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Competitive immunoassay for clenbuterol using capillary electrophoresis with laser-induced fluorescence detection

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Abstract

A competitive immunoassay for detecting clenbuterol in urine was established by capillary electrophoresis (CE) with laser-induced fluorescence (LIF). The clenbuterol was conjugated with bovine serum albumin (BSA), and then the derivative was labeled with fluorescein isothiocyanate (FITC) and competes for antibody with free clenbuterol in the sample. Under the optimal conditions, Free and bound FITC labeled clenbuterol was separated within 8 min with the relative standard deviation (R.S.D.) 0.72% for migration time and 2.8% for peak area. The detection limit reached 0.7 ng/ml.

Keywords: Clenbuterol; Capillary electrophoresis; Immunoassay

1. Introduction

A wide range of additives are currently employed in veterinary medicine and in the production of food. Clenbuterol, as a β_2-agonist, has been the most frequently used since it is capable of improving growth rate, reducing fat deposition, and enhancing protein accretion [1–5]. However, the residual clenbuterol in meat enters human food chain and results in poisoning accidents time after time. It has been announced as an illegal repartitioning agent in feed by many countries. Developing rapid and precise analytical method for clenbuterol becomes world wide interesting.

In recent years, some methods have already been developed to detect CLB in the urine, eye and liver, including liquid chromatography–mass spectrometry (LC–MS) [6–8], gas chromatography–mass spectrometry (GC–MS) [9,10], and capillary electrophoresis (CE) [11–13]. However, the above-mentioned methods are carried out with tedious processes, even worse, they cost a great deal of expensive, toxic and environmental unfriendly organic agents.

Immunoassay was commonly used in clinical, pharmaceutical and chemical analyses for detection and quantification of trace analytes in biological fluids and other complex matrices [14,15]. These assays offer high selectivity due to specific antibody/antigen binding. Conventional immunoassays usually use solid-phase techniques. In the case, quantification is achieved by measuring enzyme activity, e.g. enzyme linked immunosorbent assay (ELISA). Recently, as the growing immunoassay, a sensitive chemiluminescent (CL) enzyme immunoassay for clenbuterol (CLB) analysis in bovine urine has been established [16]. Thanks to the possibility of the system optimization under non-equilibrium immunological conditions and the development of fast chemiluminescence detection with the HRP-label activity, the costs of this method has been reduced, comparing with conventional colorimetric enzyme immunoassays. Although these immunoassays show a high selectivity and sensitivity, they are still labor intensive and require a number of incubating and washing steps, that may take hours to complete. Recently, capillary electrophoresis combined with immunoassay (CEIA) has been proven to be a powerful technique for the separation and analysis of biological compounds [17–21]. This technique offers rapid analysis, small sample consumption, and easy automation. The laser-induced fluorescence detection (LIF) of CE offers high sensitivity. CEIA with LIF offers a number of advantages, the high separation efficiency of CE, the

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high selectivity of immunoassays and the high sensitivity of LIF detection. CEIA-LIF has been applied to a wide range of compounds including prion protein [17,18], alpha-fetoprotein [19], vasopressin [20], hirudin [21], etc. However, clenbuterol detection using CEIA has not been studied so far.

The objective of this work is to develop a CEIA-LIF method to detect clenbuterol. In our experiments, clenbuterol is a small molecular (0.313 kD) and there is no obvious difference in mobility between free antibody and bonded antibody when clenbuterol binds to the much heavier antibody (160 kD). The separation of free and bonded antibodies is difficult in capillary electrophoresis. Therefore, in this study a competitive immunoassay was used. Instead of purified antibody or antibody fragments, the antiserum was used to simplify the procedures.

