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Inclusion Chromatography of Albuterol in Livestock by Nano-baskets

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Albuterol, which increases the muscle mass and decreases the adipose tissue, is misused as nutrient repartitioning agent in livestock. Hence, a new sensitive method for determination of albuterol in livestock is presented. Four nano-baskets of calix[4]crown were synthesized and used to prepare nano-based bended-phases of HPLC–UV. The new synthesized bonded-phases were characterized and optimized, the bonding interactions of solute:stationary phases were examined and the main interactions were reported. The albuterol level of six samples of livestock meat including pork, pork casing, beef, beef casing, mutton, and mutton casing were analyzed and the results revealed that for the best bonded-phases, the LOD and LOQ were 0.06 and 0.20 μg/mL, respectively.

Keywords: Nano-baskets, Calix[4]-1,3-crown, Bonded-Phase, Albuterol, Livestock.

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1. INTRODUCTION

Clenbuterol, which is used for the treatment of asthma in both humans and animals, is a synthetic β_2–agonist. In high doses, clenbuterol exhibits a metabolic effect, which results in an increase in muscle mass and a decrease in adipose tissue; so, the compound is also misused as nutrient repartitioning agent in livestock by diverting nutrients from fat deposition in animals to the production of muscle tissues [1]. This misuse had caused some severe accidental poisonings in humans [2,3]. Therefore, all β_2–agonists are banned for growth promotion in animal production. In order to protect consumers, specific and sensitive methods for the identification and quantitation of clenbuterol in meat and other food are required.

There are several methods to determine clenbuterol. Among them, HPLC methods are the most effective method for selective determination of clenbuterol in a variety of samples. Chang et al. [4] used HPLC with a C18 column (2.0 × 150 mm) and a mobile phase of NaH_2PO_4 (pH=3.0) acetonitrile (80:20, v v^{-1}) solution at the absorbance at 212 nm. The recovery factors, limit of detection and the dynamic linear range were determined to be 80.9-90.6 %, 0.1 μg kg^{-1} and 0.2-1.0 μg kg^{-1}, respectively. Hamann et al. [5] described HPLC determination of clenbuterol in the presence of some degradation products of the formulation components and some common pharmaceutical preservatives such as methyl and propyl paraben. Degroodt et al. [6] presented their method for analysis of clenbuterol residue in urine and animal tissues. The HPLC system was equipped with a RP-8 column. They reported the limit of detections to be 0.25 μg L^{-1} for urine samples and 0.5 μg kg^{-1} for samples of animal tissue. Botterblom et al. [7] determined some adrenergic drugs (such as clenbuterol) in rat brain using a phosphate buffer (pH=3) acetonitrile (65:35, v v^{-1}) solution and the HPLC system equipped with a C18 column. About 10% coefficient of variation and 80-100 % recoveries were achieved.

Nano-baskets of calixarenes and calixcrowns are a versatile class of macrocycles, which have been subjected to extensive research in development of many extractants, transporters and stationary phases over the past four decades [8]. Functionalization of calix[4]arenes at both the upper rim and the lower rim has been extensively studied [9]. Attaching donor atoms to the lower rim of a calix[4]arene can improve the binding strength of the parent calixarene dramatically [10]. Calixarene–bonded stationary phases are preferable to the use as mobile–phase additives, because the UV detection of analytes is prevented by strong absorbance of calixarenes [11]. Additionally, poor solubility of most calixarenes precludes their applications as additives in aqueous eluents. With various methods for functionalizing calixarenes have been developed [12], more and more applications of different calixarene bonded stationary phases have been reported.

In the present work, the synthesis of four derivatives of diacid calix[4]-1,2-crowns and the preparation of their bonded-phases are presented. A new method for determination of clenbuterol in the livestock meat samples was set up by using the new calixcrown-based columns.

