Talanta 114 (2013) 160–166

Contents lists available at ScienceDirect

Talanta

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

$Ru(phen)_3^{2+}$ doped silica nanoparticle based immunochromatographic strip for rapid quantitative detection of β-agonist residues in swine urine

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ARTICLE INFO

Article history:
Received 9 January 2013
Received in revised form
31 March 2013
Accepted 6 April 2013
Available online 12 April 2013

Keywords:
Salbutamol
Test strip immunoassay
Fluorescent nanoparticles
Quantitative detection

ABSTRACT

A $Ru(phen)_3^{2+}$ doped silica nanoparticle based immunochromatographic strip was developed for the rapid and quantitative detection of five common β-agonist (salbutamol (SAL), cimbuterol, terbutaline, clenbuterol, and brombuterol) residues in swine urine. The broad spectrum monoclonal antibodies generated by immunizing BALB/c mice with salbutamol conjugated cationic bovine serum albumin. The fluorescence intensities (FIs) of the strip on the test line (FI_T) and control line (FI_C) were determined using a strip reader. Parameters that influenced the antibody and antigen interaction on the test strip were investigated by recording FI_T and FI_C values, and the concept of FI_T/FI_C ratio was used to offset the inherent heterogeneity of the test strips and the effect of the sample matrix. Under optimal conditions, the linear range for the quantitative detection of SAL was 0.6–5.0 ng/ml with a half maximal inhibitory concentration at 1.78 ng/ml. The limit of detection for real swine urine was 0.43 ng/ml. The recovery rates of the intraassay for spiked urine at SAL concentrations of 0.8, 1.5, and 3.5 ng/mL were 88.06% $\pm$ 3.75%, 95.77% $\pm$ 5.33%, and 94.06% $\pm$ 7.43%, whereas those for the interassay were 84.69% $\pm$ 5.0%, 95.06% $\pm$ 9.3%, and 88.34% $\pm$ 7.71%, respectively. The developed quantitative method exhibited excellent agreement with a commercially available competitive enzyme-linked immunosorbent assay kit for SAL-spiked urine samples, with a correlation of coefficient of 0.95 and a slope of 0.99 ($n = 36$). The results indicated that the developed test strip enables sensitive, reproducible, and easily implementable screening for the rapid and quantitative detection of β-agonist residues in swine urine.

1. Introduction

β-agonist residues in animal tissues can increase toxicological risks to consumers [1,2]. Thus, many classical confirmatory methods, including liquid chromatography [3–7], gas chromatography [8,9], liquid chromatography–mass spectrometry [10–13], and gas chromatography–mass spectrometry [14–16], have been developed to monitor the illicit use of such residues. These methods are highly sensitive, reliable, and widely used, but require extensive sample preparation and skilled analysts to operate complicated instruments. Moreover, these techniques entail high costs and time-consuming procedures [17].

An immunochromatography test strip (ICTS) is a popular screening tool for conducting onsite testing because of its acceptable sensitivity, user-friendliness, and rapidity (5–10 min). Gold nanoparticles, a type of metal nanoparticle-based sensors, are popular tools for detecting various types of β-agonists [18]. However, its performance depends on the amount of molecules gathered; it is also susceptible to optical interference, thereby exhibiting relatively low sensitivity and inaccurate qualitative or semiquantitative results. Quantum dots and dye doped nanoparticles are representative fluorescent nanoparticle probes of increasing research focus [19,20]. Dye doped nanoparticles vary in diameter from 2 to 200 nm, contain a large quantity of dye molecules embedded in a polymer or silica matrix, and emit more intense fluorescence signals than do organic fluorophores [21]. Their excellent brightness makes them especially suitable for trace bioanalysis without additional reagents or signal amplification steps [22]. Compared with polymer nanoparticles, fluorescent silica nanoparticles (FSNPs) possess several advantages, such as easy surface modification and solution treatment processes, and simple separation through centrifugation during particle preparation because of the high density of silica [21]. Silica nanoparticles are more hydrophilic and biocompatible; they do not suffer from microbial degradation and maintain their stable structure even with environmental changes (e.g., changes in pH). Thus, FSNPs

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http://dx.doi.org/10.1016/j.talanta.2013.04.013

present promising potential for use in various biological applications and are predicted to be ideal reporters for fluorescence-based ICTS because of the aforementioned advantages and excellent photo-physical properties.

