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RESEARCH PAPER

Lateral flow biosensor for multiplex detection of nitrofuran metabolites based on functionalized magnetic beads

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Abstract The use of potential mutagenic nitrofuran antibiotic in food animal production has been banned world-wide. Common methods for nitrofuran detection involve complex extraction procedures. In the present study, magnetic beads functionalized with antibody against nitrofuran derivative were used as both the extraction and color developing media in lateral flow biosensor. Derivatization reagent carboxybenzaldehyde is firstly modified with ractopamine. After reaction with nitrofuran metabolites, the resultant molecule has two functional groups: the metabolite moiety and the ractopamine moiety. Metabolite moiety is captured by the antibody that is coated on magnetic beads. This duplex is then loaded onto biosensor and ractopamine moiety is further captured by the antibody immobilized on the test zone of nitrocellulose membrane. Without tedious organic reagent-based extraction procedure, this biosensor was capable of visually detecting four metabolites simultaneously with a detection limit of 0.1 μg/L. No cross-reactivity was observed in the presence of 50 μg/L interferential components.

Keywords Nitrofuran metabolites · Magnetic separation · Lateral flow biosensor · Fast pre-treatment

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Introduction

Nitrofuran antibacterial antibiotics, including nitrofurantoin, furazolidone, furaltadone, and nitrofurazone, have been used to prevent and treat gastrointestinal infections in breeding industry. Due to their potential mutagenic, carcinogenic, and teratogenic effects on human health, the use of the nitrofuran drugs in food animal production has been banned in the European Union, USA, and China [1–3].

Owning to the instability of nitrofurans, detection of drug abuse is mainly based on the monitoring of their metabolites [4]. Four metabolites of nitrofurans, 3-amino-2-oxazolidinone (AOZ), 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ), 1-aminohydantoin (AHD), and semicarbazide (SEM), are considered as the biomarkers of furazolidone, furaltadone, nitrofurazone, and nitrofurantoin, respectively [1].

Since cases of nitrofuran antibiotics abuse have been reported frequently, analytical methods capable of rapid and large-scale screening are necessary. Traditional liquid chromatography mass spectrometry (LC-MS) [5] and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [6, 7] are highly accepted and reliable methods. However, they are more expensive and require stronger operational skills than immunological methods that are rapid, simple, and economical. Many enzyme-linked immunosorbent based assays (ELISA) have been employed successfully for the detection of AHD [8], AOZ [9, 10], SEM [11, 12], and AMOZ [13] in recent years. A more convenient method, the lateral flow assay (LFA), has also been applied in the detection of AHD [14]. Lateral flowing of aqueous solution along the strip involves the incubation and washing processes that are inevitable in ELISA. In spite of labor and time saving by LAF, sample pre-

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treatment still costs a few hours during the measurement, which involves extraction, purification, and concentration. Thus, a simpler and more efficient LFA for nitrofuran metabolites detection is needed.

In a previous study, we developed a competitive ELISA for SEM analysis using a monoclonal antibody against SEM derivative [12]. Then, a direct ELISA for quantitative analysis of SEM was further established using biotinylated 4-carboxybenzaldehyde (CBA) [15]. This assay simplified the procedure of sample pre-treatment and improved the sensitivity. Another convenient sample pre-treatment method is the magnetic beads-based separation. Magnetic separation-based biosensors are now widely used for the detection of many analytes, such as bacterial [16, 17], heavy metal ion [18], and DNA [19]. Due to the strong colored property, magnetic beads can also be used as labels on lateral flow assay. In the present study, we introduced a simpler and more time-saving assay for quadruple and visual detection of four nitrofuran metabolites simultaneously by coupling LFA with magnetic separation.

