Preparation and Characterization of an Immunoaffinity Column for the Selective Extraction of Salbutamol from Pork Sample

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Abstract

A rapid, simple, and reliable determination method for salbutamol in pork was developed with immunoaffinity column (IAC) extraction followed by HPLC analysis. The salbutamol immunoaffinity column was prepared by coupling CNBr-activated Sepharose-4B with the anti-salbutamol polyclonal antibody which was purified by caprylic acid-ammonium sulfate. The coupling rate of the antibody and Sepharose-4B was 98.6%, and the dynamic column capacity of IAC was 400 ng/mL gel. The average recoveries of salbutamol from spiked pork samples at levels of 2, 10, 20, and 50 ng/g ranged from 83.3% to 92.2%, with the relative standard deviations of 2.8–7.0% (n = 5), and the limits of detection and qualification were 0.25 ng/g and 0.5 ng/g, respectively.

Introduction

Salbutamol (SAL), also known as albuterol, is a β_2-receptor agonist which can selectively activate the β_2-receptor of bronchial smooth muscle and cause certain broncho-dilating effects. In a clinic, SAL is usually used to treat bronchial asthma, asthmatic bronchitis, and bronchial spasm of emphysema patients (1). However, an overdose of SAL would result in severe side effects. In animal husbandry, SAL has been found to be able to promote the growth of skeletal muscle and reduce the fat accumulation. For this reason, it has still been used as feed additive to increase the carcass meat-fat ratio by farmers in China and other countries in spite of having been banned for use in animal production around the world. This would lead to SAL residue in animal-origin food and pose a potential hazard to human health (2). Therefore, it is very necessary to establish a rapid, simple, and reliable determination method to monitor the illegal usage of SAL and its residue in food.

A variety of analytical methods, such as liquid chromatography (LC) (3–5), gas chromatography–mass spectrometry (GC–MS) (6,7), LC–MS–MS (8,9), and enzyme linked immunosorbent assay (ELISA) (10, 11), have been reported to determine SAL residue in different matrixes. ELISA is usually used as screening method with relatively poor accuracy and precision. By comparison, instrumental methods including LC, LC–MS–MS and GC–MS have better quantitation ability, but they generally require extensive sample preparation to reduce matrix interference. Solid-phase extraction (SPE) and liquid–liquid extraction would be the most often used approaches for sample purification, but immunoaffinity chromatography provide a better choice by selective extracting target molecule from complex sample. Since Axen et al. (12) employed cyanogens bromide (CNBr) to activate agarose gel and successfully prepared affinity adsorbent medium in 1967, immunoaffinity chromatography has been moved toward practical application from a research means, and become one of the most effective separation methods in immunochemistry. So far, immunoaffinity chromatography is widely used in residue analysis of pesticides and veterinary drugs, such as diazinon (13), avermectins (14), quinolones (15), and chloramphenicol (16). Moreover, in previous reports, immunoaffinity column (IAC) was used to purify SAL residues in bovine liver, pig liver, and swine urine samples, followed by LC analysis with fluorescence detection (4,5) or GC–MS analysis (7).

However, poor recoveries were obtained by these methods (5), and the limit of detection (LOD) was 1 ng/g of SAL remaining in pig liver (4) which could not meet the requirement for quantitative analysis of trace SAL in samples. The objective of this study was to develop an improved IAC purification procedure with specific and simple sample preparation, and increase the sensitivity and recovery for the detection of SAL residue by HPLC method.

Experimental

Chemicals and materials

SAL was purchased from China Institute of Veterinary Drugs Control (Beijing, China). Mixed enzymatic solution including 94400 U/mL of β-Glucuronidase and 1079 U/mL of aryl sulfatase was obtained from Sigma-Aldrich (St. Louis, MO). Sepharose 4B

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was purchased from GE Healthcare (Chicago, IL). Acetonitrile and NaH_2PO_4 (HPLC grade) were purchased from Fisher, Inc. (Pittsburgh, PA). All other chemicals and solvents were of analytical grade or higher, and commercially obtained from Beijing Chemical Reagent Co. (Beijing, China). Deionized water (18.2 MΩ cm, Milli-Q Element, Millipore, Billerica, MA) was used as pure water in this study.