2. Experimental

2.1. Materials

Fluorescein isothiocyanate (FITC), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), bovine serum albumin (BSA) and Sephadex-G25 M gel column were purchased from Sigma (St. Louis, MO, USA). Clenbuterol ELISA test kit was purchased from Shanghai research center of biotechnology, Chinese Academy of Sciences. Clenbuterol and polyclonal antiserum anti-clenbuterol (rabbit IgG) were kindly provided by Professor Jian Jin (Southern Yangtze University, China). Ultra-pure water was obtained from a Milli-Q Plus water purification system (Millipore Corp., Bedford, MA). All other chemicals used were of analytical grade.

2.2. Apparatus

Beckman P/ACE MDQ Capillary Electrophoresis System (Beckman Instruments, Fullerton, CA, USA) equipped with a laser-induced fluorescence (LIF) detector (Beckman P/ACE System Laser Module 488; excitation wavelength at 488 nm, emission wavelength at 520 nm) was used. A Thermo Finnigan LCQ Deca XP ion trap mass spectrometer (Thermo-Electron, San Jose, CA, USA) equipped with an electrospray interface was used for analyzing the protein CLB conjugates. A 318 MC microtiter reader (Zhengzhou, China) was used to carry out ELISA tests.

2.3. Synthesis of clenbuterol-BSA conjugates (CLB-BSA)

The synthesis of CLB-BSA was performed according to the method described by Bacigalupo et al. [22] with slight modifications. Briefly, 5 mg of CLB was dissolved in 0.5 ml of 0.2 M HCl and cooled to 0 °C. After 20 min, 0.5 ml of $NaNO_2$ (20 mg/ml) was added dropwise to this mixture under stirring in the dark at 4 °C. This solution was added to 20 mg of BSA, dissolved in 3 ml of 0.1 M phosphate-buffered saline (PBS), pH 7.4. During this operation, the pH was maintained at 7.4 by addition of 1 M NaOH. The reaction mixture was incubated at 0 °C for 8 h. The conjugated CLB-BSA was purified by gel filtration on Sephadex-G25 with PBS (pH 7.4).

2.4. Hapten/protein analysis

Hapten/protein densities of CLB-BSA conjugates were determined according to the method previously reported by Roda et al. [16] with some modifications. Hapten/protein densities of CLB-BSA conjugates were determined by electrospray mass spectrometry (ESI-MS) by comparing the molecular weight of the standard BSA with that of the conjugate. ESI-MS spectra were obtained in the positive ion mode (ES^+) by direct injection of samples (0.1 mg/ml in H_2O/CH_3CN 50/50 v/v + 0.1% formic acid) flowing at 5 μl/min. Ionization parameters were as follows: sheath gas flow rate 45 units (no auxiliary or sweep gas flow was employed); spray voltage 4.5 kV; capillary voltage 15 V; capillary temperature 200 °C; tube lens offset 30 V; entrance lens −60 V; acquisition range 500–2000 *m/z*.

2.5. Preparation of FITC labeled CLB-BSA

FITC was dissolved in acetone (0.2 mg in 200 μl) and added dropwise to a 2.0 ml solution containing 10 mg of CLB-BSA dissolved in a 100 mM carbonate buffer (pH 8.5). The mixture was vortex-mixed and kept in the dark at room temperature for 20 h. The reaction was stopped by gel filtration on Sephadex-G25 with PBS (pH 7.4).

2.6. Immunocomplex formation

CLB was firstly conjugated with BSA, and subsequently, the conjugated CLB-BSA was labeled with FITC to form a competitor. The mixture of FITC-CLB, CLB and antibody was injected into the positive end, and then it was separated under the optimal conditions. In detail, FITC-CLB, CLB and antiserum solutions were diluted to the appropriate concentrations with PBS at pH 7.4, respectively. To perform competitive assay, 5 μl of 300 ng/ml FITC-CLB was mixed with 25 μl of 0 to 5 μg/ml CLB according to the requirement. To each above mixed solution, 5 μl of antiserum was added. After 5 min of incubation at room temperature, the samples were analyzed by CE-LIF. To construct the standard curve, a stock solution of CLB in distilled water (2 mg/ml) was diluted with PBS (pH 7.4) into concentrations of 0, 5, 20, 100, 200 and 500 ng/ml.