2. EXPERIMENTAL PROCEDURE

2.1 Materials and Apparatus

All solvents and analytes used were of analytical grade and obtained from Merck Chemical Company unless specially mentioned. Silica gel (with particle size of 5 μm, pore size of 100 Å and specific surface area of 300 m^2 g^{-1}), HPLC–grade methanol (MeOH) and γ-glycidoxypropyltrimethoxysilane (KH–560) were used. A phosphate buffer (0.005 w w^{-1}, pH 4.5) was prepared by mixing KH_2PO_4 with ultra–high quality pure water, and filtered through a 0.45 μm filter before use. Water was purified by using Milli–Q purification equipment. Clenbuterol was purchased from Sigma-Aldrich (MO,

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USA), terbutaline was obtained from the Laboratory for Residues of Veterinary Drugs (Berlin, Germany) and ractopamine was purchased from Eli Lilly Co. (IN, USA).

The stock solution of clenbuterol (250 mg mL^{-1}) was prepared by dissolving the reference substance in MeOH and stored in the refrigerator. The standard working solutions were prepared by diluting aliquots of the stock solution to obtain concentrations ranging from 0.1 to 2.0 µg mL^{-1}. The calibration graph was constructed by plotting the peak areas obtained at wavelength 243 nm versus the corresponding injected concentrations.

The samples were treated according to the following method: 2.000 g tissue samples was transferred to a 50 mL polypropylene centrifuge tube with stopper, 10 mL ammonium acetate (20 mmol) buffer solution was added to the tube, then 50 µL *β*–glucuronidase–aryl sulfatase (containing 134600 U mL^{-1} *β*–glucuronidase and 5200 U mL^{-1} aryl sulfatase) was added; after that, oscillate on the vortex oscillator for 2 min and sonicated for 20 min by using a ultrasonic processor, then put into an incubator for 16 h at 37 °C. After the tube was centrifuged at 4000 r min^{-1} for 10 min, the supernate was transferred into another tube.

Then, 5.0 mL chloroform was added, oscillated and sonicated again. The supernate was applied to Waters Oasis MCX cartridge, which was activated with 5 mL of methanol, 5mL of water, followed by 5 mL hydrochloric acid (0.01 mol) solution. The cartridge was washed with 6 mL ethyl acetate/ammonium hydroxide (97:3 v v^{-1}), the eluate was collected into a glass tube, and evaporated until dryness under a stream of nitrogen at 40°C water bath, the residue was reconstituted in 1.0 mL of 0.2 % formic acid in water/acetonitrile (90:10 v v^{-1}). The resulting solution was filtered through 0.22 µm filter and 10 µL of the filtrate was ready for analysis.

Chromatographic analyses were carried out by using an Agilent 1200 series system equipped with a 1200 model quaternary pump, a G1314A model Multiple Wavelength UV–vis detector, a G1316A model thermostated column compartment, a 1322A model vacuum degasser, and an Agilent Chemstation B.03.02 Patch data processor. The homemade calixcrown columns were filled using a packing machine under the pressure of 50 MPa. An Eclipse XDB–C_{18} column (Agilent, 150 mm × 4.6 mm i.d., 5 µm) was used as a comparison with the homemade calixcrown columns.

^{1}H NMR studies were carried out to determine the possible mechanism for the binding reactions.^{1}H NMR spectra were recorded in D_2O on a Bruker-DKX-300MHz spectrometer.

2.2 Calixcrown:Clenbuterol in Solution

A 1-mL aliquot of the stock solution (1.25×10^{-3} mol) of clenbuterol was transferred into a 25 mL volumetric flask, and an appropriate amount of calixcrown derivatives (2.5×10^{-2} mol) were added. The pH of solution was controlled by 0.5 mol phosphate buffer. Using distilled water, the mixed solutions were diluted to the final volume and were shaken thoroughly, then equilibrated for 20 min at 20±1 ◦C.

2.3 Preparation of Stationary Phases

Active silica gel (5.0 g) was suspended in 50 mL dry toluene (freshly distilled), and then KH–560 (6.0 mL) and 1.0 mL triethylamine (used as a catalyst) was added to this suspension. The mixture was stirred and heated to 80°C under the protection of nitrogen atmosphere for 8 h. After the reaction finished, the solid was filtered by 1.5 μm filter, and washed in sequence with toluene and acetone, then dried at 80°C under vacuum for 8 h. Finally, *γ*–glycidoxypropyl bonded silica gel (γ-GBSG) was obtained and used as a precursor in the following reaction.