Apparatus

Silentcrusher M homogenizer was obtained from Heidolph Group Inc. (Schwabach, Germany). Sigma 3-30k high-speed centrifuge was used (Sigma Group Inc., Munich, Germany). UV-2401PC ultraviolet-visible (UV–vis) spectrometer was obtained from Shimadzu Co., Ltd (Shimadzu, Tokyo, Japan). Oasis MCX SPE column (3 mL, 60 mg) was purchased from Waters Inc. (Milford, MA).

SAL standard solution

Stock solution of SAL standard (100 mg/L) was prepared in 100 mL of methanol, and then intermediate standard solution (1 mg/L) was obtained from the stock solution with methanol at a dilution ratio of 1:100. Working solutions of SAL standard (10–50 ng/mL) were freshly prepared from the intermediate solution by dilution with PBS buffer (pH 7.0) before use. All standard solutions were stored in a fridge at 4°C.

Coupling buffer solution

Coupling buffer solution was prepared by dissolution of 4 g of NaCl, 0.1 g of KCl, 0.1 g of K_2HPO_4, 1.45 g of Na_2HPO_4, and 250 µL of Tween into 1000 mL of pure water.

Other solutions including 0.1 mol/L of Tris-HCl (pH 8.0), 0.1 mol/L of HAc-NaAc (pH 4.0), 0.05 mol/L of HAc-NaAc (pH 4.0), 0.02 mol/L of HAc-NH_4Ac (pH 5.2), 0.137 mol/L of PBS (pH 7.4), 0.01 mol/L of PBS (pH 7.2), 0.02 mol/L of NaH_2PO_4 (pH 2.7), $(NH_4)_2SO_4$ saturated solution, and 0.1 mol/L of $NaHCO_3$ (pH 8.3) were prepared before experiment.

Pretreatment of polyclonal antibodies

The polyclonal antibodies of SAL were prepared according to the method of Lei et al. (10), and purified by caprylic acid–ammonium sulfate method (17). The purified antibodies were dissolved into the coupling buffer, and then dialysed in this buffer with 50–100 fold volume at 4°C for 3 d. The dialysis buffer was refreshed twice per day. After dialysis, the antibodies were diluted to a certain concentration for UV full-spectrum scan with the coupling buffer as reference, resulting in an UV–vis absorption spectrum. Based on the absorbance values at 280 nm (A_{280}) and 260 nm (A_{260}), the concentration of the antibody could be calculated by the empirical formula (protein concentration = $1.45A_{280} - 0.74A_{260}$) (18).

Preparation of IAC

CNBr-activated Sepharose-4B matrix powder (1 g) was dissolved into 1 mmol/L of HCl, and then placed into a sintered-glass filter and washed 15 min with the HCl solution. The activated Sepharose-4B was quickly transferred into the coupling buffer with 20.8 mg of the polyconal antibodies, and was thoroughly mixed and stirred at room temperature for 2–4 h. After finishing the coupling reaction, the mixed solution was centrifuged at 2000 rpm for 1 min, and the protein concentration in the supernatant was determined to calculate the coupling ratio. The coupling products at the bottom of the centrifuge tube were taken out and washed with the coupling buffer to remove free ligands, and then transferred into 0.1 mol/L of Tris-HCl buffer (pH 8.0) to stand at room temperature for 2–4 h. To remove the uncoupled ligands, the coupling products were sequentially washed with 0.1 mol/L of HAc-NaAc (pH 4.0) and Tris-HCl buffer for at least 3 cycles. The coupling ratio could be calculated by the following equation.

$$\text{Coupling ratio} = \frac{\text{SAL antibody} - \text{supernatant proteins}}{\text{SAL antibody}} \times 100\%$$

The coupling products were washed with 5-fold volume of 0.137 mol/L of PBS to prepare suspension, and the sedimentary matrix was mixed with the buffer at the ratio of 3:1. Finally, 1 mL of the previously described mixture was transferred to the polypropylene column (10 × 0.8 mm i.d.), and stored in 0.137 mol/L of PBS containing 0.01% NaN_3 solution at 4°C.

