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Analytical Methods

Development of molecular imprinted column-on line-two dimensional liquid chromatography for selective determination of clenbuterol residues in biological samples

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ABSTRACT

A novel method coupling molecular imprinted monolithic column with two-dimensional liquid chromatography was developed and validated for the analysis of clenbuterol in pork liver and swine urine samples. The polymers were characterized by using Fourier transform infrared spectroscopy, nitrogen adsorption desorption analyses, frontal analysis and the adsorption of selectivity. The results indicated that the imprinted columns were well prepared and possessed high selectivity adsorption capacity. Subsequently, the MIMC-2D-LC (molecular imprinted monolithic column-two dimensional liquid chromatography) method was developed for the selective analysis of clenbuterol in practical samples. The accuracy ranged from 94.3% to 99.7% and from 93.7% to 99.6% for liver and urine, respectively. The relative standard deviation (RSD) of repeatability was lower than 8.6% for both analyses. The limit of detections was 16 $ng \cdot mL^{-1}$ for liver and 25 $ng \cdot mL^{-1}$ for urine, respectively. Compared with the reported methods, the disturbance of endogenous impurity could be avoided by the 2D-LC method.

1. Introduction

β_2-Agonists which can bind to the bronchial smooth muscle β_2-adrenoceptor, were originally used as remedial medicine for asthma and other related diseases in humans (Wu et al., 2015). However, these compounds have also been misapplied as growth stimulants in stockbreeding, which can transform nutrients from fat tissues to the muscular tissue (Du, Zhao, et al., 2014). The illegal use of β_2-agonists, especially the illegal used of clenbuterol has caused serious unexpected toxicoses in humans (Tang et al., 2016). Accordingly, all β_2-agonists, as the feed additives for growth stimulation, are forbidden in livestock in both China and European Union (Gao et al., 2014). However, it still permits such drugs to be used as the feed additive for livestock both in United States and Canada (Du, Lei, et al., 2014). Therefore, it is essential to monitor the abuse of β_2-agonists in graziery (Du et al., 2013).

Currently, lots of analytical methods have been developed for the determination of β_2-agonists in animal tissues, feeds and urine: enzyme-linked immune sorbent assay (ELISA), gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and high performance liquid chromatography (HPLC). ELISA is easy to operate, while it is easily affected by environmental factors which lead to a high rate of false positive results (Cheng et al., 2014). GC–MS and LC–MS have the advantages of high sensitivity, nevertheless, the instruments are expensive and need to be equipped with professional technical personnel (Guo et al., 2015). HPLC is the most common used method, whereas conventional one-dimension detection models is liable to be interfered by endogenous contaminants in complex biological samples (Thippani, Pothuraju, Ramisetti, & Shaik, 2013). In addition, the routine sample pretreatment process is time-consuming and low selectivity (Luo et al., 2014). Therefore, it is desired extremely to develop a rapid, effective and specificity method for the detection of β_2-agonists residues in biological samples.

Compared with routine chromatography, multi-dimensional liquid chromatography (MDLC) provides a better separation effectiveness (Sarrut, Crétier, & Heinisch, 2014). Therefore, multi-dimensional liquid chromatography, especially two-dimensional liquid chromatography (2D-LC), has been extensively applied in complex specimens' analysis (Bailey & Rutan, 2011). Two-dimensional liquid chromatography is a technique in which two independent chromatography columns are involved to analysis

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the samples. According to the separation patterns, 2D-LC can also be classified as off-line 2D-LC and on-line 2D-LC. Compared with off-line 2D-LC, on-line 2D-LC has the advantage of automation and better reproducibility. On-line 2D-LC can be further classified as comprehensive 2D-LC and heart cutting 2D-LC, which depending on the first dimension sections are completely transferred into the second dimension or not (Wang et al., 2015). Therefore, on-line heart cutting 2D-LC is an ideal mode for the analysis of the specific categories compounds in complex matrixes due to its advantages of rapid and reliability (Ginzburg, Macko, Dolle, & Brüll, 2011).

Furthermore, the β_2-agonists are usually existed in biological matrixes with trace amounts (Xiong, Gao, Li, Yang, & Shimo, 2015). The complex matrixes can cause the decreasing of sensitivity and reduce the life cycle of HPLC columns. Accordingly, some sample pretreatments, such as solid phase extraction, is an essential procedure. Nevertheless, routine commercial SPE packing material suffers from the disadvantage of poor selectivity and low recovery, which lead to the sample pre-treatments is time-consuming and tedious. Consequently, specific recognition adsorbents for SPE use are in demand.

In order to solve this problem, molecular imprinting technology has attracted more attention (Luo et al., 2015). Since the recognition process of molecular imprinted polymers (MIPs) are comparable with the specificity identification of the antigen-antibody, it has a comprehensive application prospects (Kamra et al., 2016).

In this study, we established a novel MIMC-2D-LC method, which coupled molecular imprinted column with C18 column for the determination of clenbuterol in complex matrixes. In situ method was used to prepare the molecular imprinted polymers for selectively recognizing clenbuterol in biological matrixes. FT-IR, nitrogen gas adsorption and desorption analysis and frontal chromatographic was used to characterize the physicochemical properties and the recognition properties. And, we successfully analyzed the clenbuterol in liver and urine samples with the developed method.

