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Determination of clenbuterol in porcine tissues using solid-phase extraction combined with ultrasound-assisted dispersive liquid–liquid microextraction and HPLC–UV detection

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ABSTRACT

A new pretreatment method, solid-phase extraction combined with dispersive liquid–liquid microextration (SPE–DLLME), was proposed in first time for the determination of clenbuterol (CLB) in porcine tissue samples. The tissue samples were firstly extracted by SPE, then its eluents were used as dispersant of the followed DLLME for further purification and enrichment of CLB. Various parameters (such as the type of SPE sorbent, the type and volume of elution solvent, the type and volume of extractant and dispersant, etc.) that affected the efficiency of the two steps were optimized. Good linearity of CLB was ranged from 0.19 μg/kg to 192 μg/kg with correlation coefficient (r^2) of 0.9995. The limit of detection (LOD) was 0.07 μg/kg (S/N = 3) and the recoveries at three spiked levels were ranged from 87.9% to 103.6% with the relative standard deviation (RSD) less than 3.9% (n = 3). Under the optimized conditions, the enrichment factor (EF) for CLB could up to 62 folds. The presented method that combined the advantages of SPE and DLLME, had higher selectivity than SPE method and was successfully applied to the determination of CLB in tissue samples.

1. Introduction

Clenbuterol (4-amino-3,5-dichloro-a-tert-butylamino-methylbenzyl alcohol hydrochloride, CLB), a representative of the class of beta-adrenergic agents, has been used as a tocolytic, bronchodilator, and heart tonics in human and veterinary medicine [1,2]. It also possesses physiological effects similar to anabolic steroids, which promotes the growth of the muscular tissue and reduction of body fat [3]. As a consequence, CLB has been extensively used as a growth promoter in feeds for farm animals to improve its lean meat yield [4]. However, its long term or high dose use had been associated with serious side effects [5,6] and acute toxic responses [7]. So CLB was prohibited to use as growth promoter for livestock in the European Union (EU), China and many other countries [8]. It illegally misused lead to the elevated level in animal tissues which had caused serious outbreaks of human poisoning in Spain, Italy, France, and China [9–13]. Therefore, simple, accurate and reliable methods for the determination of trace levels of CLB in meat products were required for the assurance of consumer healthy.

At present, among the analytical techniques for CLB residues in biological matrixes, immunoassays [14], liquid chromatography–ultraviolet detection (LC–UV) [15,16], liquid chromatography–mass spectrometry (LC–MS) [17–19], liquid chromatography–electrochemical detection (LC–ECD) [20], liquid chromatography–fluorescence detection (LC–FD) [21], gas chromatography–mass spectrometry (GC-MS) [22–24] and capillary zone electrophoresis (CZE) [25] were mainly used methods. Owing to the complexity of sample matrices and the trace levels of CLB in meat products, a suitable sample pretreatment procedure was necessary to purification the sample matrix and enrichment of the target analyte before applied for instrumental analysis. Until now, several procedures have been developed for the purification and preconcentration of CLB from sample matrices including liquid–liquid extraction (LLE) [26], solid-phase extraction (SPE) [27], diphasic dialysis [28], matrix solid-phase dispersion [29], solid-phase microextraction [4] and liquid–liquid microextraction [30]. Among them, SPE based on different sorbents such as C_{18} [31], Alumina [27], and mixed sorbents [32] was the most widely used method. However, all the above methods suffered from the low selectivity for the extraction of analytes from complex matrix. Although molecularly imprinted polymer had been applied to improve the selectivity, low recoveries of CLB did not fit the EU criteria of the C.D.2002/657/EC [24]. Selectivity was also improved by using expensive immunoaffinity sorbents, but the time-consuming

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procedure and specific conditions limited its further application [15,33]. Recently, SPE combined with an additional clean-up procedure show a potential way to purification and concentration of trace analytes from real samples [34].

