^{5-}$ but also the medium of subsequent CL reaction. These effects could be further divided into $Cu(NO_3)_2$ concentration and the medium of electrolyte on the relative CL intensity for the determination

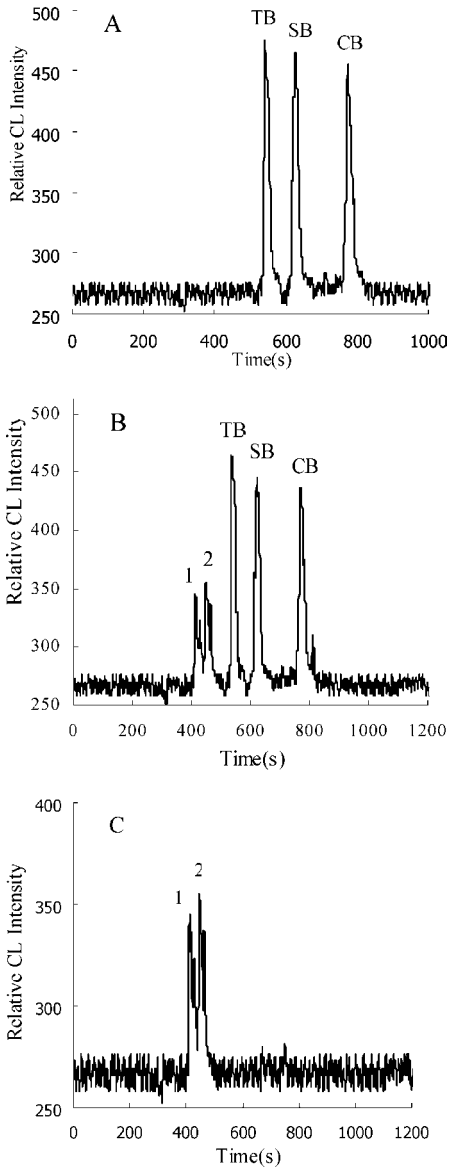

Figure 4. Typical chromatograms of (**A**) a standard mixture solution of β_2-agonists (1.0 ng mL^{-1}), (**B**) extract from pig liver spiked at 0.21 μg kg^{-1}, and (**c**) blank pig liver extract using postcolumn CL detection. HPLC conditions: 5 μm C_{18} column (250 mm × 4.6 mm) with acetonitrile–aqueous ammonium acetate (20 mmol L^{-1}, pH 4.0) (90:10, v/v) as a mobile phase; mobile phase flow rate, 1.0 mL min^{-1}; and temperature, 20 °C. CL conditions: electrolysis current, 0.8 mA; $Cu(NO_3)_2$, 0.01 mol L^{-1}; KIO_4, 0.08 mol L^{-1}; KOH, 0.8 mol L^{-1}; luminol, 10 μmol L^{-1}; CL reagents flow rate, 1.5 mL min^{-1}; peaks 1 and 2, unknown; and Peaks TB (9.0 min), SB (10.3 min), and CB (12.9 min).

of β_2-agonists. The effect of $Cu(NO_3)_2$ concentration on the relative CL intensity was investigated in the range of 1.0×10^{-3} to 1.6×10^{-2} mol L^{-1} in 0.1 mol L^{-1} KIO_4−0.5 mol L^{-1} KOH. The results are shown in **Figure 3B**. The relative CL intensities for TB, SB, and CB increased with increasing $Cu(NO_3)_2$ concentration up to 0.01 mol L^{-1}, above which the relative CL intensity remained almost constant. Hence, the optimal concentration of $Cu(NO_3)_2$ was chosen as 0.01 mol L^{-1}. According to the reference (*24*, *25*) in basic solutions, the $[Cu(HIO_6)_2]^{6-}$ complex anion can exist stably only when there was extra IO_4^-. Otherwise, Cu(II) will combine with OH^- to form $Cu(OH)_2$ precipitate. The preliminary experiments showed that when the IO_4^- concentration was higher than 0.2 mol L^{-1},

Table 2. Calibration Curves, Detection Limits, and Precisions of β_2-Agonists[a]

compound	linear range (ng mL^{-1})	ng g^{-1} LOD	ng g^{-1} LOQ	regression equation	R^2	precision (RSD) intraday (%)		precision (RSD) interday (%)
TB	0.1–40	0.007	0.023	$I = 167.5C + 30.56$	0.9991	3.8 a	1.8 b	2.8 b
SB	0.1–50	0.008	0.027	$I = 142.7C + 34.76$	0.9988	4.2 a	2.6 b	3.6 b
CB	0.08–30	0.01	0.033	$I = 151.2C + 29.40$	0.9989	4.5 a	2.2 b	3.0 b