Each of the diacid calix[4]-1,2-crowns no. **10-13** (3.1 mmol), NaH (0.6 g), and toluene (60 mL, freshly distilled) were stirred at 80ºC for 30 min, then the supernatant liquid was transferred to a 100 mL three–neck flask, 3.0 g γ-GBSG was added, and the mixture was refluxed with the catalyst for 48 h. The whole process was carried out under nitrogen atmosphere. After the reaction finished, the product was filtered and washed in sequence with toluene, acetone, methanol and distilled water. Subsequently, stationary phases were obtained and dried at 100ºC under vacuum for 8 h, then cooled to room temperature in a desiccator.

2.4 Characterization of Stationary Phases

The characterization of the developed stationary phases was carried out by elemental analysis, IR and thermal gravimetric analysis. Thermal gravimetric analysis (TGA) was carried out with a Shimadzu DT–40 thermal analyzer, the analysis was performed from 40°C to 650°C at heating rate of 10°C min^{-1} in argon atmosphere with a gas flow rate of 20 mL min^{-1}.

Table 1 shows the elemental analysis results of γ-GBSG and stationary phases. The result indicates stationary phases own higher content of carbon, nitrogen and sulfur than that of γ-GBSG, which confirmed that the calixcrowns were successfully immobilized onto the silica gel. The bonded amount of calix[4]crown derivatives onto the silica gel was calculated by subtracting that of γ-GBSG. According to the carbon content of the bonded silica gel stationary phases, the resulting stationary phases contain about 10% carbon corresponding 0.05 mmol calix[4]crown per gram silica gel.

Table 1 – Elemental analysis of bonded phases.

Bonded phase	C%	H%	Bonded amount (mmol g^{-1})
γ-GBSG	7.1	1.2	0.74
stationary phases **10**	9.8	1.5	0.05
stationary phases **11**	9.7	1.5	0.05
stationary phases **12**	9.9	1.5	0.05
stationary phases **13**	9.8	1.5	0.05

Moreover, the bonded stationary phases were characterized by infrared spectroscopy. From IR spectra, the characteristic absorption band of peaks around 2960 and 2865 cm^{-1} are assigned to C–H stretching frequency. The peaks around 1712 cm^{-1} are assigned to the absorption frequency of the –C=O groups. The peaks around 1205 cm^{-1} correspond to the groups of Si–O–Si and C–O–C. All IR spectra indicate that the organic ligands were bonded onto silica gel.

The thermal stability of γ-GBSG and stationary phases has been investigated by TG analysis. The results show that the temperatures of weight loss for γ-GBSG and stationary phases are both more than 300ºC. It indicates that the new bonded phases possess high thermal and chemical stability. Moreover, the weight losses in 25–650ºC were 7 and 13 % for γ-GBSG and stationary phases, respectively, which was in line with the results of elemental analysis.

The stability of the columns was evaluated over two months of being used under different chromatographic conditions. The relative standard deviations (RSDs) of retention time of analyte were less than 2 % (n =6) during that time. The prepared columns showed high chemical stability when methanol mixtures and water (phosphate buffer, pH from 3.5 to 7.5) were used as mobile phases.

2.5 Chromatographic Procedures

Four columns (150 mm×4.6 mm i.d.) were packed with modified calix[4]crown–silica gels according to a slurry packing procedure by using methanol as the displacing agent (50 MPa, 1 h). The mobile phases used were methanol–water and methanol–phosphate buffers (0.005 w w^{-1}, pH 4.5). Analytes were dissolved in the mobile phase at the concentration in range of the 5–100 μg mL^{-1}, and 20 μL of the solution were injected into the chromatographic column. The void time (t_0) for the calculation of the retention factor was determined by injecting 0.05 M sodium nitrate ($NaNO_3$) at UV detection 210 nm, with MeOH–H_2O (70:30, v v^{-1}) as mobile phase. All measurements were carried out at 25°C and repeated three times.