The detection processes is schematically showed in Fig. 1. 4-Carboxybenzaldehyde is firstly modified with ractopamine, which is used for the derivatization of AHD, AOZ, SEM, or

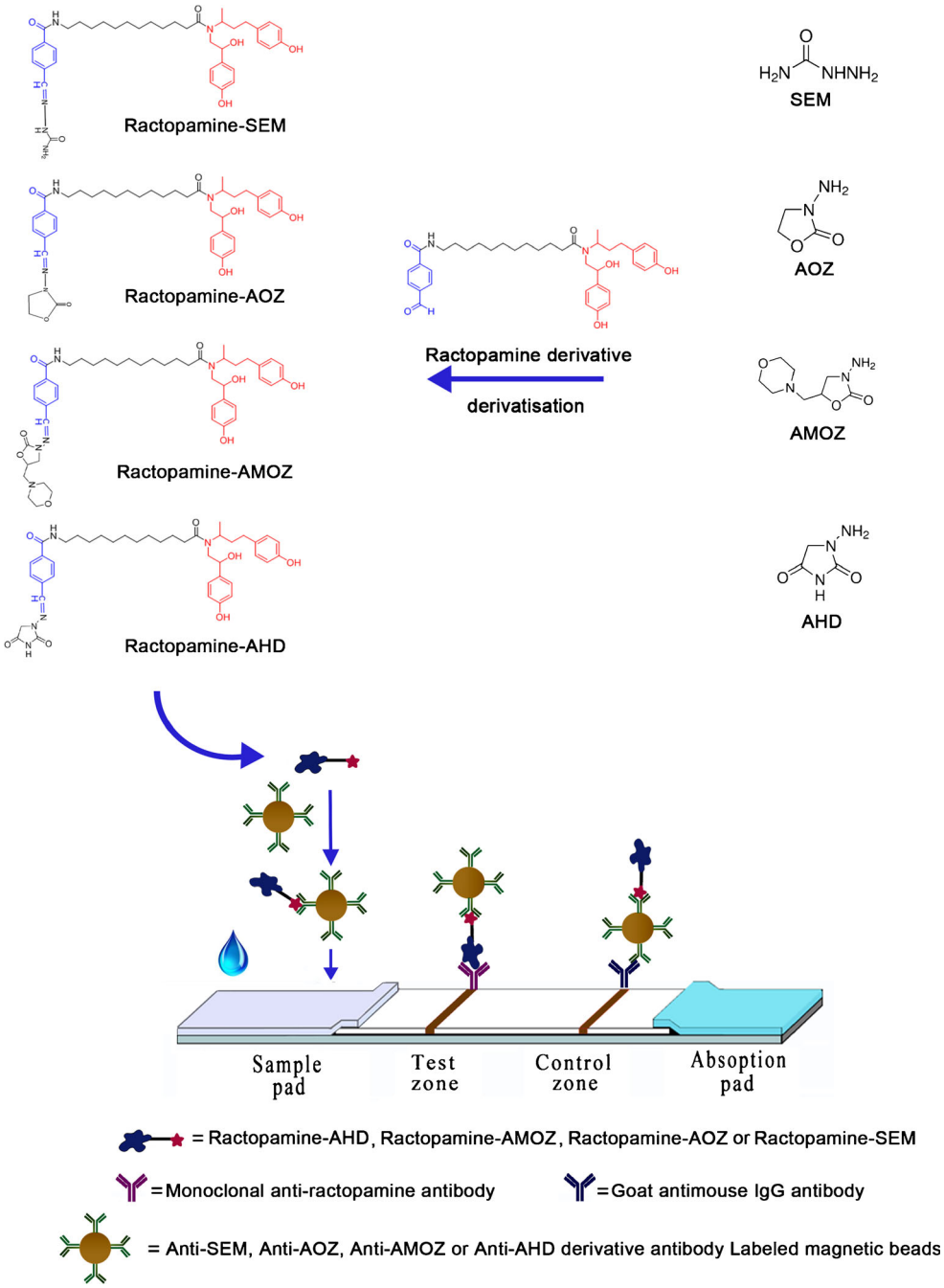


Fig. 1 Derivatization of nitrofuran metabolites (AHD, AOZ, SEM, or AMOZ) and LFA detection of the derivative products

AMOZ. The derivative product has two functional groups: the SEM-CBA moiety and the ractopamine moiety. SEM-CBA (AOZ-CBA, AMOZ-CBA, or AHD-CBA) moiety is captured by the antibody that coated on magnetic beads (MBs). Ractopamine moiety is captured by the antibody that immobilized on the test zone of nitrocellulose membrane. Thus, the assay is established in a sandwich format.