[a] Precision data are presented as the RSDs ($n = 11$) (see the text). Concentration of the analytes: a, 0.5 ng mL^{-1}; b, 1.0 ng mL^{-1}. R^2, correlation coefficient.

the relative CL intensity declined, probably because of too high a concentration of IO_4^- was not suitable for the HPLC-CL system. The effect of KIO_4 concentration on the relative CL intensity was examined in the range of 0.02−0.2 mol L^{-1} (**Figure 3C**). From **Figure 3C**, it can be seen that the relative CL intensity of β_2-agonists increased in the range of 0.02−0.08 mol L^{-1}, remained constant in the range of 0.08−0.12 mol L^{-1}, and decreased in the range of 0.12−0.2 mol L^{-1}. Thus, the optimal concentration of KIO_4 was chosen as 0.08 mol L^{-1}. The effect of KOH concentration on the relative CL intensity was investigated in the range of 0.1−1.0 mol L^{-1} (**Figure 3D**). The relative CL intensities for TB, SB, and CB increased with increasing KOH concentration up to 0.8 mol L^{-1}, above which the relative CL intensity remained almost constant. Hence, 0.8 mol L^{-1} was the optimal concentration of KOH for the determination of β_2-agonists and chosen for the subsequent studies.

The concentration of luminol had a very important effect on the relative CL intensity for the determination of β_2-agonists. The effect of luminol concentration on the relative CL intensities was investigated from 1.0 to 20.0 μmol L^{-1}, and the results are shown in **Figure 3E**. When the concentration of luminol was lower or higher than 10 μmol L^{-1}, the relative CL intensity for all of the tested β_2-agonists decreased. Therefore, the optimum concentration of luminol was 10 μmol L^{-1}.

On the basis of the principle of FIA, the spatial distribution of the concentration of the on-line electrogenerated $[Cu(HIO_6)_2]^{5-}$ in the near surface of platinum electrode was dependent more on the flow rate of electrolyte and HPLC mobile phase. When the electrolytic currents and HPLC mobile phase were fixed at 0.8 mA and 1.0 mL min^{-1}, the effect of flow rate of electrolyte on the CL intensity was investigated in the range from 0.5 to 2.5 mL min^{-1} and reached its maximum values at 1.5 mL min^{-1} (**Figure 3F**). Above 1.5 mL min^{-1}, the CL signal decreased with the increase of electrolyte flow rate. Thus, 1.5 mL min^{-1} was selected as the optimum flow rate of electrolyte.

Optimization of HPLC System. As for HPLC-CL detection, the mobile phase of HPLC should be not only suitable for the separation of β_2-agonists but also compatible with the CL reaction. Several mobile phases have been reported for the separation of β_2-agonists on RP-C_{18} column, such as methanol−acetic acid (*1*), acetonitrile−ammonium acetate (*2*, *26*), methanol−0.1% formic acid (*27*), and methanol−ammonium acetate (containing 3.15 g L^{-1} ammonium acetate and 5.49 g L^{-1} 1-octanesulfonic acid sodium salt and formic acid, pH = 3) (*28*). Among these mobile phases, the mixture of methanol or acetonitrile with other reagents was currently used as the mobile phase in separating β_2-agonists. The CL characteristic of these mobile phases was tested, and it was found that the CL intensity was intensely inhibited by methanol, but acetonitrile was suitable for good separation of these compounds and compatible with the HPLC-CL system. To improve the peak shape and completely separate these compounds, the aqueous ammonium acetate was used. Under the condition of complete separation, the effect of mobile phase composition on the relative CL intensity was studied. The effect of ammonium acetate concentration ranged from 1 to 30 mmol L^{-1}. When the concentration of ammonium acetate was 20 mmol L^{-1}, the separation was good, and the CL intensity of β_2-agonists was almost maximal. The effect of changing the pH value of the buffer solution ranged from 3.0 to 5.2. The chromatographic peaks of TB and SB overlapped when the pH was lower than 3.0. When the pH increased, good separation could be achieved, and the relative CL intensity reached its maximum at pH 4.0. The effect of acetonitrile concentration ranged from 100 to 80%. When the concentration of acetonitrile in a mixture of acetonitrile and aqueous ammonium acetate (20 mM, pH 4.0) decreased from 100 to 80%, the CL intensity of β_2-agonists almost increased. When the concentration of acetonitrile was below 90%, complete separation of TB and SB could no longer be obtained. Therefore, the mobile phase containing 90% acetonitrile and 10% aqueous ammonium acetate (20 mM, pH 4.0) was considered optimal for isocratic elution and for the CL intensities of the β_2-agonists.