2.7. Electrophoresis conditions

Untreated fused-silica capillary with an inner diameter of 75 μm and total length of 60 cm (50 cm to the detector) were purchased from YongnianRuifeng Chromatographic Apparatus (Hebei, China), which was preconditioned by successively flushing with 0.1 M HCl, 0.1 M NaOH, H_2O, and running buffer for 20 min each. Electrophoresis was performed at 25 °C using 50 mM borate acid and 20 mM CAPS (pH 9.3) containing 40 mM SDS. The samples were pressure injected at 0.5 p.s.i. for 10 s (1 p.s.i. = 6894.76 Pa). The applied voltage was 30 kV. Between runs the capillary was rinsed with running buffer for 2 min.

2.8. ELISA procedures

According to the ELISA test kit's instruction, 50 μl of sample or standards (over a range of 0.1–100 ng/ml) and HRP-labeled clenbuterol (100 μl) were added to the assay wells, and the mixed solution was incubated with gentle shaking for 2 h at 20 °C. After washing with PBS, substrate/chromogen solution (3,3′,5,5′-tetramethylbenzidine/hydrogen peroxide in acetate buffer, pH 5.5, 50 μl) was added to all the testing wells, and the plate was incubated for 30 min. After this, 1.25 M sulfuric acid (40 μl) was added to end the reaction. When the microtiter reader was used, the signal was measured in each well at 450 nm.

3. Results and discussions

3.1. Choice of separation modes

Efficient separation of free tracer and its immunocomplex is of vital important in CEIA. Immune reagents adsorb to the capillary wall, which is a common problem in protein CE analysis. Several strategies have been developed to overcome these problems, including changing the pH of running buffer (<3 or >9), coating the inner surface of the capillary with a hydrophilic polymer, and using additives in the buffer solution. In this study, competitive CEIA was first tested in dynamically coated capillary and coated capillary permanently bonded with polyacrylamide. Both methods were not only increase the migration time but also undermine the separation. It may be attributed to the fact that while most coatings prevent protein adsorption, they also reduce or eliminate electroosmotic flow (EOF), which prolongs the CE separation time and increases the likelihood of immunocomplex dissociation. This phenomenon was also observed in the CE of horseradish peroxidase (HRP) and HRP-labeled Ab [23]. We finally focused on the electrophoretic migration of FITC-CLB and its immunocomplex in alkaline buffer, and got their efficient separation, as shown in Fig. 1.

3.2. Effect of incubation conditions on immunocomplex formation

In order to determine the effect of the incubation temperature and time on the immunocomplex formation, before injection into the capillary, FITC-BSA and antiserum solution were incubated for 0, 2, 5, 10, 30 and 60 min at 4, 20 and 35 °C, respectively.

The effect of the incubation temperature and the time on the immunocomplex formation was illustrated in Fig. 2. Among the three incubation temperatures, 35 °C took the shortest time to reach equilibrium. At 4 °C, higher immunocomplex yields were obtained but it took longer to reach equilibrium. After 5 min incubation, the peak area of immunocomplex did not changed at 20 °C. This indicates that equilibrium was reached after 5 min when the incubation temperature was 20 °C. There was also a report showing that reliable result can be achieved within less than 1 min incubation, and suggesting that prolonged incubation time was not necessary [24].

