

Original Article

Development of a lateral-flow assay for rapid screening of the performance-enhancing sympathomimetic drug clenbuterol used in animal production; food safety assessments

Weihua Lai PhD^{1,2}, Yang Xu PhD¹, Daniel YC Fung PhD² and Yonghua Xiong PhD¹¹The Key Laboratory of Food Science (Nanchang University), Ministry of Education, 330047, China²Department of Animal Science and Industry, Kansas State University, U.S.A. 66506

A lateral-flow assay that could provide visual evidence of the presence of clenbuterol in swine urine was developed. Colloidal gold was prepared and conjugated with anti-clenbuterol monoclonal antibody. Immunochromatographic test strips were produced, and then, 210 samples were tested on these strips. Analysis was completed in 10 min. Detection limit was 3 ppb of clenbuterol. Parallel GC-MS data indicated that clenbuterol rapid detection strip had no false negative. The false positive rate was 4.4%. Immunochromatographic strip has great applied value in the food safety field because it possesses benefits of sensitivity, stability, reproducibility, ease of use and inexpensive.

Key Words: lateral-flow, strip, screen, clenbuterol

Introduction

Clenbuterol (4-amino-(*t*-butylamino)methyl)-3,5-dichloro benzylalcohol hydrochloride), belonging to the family of β -agonists, is currently used as bronchodilators for the treatment of asthma in humans, as well as tocolytic agents in veterinary medicine. However, clenbuterol has been used illegally at higher dosages (ten to hundred times the clinically active dose) to promote animal growth in muscular mass, and at the same time decreasing fat accumulation. Although these properties were known in 1984, the use of clenbuterol did not become widespread until 1988 in response to the European directive forbidding the use of hormones in animal production. Even so, no standard was established for clenbuterol, or other β -adrenergic agonists, for such purposes. On the contrary, clenbuterol and similar products were included in the list of substances formally banned for zootechnical use.

Proliferation of the illegal use of clenbuterol to promote animal growth raised new concerns about its safety/toxicity, particularly regarding consumers. Various intoxications have been described as being due to the ingestion of liver and meat containing clenbuterol residues in some countries. An outbreak of illness in 1992 totaling 113 persons who consumed contaminated beef liver was took place in Spain. Clenbuterol was detected in 47 urine samples in amounts ranging from 11 to 486 ppb.¹ Victims were hospitalized with reversible symptoms of increased heart rate, muscular tremors, headache, nausea, fever, and chills. In August 1996, 62 persons asked for medical help in Italy. All patients had beef meat consumption 10-30 min to 2-3 h before symptoms developed. Concentrations in the meats ranged from 0.8 to 7.4 mg/kg.² A similar incident with 22 cases, also traced to beef liver, was reported from France.³ Clenbuterol was also a concern in Portugal.⁴

In China, over 1000 people were ill in Guangdong province in 2001 after consumption of contaminated swine liver and heart. A person was died in Guangdong province in March 19 2006. The death is the first report related to clenbuterol in the world. Also 300 people were poisoned in Shanghai in September 15, 2006.

A wide variety of analytical methods for the determination of clenbuterol in different biological matrices have been described. Enzyme-linked immunosorbent assay⁵⁻⁷ is the most sensitive detection systems for this compound and they have been used for screening purposes. Quantification and confirmation have been usually made by using methods based on HPLC,⁸⁻¹⁰ liquid chromatography coupled with mass spectrometry¹¹ and gas chromatography coupled with mass spectrometry.¹²⁻¹⁶

Lateral-flow assays, which to date have been used as diagnostic tools for monitoring drugs,¹⁷ toxins,¹⁸⁻²⁰ hormones²¹ and pathogens;²² allow a rapid, qualitative determination of analytes. This technique is based on an immunochromatographic procedure that utilizes antigen-antibody properties in a novel way and provides rapid detection of analyte. They possess four benefits of user-friendly format, a very short period (10 min) to get test results, long-term stability over a wide range of climates and relatively inexpensive to make. These characteristics render it ideally suited for on site testing by untrained personnel.

Corresponding Author: Associate Professor Weihua Lai, The Key Laboratory of Food Science (Nanchang University), Ministry of Education, 330047, China
Tel: 086-791-8182405; Fax: 086-791-8333708
Email: talktolaiwh@163.com

In this report, we used a high-affinity anti-clenbuterol monoclonal antibody to develop a rapid and sensitive immunochromatographic assay for clenbuterol detection.