As a summary, all of the optimum values for the variables involved in the CL system are included in **Table 1**. Under the optimum conditions described above, an HPLC system equipped with a Nucleosil C_{18} column was used to separate β_2-agonists. Using an isocratic mobile phase composed of 90% acetonitrile and 10% aqueous ammonium acetate (20 mM, pH 4.0), good separation was achieved within 15 min. The retention times of β_2-agonists were as follows: 9.0 min for TB, 10.3 min for SB, and 12.9 min for CB. **Figure 4** showed the chromatograms of (**A**) the mixture of 1.0 ng mL^{-1} β_2-agonists standard solution, (**B**) blank pig liver matrix spiked at the same concentration level (0.21 μg kg^{-1} sample), and (**C**) blank pig liver extract, which made it possible to identify β_2-agonists in the sample and to evaluate its concentration. From **Figure 4A−C**, it can be seen that β_2-agonists can be well-separated without the interference of other compounds in the samples. Peak identification was carried out by the standard addition method and the retention time of β_2-agonists. Next, the interference from other β_2-agonists, such as mabuterol, cibuterol, tulobuterol, pirbuterol, and clenproperol for the determination of TB, SB, and CB, was investigated. Their solutions of 1.0 ng mL^{-1} prepared in the mixture of standard TB, SB, and CB (at 1.0 ng mL^{-1}) samples were injected into HPLC individually and detected by this proposed method. No conspicuous peaks were detected within 45 min, which means no interference of the other β_2-agonists would occur and the method of coupling CL detector to HPLC was a method of high selectivity. These chromatograms demonstrated that the application of the HPLC-CL method to the determination of β_2-agonists in biological samples was possible.

Method Validation. In the present work, the HPLC-CL method for the determination of β_2-agonists was validated by determining its performance characteristics regarding linearity, limit of detection (LOD), limits of quantification (LOQ), and reproducibility (precision). To test the CL response linearity, a

Table 3. Comparison of β_2-Agonists Detection with Different Methods

β_2- agonists	method	linear range (ng mL^{-1})	detection limit (ng g^{-1})	samples	literature cited
TB	this CL method	0.1–40	0.007	liver	this work
	GC-MS–MS			urine and liver	*26*
	LC-MS–MS		0.1		
	SPR biosensor	0.02–0.3	1.27	liver	*29*
	HPLC-ED	20–100	0.8	plasma	*30*
	HPLC-FD	25–100	1.0	plasma	*9*
SB	this CL method	0.1–50	0.008	liver	this work
	GC-MS–MS		0.1	urine and liver	*26*
	LC-MS–MS		0.05		
	SPR biosensor	0.02–0.3	0.19	liver	*29*
	HPLC-ED	20–100	1.0	human plasma	*30*
	HPLC-FD	25–100	2.5	human plasma	*9*
	immunoassay		5.0	urine	*31*
CB	this CL method	0.08–30	0.01	liver	this work
	GC-MS–MS		0.1	urine and liver	*26*
	LC-MS–MS		0.05		
	SPR biosensor	0.02–0.3	0.11	liver	*29*
	immunoassay		1.0	urine	*31*
	immunoassay	0.01–5.18		foodstuffs	*32*

series of β_2-agonists standard solutions at concentrations ranging from 0.1 to 40 ng mL^{-1} for TB, from 0.1 to 50 ng mL^{-1} for SB, and from 0.08 to 30 ng mL^{-1} for CB were determined. Linear regression analysis of the results was summarized in **Table 2**. From these calibration curves, the LODs at a signal-to-noise ratio of 3 ranged from 0.007 to 0.01 ng g^{-1}, and the LOQs at a signal-to-noise ratio of 10 ranged from 0.023 to 0.033 ng g^{-1}. The linear ranges and detection limits were compared with those obtained by different methods in the literature. **Table 3** indicates that the proposed method has a wider linear range and lower detection limit than those of the HPLC methods using fluorescence (*9*) detection and gas and liquid chromatography coupled to tandem mass spectrometry-(GC-MS−MS and LC-MS−MS) (*26*).

The intraday precision was tested with 11 repeated injections of two sample solutions containing β_2-agonists at two concentration levels (0.5 and 1.0 ng mL^{-1}). The relative standard deviations (RSDs) were always less than 4.5%. The interday precision of the proposed method was studied by analyzing two identical samples (β_2-agonists at 1.0 ng mL^{-1}), injected six times every day, on five consecutive days. The RSDs were below 3.6%.

To check the influence of the extraction process to be applied for the analysis of real samples on the CL signals, other calibration curves were established using the same standard solutions of β_2-agonists but applying the extraction process to each standard solution. By statistically comparing both curves, no significant differences were obtained from the intercepts and the slopes. This comparison ensures that there are no significant losses of analytes due to the extraction process, being possible to use directly the calibration curves for quantification purposes. Additionally, from the HPLC-CL chromatograms of a pig liver blank extract spiked with the β_2-agonists at concentration levels corresponding to the LOQs, peaks of β_2-agonists were well-resolved and showed that no matrix effect had been found. Therefore, the present method does offer an alternative, sensitive, and simple approach to the simultaneous detection of β_2-agonists by HPLC.