Dispersive liquid–liquid microextraction (DLLME) as a new microextraction technique was developed by Assadi et al. [35,36], which was based on a ternary solvent system like homogeneous LLE and cloud point extraction. In this method, the appropriate mixture of extraction solvent and dispersive solvent was injected rapidly into an aqueous solution, resulting in a cloudy state consisting of fine droplets of the extraction solvent dispersed in the aqueous phase, which markedly increased the contact surface between phases and reduce extraction time with the increasing enrichment factors. The advantages of DLLME were simplicity, rapidity, low cost, high recovery and enrichment factors, however, the applications of DLLME in most cases were limited for water samples [37–39]. Recently, few attempts about DLLME combined with SPE and LLE to improve the selectivity of the pretreatment process and/or to reduce the limits of quantification (LOQs) for water samples were reports [40–42]. Unfortunately, its applications for complex biological matrices are not available.

The aim of this study was to present the first attempt at combining the advantages of SPE and DLLME to develop a new pretreatment method for the extraction of CLB from porcine tissues. The samples were directly extracted using SPE procedure and the eluents of SPE were used as dispersive solvent of the followed DLLME procedure for further purification and enrichment of CLB before HPLC analysis. The enrichment factor (EF) of the SPE–DLLME–HPLC method could be improved more than 62 folds. Compared with the conventional SPE procedure, the SPE–DLLME–HPLC method provided higher purification ability and selectivity.

2. Experimental

2.1. Chemicals

CLB was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China) and its molecular structure was shown in Fig. 1. Chloroform ($CHCl_3$), dichloromethane (CH_2Cl_2), 1,2-dichloroethane ($C_2H_4Cl_2$), 1,1,2,2-tetrachloroethane ($C_2H_2Cl_4$), tetrachloroethylene (C_2Cl_4), chlorobenzene (C_6H_5Cl) and tetrachloromethane (CCl_4) were obtained from Huaxin Chemical Reagent Co. (Baoding, China). Acetone, methanol, ethanol, acetonitrile, isopropanol, ammonia and hydrochloric acid were purchased from Huadong Chemical Reagent Co. (Tianjin, China). PCX cartridges and C_{18} cartridges (3.0 mL, 60 mg) were obtained from Varian Co. (Palo Alto, CA, USA). OASIS HLB (3.0 mL, 60 mg) cartridges were obtained from Sigma (Louis, MO, USA). All the other reagents used in the experiment were of the highest grade commercially available. Double deionized water was filtered through a 0.45-μm fiber membrane before using.

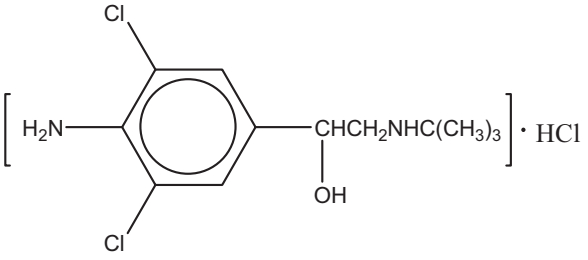

Fig. 1. Molecular structure of clenbuterol hydrochloride.

2.2. Instrumentation

HPLC analysis was performed using a Shimadzu HPLC system equipped with two LC-20AT Solvent Delivery Units, a SUS20A gradient controller, and a SPD-20A UV-VIS Detector (Shimadzu, Kyoto, Japan). An N-2000 Chromatographic workstation (Zheda Zhineng Co. Ltd., Hangzhou, China) was used as a data acquisition system. The analytical column was purchased from RStech Co., Korea (150 mm × 4.6 mm I.D., C_{18}, 5.0 μm). The mobile phase was water–methanol (64:36, v/v, containing 0.2‰ trifluoroacetic acid, pH 2.8) and its flow rate was set at 1.0 mL/min. The detection wavelength of the detector was set at 210 nm. The HSE-12D SPE Apparatus was obtained from Hengao Tech. Co. Ttd. (Tianjin, China) and a KQ3200E ultrasonic oscillator (Kunshan Instrument Co., Jiangsu, China) was set at 40 kHz and 25 °C for emulsification. Two centrifuges were obtained from Medical Devices Co. Ltd. (0406-1, Shanghai, China) and Jintan Instrument Co. (TGL-16C, Jintan, China) for phase separation.

