Food Control 34 (2013) 725–732

Contents lists available at SciVerse ScienceDirect

Food Control

journal homepage: www.elsevier.com/locate/foodcont

Development of an immunochromatographic assay for rapid and quantitative detection of clenbuterol in swine urine

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ARTICLE INFO

Article history:
Received 29 March 2013
Received in revised form
10 June 2013
Accepted 12 June 2013

Keywords:
Immunochromatographic assay
Rapid and quantitative detection
Clenbuterol
Swine urine

ABSTRACT

A lateral flow strip test was developed for quantitative detection of clenbuterol (CLE) in swine urine in a 10 min total assay time. This new quantitative system for CLE was developed based on the concept of using the ratio of the optical densities (ODs) of the test line (A_T) to that of the control line (A_C) to offset the inherent heterogeneity of lateral flow test strips ($LFTS_{AT/AC}$) and the effect of sample matrix. Various parameters such as immunoreaction time, the concentration of analyte, reaction temperature and pH of the reactions, as well as the ionic strength were optimized in urine samples. The assay kinetics based on the immunological reactions indicated achievement of constant signal within 5–10 min of incubation depending upon the CLE concentration. Using the optimized parameters, the linear range for the quantitative detection of CLE using the $LFTS_{AT/AC}$ was between 100 pg/mL and 2500 pg/mL over a 10 min total assay time. The CLE $LFTS_{AT/AC}$ showed a half maximal inhibitory concentration (IC_{50}) of 460 ± 60 pg/mL ($n = 5$) and a reliable coefficient of correlation ($R^2 = 0.996$). The CLE test strip exhibited insignificant cross-reaction (Cr) to mabuterol, isoproterenol, salbutamol, terbutaline, brombuterol, ractopamine and bambuterol. The limit of detection (LOD) for swine urine was 220 pg/mL, and recoveries for spiked sample were 102.35% ± 7.66% at 500 pg/mL, 95.07% ± 7.14% at 1000 pg/mL and 100.65% ± 10.21% at 1500 pg/mL, respectively. The intra-and inter-assay precision at 500–1500 pg/mL CLE concentrations showed coefficients of variation (CVs) at <11%. This rapid quantitative $LFTS_{AT/AC}$ system for CLE in spiked urine samples exhibited a coefficient of correlation with traditional ELISA at 0.97 ($N = 50$). Furthermore, 96% of 90 samples gave a percent recovery of 100 ± 30%. These results indicated that the 10-min colloidal gold-based $LFTS_{AT/AC}$ system is adaptive, sensitive, reproducible, and comparable to traditional ELISA method with the added advantage of being easy to use, inexpensive, and requires non-skilled personnel. The $LFTS_{AT/AC}$ holds promise for field use an on-site detection of CLE in swine urine samples.

1. Introduction

Clenbuterol (CLB) is a beta-adrenergic drug and typically employed as bronchodilator for treatment of asthma patients. CLB enhances the lean meat/fat ratio and increases the efficiency of feed conversion by inhibiting fat synthesis, improving muscular mass, and decreasing adipose tissue deposition in livestock production at a dose ten to hundred times higher than clinical dosage. Thus, CLE is also called as a "leanness enhancer" and illegally used as a feed additive in meat industry. The CLE residues in animal tissues can cause acute poisoning in consumers, especially in the cardiovascular and central nervous systems (Hoey, Matthews, Badran, Pegg, & Sillence, 1995). Hence, many countries including China, the United States and most European countries have forbidden the use of CLB as feed additives.

Various methods have been developed for the confirmation or screening of the presence of CLE in different biological matrices. The common detection methods include liquid chromatography (Choi et

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http://dx.doi.org/10.1016/j.foodcont.2013.06.021

al., 2004; Hendricus, Robert, Willem, & Dirk, 1991), liquid chromatography coupled with mass spectrometry (Crescenzi, Bayoudh, Cormack, Klein, & Ensing, 2001; Thevis, Thomas, Thomas, & Schanzer, 2005), gas chromatography coupled with mass spectrometry (Bocca, Fiori, Cartoni, & Brambilla, 2003) and capillary electrophoresis with electrochemical detection (Wang, Zhang, Wang, Shi, & Ye, 2010). These methods are very specific, sensitive and widely used but are unsuitable for on-site or real-time monitoring of CLE. Furthermore, these methods use expensive instruments, require complicated and tedious sample pre-treatment, and involve time-consuming procedures (Shen et al., 2006). Although some new screening methods, such as electrochemical methods (Abera & Choi, 2010; Liu et al., 2011), surface plasmon resonance (Bao, Wei, Meng, & Liu, 2012) and surface-enhanced Raman scatter immunoassay (Zhu, Hu, Gao, & Zhong, 2011) have been established to detect CLE residues, the enzyme-linked immunosorbent assay (ELISA) and colloidal gold immunochromatographic assay (CGIA) are still most successful owing to their stability, selectivity, and low cost. ELISA is used for CLE detection because of its high sensitivity and ability for quantification (Degand, Duyckaerts, & Rogister, 1992). However, this method has some disadvantages, such as time-consuming process (60–90 min), requires skilled personnel, and detection instrument making it unsuitable for on-site detection. Compared with ELISA, CGIA is one of the most preferred real-time analytical tools because of simplicity, rapidity (5–10 min), selectivity and cost effectivity. Many research groups have developed CGIA methods and many commercial products are available for the rapid detection of CLE in urine or tissue samples (Vanoosthuyze, Arts, & Van Peteghem, 1997; Zhang et al., 2006, 2009). However, the results of these strips are purely qualitative (positive or negative results only) because the results are based on the visual appearance of the test line that is dependent upon a range of analyte level. These types of strip tests are less sensitive and cannot be used for low level CLE.