Applications. Following the procedure for the determination of β_2-agonists detailed in section 2, the proposed method was applied to determine β_2-agonists in pig liver samples. Five different kinds of pig liver samples were detected. Three of them were not certified, one had CB residues, and the other had SB.

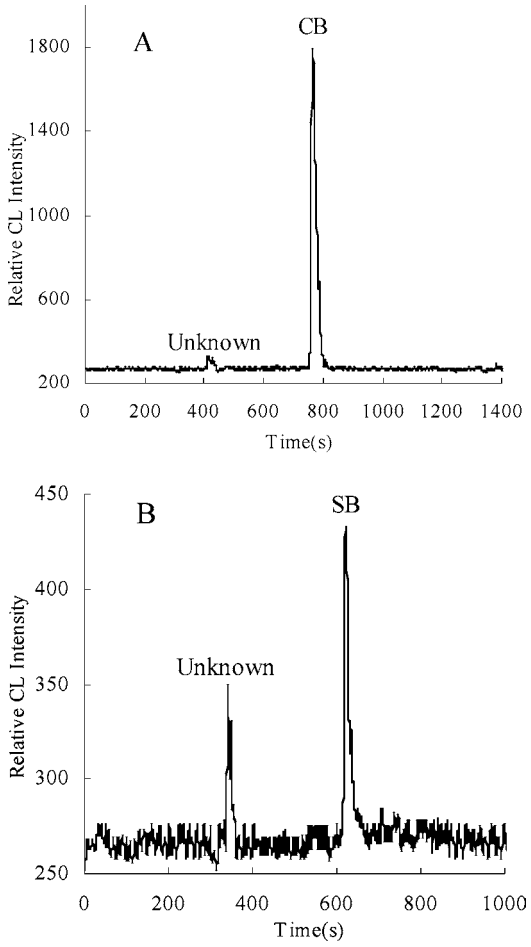

Figure 5. Chromatograms of (**A**) CB (containing 2.32 ng g^{-1}) and (**B**) SB (containing 0.18 ng g^{-1}) in the real samples with postcolumn CL detection. HPLC-CL conditions are the same as those of **Figure 4**.

The CB and SB contents of these samples were calculated by the calibration formula. The concentrates of CB in sample (#4) and SB in sample (#2) were 2.32 and 0.18 ng g^{-1}, respectively. Of course, this kind of pig liver was harmful to people's health. The chromatograms of two samples are shown in **Figure 5**.

To evaluate the validity of the proposed method for the determination of TB, SB, and CB in pig liver samples, a

Table 4. Determination of β_2-Agonists in Pig Liver Samples

samples pig liver	β_2-agonists	ng g^{-1} detected	ng g^{-1} added	ng g^{-1} found[a]	% RSD	% recovery
1	TB	0	0.05	0.042	5.8	84.0
	SB	0	0.05	0.045	7.2	90.0
	CB	0	0.05	0.046	6.3	92.0
2	TB	0	0.1	0.96	3.9	96.0
	SB	0.18	0.1	0.29	4.2	110.0
	CB	0	0.1	0.89	4.5	89.0
3	TB	0	0.5	0.52	1.9	104.0
	SB	0	0.5	0.48	2.4	96.0
	CB	0	0.5	0.53	2.1	106.0
4	TB	0	1.0	0.93	1.6	93.0
	SB	0	1.0	1.07	2.0	107.0
	CB	2.32	1.0	3.38	1.9	106.0
5	TB	0	5	4.86	1.7	97.2
	SB	0	5	4.63	1.8	92.6
	CB	0	5	5.12	1.6	102.4

[a] Mean of three determinations.

recovery experiment was carried out by adding the known amounts of β_2-agonists to the samples, extracted and analyzed by using the described method, and the recoveries varied from 84.0 to 110.0%. The mean recovery percentages and the RSDs of the five kinds of samples are shown in **Table 4**. The RSDs of the quantitative results were in the range of 1.6−7.2% with triplicate measurements. It could be concluded from the above experiments that the proposed HPLC-CL approach could be used for the sensitive quantification for TB, SB, and CB in pig liver samples.

Conclusion. In this paper, a novel method based on HPLC with CL detection using on-line electrogenerated $[Cu(HIO_6)_2]^{5-}$ has been developed for the determination of β_2-agonists including TB, SB, and CB. The method allows for the simultaneous and sensitive detection of β_2-agonists in animal tissues and offers a wider linear range, lower detection limit, and shorter analysis time. Moreover, the CL reaction is very compatible with the mobile phase of HPLC. The result indicates that the proposed HPLC-CL method could be potentially used for the routine monitoring of β_2-agonist residues in animal tissues.

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