Materials and methods

Chemicals

Antibodies against AOZ, AMOZ, and AHD were obtained from Abcam (Cambridge, UK). Anti-CPSEM monoclonal antibody was produced using lymphocyte hybridoma technique [12]. Carboxylated magnetic beads and all chemical reagents (analytical grade purity) were purchased from Aladin (Shanghai, China). $HAuCl_4$ was from Sigma-Aldrich (Steinheim, Germany). Bovine serum albumin (BSA) was obtained from Dingguo Biotech (Beijing, China). Nitrocellulose membrane was purchased from Sartorius (Goettingen, Germany). Fiberglass and absorbent paper were purchased from Shanghai Kinbio (Shanghai, China). All buffer solutions used in this study were prepared in our lab.

Synthesis of ractopamine derivative 5

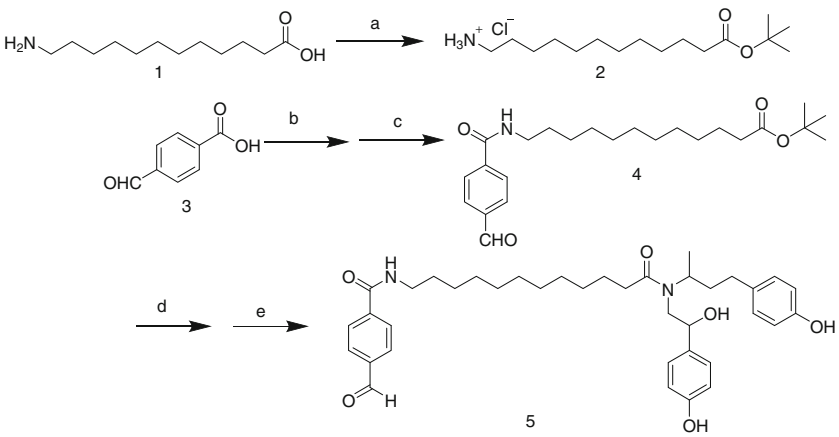
The synthetic route of the ractopamine derivative 5 is outlined in Scheme 1. 12-Aminododecanoic acid 1 was esterified by treatment with $SOCl_2$ in tert-butyl alcohol [20]. 4-Formylbenzoic acid 3 was transformed into the corresponding acyl chloride, which was treated with the tert-butyl ester 2 to give compound 4. At last, the obtained intermediate 4 was de-protected to give the corresponding carboxylic acid, which was then coupled with ractopamine using TBTU/DIPEA as coupling reagents to afford the target amide 5 [14].

Preparation of magnetic beads

Twenty microliters of magnetic beads (1 μg/L) were washed with phosphate buffer saline (PBS) twice and activated by adding 50 μL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC, 2 μg/L). The reaction mixture was vortexed gently for 30 min at room temperature. Beads were collected by magnetic separation and washed twice with PBS. The beads were then re-suspended in 50 μL of antibody (0.5 μg/L in PBS) and vortexed gently for 30 min at room temperature. After coating with antibody, excess active carboxy groups on beads were blocked with 10 μL of BSA (3 μg/L in PBS). The prepared beads were washed twice with PBST (0.1 % Tween-20 in PBS) to reduce non-specific binding. The beads were further washed with PBS twice and re-suspended in 100 μL of stock buffer (2 % BSA in PBS). The buffered beads were stored at 4 °C before use. Typical scanning electron microscopic (JEOL Ltd., Japan) image of the antibody conjugated MSNs with an average diameter of 180 nm was shown in Fig. S2 in the Electronic Supplementary Material (ESM).

