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Comparison of time-resolved fluoroimmunoassay for clenbuterol residues in pig liver with enzyme-linked immunosorbent assay

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ABSTRACT

A time-resolved fluoroimmunoassay (TR-FIA) for determination of clenbuterol (CLB) in pig liver was developed. The limit of detection (LOD) was 0.02 ng g^{-1} and limit of quantification (LOQ), 0.08 ng g^{-1}. Recoveries ranged from 89.3 to 117.9% for pig liver at spiked levels of 0.1-5 ng g^{-1}. The results obtained by the TR-FIA and enzyme-linked immunosorbent assay (ELISA) showed a good correlation. The established TR-FIA was applied for screening pig liver from the local market and confirmed by gas chromatography-mass spectrometry (GC-MS). This proposed technique could be used for routine screening for drug residues.

KEY WORDS: time-resolved fluoroimmunoassay, clenbuterol, ELISA, liver, pig

INTRODUCTION

Clenbuterol (4-amino-α-[(tert-butylamino)methyl]-3,5-dichlorobenzyl alcohol hydrochloride, CLB), a sympathomimetic drug with potent β-adrenoceptor stimulating properties, can effectively prevent and reverse bronchoconstriction and has been used for the treatment of the pulmonary diseases in veterinary and clinical medicine. Some studies have reported that β_2-agonists, including clenbuterol, can promote muscle growth and reduce body fat (Hooijerink et al., 1991). Recently,

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the misuse of β_2-agonists in animal feeds and the residues of these compounds in animal tissue have drawn substantial attention. When animals are treated with a β_2-agonist, residues can accumulate in their muscles and liver, and may have a pharmacological effect in humans (Navarro-Martinez, 1990; Salleras et al., 1995). Therefore, the use of β_2-agonists in meat-producing animals is now banned.

Consequently, the development of highly specific, reliable and low-cost methods for rapid determination of clenbuterol in diverse matrices is urgently needed. Various analytical methods have been reported for detecting clenbuterol in animal tissues or urine (Polettini, 1996; Mitchell and Dunnavan, 1998). The method currently used by most laboratories is gas chromatography-mass spectrometry (GC-MS) (Ramos et al., 2003) and high performance liquid chromatography (HPLC). Ultraviolet (UV) detection is the most popular detection method applied in HPLC, but it is limited due to low sensitivity (Song et al., 2003). HPLC-MS-MS is a powerful analytical method for the detection of clenbuterol, however, the complicated and expensive instrument does not belong to the facilities of the average investigator (Van Hoof et al., 2005).

In China, inspection of animal tissues plays an important role, but it has not brought satisfactory results due to the low levels of substances in tissues and complex matrices. Recently, some studies in China were carried out on β_2-agonist residues in domestic animal food. Some surprising results were obtained, for example, some meat samples have above 0.1 mg kg^{-1} clenbuterol (CLB) residues. CLB residues are always found in pig livers in China. Developing more sensitive methods for screening animal food products for β_2-agonist residues in China is worthwhile.

While residue levels can be detected by instrumental analysis, the large number of samples and labour- and time-intensive analysis required for thorough monitoring led us to develop an alternative method, suitable for trace element testing by technically competent personnel. Immunoassay is the most suitable testing method for rapid field analysis of agrochemical residues (Dykman and Bogatyrev, 1997).

Enzyme-linked immunosorbent assays (ELISA) for laboratory analysis of clenbuterol in microwells have been developed. These laboratory assays were applied to the quantitation of clenbuterol in animal tissues. Enzyme immunoassay (EIA) methods have their disadvantages. The operation is quick and simple, but the limit of detection of the present colorimetric ELISA is about 0.1 ng g^{-1} (Xu et al., 2005).

Time-resolved fluoroimmunoassays (TR-FIAs) have been utilized in the analysis of veterinary drugs (Elliott et al., 1994; Tuomola et al., 2002). In this paper, we developed and validated a novel, rapid, more sensitive, trace method for CLB residue screening of pig liver tissues by using TR-FIA and compared it with ELISAs.

MATERIAL AND METHODS

Reagents and apparatus

Clenbuterol hydrochloride (CLB), ovalbumin (OVA), and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO, USA) and goat anti-rabbit IgG, acetonitrile and methanol from Boao Biotech Co., Ltd. (Shanghai, China). Dry N, N-dimethylformamide was obtained from Fluka (Buchs, Switzerland), Germall II and intrinsically fluorescent europium chelate [2,2′,2″,2‴({ 4 - [(4 - isothiocyanatophenyl) ethynyl] pyridine-2,6-diyl}bis- (methylenenitrilo)) tetrakis (acetato)]-europium(III) from the Chinese Academy of Inspection and Quarantine (Beijing, China). DELFIA Assay Buffer, Wash Solution, which is intended for *in vitro* use with the DELFIA® Platewash or the AutoDELFIA Automatic Immunoassay System after dilution with distilled water, Enhancement Solution, and Victor 1420 Multilabel Counter were obtained from PerkinElmer Life and Analytical Sciences (Shanghai, China). Non-coated, yellow (UV-quenched, low-fluorescence) MaxiSorp microtitration wells were purchased from Nunc A/S (Roskilde, Denmark). N,O-*bis*(trimethylsilyl) trifluoroacetamide (BSTFA), 0.1 ml ampules (lot: LB 10799), was obtained from Supelco Inc.(Oakville,ON,USA). Other reagents were of analytical quality and were supplied by Shanghai Reagent Corporation (Shanghai, China).