3. RESULTS AND DISCUSSION

In this section, the column factors are determined and followed by description of analytical results and analyte:bonded-phase interactions. Finally, the effect of organic modifier (methanol) is discussed.

3.1 Analytical Results

The graph of the peak area (y) against concentration (x, μg mL^{-1}) proved the linearity in the range of 0.2 to 20.0 μg mL^{-1} and the regression coefficient of R^2 = 0.996. The limit of detection (LOD), was defined as the injected quantity giving S/N of 3 (in terms of peak area), was found to be 0.06 μg mL^{-1}. The limit of quantitation (LOQ), was defined as the injected quantity giving S/N of 10 (in terms of peak area), was found to be 0.20 μg mL^{-1}. Inter–day precision was assessed by injecting the standard solution of different concentration (0.2, 5.0, 10.0, 15.0 and 20 μg mL^{-1}) and on each day for 5 days.

The results show that there were high inter–day precisions, the RSD% of retention times was within 0.03 and the RSD% of peak areas was within 2.5. Intra–day precisions were assessed by injecting the standard solution at the three concentrations five times during a day, and the intra–day RSD% of retention time was within 0.03 and the RSD% of peak area was within 1.5. The accuracy of the method was determined by recovery experiments. The analysis of clenbuterol in the livestock meat showed high accuracy with a recovery of 95 % in average. Using the stationary phases, the chromatogram representing the clenbuterol in the livestock was obtained. According to Figure 1, clenbuterol was distinctively separated from the matrix of livestock meat. The residues of clenbuterol in the livestock meat were 0.10 μg g^{-1}.

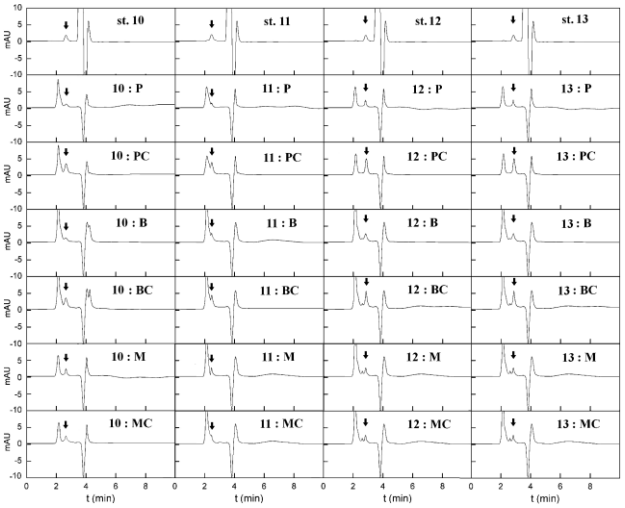

Fig. 1 – Chromatogram of clenbuterol in six livestock meat (pork, pork casing, beef, beef casing, mutton, and mutton casing) using stationary phases **10-13**

In Figure 1, the upper chromatograms (st.) depict the standard solution containing 0.2 μg mL^{-1} clenbuterol and the other traces show the chromatograms of the following samples: pork (P), pork casing (PC), beef (B), beef casing (BC), mutton (M) and mutton casing (M), respectively.

3.2 Simultaneous Determination of Salbutamol, Clenbuterol, Ractopamine and Terbutaline

In order to demonstrate the application area and chromatographic performance of bonded phases **10-13**, more compounds such as ractopamine and terbutaline, which are similar to clenbuterol, were determined. Owing to the pre-separation step (solid phase extraction, Waters Oasis MCX cartridge) before chromatography, ractopamine and terbutaline were removed from the sample of clenbuterol and the chromatogram just showed the clenbuterol's signal.