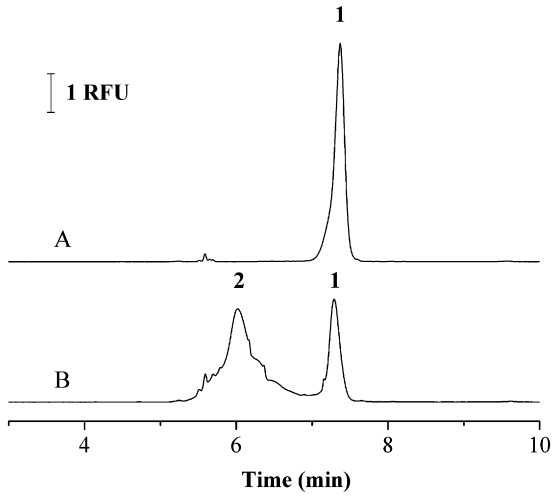

Fig. 1. Electropherograms of CLB labeled with FITC. Samples: (A) without antiserum and (B) with antiserum. Buffer: 50 mM borate and 20 mM CAPS containing 40 mM SDS at pH 9.30; injection: 0.5 p.s.i., 10 s; applied voltage: 30 kV; untreated fused-silica capillary: 60 cm length (50 cm effective length), 75 μm i.d., 360 mm o.d., at 25 °C. Peaks: 1 = free FITC-CLB, 2 = immunocomplex.

3.3. Optimization of the separation conditions

The electrophoretic separation is unfavorable to the association of immunocomplex. The differences in electrophoretic mobilities of the components in the immunocomplex cause them to move apart in the electrical field, resulting in dissociation of the immunocomplex. In this experiment, we addressed these problems by using a high as possible separation voltage to ensure enough separation efficiency and resolution.

The buffer pH is an important parameter in CEIA. The buffer with borate acid 50 mM, CAPS 20 mM at different pH was used. The applied voltage was 30 kV. As shown in Fig. 3, the resolution improved as the pH increased to 9.3. Above pH 9.6, peak broadening was obvious, and the separation time was prolonged. Therefore, the pH of the borate buffer in the subsequent experiments was chosen to 9.3.

It is a common problem in protein CE analysis that proteins adsorb to the capillary wall [19,25]. Adding SDS to the buffer has been proven to be an effective way to solve this problem

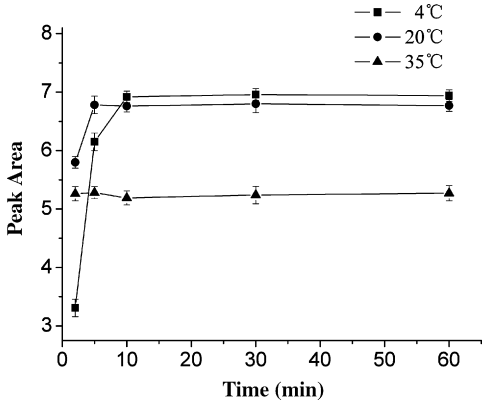

Fig. 2. Effect of incubation temperature and time on the immunocomplex formation ($n = 3$). Other electrophoresis conditions were as in Fig. 1.

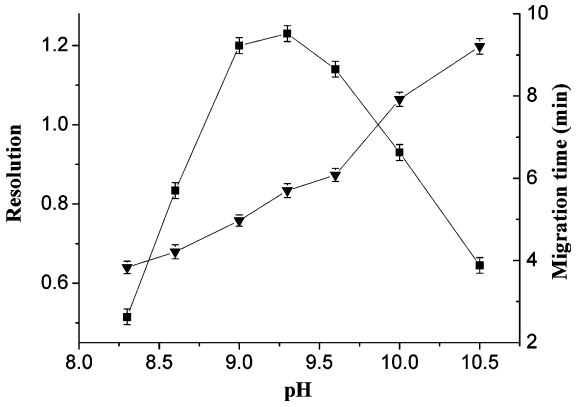

Fig. 3. Effect of buffer pH on the resolution (■) and migration time (▼) ($n = 3$). Other electrophoresis conditions were as in Fig. 1.