Preparation of strip biosensor

Monoclonal antibody against ractopamine (0.8 μg/L in PBS) and goat anti-mouse IgG antibody (1 μg/L in PBS) were dispensed onto nitrocellulose membrane at 1 cm/s simultaneously to form the test and control line with the aid of a lateral flow dispenser (Kinbio, Shanghai, China). Untreated fiberglass was used as sample pad. Sample pad, nitrocellulose membrane, and absorbent pad were attached along the long axis of an adhesive plate with an overlap of 2–3 mm. The plate was then cut into 3-mm-wide strips using a strip cutter (Kinbio, Shanghai, China) and stored under dry condition.



Scheme 1 Synthetic route of ractopamine derivative 5. Reagents and conditions: (*a*) t-BuOH, $SOCl_2$, 0-70 °C; (*b*) $SOCl_2$, CH_2Cl_2, Toluene, 80 °C; (*c*) Compound 2, NEt_3, DMF; (*d*) TFA, CH_2Cl_2, 0–25 °C; (*e*) ractopamine, TBTU, DIPEA, DMF, r.t

Derivatization and detection of metabolites

Ten microliters of derivatization stock solution (20 g/L in methanol) and 15 μL of 0.1 M HCl were added into 1 mL of metabolites standard solution. The mixture was incubated at 65 °C for 1 h. Then, 20 μL of Tris-base buffer (300 mM, pH = 8) was added to the above solution to inactivate aldehyde groups. After gentle vortex, 100 μL of this solution was