Materials and method

Materials

Tulobuterol hydrochloride, terbutaline hemisulfate, clenbuterol hydrochloride, salbutamol hemisulfate, ritodrine hydrochloride and fenoterol hydrobromide, bovine serum albumin (BSA) and Goat anti-mouse antibody were obtained from Sigma (St. Louis, MO, USA). Monoclonal antibody (R6) was produced in our laboratory and purified by protein A affinity column (Pharmacia, Uppsala, Sweden). Hydrogen tetrachloroaurate trihydrate was obtained from Aldrich (Milwaukee, WI, USA). N-methyl-N-trimethylsilyltrifluoroacetamide (MSTFA) was purchased from Fluka (Buchs, Switzerland). Sample pad, the conjugate release pad, the analytical membrane and the absorbent pad were obtained from Schleicher&Schuell GmbH (Dassel, Germany). All solvents and other chemicals were analytical reagent grade.

BioDot

The BioDot XYZ Platform combining motion control with BioJet Quanti3000k dispenser and AirJet Quanti3000k dispenser was invented by BioDot (Irvine, CA) The BioJet Quanti3000k dispenser is a non-contact, quantitative dispenser which couples BioJet drop-on-demand valve with a high resolution syringe pump to meter precise amounts of capture clenbuterol-BSA or anti-mouse IgG to the NC membrane. The AirJet Quanti3000k is a non-contact, quantitative aerosol dispenser. It is used to dispense detector antibody on the conjugate release pad.

Preparation of colloidal gold

5 mL of a 1% (w/v) stock solution of hydrogen tetrachloroaurate trihydrate was added to 500 mL of distilled water and heated to boiling point. 5 mL of a freshly made 1% solution of sodium citrate was added to the gold solution under constant stirring and the mixture was boiled until it turned red. After an additional 5 min boiling, the solution was cooled to 4°C for further processing.

Preparation of colloidal gold probe

Colloidal gold was used for conjugation of IgG. Protein A gel purified anti-clenbuterol monoclonal antibody (2 mL, 0.5 mg/mL, in 5 mM Tris-HCl, pH 7.5) was added to 20 mL pH-adjusted colloidal gold solution and was agitated for 30 min. Then 2 mL of 1% (w/v) BSA solution was added and was agitated for 15 min. the mixture was centrifuged at 6000 rpm for 30 min. After centrifugation, the gold pellets were dissolved in 50 mM Tris/HCl buffer.

Preparation of immunochromatographic test strips

The sample pad was treated with 50 mM borate buffer, pH 7.4, containing 1% BSA, 0.5% Tween-20, and 0.05% sodium azide, and dried at 60°C. Clenbuterol-BSA (0.038 mg/mL) and goat anti-mouse antibody (1.123 mg/mL) were applied to the nitrocellulose membrane as the test and control lines, respectively, and dried at 35°C. An absorption pad was used without treatment. The colloidal gold probe was applied to an untreated glass-fiber

membrane and completely dried at 35°C. The nitrocellulose membrane, absorption pad, glass fiber membrane, and pretreated sample pad were assembled as the strip.

Assay of clenbuterol on test strip

A urine sample was pipetted into the reaction holder to evaluate the results.

Sample preparation for GC-MS analysis

Internal standard solution (20 µL of 50 ng/mL penbutolol in methanol) was added to 5 mL of sample or clenbuterol-spiked blank urine, followed by addition of 5 mL of pH 2.2 potassium hydrogen phthalate buffer solutions. The urine solution was then transferred to a 3 mL polypropylene SPE cartridge which contained a 0.5 cm deep bed of AG-MP 50 resin. After allowing the sample to pass through to waste under gravity, the resin was washed with 1 mL of water and 1 mL of 2N sodium hydroxide solution. Finally the analyte was eluted into a capped test tube with 2 mL of 10% methanol in ethyl acetate. 1 mL of water and 7 mL of ethyl acetate were added to the tube and it was shaken for 30 min. The upper layer was transferred into a Quickfit test tube and evaporated to dryness under nitrogen at 40°C. Silylation was performed by adding 50 µL of MSTFA/dithio reagent (0.4%, dithioerythritol, 0.2% ammonium iodide in MSTFA) to the test tube sealing it with a glass stopper, and heating for 15 min at 60°C. The solution was transferred to a low volume auto sampler vial for GC-MS analysis.