2.3. Standard and sample solution preparation

Stock solution of CLB was prepared by dissolving an appropriate amount of the drug in methanol to get the concentration of 0.2 mg/mL and stored in dark at −4 °C and the working standard solutions of CLB (0.00096–1.92 mg/L) were prepared by gradual dilution with methanol. Fresh meat and liver samples were collected from local market of Baoding. After homogenized by a disintegrator, 1.0-g sample was put into a 5.0-mL conical tube and extracted by ultrasonic vibration for 10 min with 2.0 mL anhydrous alcohol as extraction solvent. The extraction procedure was repeated in three times and the supernatants obtained by centrifugation at 16,000 rpm for 5 min were combined together and defatted using 1.0 mL of hexane. After centrifuged at 4000 rpm for 5.0 min and removed the degreasing phase, the solution was concentrated to 1.3 mL for further SPE–DLLME.

2.4. SPE–DLLME procedure

The PCX cartridge was preconditioned with 5.0 mL methanol and hydrochloric acid (30 mmol/L) at the flow rate of 1.0 mL/min, respectively. After the sample was loaded by gravity, the cartridge was washed with 5.0 mL of water and methanol at a flow rate of 1.0 mL/min to remove the matrix interferences. Finally, the analyte was eluted into a 10-mL conical tube by 5.0 mL of methanol-ammonia solution (95:5, v/v) and concentrated to 0.5 mL at 35 °C under decompression. Finally, 5.0 mL water, 50 μL ammonia [29] and 150 μL $C_2H_2Cl_4$ were added into the eluate and then ultrasonicated for 2.0 min to get the fine cloud solution. The phase separation was performed by centrifugation at 4000 rpm for 5.0 min. The sediment phase was evaporated to dryness and reconstituted in 20 μL of mobile phase for further HPLC analysis. The extraction efficiency was calculated as the percentage of the total analyte (n_0) and the analyte (n_a) extracted to the sediment phase for HPLC analysis.

3. Results and discussion

3.1. The choice of extraction solvent

The extraction efficiency of CLB was directly related to the extraction solvent used for tissue sample. Considering the chemical properties of CLB and the previous reports [42–45], 0.1 mol/L hydrochloric acid, 0.1 mol/L perchloric acid, and anhydrous alcohol as the common solvent for extracting CLB from animal tissues were investigated respectively. The results showed that the best extraction efficiency (>96%) with less matrix interferences was obtained from anhydrous alcohol extraction system. In addition, anhydrous

alcohol had lower toxicity and could be used as protein precipitant to precipitate protein simultaneously. Therefore, anhydrous alcohol was employed as the extraction solvent in this work.

3.2. Optimized SPE procedure

The type of SPE sorbent is one of the key factors for isolation and purification of the target analytes from complex samples. PCX, C_{18} and HLB sorbents were investigated and the best recovery for CLB was obtained from PCX sorbent. The PCX sorbent revealed a unique polarity gradient surface, which caused the proteins and phospholipids in samples could not enter the center of the hydrophobic hole and the hydrophilic surface, thereby it effectively reduced the matrix interferences of tissue samples. For PCX cartridge, CLB was adsorbed strongly on the sorbents under acidic condition, which owing to it converted into the corresponding quaternary ammonium salts (NH_2RR^+) [17]. Therefore, it was necessary to keep an acidic condition to make sure CLB strongly fixed on the sorbent by connecting with the SO_3^- groups of PCX in the form of ammonium salts. Based on the previous works, water and methanol were selected as the washing solvent and its volumes were evaluated in a range of 1.0–10.0 mL for spiked samples. The recovery of CLB almost constant with clearer eluates was observed with increasing the volume of washing solvent from 1.0 to 5.0 mL and then the recovery of CLB decreased gradually with further increasing the washing solvent from 5.0 to 10.0 mL. Considering the extraction efficiency and matrix effect, 5.0 mL of washing solvent was chosen for further work. Due to the eluent of SPE also played as the dispersant in the followed DLLME procedure, methanol, ethanol, acetonitrile, acetone, and isopropanol containing varies amounts of ammonia (1.0–9.0%) as elution solvent were investigated. The results in Fig. 2 showed that the best recovery of CLB was obtained using methanol–ammonia (95:5, v/v) as the elution solvent, which was due to the ammonia could neutralize the dissociated CLB and break the electrovalent bond between CLB and PCX sorbent. Different volumes of elution solvent in a range of 1.0–9.0 mL were investigated and the result showed that the recoveries obviously increased with the volume increasing from 1.0 to 4.0 mL, and then it keep constant even further increased the volume up to 9.0 mL. Considering the extraction time, recoveries and level of CLB in samples, 5.0 mL was used as the optimized volume of elution solvent.