Preparation of immunosensitive microtitration wells

The microtitration wells were coated with activated anti-rabbit IgG (120 µl per well) and incubated overnight at room temperature in a humid environment. The wells were washed twice with DELFIA Wash Solution and then saturated with 50 mM NaH_2PO_4, pH 4.5, containing, 6 sorbitol, 0.1 BSA and 0.05% Germall II (250 µl per well). After overnight incubation at room temperature in a humid environment, the wells were aspirated to dryness and stored in sealed packages with desiccant at 4°C.

Preparation of labelled analyte

The CLB-BSA conjugate was labelled overnight at room temperature with a 50-fold molar excess of europium chelate in a volume of 1 ml in 50 mM carbonate buffer (pH 8.5, 50 mM Na_2CO_3-$NaHCO_3$, 0.155 M NaCl). The conjugate was purified by gel filtration on a Sepharose CL-6B column (Boao Biotech Co., Ltd., Shanghai, China) with 50 mM Tris-HCl, pH 7.8, containing 0.9% NaCl, 0.05% NaN_3. Fractions of 1.0 ml were collected. The fractions from the first peak with the highest labelled analyte counts were pooled to form the label stock and characterized.

Sample pretreatment

The stock solution of CLB (1 mg ml^{-1}) was prepared in methanol. The stock solution was stable for at least 3 months when stored at 4°C. The working solutions were prepared daily in deionised water.

Pig liver was collected from the lab farm of our university and known to have been reared under CLB-free conditions. These tissues were stored at -40°C until required. Into a glass universal (25 ml) finely chopped tissues (2.5 g) were weighed and acetonitrile (7.5 ml) added, and spiked with appropriate concentrations of CLB when necessary. The samples were homogenized for 30 s and 1 M HCl (2.5 ml) added. Petroleum ether (5 ml) was added and the samples mixed for 15 min using a mixer, followed by centrifugation at 3450 *g* for 10 min. The ether layer was discarded and the liquid phase was decanted into a glass test-tube and reduced to approximately 5 ml under a gentle stream of nitrogen on a Driblock and sample concentrator at 100°C. The reduction took approximately 20 min. The supernatant was transferred to a clean test-tube and NaOH (5 M, 3 ml) added. Dichloromethane (10 ml) was added and the samples mixed using an end-over-end mixer for 15 min, followed by centrifugation at 3450 *g* for 10 min. The extracts were passed through phase separation columns (6 ml) and the dichloromethane layer was collected in clean test tubes. The dichloromethane layer was evaporated to dryness (approx. 10 min) under nitrogen, using a Driblock and sample concentrator at 80°C, resuspended in ethanol (250 µl) and vortexed for 15 s. Finally, DLEFIA dilution buffer (2250 µl) was added.

Time-resolved fluoroimmunoassay for CLB

Anti-CLB monoclonal antibodies were produced using the hybridoma technique (Xu et al., 2005). The CLB antibody was diluted to a suitable working titer with DELFIA Assay Buffer and attached to the anti-rabbit IgG coated microtitration wells in a volume of 50 µl per well. After a 1-h incubation, the wells were washed four times with DELFIA Wash Solution. The extracted samples (25 µl per well) and labelled analyte (25 µl per well), which was diluted to an appropriate concentration with 100 mM phosphate buffer, pH 7.2, containing 0.4% BSA, 0.05% NaN_3 and 20 µM DTPA, were added to the wells and incubated for 30 min. The wells were washed six times, after which 200 µl of DELFIA Enhancement Solution was dispensed to each well. After a 15-min incubation period, the fluorescence signal was measured with a Victor 1420 Multilabel Counter using the default settings for europium measurement.

Determination by GC-MS

The sample and standards were evaporated to dryness at 55°C under a mild stream of nitrogen and dissolved in 100 µl toluene (votexed for about 10 s). Then 100 µl BSTFA were added (votexed for about 10 s), reacted at 80°C for 1 h to produce trimethylsilyl (TMS) derivatives of CLB. After cooling to room temperature, 300 µl toluene were added (votexed for about 10 s) for GC-MS analysis.

A Perkin-Elmer AUTOSYSTEMXLGC with autosampler system was coupled to a Perkin-Elmer TURBOMASS quadrupole mass spectrometer. Chromatographic separation was performed using a capillary column (HP-5MS; cross-linked 5% phenyl-methylpolysiloxane; column length (30 m×0.25 mm with a 0.25 µm film thickness) from Agilent Technologies (Palo Alto, CA). The GC temperature program was as follows: initial temperature was 110°C for 1 min, then the temperature was programmed to rise by 30°C min^{-1} to 210°C, and then increased at 5°C min^{-1} to a final temperature of 280°C, which was held for 4 min. The injector temperature was set at 250°C. Helium was used as carrier gas at a flow rate of 1.3 ml min^{-1}. The interface temperature was 280°C, and the ion source temperature was 230°C. Samples (1 µl) were injected in the splitless mode.

