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Development of an immunoaffinity chromatography column for selective extraction of a new agonist phenylethylamine A from feed, meat and liver samples

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ABSTRACT

Phenylethanolamine A (PA) is a new emerged β-adrenergic agonist that has been illegally used as an animal feed additive for growth promotion in China. In this study, an immunoaffinity chromatography (IAC) column for selective extraction of PA from swine feed, meat and liver samples was developed. The IAC column was constructed by covalently coupling specific polyclonal antibody (Ab) against PA to CNBr-activated Sepharose 4B and packed into a common solid phase extraction (SPE) cartridge. The extraction conditions including loading, washing and eluting solutions were carefully optimized. Under optimal conditions, the IAC column was characterized in terms of maximum capacity, selectivity, extraction recovery and stability. The maximum capacity of the ICA for PA extraction was found to be 239.4 ng. For selectivity testing, 100 ng of other three β-adrenergic agonists (clenbuterol, ractopamine and salbutamol) was separately loaded onto the column, and it was observed that the tested compounds could not be captured on the column, e.g. the column could only selectively recognize PA. The recovery of the IAC for PA extraction was found within 96.47–101.98% when 10, 50 and 100 ng PA were separately loaded onto IAC column. The IAC column was also applied to real sample extraction. Swine feed, meat and liver samples were collected and spiked with PA in range of 1.0–20 ng g^{-1}. The spiked and unspiked samples were extracted by IAC column and measured by high performance liquid chromatography (HPLC). It was found that there was no detectable PA in the blank samples, and the extraction recoveries of the IAC for PA from the spiked samples were within 89.48–104.89%. The stability of the column was also tested. It was showed that after 35 times repeated usage, 60% of the maximum capacity was still remained. The proposed IAC was proven to be a feasible extraction method for PA from different matrices with the properties of high maximum capacity, selectivity, extraction efficiency and stability.

1. Introduction

With the development of society and the increasing of living standard, people pay more attentions to the food safety, especially to the risk of the residues of β-adrenergic agonists in animal production. β-Adrenergic agonists are synthetic phenethanolamine compounds widespread used as bronchodilatory agents for therapeutic purposes. When the drugs were given in doses five to ten times higher than the requirement of therapeutic treatments, they could sharply increase the promotion of muscle growth and the protein-to-fat ratio [1], therefore β-adrenergic agonists were often illicitly abused as growth-promoting agents in animal feeds to enhance the lean meat-to-fat ratio for livestock and as doping drugs to enhance the performance of human athletes [2]. However, their illegal use in livestock production may cause deleterious physiological side-effects such as cardiac palpitation, tachycardia, nervousness, muscle tremors and confusion after human consumption of meat products. Poisoning incidents caused by high concentrations of β-agonists residues in edible tissue occurred in many countries [3,4]. Therefore, β-agonists are banned as feed additives for growth promotion in animals. The most commonly abused β-adrenergic agonists are clenbuterol, ractopamine and salbutamol (the molecular structures are shown in Fig. 1). Unfortunately, with the crackdown of banned β-agonists, some other new β-agonists were emerged. Recently a new alternative of β-adrenergic agonist named phenylethanolamine A (PA, 2-(4-(nitrophenyl) butan-2-ylamino)-1-(4-methoxyphenyl) ethanol,

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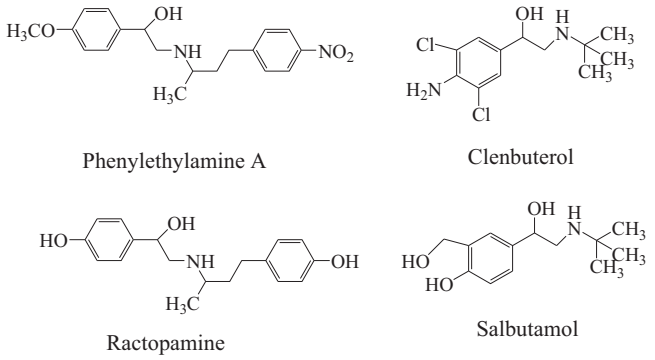

Fig. 1. The molecular structures of phenylethanolamine A and other three β-adrenergic agonists (clenbuterol, ractopamine and salbutamol) used for selectivity testing.

$C_{19}H_{24}N_2O_4$, Fig. 1) has been illegally used in livestock in China, and in 2010, PA was prohibited from being used in animal feeds and drinking water in China [5].

The main analytical methods for the determination of β-adrenergic agonists in biological and feed samples are chromatographic ones including gas chromatography/mass spectrometry (GC–MS) [6], liquid chromatography (LC) [7], liquid chromatography/mass spectrometry (LC–MS) [8] and liquid chromatography/tandem mass spectrometry (LC–MS/MS) [9,10]. In 2010, the Ministry of Agriculture of China issued a standard analytical method for the detection of PA in feed using high-performance liquid chromatography tandem mass spectrometry (HPLC–MS/MS) [11]. Recently there was a report of the LC–MS/MS for the detection PA in animal hair, tissues and feeds [12]. Although LC–MS/MS is accurately quantitative method, the instrument is very expensive. In most cases, these chromatographic methods could detect trace levels of target analytes when an extensive pre-treatment step such as solid phase extraction (SPE) was coupled with. However, current SPE is based on physicochemical retention on functionalized surface which captures not only the target analyte but also other matrix components [13,14]. The drawbacks of routine SPE techniques are their low selectivity toward the particular target analyte. New efficient cleanup techniques employing the sorbents with the high selectivity for analyte are indispensable.