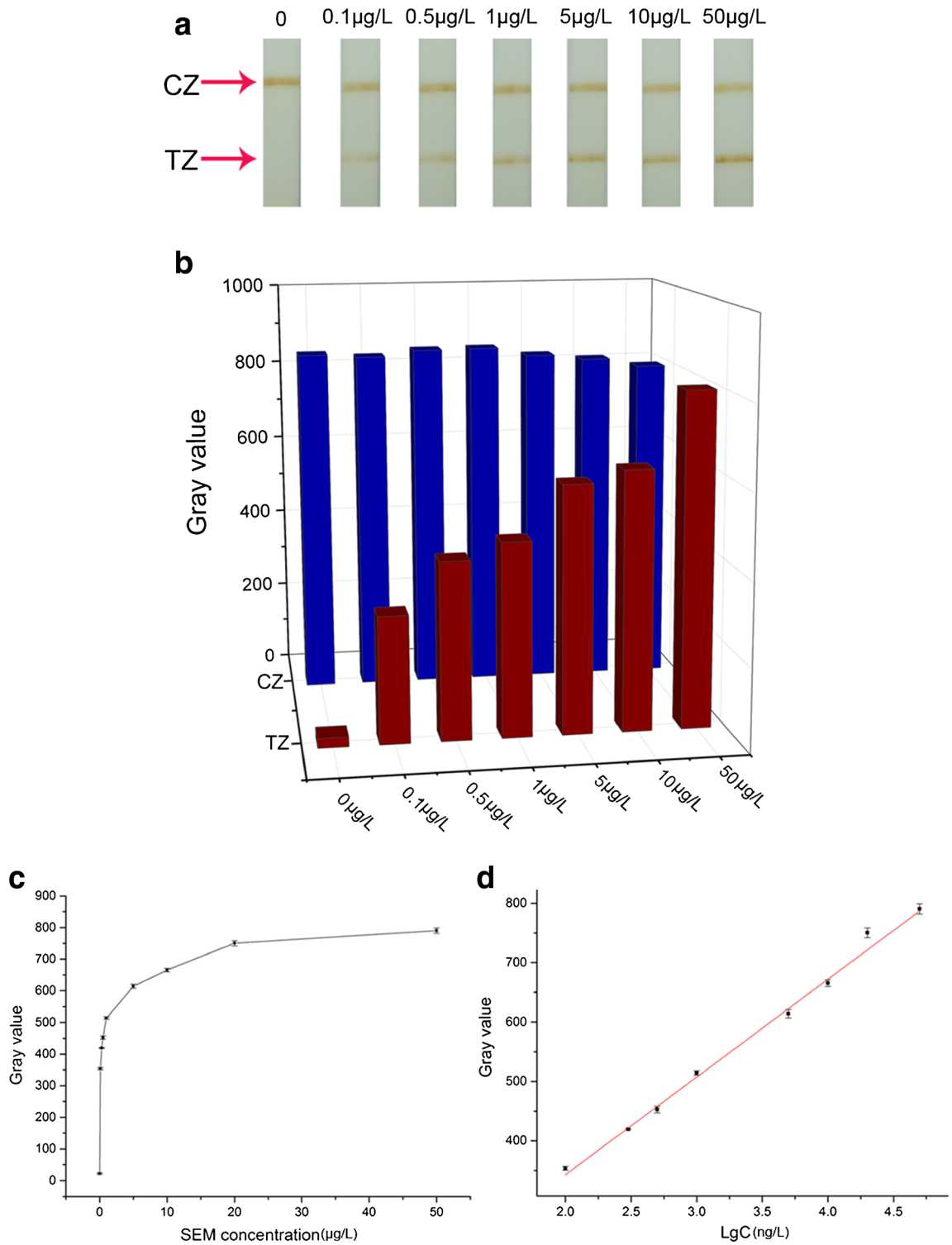


Fig. 2 Results of biosensor for SEM detection. **a** Photographs of strip covering test zone (TZ) and control zone (CZ). **b** Quantitative analysis of color intensity of the brown band on test zone. **c**) The optical intensity versus SEM concentrations plot. **d** Calibration curve for SEM detection

mixed with 5 μL of as-prepared MBs, followed by incubating at room temperature for 10 min with gentle vortex. After then, MBs was separated in magnetic field and washed twice with PBS to remove excess derivative. It was re-suspended in 100 μL of PBS and loaded onto the sample pad.

Results and discussion

Sensitivity of the assay

Sensitivity and dynamic range of the present assay was firstly evaluated using serial diluted SEM standard solutions (SEM spiked ultra-pure water). As shown in Fig. 2a, color intensity of brown bands on the test zones increased with the increment of SEM concentration. There was no brown band observed on the test zone in the absence of SEM (0 μg/L). For quantitative detection, optical intensities (peak areas) of test lines were further analyzed with the aid of a portable strip reader (Kinbio, Shanghai, China). The optical intensity versus SEM concentration plot is shown in Fig. 2c. Calibration curve shows that optical intensity is proportional to the logarithm of SEM concentration in the range from 0.1 to 50 μg/L (Fig. 2d). Brown band on the test zone is quite visible even at 0.1 μg/L of SEM, which can be used as the threshold for visual determination of SEM. The detection limit of SEM for real sample analysis was also 0.1 μg/L (ESM Fig. S6, ESI†). Compared to previously reported competitive immunochromatographic assay-based LFA [14, 21], the new method developed here is more sensitive and convenient. As the maximum contamination level of SEM in food defined by the United States Environmental Protection Agency (EPA) is 1 μg/kg, this lateral flow biosensor holds great promise in on-site monitoring.