Gas chromatography-mass spectrometry

GC-MS analyses were carried out with a Hewlett-Packard 5890 Series II gas chromatograph and HP 5970 mass selective detector. GC was equipped with electronic pressure control unit, autoinjector (HP18593B) and autosampler (HP18596 BX). The system was under computer control with a software of HP 59940C UNIX version A.01.04. A cross linked Ultra-2% 5 phenylmethylsiloxan capillary column (length 17 m, i.d. 0.2 mm, film thickness 0.33 µm) (Hewlett-Packard) was connected into the ion source. Samples were injected in the split mode with the split ratio 1:10. Helium was used as the carrier gas at the flow rate 0.7 mL/min. Injector and detector temperatures were set at 280 and 290°C, respectively. The column temperature was programmed to start at 150°C, increase at a rate of 15°C/min up to 280°C. The mass spectrometer conditions were as follows: electron impact ionization voltage 70 eV for both SCAN and selected ion monitoring (SIM) mode.

Results

Determination of test results

This test is a competitive binding immunoassay. The clenbuterol in the urine specimen competes with the antigen coated on the nitrocellulose membrane for the limited binding sites of the antibody in the conjugate pad. When an adequate amount of urine specimen is applied to the sample pad of the device, the urine migrates by capillary action through the test strips. If the clenbuterol level in the specimen is below the cutoff level, the red-colored conjugate will bind to the antigens coated on the nitrocellulose membrane (the test line). A red T line will be

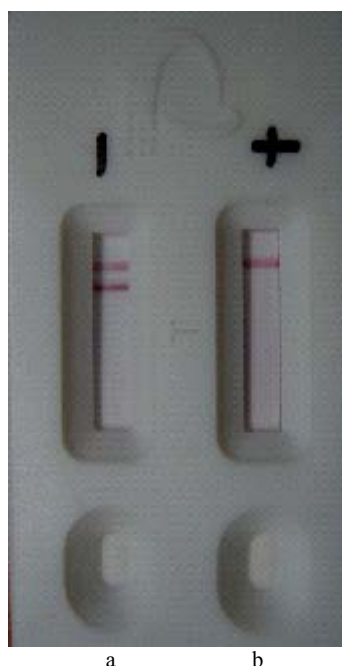


Figure 1. Schematic diagram of a test strip. a: negative urine for clenbuterol b: positive urine (3ppb) for clenbuterol

Table 1. The cutoff level of the test strip for clenbuterol

Sample	Result of detection of the test strip					
	1	2	3	4	5	6
1ppb	-	-	-	-	-	-
2ppb	-	-	-	-	-	-
3ppb	+	+	+	+	+	+
4ppb	+	+	+	+	+	+
5ppb	+	+	+	+	+	+
10ppb	+	+	+	+	+	+

Table 2. Comparison of test result of rapid test strip with that of GC-MS for clenbuterol

Rapid test strip	GC-MS		Total
	Positive (≥ 3 ppb)	Negative (< 3 ppb)	
Positive	52	7	59
Negative	0	151	151
Total	52	158	210
False positive rate			4.4%
False negative rate			0%

formed, which indicates a negative result. If the clenbuterol is present in the urine specimen at a cutoff level or higher, it will bind to antibodies in the conjugate pad, so that no red line develops in the test region (T line), which indicates a positive result. The colored gold-antibody conjugate should bind to the C line and form a red-colored band regardless of the presence of clenbuterol (Fig 1).

Detection limit of clenbuterol test strip

Six negative urine samples, which were verified by GC-MS spiked with various concentrations of clenbuterol, were assayed by clenbuterol test strip. Analysis was complete in less than 10 min, The cutoff level was 3 ppb of clenbuterol (Table 1).

Table 3. Cross reactivity of clenbuterol test strip for clenbuterol

β -agonist compounds	Result of the test strip					
	1ppb	3ppb	5ppb	10 ppb	50 ppb	100 ppb
Tulobuterol	-	-	-	-	-	-
Terbutaline	-	-	-	-	-	-
salbutamol	-	-	-	-	-	-
ritodrine	-	-	-	-	-	-
fenoterol	-	-	-	-	-	-

Accuracy of the test strip

210 urine samples collected from Jiangxi and Zhenjiang province were detected by the test strip and GC-MS. There are 52 positive samples (≥ 3 ppb) and 158 negative samples (< 3 ppb) which was determined by GC-MS. The 52 positive samples and 7 out of 158 negative samples were positive detected by the test strip, while 151 out of 158 negative samples were negative detected by test strip. The test strip compared to GC-MS gave a false positive rate of 4.4%, false negative rate of 0 % (Table 2), while the overall relative accuracy obtained was 203/210 or 96.7%.