[20,26]. The effect of SDS concentration on the separation is shown in Fig. 4. With the use of SDS, the resolution was improved and the best separation was achieved when 40 mM SDS was added. On the other hand, with the increase of SDS concentration, migration time slightly prolonged. As the increase of SDS concentration, the current increased that would affect the efficiency and resolution. Though SDS tends to open the tertiary structure of protein, some investigators reported that antibody-antigen binding remains unaffected by up to 75 mM SDS [19,27]. Thus, 40 mM SDS was used in running buffer. The electrophoretic results demonstrated that adding SDS in the running buffer not only facilitated the separation but also enhanced the reproducibility.

3.4. Effect of hapten/protein ratio on detection

The diazo-derivative of CLB reacts with the phenol group of tyrosine residues and heterocyclic group of histidine residues present in the BSA, yielding a very stable compound. Based on literature reports, BSA contains nineteen tyrosine residues and seventeen histidine residues. In this work, two kinds of FITC labeled CLB-BSA competitors were synthesized, one with a low molar ratio of CLB to BSA (10:1) and the other with a high ratio

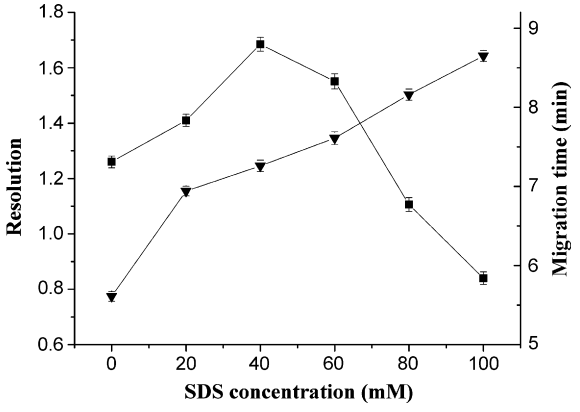

Fig. 4. Effect of SDS concentration on the resolution (■) and migration time (▼) ($n = 3$). Other electrophoresis conditions were as in Fig. 1.

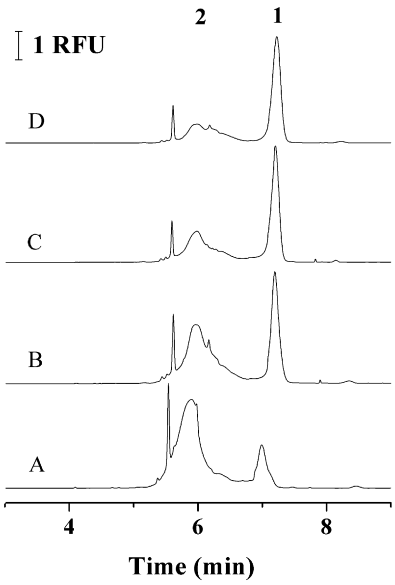

Fig. 5. CEIA-LIF profiles. Peaks: 1 = free FITC-CLB, 2 = immunocomplex. (A) 20 ng/ml; (B) 100 ng/ml; (C) 200 ng/ml; (D) 500 ng/ml. Electrophoresis conditions were as in Fig. 1.

(31:1), to study the relationship between competitor's character and the detection sensitivity. The lower detection limit for CLB was observed when the high ratio competitor (CLB:BSA = 31:1) was used. This was caused by the fact that the high ratio competitor has a weaker binding ability to antibody than the low ratio competitor [28], and thus gives a lower detection limit. This result suggests that the character of the competitor affects the sensitivity significantly, which is similar to the report by Jockers et al. [29].

3.5. Competitive immunoassay of CLB

Different amounts of CLB (from 0 to 500 ng/ml) were analyzed under the optimal CEIA conditions. The immunocomplex was well separated from the FITC-CLB. As the concentration of free CLB increased, the peak height and area of the immunocomplex in the electropherogram decreased, while that of the free FITC-CLB increased (see Fig. 5). The multiple immunocomplex peaks were due to the impurity of the polyclonal and the heterogeneous labeling of FITC or CLB to the BSA.