3.3. Optimized DLLME procedure

The performance of DLLME is mainly determined by the type and volume of the extractant, which should higher density than water, high extraction capability of the analytes, and low solubility in water. Considering the principles of DLLME and the previous works [36,38], different volume of $CHCl_3$, CH_2Cl_2, $C_2H_2Cl_4$, $C_2H_4Cl_2$, C_2Cl_4, C_6H_5Cl, CCl_4 in a range of 50–200 μL were investigated and the results in Fig. 3 showed that 150 μL $C_2H_2Cl_4$ as extractant achieved the best recovery for CLB. The volume of dispersant is another important factor to be considered in DLLME. Therefore, various volumes of methanol–ammonia dispersant (0.3, 0.5, 0.8, 1.0, 1.2 and 1.5 mL) were investigated and the results showed that the EF initially increased and then obviously decreased when the volume of dispersant more than 0.5 mL. Moreover, the extraction recovery of CLB also decreased with the increasing volume of dispersant over 0.5 mL, which caused by dispersant could increase the solubility of analyte in water and accelerate the droplets of extractant into aqueous phase. Therefore 150 μL $C_2H_2Cl_4$ as extractant and 0.5 mL methanol–ammonia as dispersant were employed for further work.

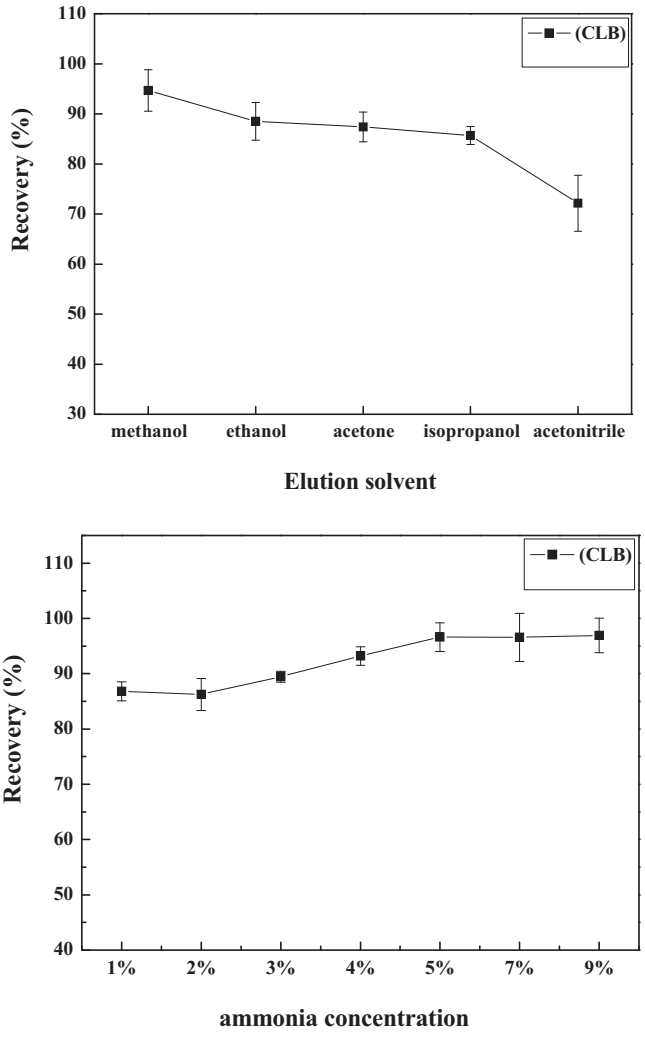

Fig. 2. Effect of elution solvent on extraction recovery of CLB.

3.4. Method validation