Cross reactivity of clenbuterol test strip

Tulobuterol, terbutaline, salbutamol, ritodrine and fenoterol are most frequently associated with clenbuterol. The cross reactivity of clenbuterol test strip with tulobuterol, terbutaline, salbutamol, ritodrine and fenoterol was also examined. These β -agonist compounds (from 1ppb to 100ppb) were tested. Red band was revealed in the test region. The cross-reactivities of the test strip with the different compounds are given in Table 3.

Stability studies

The test strips were subjected to accelerated stability studies. They were kept at 37°C, room temperature and 4°C and taken out at different time intervals and tested for sensitivity and workability. From this study, it was concluded that they can be stored at room temperature for 12 months.

Discussion

China is a developing country with a population of 1.3 billion, and its economy has grown relatively fast in the late 20 years. With the increase of economy and improvement of life quality consumer has paid more attention to food safety and quality. The Chinese government is facing challenges for food safety.

Outbreak of illness concerning clenbuterol was reported at Hong Kong in 1997.²³ Limits to the tissue content of clenbuterol residue in meat products have been established by law in China. However, it is difficult for Chinese government to control the illegal use of clenbuterol in swine production because there are too many individual farmers and small farmers are breeding swine in China. Consequently, it is essential to have an inexpensive, convenient and reliable technique to detect the presence of these residues on the spot.

A wide variety of analytical methods have been described for the determination of clenbuterol in different

biological matrices. ELISA is the most sensitive detection system for this compound and has been used for screening purposes. Quantification and confirmation have been usually made by using methods based on GC-MS. The advantages of the rapid strip test over ELISA include that it is faster, requires less sample processing, often cheaper, and generates yes/no answers without using an instrument, it is often used in the field by non-laboratory people to test whole samples. However, rapid strip test are neither sensitive nor can be used to accurately quantitate an analyte. This assay provides only a preliminary analytical test result. In spite of its drawbacks, the strip has great applied value in food safety field especially in developing countries. Under this situation, the way, screening clenbuterol in urine-colloidal gold immunochromatographic assay, was certificated by Chinese Ministry of Agriculture (NY/T933-2005) in 2005 and has been extended in China from then on.

Control of clenbuterol usage in live animals may be focused on analysis of urine samples because of the easiness of sampling and extractability of the residues. The elevated drug concentration in urine is well above detection limits of the usual analytical techniques during treatment at growth-promoting dosages.

Different sample volumes applied on the test strips were tested. Sample volumes of 50, 80, 100, 120, and 150 μL were employed on the sample well of the cassette holder. An 80 μL sample was selected as the optimal sample volume throughout the study.

This assay may generate false positive results. False positive results are frequently caused by the failure of the sample to release the gold conjugate, or even to move along the membrane strip. A number of possible causes can result in such a symptom. There could be insufficient sample to make the test run, poor contact between the gold-conjugate pad and the membrane, insufficient surfactant in the system, or the wrong type of conjugate pad material. The most likely cause of gold-conjugate not moving onto the membrane is its accumulation following exposure to excess salt in the urine.

A different antigen/antibody reaction is added to the membrane strip at the control region (C) to indicate that the test has been performed properly. This control line should always appear, regardless of the clenbuterol status in the urine. This means that negative urine will have two pink colored bands while positive urine will have only one pink colored band. The pink colored control band serves as an indicator that 1) sufficient volume has been added, and 2) that proper flow was obtained

In conclusion, rapid lateral-flow strip can play an important role in preliminary screening clenbuterol in China.

Acknowledgements

This work was supported by Innovation Fund for Technology Based Firms of China (05C26213600918) and the China Scholarship Council.