3.6. Validation of the method

Under the optimized conditions, a calibration curve (see Fig. 6) for CLB was constructed by plotting the peak areas of free FITC-CLB versus the CLB concentration. Although competitive assay inherently yields nonlinear calibration plots, it is possible to fit our data to linear models within the range of 1–200 ng/ml of CLB with a correlation coefficient $R = 0.989$ ($Y = 0.1339 + 0.0084X$). The detection limit was 0.7 ng/ml at three times of signal-to-noise ratio. The reproducibility (expressed as R.S.D. values of relative migration times

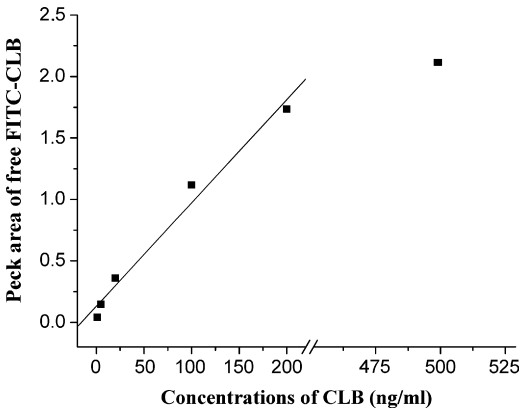

Fig. 6. Calibration curve of CLB. Electrophoresis conditions were as in Fig. 1.

Table 1
Recovery of CLB in urine ($n = 5$)

Sample concentration (ng/ml)	Detected (ng/ml)	R.S.D. (%)	Recovery (%)
5	4.4	1.2	88.0
60	56.2	0.88	93.7

Electrophoresis conditions are the same as in Fig. 1.

and R.S.D. values of relative peak areas) were calculated by analyzing standard solutions of CLB ($n = 5$). In general R.S.D. values of relative migration times (RMTs) were lower than 0.72%. The R.S.D. values of the relative peak areas (RPAs) were below 2.5%.

Since a clenbuterol-positive sample is difficult to obtain, the simulated samples were analyzed with the proposed method. Two samples, one with low concentration (5 ng/ml) and the other with high concentration (60 ng/ml) of CLB were prepared with CLB-free urine. Five repetitive experiments were made under the same conditions. The analytical results are summarized in Table 1. The recoveries of CLB for the low and high samples were 88 and 93.7% with relative standard deviation of 1.2 and 0.88%, respectively.

3.7. Comparison with ELISA analysis

Two standard samples, one with 6 ng/ml of CLB and the other with 120 ng/ml of CLB in ultra-pure water were analyzed by CE-LIF. An average of these values was taken and compared with an average value of ELISA results (see Table 2). The results indicate a good agreement between CE-LIF and ELISA assays, and suggest that the assay can be used for screening CLB analysis. The overall simplicity of the CE-LIF test coupled with no cleaning and washing steps and rapidness makes the test very attractive.

Table 2
Comparison of CLB analyses of standard samples using CE-LIF and ELISA ($n = 4$)

Sample content (ng/ml)	CE-LIF		ELISA	
	Average (ng/ml)	R.S.D. (%)	Average (ng/ml)	R.S.D. (%)
6	6.13	0.9	6.8	3.3
120	117	1.1	128	5.6

Electrophoresis conditions are the same as in Fig. 1.

4. Conclusion

A simple, accurate and sensitive method for the determination of free CLB was developed using a competitive immunoassay based on CE-LIF. CE-LIF was performed in an uncoated fused-silica capillary with a buffer solution of 50 mM borate, 20 mM CAPS buffer (pH 9.3) and 40 mM SDS. This assay had good selectivity, high separation efficiency and sensitivity. Comparing with other immunoassay methods, CEIA-LIF shows such advantages as shortening analysis time, reducing reagent consumption, and simplifying assay methodology by eliminating washing steps. It is possible to apply this method in analysis of CLB in urine and other biofluids.

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