In this study, $Ru(phen)_3^{2+}$ doped silica nanoparticles as fluorescent reporters were introduced into an immunochromatographic test strip for rapid, sensitive, quantitative, and broad spectrum screening detection of five common β-agonist (salbutamol (SAL), cimbuterol, terbutaline, clenbuterol, and brombuterol) residues in swine urine. The effects of immunoreaction time, pH, and ionic strength on the antibody and antigen interaction of the strip were analyzed. The fluorescence intensities (FIs) of the test line (FI_T) and control line (FI_C) were measured and the ratio of FI_T/FI_C was set to offset the interference from sample matrix and inherent heterogeneity of the test strips [19,23]. The performance of the proposed quantitative method, including its half maximal inhibitory concentration (IC_{50}), limit of detection (LOD), detection range, precision, and reliability, were evaluated. Experimental results revealed that the $Ru(phen)_3^{2+}$-doped silica nanoparticle-based immunochromatographic test strip has satisfactory sensitivity, as well as acceptable accuracy and precision.

2. Experimental

2.1. Reagents and materials

All β-agonists, namely salbutamol (SAL), cimbuterol, terbutaline, clenbuterol, cimaterol, ractopamine, mabuterol, bambuterol, tulobuterol, clorprenaline, penbutolol, and brombuterol; 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl); N-hydroxysulfosuccinimide (NHSS); bovine serum albumin (BSA); Freund's complete adjuvant (FCA); and Freund's incomplete adjuvant (FIA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Cationic BSA (CBSA) was provided by ShiYi Biotechnology, Inc. (Shanghai, China). Goat-anti-mouse IgG and the enzyme-linked immunosorbent assay (ELISA) kit for SAL were provided by Wuxi Zodoboer Biotech. Co., Ltd. (Wuxi, China). Dichlorotris (1, 10-phenanathroline) ruthenium (II) hydrate ($Ru(phen)_3^{2+}$) was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Tetraethoxysilane (TEOS) was obtained from Tiantai Chemical, Int. (Tianjin, China) and distilled under reduced pressure before use. The nitrocellulose membrane, sample pad, conjugate release pad, and absorbent pad were obtained from Millipore (Bedford, MA, USA). Ultra-pure water was prepared by Elix-3 and Milli-QA (Molsheim, France). All other reagents are of analytical grade and purchased from Sinopharm Chemical Corp. (Shanghai, China).

Swine urine samples, ascertained to be free of SAL, cimbuterol, terbutaline, clenbuterol, and brombuterol by liquid chromatography–tandem mass spectrometry (LC-MS/MS), were obtained from different local farms. The blank urine mixture was obtained by combining 20 randomly selected urine samples and stored at −20 °C for later use. All swine urine samples used for the strip analysis were centrifuged at 8000*g* for 5 min to remove any precipitate. SAL stock solution was prepared by dissolving 1.0 mg SAL in 1.0 ml methanol. The fortified urine samples (0–5 ng/ml of SAL) were prepared by spiking a stock solution into a blank-mixture urine sample.

2.2. Apparatus

The microplate reader (DNM9602) was from Perlong Instruments, Ltd. (Beijing, China). The Bio-Dot XYZ platform and CM 4000 cutter were invented by Bio-Dot (Irvine, CA).