Immunoaffinity chromatography (IAC) is a separation method that takes advantage of the specific recognition binding between antibody and antigen [15,16]. Antibodies against a target analyte (antigen) are immobilized on a solid support. The immobilized antibodies will specifically retain the antigen from a solution passed through the support. Bound antigen can then be eluted and the support regenerated for reuse. IAC offers unique and powerful techniques, which enables selective extraction and enrichment of individual compounds or classes of compounds in one step. Additionally, IAC uses little or no organic solvents and columns may be reused and easily automated. IAC techniques have been widely used in clinical, biological, food and environmental areas for the extraction and enrichment of target analytes from different matrices prior to analysis by on-line and off-line HPLC [17–22].

So far, there are many reports of IAC for β-agonist extraction such as clenbuterol [23], ractopamine [24] and salbutamol [25], but it is still no report of IAC for PA. In our previous study, we properly modified the PA molecule and produced polyclonal antibody against PA, then coupled with SPE, we successfully developed a sensitive and specific ELISA for PA detection [26]. To overcome the non-specific absorption of SPE column, taking advantage of the specific binding of the antibody against target analyte (PA) that was produced in our lab, the aim of this work is to develop an IAC column for the selective and effective extraction of PA from feed, meat and liver samples prior to the analysis by HPLC. The purified Ab was covalently coupled to CNBr-activated Sepharose 4B particles and packed into a SPE column. The extraction conditions of the IAC column for PA were optimized and the IAC column was characterized in terms of maximum capacity, selectivity, extraction recovery and stability. Then IAC column was also applied for the extraction of PA in real samples. To the best of our knowledge, it is the first report for the extraction of PA in samples with IAC column.

2. Experimental

2.1. Reagents and apparatus

CNBr-activated Sepharose 4B was purchased from Pharmacia Biotech (Uppsala, Sweden). Phenylethylamine A (PA, 98%) was purchased from Toronto Research Chemicals Inc. (Toronto, Canada). Methanol (MeOH) and acetonitrile (HPLC grade) were purchased from J&K Scientific Ltd. (Shanghai, China). Sodium acetate (NaAc), sodium chloride (NaCl), sodium dihydrogen phosphate, disodium hydrogen phosphate, glacial acetic acid, hydrochloric acid (HCl), sodium bicarbonate and potassium dihydrogen phosphate were purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China).

Ultraviolet visible spectrophotometer (UV-2300) was purchased from Techcom Com. (Shanghai, China). High speed refrigerated centrifuge was purchased from Anhui USTC Zonkia Scientific Instruments Co. Ltd. (Hefei, China). Nitrogen evaporator with 12 channels and temperature controller for water bath was purchased from ANPEL Scientific Instrument Co. Ltd. (Shanghai, China). Extraction device (Supelco visiprep) with 12 channel settings and vacuum pump was purchased from Sigma–Aldrich Biotechnology Co. Ltd. (Bellefonte, USA). Deionized-RO water supply system (Dura 12FV) was purchased from THE LAB Com. (USA).

2.2. Detection of PA by HPLC–UV

To develop an IAC column for selective extraction of PA from different matrices, it is required firstly to establish an accurately quantitative analytical method for the detection PA in order to evaluate the prepared IAC column. In this study, the HPLC coupled with ultraviolet detector (HPLC–UV) for the detection of PA was established. A HPLC system from Alltech Associates, Inc. (Deerfield, IL, USA) with a C_{18} column (250 mm × 4.6 mm, 5.0 μm particle size) and the HPLC work station software used for the instrument control and data analysis was employed for detecting PA. The mobile phase was 7.5 mmol L^{-1} KH_2PO_4 (pH 3):acetonitrile (13:87, v/v) at a flow rate of 1 mL min^{-1} [27]. PA standard solutions or sample extracts was passed through a 0.2 μm cellulose acetate membrane filter prior to HPLC detection. The detection wavelength was set at 215 nm with 20 μL sample loop and the peak areas were used for quantification. The standard curve for PA was constructed in concentration range of 0.1, 0.2, 0.5, 1, 2, 5 and 10 μg mL^{-1} prepared by diluting PA stock solution (1 mg mL^{-1}, by dissolving PA in methanol) with mobile phase. The retention time of PA was 5.96 min. The linear equation for the HPLC–UV for PA was found to be: $y = 1158.7 + 45{,}378.4x$ ($R^2 = 0.9999$, $n = 8$) with the detection limit of 48.7 ng mL^{-1}.

2.3. Production and purification of polyclonal antibody

The polyclonal Ab against PA was produced according to the procedures described in our previous study [26]. Briefly, the nitryl group on the PA molecule was changed to amino group via a reduction reaction, then the PA derivative with the amino group (PA-NH_2) was covalently coupled to carrier protein (bovine serum albumin, BSA) by diazobenzidine method. The PA-BSA conjugate