The mass instrument was operated in the electronic impact ionization mode. Scan range: 50-550 amu; all data for quantification were collected in the selected ion monitoring (SIM) mode at *m/z* 86, 243, 262 and 277 for CLB.

RESULTS AND DISCUSSION

Cross-reactivity. The specificity of the MAb was evaluated using similar compounds structurally related to CLB and the results are presented in Table 1.

Table 1. Cross-reactivity(CR) of MAb to CLB and its analogs

Compound	CR, %
Clenbuterol	100
Cimaterol	1.0
Salbutamol	0.8
Terbutaline	0.2
Epinephrine	<0.1
Norepinephrine	<0.1
Fenoterol	<0.1
Ambroxol	<0.1
Phenylbutazone	<0.1
Isoproterenol	<0.1
Pirbuterol	<0.1
Adrenaline	<0.1
Fenoterol	<0.1

The MAb exhibited high cross-reactivity (CR) with CLB. Therefore, it was concluded that the MAb was specific to CLB and could be used in immunoassays for CLB screening of animal tissues.

Reproducibility and accuracy. The standard curve for CLB prepared in the assay buffer is illustrated in Figure 1. Recovery experiments were conducted to evaluate the accuracy and precision of the TR-FIA. Recoveries were determined using blank pig liver samples fortified with 0.1, 1, and 5 ng g^{-1} CLB. Results of the recovery experiments are shown in Table 2. The average recoveries from pig liver tissues were 89.3-117.9%. The intra- and inter-assay coefficients of variation were less than 11.3 and 14.5%.

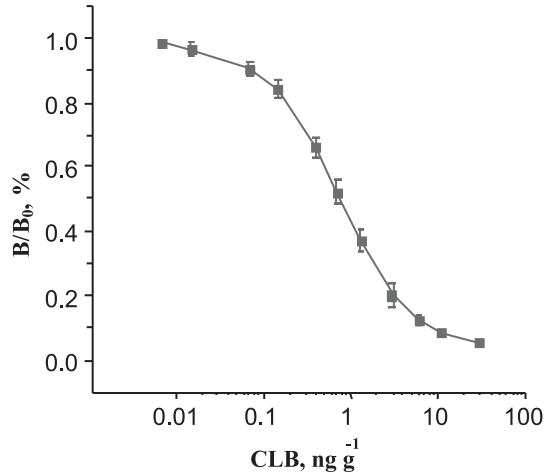

Figure 1. The standard curve for CLB by TR-FIA under optimized conditions

Table 2 Accuracy and precision of the TR-FIA CLB in spiked pig liver samples

Sample	Added ng g^{-1}	Recovery %, n=6	Intra-assay CV, %, n=6	Inter-assay CV, %, n=6
Pig liver	0.1	89.3	11.3	14.5
	1	117.9	10.3	13.1
	5	105.4	7.8	8.3

LOD and LOQ. The limit of detection (LOD), defined as mean + 3 × SD for CLB was determined by repeated analysis (n=6) of CLB-free pig liver samples. The LOD was 0.02 ng g^{-1}. The LOQ was 0.08 ng g^{-1} for pig liver tissues.

The performance of the new TR-FIA method was compared with that of ELISA kits using CLB-spiked samples (n=25). CLB concentrations measured by the TR-FIA and the ELISA were comparable. Linear regression analysis showed good correlation, with r^2 values of 0.97 for pig liver.

Comparison with GC-MS. To further demonstrate reliability of the TR-FIA for the determination of CLB residues in animal tissues, 15 pig liver samples from local food markets were analysed using the TR-FIA and GC-MS methods. CLB was found in 5 out of 15 pig liver samples by both methods. CLB concentrations in these samples are presented in Table 3 and the trend line with R^2=0.89 indicating that the TR-FIA and GC-MS results are more in agreement and show a higher degree of correlation.

Table 3. CLB concentrations in pig liver samples purchased from local food markets (n=6)

Sample	CLB, ng g^{-1}, mean ± SD	
	TR-FIA	GC-MS
1	0.7 ± 0.1	0.5 ± 0.2
2	4.6 ± 0.3	2.1 ± 0.4
3	5.8 ± 0.6	4.3 ± 0.3
4	1.9 ± 0.3	1.5 ± 0.4
5	6.3 ± 0.7	4.9 ± 0.6

CONCLUSIONS

In this study, a simple and rapid method for the analysis of low levels of CLB residues in pig liver tissues were established. The TR-FIA method offers significant improvement in sensitivity compared with conventional ELISA. Analysis of fortified and field samples demonstrated that the TR-FIA method is suitable for screening for CLB residues in animal tissues. This sensitive and stable immunoassay could be applied as a rapid screening method in routine drug residue analysis.

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