Under the above-optimized conditions, the SPE–DLLME–HPLC method was validated by linearity, precision, detection limit, recovery, inter-assay and intra-assay deviation. Calibration curve was constructed using the areas of the chromatographic peaks measured at nine increasing levels, in a range of 0.19–192 μg/kg. Good

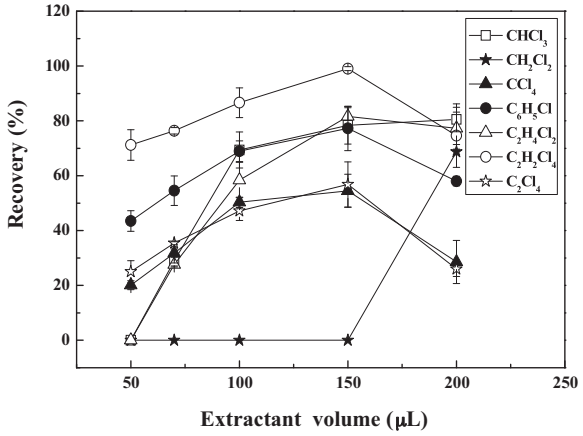

Fig. 3. Effect of DLLME extractant on extraction recovery of CLB.

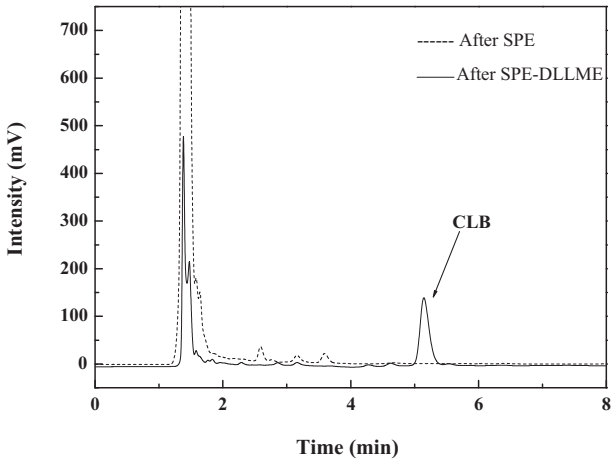

Fig. 4. Chromatograms of porcine meat sample after SPE and SPE–DLLME.

linearity was obtained for CLB throughout the concentration range and the calibration equation was $y = 7.11 \times 10^5 x - 7.13 \times 10^3$ with correlation coefficient (r^2) of 0.9995. Intra-assay and inter-assay precision expressed as the relative standard deviation (RSD) of concentrations calculated from the control samples at same day and five different days were less than 4.0% and 5.9%. Under the optimum extraction conditions, the EF of CLB was 62 folds. Based on signal-to-noise ratio (S/N) of 3, the limit of detection (LOD) was 0.07 μg/kg, which was below the maximum residue limits established by FDA and WHO. Additionally, five blank samples were extracted and analyzed by the SPE–DLLME–HPLC procedure to assessment of the potential interferences. No interfering peaks from the sample matrix were observed at the retention time of CLB, which demonstrated the good practicability of the SPE–DLLME–HPLC method. Moreover, the clearer chromatograms were observed by the SPE–DLLME procedure (compared with SPE), which indicated the developed method obviously improved the selectivity of the sample pretreatment process (Fig. 4).