Determination of column capacity

Two milliliters of intermediate solution of SAL standard were transferred into the IAC at a flow rate of 1 mL/min. The saturated column was washed with 5 mL of pure water, and then eluted with 2 mL of methanol. The eluate was collected and determined by HPLC, and thus the amount of SAL in the eluate was the capacity of the column. The column was regenerated by 0.137 mol/L of PBS.

Optimization of elution conditions

Five milliliters of SAL standard working solution (20 ng/mL) were transferred into every one of the six IACs. All of the columns were washed with 5 mL of pure water, and then eluted with 2 mL of 50%, 60%, 70%, 80%, 90%, and 100% methanol solutions, respectively. The eluates were collected and determined by HPLC, and the methanol solution with the highest elution rate was selected as the eluent.

Five milliliters of SAL standard working solution (20 ng/mL) were transferred into the IAC. The column was washed with 5 mL of pure water, and then eluted with methanol. The eluates were colleted every 0.5 mL from 0.5 to 6 mL of eluent, and determined by HPLC to select the volume of eluent.

Sample preparation with IAC

Ten milliliters of HAc-NH_4Ac (0.02 mol/L) were added into 10.0 g of homogenized pork sample, and further homogenized for 2 min at high speed. Then, 50 µL of the mixed solution of β-glucuronidase and aryl sulfatase was added into the sample solution, and decomposed in a 60°C incubator for 2 h after ultrasonic treatment for 15 min, and centrifuged at 3000 rpm for 10 min. Four milliliters of the supernatant was taken out and diluted to a final volume of 20 mL by 0.137 mol/L of PBS. After filtered by glass microfiber filters, the filtrate was collected in a clean beaker. Five milliliters of the filtrate was loaded into the IAC at a flow rate of 1 drop/s until air went into the column, and washed by 5 mL of PBS (0.137 mol/L). The IAC was eluted by 2.0 mL of methanol at a flow rate of 1 drop/s. The eluate was col-

lected in a glass test tube and evaporated to dryness at 40°C under a gentle stream of nitrogen, and dissolved with mobile phase to a final volume of 0.5 mL for HPLC analysis.

HPLC analysis

Agilent Technologies 1200 Series High performance liquid chromatograph comprised a G1322A degasifier, a G1311A pump, and a G1329A sample injector (Agilent Group Inc., Greensboro, NC). The separation of SAL from the matrix was achieved in an Eclipse XDB-C_{18} column (150 × 4.6 i.d., 5 µm, Agilent) with a mobile phase of acetonitrile–NaH_2PO_4 (10 mmol/L, pH 3.0) (8:92) at a flow rate of 0.8 mL/min. An isocratic elution mode was employed, and the retention time for SAL was about 4.66 min under these conditions. A fluorescence detector was used to detect SAL with excitation wavelength and emission wavelength at 225 nm and 320 nm, respectively. For each analysis, 50 µL of the sample solution was injected.

Results and Discussion

The performance of the IAC

The performance of the IAC includes coupling rate of antibody, column capacity, and specificity. Based on the weight of the SAL antibodies before coupling and the weight of the protein in the supernatant after centrifugation, the coupling ratio was calculated to be 98.6% and the column capacity of the prepared IAC in this study was found to be 400 ng/mL gel. As a polyclonal antibody, the SAL antibody used in this study can also recognize clenbuterol and terbutaline besides SAL, and the cross-reactivities of SAL, terbutaline, and clenbuterol were 100%, 100%, and 205%, respectively. This implied that the prepared IAC would have the potential to purify the three drugs. In addition, some commonly-used drugs including tetracycline, sulfanilamide, chloramphenicol, and β-lactam were also purified using the prepared IAC, no drugs were found to be able to absorb on the IAC, which indicates that the retention of SAL on the IAC was specific.