References

- Salleras L, Dominguez A, Mata E, Taberner JL, Moro I, Salva P. Epidemiologic study of an outbreak of clenbuterol poisoning in Catalonia, Spain. *Public Health Rep* 1995; 110:338-42.
- Sporano V, Grasso L, Esposito M, Oliviero G, Brambilla G, Loizzo A. Clenbuterol residues in non-liver containing meat as a cause of collective food poisoning. *Vet Hum Toxicol* 1998; 40:141-3.
- Pulce C, Lamaison D, Keck G, Bostvironnois C, Nicolas J, Descotes, J. Collective human food poisoning by clenbuterol residues in veal liver. *Vet. Hum. Toxicol* 1991; 33: 480-1.
- Barbosa J, Cruz C, Martins J, Silva JM, Neves C, Alves C, Ramos F, Da Silveira ML. Food poisoning by clenbuterol in Portugal. *Food Addit Contam* 2005; 22: 563-6.
- Posyniak A, Zmudzki J, Niedzielska J. Screening procedures for clenbuterol residue determination in bovine urine and liver matrices using enzyme-linked immunosorbent assay and liquid chromatography. *Analytica Chimica Acta* 2003; 483:61-7.
- Sawaya WN, Lone K, Husain A, Dashti B, Saeed T. Screening for β -agonists in sheep urine and eyes by an enzyme-linked immunosorbent assay in the state of Kuwait. *Food Control* 2000; 11:1-5.
- Podesta A, Luppi A, Benatti L, Villani C.A Source of False-Negative Results in Clenbuterol Analysis in Tissues of Veal Calves. *Journal of Pharmacological and Toxicological Methods* 1998; 40: 27-32.
- Grzegorz B, Luc J N. Simultaneous high-throughput determination of clenbuterol, ambroxol and bromhexine in pharmaceutical formulations by HPLC with potentiometric detection. *Journal of Pharmaceutical and Biomedical Analysis* 2003; 32: 887-903.
- Gianfranco B, Maurizio F, Barbara R, Vittorio C. Use of molecularly imprinted polymers in the solid-phase extraction of clenbuterol from animal feeds and biological matrices. *Journal of Chromatography B* 2001; 759: 27-32.
- Zhang XZ, Gan YR, Zhao FN. Determination of clenbuterol in pig liver by high-performance liquid chromatography with a coulometric electrode array system *Analytica Chimica Acta* 2003;489: 95-101.
- Philippe A G, Marie-Claude S, Richard H S. Quantitative analysis of clenbuterol in meat products using liquid chromatography-electrospray ionisation tandem mass spectrometry *Journal of Chromatography B* 1999;736: 209-19.
- Ramos F, Cristino A, Carrola P, Eloy T, Silva J M, Castilho MC, Silveira MIN. Clenbuterol food poisoning diagnosis by gas chromatography-mass spectrometric serum analysis. *Analytica Chimica Acta* 2003; 483: 207-13.
- Amendola L, Colamonic C, Rossi F, Botre F. Determination of clenbuterol in human urine by GC-MS-MS-MS: confirmation analysis in antidoping control. *Journal of Chromatography B* 2002; 773: 7-16.
- Keskin S, Ozer D, Temizer A. Gas chromatography-mass spectrometric analysis of clenbuterol from urine. *Journal of Pharmaceutical and Biomedical Analysis* 1998; 18: 639-44.
- Hernández-Carrasquilla M. Gas chromatography-mass spectrometry analysis of β -agonists in bovine retina *Analytica Chimica Acta* 2000; 408: 285-90.
- Whaites LX, Murby EJ. Determination of clenbuterol in bovine urine using gas chromatography-mass spectrometry following clean-up on an ion-exchange resin. *Journal of Chromatography B* 1999; 728: 67-73.
- Wong R. The effect of adulterants on urine screen for drugs of abuse: detection by an on-site dipstick device. *American Clinical Laboratory* 2002; 21: 37- 9.
- Wittmann C, Bilitewski U, Giersch T, Ketting U, Schmid RD. Development and evaluation of a dipstick immunoassay format for the determination of atrazine residues on-site. *Analyst* 1996; 121: 863-9.

19. Wittmann C, Schreiter PY. Analysis of terbutylazine in soil samples by two test strip immunoassay formats using reflectance and luminescence detection. *Journal of Agricultural and Food Chemistry* 1999; 47: 2733–37.
20. Yeoh HH, Sun F. Assessing cyanogen content in cassava-based food using the enzyme-dipstick method. *Food and Chemical Toxicology* 2001; 39: 649–53.
21. Henderson K, Stewart J. A dipstick immunoassay to rapidly measure serum oestrone sulfate concentrations in horses. *Reproduction Fertility and Development* 2000;12: 183–9.
22. Brandonisio O, Fumarola L, Maggi P, Cavaliere R, Spinelli R, Pastore G. Evaluation of a rapid immunochromatographic test for serodiagnosis of visceral leishmaniasis. *European Journal of Clinical Microbiology and Infectious Diseases* 2002; 21: 461–4.
23. Chan TYK. Food poisoning following the consumption of clenbuterol-treated livestock. *Hong Kong Practitioner* 1998; 20: 366-9