2.3. Preparation of immunogen and coating antigen

The SAL succinate was synthesized by the mixed anhydride method as previously described with minor modifications [24], and the immunogen of the SAL–CBSA conjugate was prepared by an ester activation method [25]. In brief, 20 mg SAL in 4.0 ml anhydrous ethanol was mixed with 9 mg succinic anhydride in 1 ml anhydrous toluene. After the mixture was stirred at room temperature for 2 h, the carboxyl SAL was isolated by centrifugation at 8000*g* for 10 min and washed with anhydrous ethanol three times. Then, 5 mg of carboxyl SAL, 2.5 mg of EDC·HCl, and 2.0 mg of NHSS were mixed with 20 mg of CBSA and BSA in 2 ml of 0.01 M phosphate buffer saline (PBS, pH 5.5), and stirred at 4 °C overnight. The SAL–CBSA and SAL–BSA conjugates were dialyzed against 0.01 M in pH 7.4 PBS to remove free hapten.

2.4. Production of anti-SAL monoclonal antibodies

Four eight-week old female BALB/c mice were subcutaneously injected at multi-points with SAL–CBSA conjugates. The first dose consisted of 100 μg of immunogen in 0.1 ml PBS and mixed with an equal volume of FCA. Four subsequent injections were performed every 3 weeks with the same dosage of immunogen emulsified in FIA. Ten days after the fifth immunization, the sensitivity of the antiserum was determined by an indirect competitive ELISA (icELISA). The mouse with serum that showed the highest inhibition was given the sixth boost injection. Fusion was performed on the third day after the last injection. Twelve days later, the culture supernatant was tested with ciELISA to screen antibody-producing cells. Stable hybridomas-secreting anti-SAL monoclonal antibodies (anti-SAL mAbs) were selected and cloned by limited dilution. Ascitic fluid was produced in paraffin-primed BALB/c mice. Antibodies were purified from this fluid using ammonium sulfate precipitation [26].

2.5. Preparation and characterization of FSNPs

The FSNPs were prepared according to a previously reported procedure [27,28]. Briefly, 2.4 ml TEOS was added to an ethanol solution (30 ml) containing 1.0 ml ammonia and 1.125 ml pure water. The reaction mixture was kept at 25 °C and stirred for 12 h; 0.5 ml of $Ru(phen)_3^{2+}$ ethanol solution (1 mg/ml) was added at 3 h reaction time. The $Ru(phen)_3^{2+}$ doped silica nanoparticles were further functionalized with the amino group by adding 0.3 ml of (3-aminopropyl) triethoxysilane under vigorous stirring for 12 h. The resultant nanoparticles were isolated by centrifugation at 15,000*g* for 10 min and washed with 10 ml ethanol three times. The $Ru(phen)_3^{2+}$ doped SiO_2–NH_2 was resolved in 15 ml DMF solution and then added dropwise to 20 ml of 0.1 M succinic anhydride. The mixture was stirred for 24 h. The carboxyl group modified $Ru(phen)_3^{2+}$ doped silica nanoparticles (FSNPs) were cleaned as described above and kept in pure water for later use. Transmission electron microscopy (JEM-2100, JEOL, Japan) was performed to characterize the diameter of the FSNPs.

2.6. Preparation and characterization of mAb-labeled FSNPs

The mAb-labeled FSNPs (FSNPs–mAbs) were prepared according to previous methods with some modifications [29]. Briefly, 1 mg of FSNPs, 0.75 mg of EDC·HCl, and different volumes (30, 40, and 50 μl) of anti-SAL mAbs (1.0 mg/ml) were added to 2 ml of 0.02 M PB solution (pH 5.0) with a magnetic stirrer. After reaction at room temperature for 2 h, the mixture was blocked with 200 μl of 10% BSA (w/v) for 30 min and then separated by centrifugation at 7500*g* for 5 min. The FSNPs–mAbs were washed twice with 2 ml of 0.02 M PB (pH 5.0) and then resuspended in a 200 μL solution containing