2. Experimental

2.1. Reagents and chemicals

Clenbuterol (CLB, 99.0%) was purchased from Jinhe Pharmaceutical Co. Ractopamine and terbutaline sulfate were purchased from Gangzheng Pharmaceutical Co. (Wuhan, China). Ambroxol hydrochloride, noradrenaline Bitartrate and isoprenaline hydrochloride were purchased from Hefeng Pharmaceutical Co. (Shanghai, China). Dopamine was purchased from J&K China Chemical Ltd. (Beijing, China). The chemical structures of these compounds were illustrated in Fig. S1. Methacrylic acid (MAA) was purchased from Tianjin Chemical Reagent Plant (Tianjin, China). Ethylene glycol dimethacrylate (EGDMA) was obtained from J&K China Chemical Ltd. (Beijing, China). 2,2′-Azobisisobutyronitrile (AIBN) was purchased from Shanghai Shanpu Chemical Ltd. (Shanghai, China). Methanol and acetonitrile were of HPLC grade, purchased from Tianjin Kemiou Chemical Reagent Co. (Tianjin, China). Water was purified with Molement 1805b (Shanghai, China). All other chemicals were of analytical grade and obtained from local suppliers.

2.2. HPLC conditions

An on-line two-dimensional liquid chromatography system, including two CMC I-PUMP chromatography pumps (Zhejiang FULI analytical instruments Co. Ltd, China), an Agilent 1260 diode array detector (Agilent Technologies Inc., USA), a CMC I-UV UV detector (Zhejiang FULI analytical instruments Co. Ltd, China) and a ten-port valve switching system. A LC-20A liquid chromatograph system was composed of a LC-20A pump, a SPD-20AT spectrophotometer, a CBM communications bus module and a LC Solution work station (all from Shimadzu Co., Japan). LC-2010A$_{HT}$ high performance liquid chromatograph (Shimadzu Co., Japan).

High-performance liquid chromatography was performed on an LC-20 HPLC system. The UV detector was set at 213 nm with a flow rate of 1.0 mL·min^{-1}. The column void volume was measured by injecting 20 μL of acetone (0.1%, *v/v*), in a mobile phase of acetonitrile-buffer phosphate (20 mmol·L^{-1}, pH = 4.0) (65:35, *v/v*). All of the mobile phases were filtered through 0.45 μm membranes before used.

The capacity factor (k') is calculated from the equation $k' = (t_R - t_0)/t_0$, where t_R is the retention time of the eluted substances and t_0 is the retention time of the void marker. The imprinting factor (*IF*) is calculated from the equation $IF = k'_{MIPs}/k'_{NIPs}$, where k'_{MIPs} is the capacity factor of the target molecule eluted from the imprinted column and k'_{NIPs} is the capacity factor of the target molecule eluted from the non-imprinted column. The separation factors are calculated as $\alpha = k'_{CLB}/k'_2$, where k'_{CLB} and k'_2 are the capacity factors of clenbuterol and other analogues on molecular imprinted column, respectively.

2.3. Preparation of the imprinted column

CLB-molecularly imprinted columns were synthesized according to the reported method (Luo et al., 2011). MAA was used as the functional monomer, EGDMA as the cross-linking reagent, AIBN as the initiator, toluene and dodecanol as the mixed porogenic solvents. In brief, 95.6 mg clenbuterol, 136.4 μL MAA, and 762 μL EGDMA were dissolved in 3.08 mL mixed solvent of toluene and dodecanol (18:82). After the addition of 6.6 mg AIBN, the mixture was sonicated for 10 min. After being purged with nitrogen for 15 min, the mixture was introduced into a stainless-steel column (100 mm × 4.6 mm, i.d.). Then the polymerization was proceeded at 50 °C in a 101-AB oven (Tianjin Taisite Instrument Co, China) for 20 h. After polymerization, the column was elutriated with a mixture of methanol and acetic acid (4:1, *v/v*) until the baseline smoothly. Meanwhile, the non-imprinted polymers (NIPs) were prepared by same processes in the absence of clenbuterol.

2.4. Physical and morphological characterization

Fourier transform infrared spectra were performed by an FTIR-8400S spectrometer (Shimadzu, Japan) with a scanning range from 400 to 4000 cm^{-1}. Nitrogen adsorption and desorption analyses were evaluated by an Autochem 2920 (Quantachrome, USA) with a bath temperature of 77 K.

2.5. Frontal chromatography

The binding performances of the imprinted and non-imprinted monolithic columns were investigated by consecutive frontal analysis at a flow rate of 1.0 mL min^{-1}. The detector was performed at 213 nm, and mobile phases containing 3.61–36.08 μM (1–10 μg·mL^{-1}) of clenbuterol was used. The columns were first equilibrated with the mobile phase acetonitrile-buffer phosphate. Then a low concentration of solute, 3.61 μM of clenbuterol, was passed through the column until a plateau appeared. Thereafter, a second solution of higher concentration, 7.22 μM of clenbuterol, was applied directly to the column, without any regeneration of the columns with the blank mobile phase. This process was repeated until the plateau for the 36.08 μM solution was observed. Then, the chromatography conditions were adjusted and the breakthrough curves for other conditions were obtained by the same manners. The gradient delaying volume of the whole system was examined by using 0.1 mg·mL^{-1} of sodium nitrate to the columns