Delauna and Pichon (19) pointed out that it is appropriate for every one milliliter of swollen gel to couple with 4~10 mg of immobilized antibody. When the amount of the coupled antibody is above 10 mg/mL gel, the adsorption ability of IAC will be so strong that the adsorbed antigen or hapten can not be eluted; although the amount of the coupled antibody is below 4 g/L, the adsorption ability of the IAC will be too weak to remove impurities effectively. For the prepared IAC in this study, every 1 mL of swollen gel could couple with 6.0 mg of salbutamol antibodies, which showed that the coupled amount was very suitable. This result was also consistent with that reported by Wang et al. (7).

In previous studies, Cooper and Shepherd prepared an IAC using clenbuterol polyclonal antibodies and the column capacity for SAL was 270 ng/g gel (5), and another SAL affinity column prepared by Wang et al. had a column capacity of 400 ng/mL gel (7). Therefore, the column capacity in this study is closest to that obtained by Wang et al., and higher than that by Cooper and Shepherd.

Optimization of elution condition

Some conditions such as loading, washing, and elution would have a strong influence on the association and dissociation of the antigen-antibody complex (20). Generally, neutral salt solutions (4), PBS, or Ca-tris buffer (5) were used as the loading solutions and washing solvent, respectively, and the elution solution varied in its composition and volume because it would bring great effect on recovery. In this study, the elution conditions were investigated, and the results are showed in Table I and Figure 1. Table I shows that the elution rate was increased from 42.1% to 100.0% with the concentration of methanol in elution solution elevated from 50% to 100%. To ensure the best recovery and facilitate sample concentration, pure methanol was selected as the eluent. Figure 1 demonstrates that the elution rate was improved with the volume of the eluent increasing, and reached maximum (100%) when methanol volume was more than 2 mL. Therefore, the elution volume was selected to be 2 mL.

Method validation

Typical chromatographs of SAL standard solution, blank pork sample, and pork sample spiked SAL standard were shown in Figure 2. The lack of interference to SAL peak in chromatogram suggests a good purification effect of the prepared IAC. The LOD and limit of quantitation (LOQ), which were defined as signal-to-noise ratio of 3:1 and 10:1, were determined to be 0.25 ng/g and 0.5 ng/g, respectively. The sensitivity of this method is much higher than the existing methods, and close to the LOQ of enzyme immunoassay (21).

The accuracy and precision of the method were evaluated according to recovery and relative standard deviations (RSD), respectively. The recoveries of SAL from spiked pork samples are

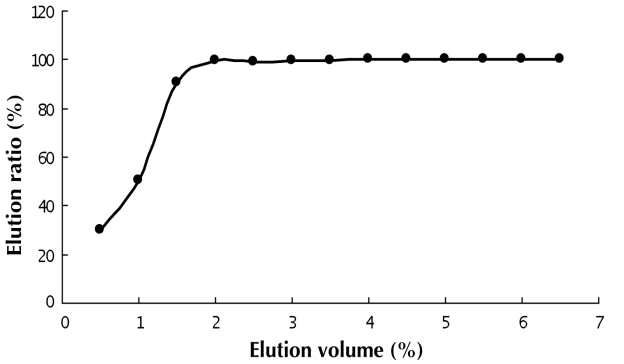

Figure 1. Effect of elution volume.

Table I. Effect of the Conc. of Methanol Solution on Elution Rate

Conc. of methanol solution (%)	Elution rate (%)
50	42.1
60	49.6
70	68.1
80	81.2
90	95.6
100	100.0