Sensitivity of the developed biosensor for AHD, AOZ, and AMOZ detection were further evaluated with the same

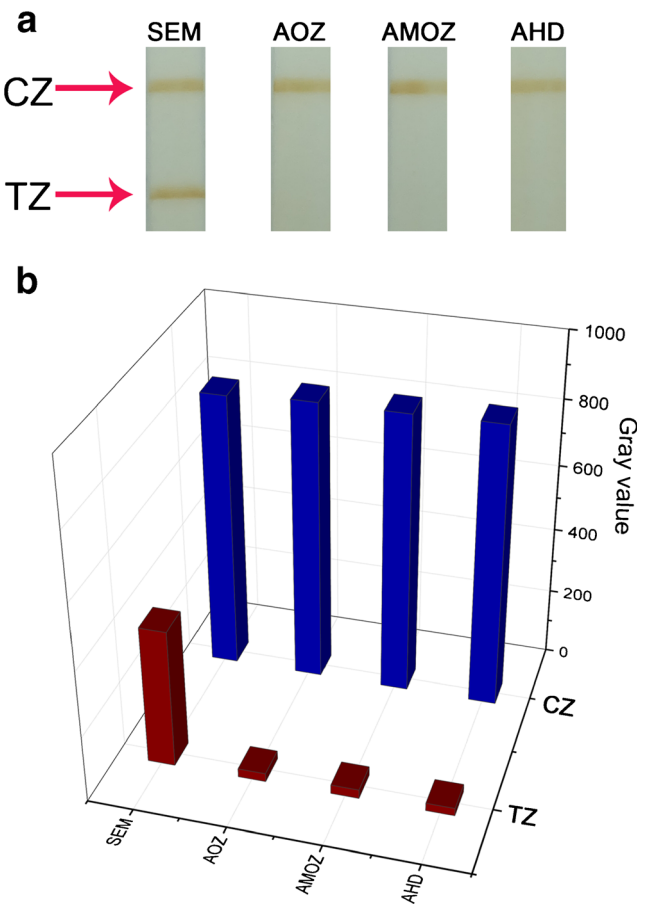


Fig. 3 Results of selectivity tests of strip biosensor for SEM detection. **a** Photographs of strip covering test zone and control zone. **b** Quantitative analysis of color intensity of the brown band on test zone

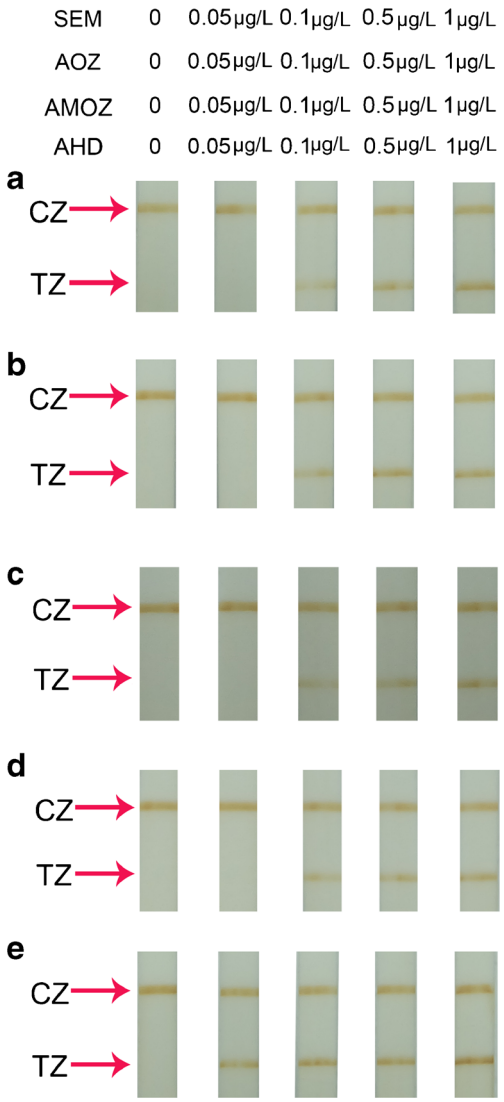


Fig. 4 Results of quadruple strip biosensor for the detection of SEM (**a**), AOZ (**b**), AMOZ (**c**), AHD (**d**) separately and a mix of four metabolites simultaneously (**e**)

procedure. The visual detection limit for AHD, AOZ, and AMOZ was 0.1, 0.1, and 0.1 μg/L, respectively (ESM Fig. S2–4, ESI†).