3.3 Effect of Organic Modifier

As methanol content of the mobile phase increasing, the retention factors decrease. This result indicates that the new bonded phases can behave as an excellent reversed–phase performance and the hydrophobicity interaction is one of the factors playing a role in the separation of clenbuterol. However, it is obvious that the relationship between log *k'* and the content of methanol is not linear. Moreover, by increasing the methanol contents to certain values, the relationship line of log *k'* and methanol content tends to be flatten, which means that there exist other interactions in the separation process when the methanol content was in the low levels. Hence, it indicates that hydrophobic was not the only main factor in the separation of the solute

and the crown-ether rings may also be responsible for the retention behavior. When the methanol content was in the low levels, all bonded phases show different *k'* values; while at the higher levels of methanol, they depict just two *k'* values. These results revealed that inclusion complexation and hydrogen bonding interactions in the bonded-phases are the most and followed by the order of **11** < **10** < 1**3** < **12**.

3.4 Free Calixarenes vs. Clenbuterol

^{1}H NMR titration experiments were undertaken to investigate the binding mode between water-calixcrown derivatives and clenbuterol at ambient temperature and pH≈7.0 in buffer solution. Concerning the ^{1}H NMR titration experiments, the change of the chemical shifts of acid–basic reactions were examined. The ^{1}H NMR spectra of 1.25×10^{-2} mol clenbuterol and its complex with different concentration of calixcrown **10** is depicted in Figure 2. According to this figure, the di-amines' proton as well as the methyl-protons almost did not shift, but the Ha, Hb, and Hc protons of benzene shifted remarkably.

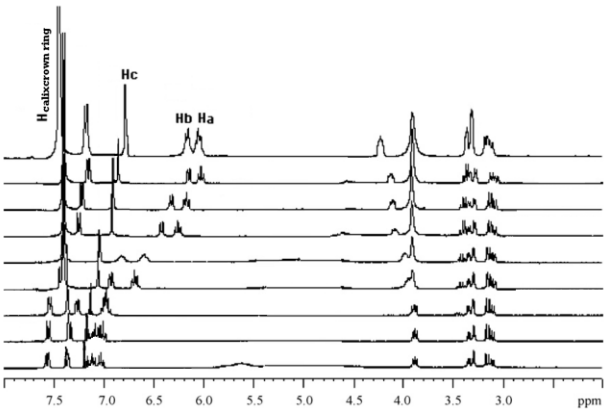

Fig. 2 – The ^{1}H NMR spectra of 1.25×10^{-2} mol clenbuterol and its complex with different concentration of calixcrown **10**

The protons in the calixcrowns ring slightly shifted. The downfield of the protons upon addition of calixcrowns is obvious around 3.9–4.0 ppm. This phenomenon may attribute to the effect between the *p-tert*-butyl groups on the upper-rim of calixarene and the di-amines' proton in clenbuterol. Owing to the reciprocity, the chiral hydrogen of the clenbuterol has been shifted obviously. It is attributed to the ring current effect of the aromatic nuclei of the host.

The proposed conformation of host–guest complex **10** is presented in Figure 3. The result clearly demonstrated that calixcrown scaffold partially accommodate benzene ring of clenbuterol in its hydrophobic cavity with a tilt-in conformation.

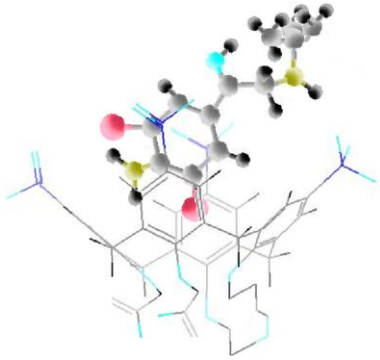

Fig. 3 – The proposed conformation of host–guest complex **10** with clenbuterol.

4. CONCLUSIONS

Four novel calixcrown-columns were prepared and characterized by FTIR, elemental and thermal analysis. The chromatographic behaviors of new developed stationary phases were investigated towards clenbuterol in six samples of livestock meat. By increasing the methanol content of the mobile phase, the retention factors decreased and showed that various chromatographic retention mechanisms occur in the separation of the above analyte, such as hydrogen bonding interaction and inclusion complexation. The columns were successfully used for the analysis of clenbuterol level in pork, pork casing, beef, beef casing, mutton and mutton casing.

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