In recent years, a higher number of quantitative strip assays have been reported based on the use of strip readers for rapid determination of CLE and enrofloxacin residue (Zhang et al., 2006; Zhao et al., 2008). Fluorescent silica nanobead and quantum dot based quantitative fluorescent strips have also been established for CLE and trichloropyridinol residue detection (Li et al., 2011; Zou et al., 2010). There reported quantitative methods were achieved by recording only the optical density (OD) of signals established on the test lines. However, the ODs on the test and control lines change not only with the concentration of analytes, but also with the immunoreaction time, operation temperature, and difference between batches of strips (Yang et al., 2011). Furthermore, pH value, ionic strength and matrix of samples also influenced the efficiency of the immunoreaction between the antibody and antigen (Li et al., 2009). Thus, the test results determined only by the OD of test lines were not very accurate, are irreproducible, and semi-quantitative at best. Thus, we worked on the development of a better quantitative strip test for CLE.

Researchers have found that the ratio of the ODs of the test line (A_T) to that of the control line (A_C) can effectively offset the effects of parameters such as the inherent heterogeneity of test strips and the matrix containing the samples (Ahn et al., 2003). Based on this concept, a few research groups have successfully developed strip reader based quantitative assays for detection of alpha fetoprotein in human serum (Yang et al., 2011), prostate specific antigen in serum, hepatitis B surface antibody in serum, fumonisins in maize (Anfossi et al., 2010) and deoxynivalenol in the cereal (Liu, Zanardi, Powers, & Suman, 2012). Based on these reports, we hypothesized that the same concept can be used for the development of a rapid, quantitative, reproducible, and easy to use CGIA strip test for CLE that can be used for on-site monitoring.

In this study, we developed a quantitative CGIA method for the rapid, easy to use, reproducible, inexpensive and quantitative detection of low concentration of CLE. A portable optical strip reader was developed for possible on-site detection of CLE residues in swine urine. The effects of immunoreaction time, operation temperature and sample conditions during the quantitative assay were analyzed. The performance of the proposed CGIA quantitative $LFTS_{AT/AC}$ method for CLE in terms of the analyte recovery, limit of detection (LOD), detection range and precision were evaluated. The reliability of the $LFTS_{AT/AC}$ was further compared with a commercially available ELISA kit.

2. Materials and methods

2.1. Reagents and equipment

$HAuCl_4 \cdot 3H_2O$, citric acid, CLE, ractopamine, salbutamol, mabuterol, terbutaline, brombuterol, bambuterol and isoproterenol were purchased from Sigma–Aldrich (St. Louis, MO, USA). The sample pad, conjugate pad, nitrocellulose membrane and absorbent pad were obtained from Schleicher and Schuell GmbH (Dassel, Germany). Goat anti-mouse IgG was obtained from Beijing Zhongshan Biotechnology Inc. (Beijing, China). Anti-CLE monoclonal antibody, CLE–BSA conjugates (the molar ratio of CLE–BSA was 15:1) and the ELISA kit for CLE were provided by Wuxi Zodoboer Biotech. Co., Ltd. (Wuxi, China). The BioDot XYZ platform combined with a motion controller, BioJet Quanti3000k dispenser and AirJet Quanti3000k dispenser for solution dispensing were supplied by BioDot (Irvine, CA, USA). The vacuum drying oven was purchased from Shanghai Sumsung Laboratory Instrument Co., Ltd. (Shanghai, China). Other reagents were of analytical grade and purchased from Sinopharm Chemical Corp. (Shanghai, China). Aqueous solutions for the immunoassay experiments were prepared using 18 MΩ water (purified by Elix-3 and Milli-QA, Millipore, Molsheim, France).

2.2. Swine urine samples

Swine urine samples ($N = 120$), which were ascertained to be free of twelve β-agonists consisting of CLE, ractopamine, salbutamol, mabuterol, terbutaline, brombuterol, cimbuterol, bambuterol, cimaterol, tulobuterol, penbutolol and isoproterenol that were established by LC–MS/MS, were collected from Jiangxi-40, Zhejiang-30, Guangdong-20, Jiangsu-15 and Henan-15 Provinces of China. The blank urine mixture was obtained by combining 40 randomly selected CLE free urine samples and stored at −20 °C for further use. To evaluate the accuracy of the system, 90 samples were spiked with CLE concentrations from 200 pg/mL to 900 pg/mL in 120 randomly selected CLE free urine samples. All urine samples were centrifuged at 8000 *g* for 5 min to remove any precipitation before a CLE $LFTS_{AT/AC}$ test.

2.3. Preparation of colloidal gold

Colloidal gold with an average diameter of 30 nm was produced by reduction of gold chloride trihydrate with 1% trisodium citrate as described previously (Hayat, 1989). Briefly, 100 mL of 0.01% gold chloride trihydrate solution in 18 MΩ water was heated to boiling and 1.0 mL of 1% sodium citrate solution was added under constant stirring. When the color of the mixture changed to red purple, the solution was kept at boiling temperature for another 10 min. The colloidal gold solution obtained was supplemented with 0.01% (w/v) sodium azide and stored at 4 °C for further use.