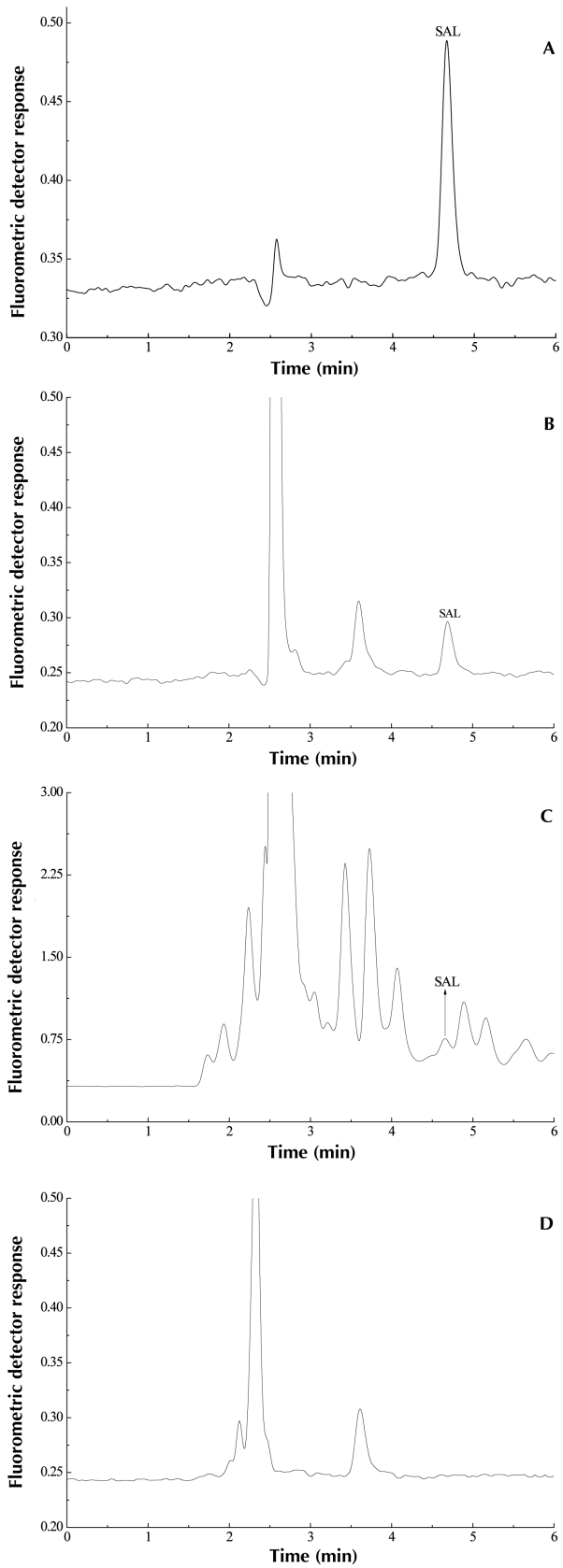

Figure 2. Chromatogram of SAL standard solution and pork samples spiked. (A) SAL standard solution (10 ng/mL), (B) pork samples spiked with 2.0 ng/g of SAL by IAC purification, (C) pork samples spiked with 2.0 ng/g of SAL by MCX column purification, and (D) blank pork sample by IAC purification.

Table II. Recovery of Pork Samples Spiked with SAL Standards*

Spiked level (ng/g)	Mean % recovery	RSD %
2.0	83.3	6.9
10.0	85.2	3.8
20.0	90.7	2.8
50.0	92.2	7.0

* $n = 5$

summarized in Table II. It can be seen that the average recoveries at fortification level of 2~50 ng/g were in the range of 83.3–92.2%, with the RSD of 2.8–7.0% (n = 5). This result demonstrated that the IAC procedure that was developed is superior to that developed by Degroodt et al. (4) and Cooper et al. (5) because both of the latter two IAC protocols gave relatively low recovery.

Comparison with SPE column

Generally, SPE column was used to cleanup SAL from complex samples. In order to compare the purification effect of the IAC with that of SPE column, the pork samples spiked with 2.0 ng/g of SAL were extracted and then purified by IAC and MCX SPE column, respectively. The obtained chromatograms indicated that the purification effect of the IAC is better than that of the MCX SPE column, because the former lead to a cleaner chromatogram and less interference to SAL peak (Figures 2).

In this study, we developed an IAC procedure followed by HPLC analysis for determining SAL residue. The cleanup effect of the IAC purification is superior to that of the existing IAC and SPE method, and the accuracy and precision of the developed method meet the requirements for quantitative analysis, thus the proposed IAC-HPLC protocol can be used as a routine method for the determination of SAL in pork samples.

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