Selectivity of the assay

Selectivity of this biosensor for SEM detection was evaluated using the other three nitrofuran metabolites (AHD, AOZ, and AMOZ). As shown in Fig. 3, 1 μg/L SEM resulted in a bright brown band on the test zone, whereas the other metabolites at a concentration of 50 μg/L did not yield visible band on the test zone. The results showed good selectivity of the present biosensor in distinguishing SEM from other metabolites.

Selectivity of the developed biosensor for AHD, AOZ, and AMOZ detection were further evaluated with the same procedure used in SEM detection. No cross-reactivity was observed in the existence of the other three nitrofuran metabolites at 50 μg/L (ESM Fig. S2–4, ESI†).

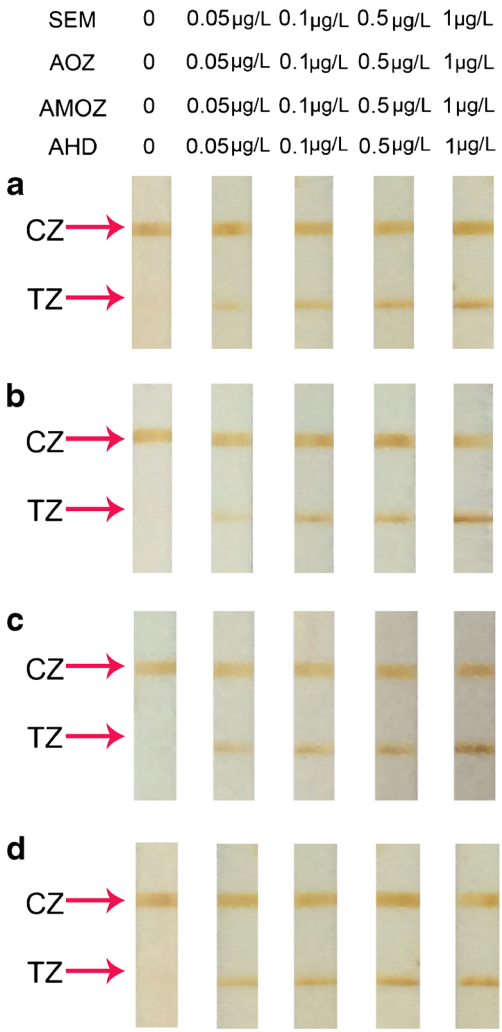


Fig. 5 Results of simultaneous detection for SEM + AOZ (**a**), AMOZ + AHD (**b**), SEM + AOZ + AMOZ (**c**), SEM + AOZ + AHD (**d**)

Sensing performance for quadruple detection of nitrofuran metabolites

The strip biosensor for separate or simultaneous detection of four nitrofuran metabolites (AHD, AOZ, SEM, and AMOZ) was established by using a mixing of four MBs (1:1:1:1) for magnetic separation. The developed strip biosensor was firstly evaluated using a mixture of AHD, AOZ, SEM, or AMOZ standard solutions. As shown in Fig. 4a–d, color intensity of the brown band on test zone increases with the elevated metabolite concentration. Brown band on the test zone is quite visible at 0.1 μg/L of AHD, AOZ, SEM, or AMOZ, respectively. The co-existence of four metabolites (0.05 μg/L each) also resulted red test line (Fig. 4e). As shown in Fig. 5, the present quadruple detection strips were capable of detecting two or more nitrofuran metabolites simultaneously.

Conclusions

In summary, we have established a strip biosensor for fast and quadruple detection of nitrofuran metabolites using a novel ractopamine derivative. By coupling LFA with MBs-based magnetic separation, this assay not only simplified the extraction procedure but also increased the sensitivity. It can be further applied in other small molecules detection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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