3.5. Analysis of samples

Nine tissue (meat and liver) samples collected from different markets of local city were applied for validating the proposed method. All the actual samples were pretreated according to Section 2.3. One liver sample was observed trace amount of CLB at level of 0.5 μg/kg, which was below the maximum residue limits (0.6 μg/kg) established by FDA and WHO (Fig. 5). To study the effect of sample matrix and the accuracy of the SPE–DLLME–HPLC method, recovery experiments were carried out by spiking three levels of CLB in tissue samples (Table 1). The average recoveries for CLB at three spiked levels were in a range of 87.9–103.6% with SRD less than 3.9% ($n = 3$), which indicated that the method was reliable and could be used for the determination of trace CLB in tissue samples.

Table 1
Recoveries of the SPE–DLLME–HPLC method for tissue samples.

Analyte	Added (μg/kg)	Found (μg/kg)	Recovery (%)	RSD (%)
CLB	0.58	0.51	87.9	3.9
CLB	1.92	1.88	97.9	3.6
CLB	4.80	4.97	103.6	3.0

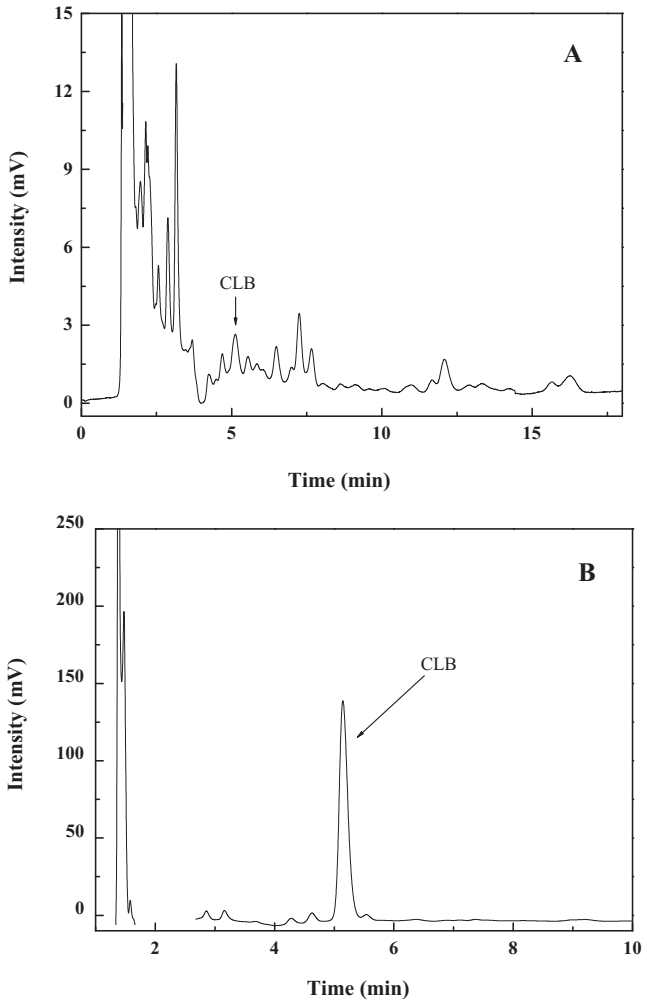

Fig. 5. Chromatograms of porcine liver (A) and spiked meat samples (B).

4. Conclusions

A new SPE–DLLME–HPLC method was developed for determination of CLB in tissue samples. The samples were firstly extracted by SPE and its eluents were used as dispersant of the followed DLLME for further purification and enrichment of the analytes before HPLC analysis. Good linearity was observed in a range of 0.19-192 μg/kg with LOD of 0.07 μg/kg (S/N = 3). The recoveries at three spiked levels were ranged from 87.9% to 103.6% with RSD less than 3.9% and the EF of 62 folds could be obtained. The proposed SPE–DLLME–HPLC method had higher selectivity and combined the advantages of SPE and DLLME, it could be used in the determination of trace analytes in biological samples.

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