Analytica Chimica Acta 927 (2016) 64–71

Contents lists available at ScienceDirect

Analytica Chimica Acta

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

A sensitive and semi-quantitative method for determination of multi-drug residues in animal body fluids using multiplex dipstick immunoassay

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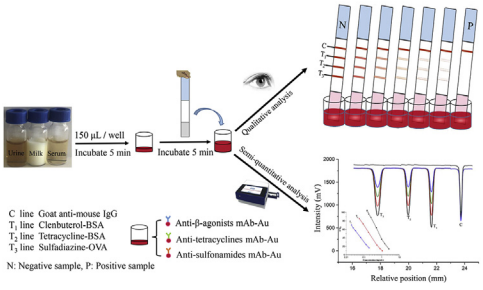
HIGHLIGHTS

- A multiplex dipstick immunoassay was developed to detect 3 families of antibiotic residues in milk, urine, and serum.
- Samples did not require pretreatment and could be directly analyzed within 10 min.
- The method showed high sensitivity and specificity with false positive ≤5%.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 14 March 2016
Received in revised form
28 April 2016
Accepted 1 May 2016
Available online 4 May 2016

Keywords:
Multiplex dipstick immunoassay
β-Agonists
Sulfonamides
Tetracyclines
Body fluids

ABSTRACT

The objective of this research was to develop a multiplex dipstick immunoassay method for the simultaneous determination of multi-veterinary drug residues, such as β-agonists, sulfonamides, and tetracyclines in milk, urine, and serum. The multiplex dipstick assay format was based on an indirect competitive approach: Three test lines (different antigens) and one control line (goat anti-mouse IgG) were located on the strip membrane. Labeled antibodies were freeze-dried in microwells. Samples did not require pretreatment and could be directly analyzed within 10 min. Threshold levels in different sample matrices were visually estimated at 0.3–0.45 ng mL^{-1} for clenbuterol; 3–4 ng mL^{-1} for sulfadiazine; and 4.5–6 ng mL^{-1} for tetracycline, respectively. The linear relationship between the concentrations of veterinary drug residues and the Au nanoparticles plasmon absorbance allowed quantitative determination of these veterinary drug residues. The recoveries of clenbuterol, sulfadiazine and tetracycline in spiked samples ranged from 78.4% to 112.6%, and the relative standard deviations were below 11.2%. Analysis of animal samples suggested that the proposed multiplex dipstick assay method was consistent with the LC-MS/MS method. The percentage of false results was less than or equal to 5%. Thus, the proposed multiplex dipstick assay is inexpensive, easy-to-use, and suitable for the purposes of rapid and comprehensive screening of 3 families of β-agonists, sulfonamides and tetracyclines including 26 drugs in animal body fluids.

1. Introduction

The use of veterinary drugs during animal production is increasingly regulated in most countries with variable regulations

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http://dx.doi.org/10.1016/j.aca.2016.05.004
0003-2670/

and directives, depending on the country. Permissible drugs and/or tissue residue levels vary according to country, but international trade has made it imperative that drug detection methodology be developed to ensure food products meet the standards of recipient countries. The β-agonists are growth-promoting agents and improve growth rate and reduce carcass fat when fed to animals [1–3]. However, β-agonists accumulated at higher levels in animal tissues can cause acute toxicity when consumed by humans with symptoms of muscular tremor, cardiac palpitation, nervousness, and headache [4,5]. Therefore, the use of β-agonists in animal production has been banned in most countries. The EU and China have prohibited the administration of all β-agonists as growth promoters [6]. Sulfonamides (SAs) and tetracyclines (TCs) are widely used in veterinary medicine for the treatment of bacterial infections [7,8]. However, there is concern about the potentially serious threats to human health resulting from widespread usage of these drugs. Such antibiotic residues in animal tissues can potentially contribute to increased drug resistance of microbial strains in human beings and cause allergic or toxic reactions among some hypersensitive individuals [9]. To protect the safety of consumers, maximum residue limits (MRL) for antibiotics in edible animal products are being continually reduced. The Codex Alimentarius Commission (CAC) and most countries in Europe and America have required the concentration of total or individual SA residues in foods and feeds to not exceed 0.1 mg kg^{-1} [10], while Japan completely banned food with SA residues at any level [11]. However, in many countries, there is still improper, if not illegal, usage of veterinary drugs in animal production, with the application of these drugs beyond their labeled use, including not following the labeled withdrawal period.

Various analytical methods have been developed to detect the level of veterinary drug residues in animal samples. Generally, these analytical strategies of residue monitoring include a two-step approach: low-cost screening as a first step, followed by a confirmatory step to verify a positive result [12,13]. The screening methods primarily include enzyme-linked immunoassay (ELISA), and the confirmatory steps mainly include chromatography coupled with mass spectrometry technology [14–16]. Each method has distinct advantages and drawbacks. Chromatography and mass spectrometry are sensitive, but time-consuming and dependent on highly skilled personnel and expensive equipment. Principal disadvantages of rapid screening methods are high rates of false positives, and the limitation of one drug per ELISA test [17,18]. Thus, they are not suitable for routine, large-scale screening.

Recently, the lateral flow immunoassay (LFA) method has been widely used for its rapidity, sensitivity and time-efficiency. The tests are usually based on a visual evaluation of the results and provide a qualitative result (positive or negative). A great variety of commercial test strips have become available, reflecting the increasing demand for screening tests that are easy to use and allow rapid determination [19]. The LFA methods for qualitative detection of single β-agonist, sulfonamide, and tetracycline residues have been reported [20–25]. Combined with portable photometric strip readers, designed to read results photometrically from LFA, qualitative or semi-quantitative analysis for drugs is also available. By analogy with chromatographic methods, the new trend for LFA technology is the development of multicomponent screening methods in which a variety of analytes can be simultaneously detected in one single immunoassay [26–28]. Performance and application of colloidal gold-based multi-residue test strips for analysis of mycotoxins have been reported [17,29]. A fluorescent multiplex dipstick for the simultaneous determination of three major β-agonists (clenbuterol (CL), ractopamine, and salbutamol) has also been recently developed [20]. However, the detection of fluorescence signals requires special instrumentations, which increases the cost and limits the usage of *in situ* detection. The need remains for the development of a simple, timely, and accurate test for multiple drug residues in livestock tissues. To our knowledge, no work has previously been reported that detects the three classes of banned or regulated veterinary drugs using biological matrices with multiplex dipstick assay. Therefore, the objective of this study was to develop a colloidal gold-based colorimetric dipstick assay method for the rapid and semi-quantitative determination of three different families of drugs in milk, blood, and urine.

2. Experimental methods

2.1. Materials and apparatus

2.1.1. Chemicals and reagents

All the β-agonists and TCs standards were purchased from Sigma (St Louis, MO). All SAs standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Bovine serum albumin (BSA), polysorbate 20, sucrose, sodium citrate and hydrogen tetrachloroaurate (III) hydrate ($HAuCl_4 \cdot 3H_2O$) were purchased from Sigma (St Louis, MO). The monoclonal antibodies (mAbs) against β-agonists, SAs, and TCs together with their conjugates antigens (CL-BSA, tetracycline (TC)-BSA and sulfadiazine (SD)-OVA), were obtained from Beijing WKHH biotechnology Co., Ltd (Beijing, China). The ratios of CL to BSA, TC to BSA, and SD to OVA are 20:1, 15.7:1, and 25:1, respectively. Hi-Flow Plus 90 Nitrocellulose (NC) membrane was obtained from Millipore Corporation (Bedford, USA). Sample pads, absorption pads, conjugate pad and PVC pads were obtained from Shanghai Jiening Biotechnology Co., Ltd. (Shanghai, China). Methanol and acetonitrile (HPLC grade) were obtained from Fisher Scientific International (Hampton, NH). Formic acid, ammonium acetate and acetic acid (HPLC grade) were obtained from Dikma Technology (Richmond Hill, Canada). Ultrapure water (Milli-Q, Millipore Corporation, Bedford, MA) was used to prepare all aqueous solutions. Other chemicals used in this research were analytical grade.

2.1.2. Instrumentation

Colorimetric measurements were performed using an ESE Quant LR3 Lateral Flow Reader (Qiagen, German). An XYZ 3030 dispensing platform and a CM4000 guillotine cutting module (Shanghai Kinbio Tech. Co., Ltd, China) were used to prepare test strips. A sealing machine was purchased from Beijing Ruimingxing Packaging Machinery Technology Co., Ltd. (Beijing, China) and a vacuum freeze-dryer was obtained from Shanghai Pudong Freeze Dryer Equipment Co., Ltd. (Shanghai, China). Ultra-high performance liquid chromatography tandem mass spectrometry was performed on an Agilent 1200 UHPLC system coupled with a 6460 Mass Selective Detector (Agilent Technologies, Fremont, CA).

2.2. Preparation of colloidal gold-antibody conjugate in microwells

Colloidal gold (CG) with a mean diameter of 40 nm was prepared according to the procedure described by Hayat et al. [30]. Briefly, 100 mL of 0.01% $HAuCl_4$ filtrated through a 0.22-μm membrane filter was heated to boil. 1.2 mL of 1% citric acid trisodium salt filtrated through a 0.22-μm membrane filter was rapidly added to the boiling solution under constant stirring. After the color changed, the solution was kept boiling for an additional 15 min, and allowed to cool down to room temperature. By detecting the absorbance value at 520 nm, the concentration of CG was about 4.1 nM. The CG solution obtained was stored at 4 °C for further use. Three CG-labeled mAbs were separately prepared. For the preparation of anti-β-agonists mAb-gold conjugates, the pH of the CG was adjusted to pH 8 with 0.2 mol L^{-1} K_2CO_3. Subsequently, 3.5 μg