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Short communication

Determination of clenbuterol in beef liver and muscle tissue using immunoaffinity chromatographic cleanup and liquid chromatography with ultraviolet absorbance detection

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Abstract

Clenbuterol, a beta-agonist, was determined in samples of beef liver and muscle. The method employed an acidic aqueous extraction followed by protein precipitation. The supernatant liquid was passed through a weak cation-exchange cartridge and then through a commercially available immunoaffinity cartridge. Clenbuterol was eluted from the immunoaffinity cartridge with 80% ethanol in water. The eluate was concentrated and analysed directly by reversed-phase liquid chromatography using gradient elution and UV detection at 245 nm. Detection limits were estimated to be 0.3 ng g^{-1} clenbuterol. A single immunoaffinity cartridge was used for ten sample extracts with no significant loss in capacity. No organic solvents other than ethanol and methanol were employed in the procedure. Recoveries of clenbuterol from samples of beef liver and muscle spiked at 2 and 5 ng g^{-1} carried through the entire procedure were 63±11% (range, 53–74%) compared to pure standards. Absolute recoveries of pure standards (30 ng clenbuterol) carried through the same analytical steps were 70±5% (n=6), the losses being primarily due to the ion-exchange step. © 1997 Elsevier Science B.V.

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1. Introduction

Clenbuterol is one of a number of beta-agonistic drugs used in veterinary medicine to treat pulmonary diseases [1,2]. However, because of its growth promoting ability, it has been extensively misused and has led to serious outbreaks of human poisoning in Spain, France and Italy, due to elevated levels in beef liver [3–6]. The illegal use of the drug in the USA is presently the subject of a federal indictment [7]. In the Netherlands and other European countries, strict control strategies have been developed in order to prevent the illegal use of beta-agonists [8]. As a result of this concern, a number of methods for the determination of clenbuterol and other beta-agonists have been developed. Several of these involve an immunochemical step in the analytical procedure, either by employing immunoassay for detection [9–12] or using immunoaffinity chromatography as a cleanup for determination by liquid chromatography [13,14] or by gas chromatography–mass spectrometry after chemical derivatization [8,14,15]. There are a number of distinct advantages to using immunochemical techniques for the determination of

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beta-agonists in animal tissues and these have been reviewed [9,14]. While immunoassay methods such as ELISA and RIA [9–12] provide fast and sensitive results, they are most useful for screening purposes and not for the unequivocal identification and quantitation of individual beta-agonist residues. Immunoaffinity chromatography, on the other hand, makes use of the same antibody–antigen interactions to selectively isolate analytes of interest from tissue extracts for qualitative and quantitative determinations by standard chromatographic techniques. It offers simplicity in terms of sample manipulations and also as an alternative to more cumbersome sample cleanup methods that involve the use of organic solvents. We have already demonstrated these advantages in direct comparisons of immunoaffinity chromatography with classical sample cleanup for the liquid chromatographic (LC) determination of phenylurea [16] and triazine [17] herbicides in plant tissues. The aim of the present work is to evaluate a commercially available immunoaffinity cartridge for the determination of clenbuterol at low ng g^{-1} concentrations in beef tissue by direct LC employing UV absorbance detection with confirmation by LC–mass spectrometry without the need for chemical derivatization. The resulting method uses no organic solvents other than ethanol and methanol.

2. Experimental

2.1. Reagents

All chemicals and reagents were analytical grade materials. Ethanol and methanol were the only organic solvents used. Doubly deionized water was used throughout the method. Clenbuterol hydrochloride (Sigma, USA) stock solution was prepared at 1 mg ml^{-1} in 10% (v/v) methanol–water. For LC injections, the stock solution was diluted with mobile phase. Phosphate buffered saline (PBS) solution was prepared by dissolving 2.68 g Na_2HPO_4 and 8.76 g NaCl in 1 l of water. The pH was adjusted to pH 7.4 with 0.1 *M* H_3PO_4. The immunoaffinity cartridges (RidaScreen, Bioman Products, Mississauga, Canada) contained enough immobilized polyclonal antibodies to bind with about 40 ng of clenbuterol. The cartridges were stored at 4°C when not in use.

2.2. Liquid chromatography

The chromatography system consisted of two pumps (Beckman, USA, Model 114M) with a gradient controller (Beckman, M421A), a solvent mixer, an autoinjector (Gynkotek, Germany) and a diode array UV detector (Hewlett–Packard, Canada, Model 1040A) set to 245 nm. The column was a Symmetry C_{18} (15 cm×3.9 mm I.D., Waters, USA). Clenbuterol was eluted with a linear gradient of 30 to 70% methanol (v/v) in water containing 10 m*M* acetic acid–ammonium acetate buffer (adjusted to pH 4.6 with acetic acid) over 5 min at a flow-rate of 0.8 ml min^{-1}. The autoinjector syringe and needle were rinsed with water between injections. Normally, 150 μl of standard or sample solution were injected.

2.3. Sample extraction

A 6-g portion of homogenized meat tissue was placed into a 50-ml polypropylene centrifuge tube. A 30-ml volume of 0.01 *M* HCl was added, the contents shaken for 20 s then placed in an ultrasonic water bath for 15 min. The mixture was shaken again and then heated in a water bath for 30 min at 80°C. The tube was then cooled for 10 min in a freezer (−15°C) and centrifuged at 9200 *g* for 20 min at 5°C. The supernatant was transferred to a clean centrifuge tube and the pH adjusted to pH 6 with 1 *M* NaOH and recentrifuged if a precipitate formed.

2.4. Ion-exchange chromatography

A weak cation-exchange solid-phase extraction cartridge (WCX–SPE) (3-ml volume, 500 mg adsorbent, Baker) was conditioned with 10 ml of ethanol followed by 3 ml of water, 3 ml 0.1 *M* NaH_2PO_4 (pH 6) and 3 ml of water. The whole sample extract (adjusted to pH 6) or standard solution was applied to the cartridge and the effluent discarded. The cartridge was rinsed with 4 ml of water followed by 4 ml of ethanol and the rinsings

were discarded. A 5-ml volume of 2% (v/v) ammonium hydroxide in ethanol was added to the cartridge and the effluent containing the clenbuterol was collected and evaporated to 0.1 ml under a stream of nitrogen at 30°C. The solution was then diluted to 1 ml with PBS (pH 7.4).

2.5. Immunoaffinity chromatography

The immunoaffinity (IA) cartridge was rinsed with 2 ml of PBS (pH 7.4). The 1-ml extract from the WCX–SPE step was then applied and the effluent discarded. The cartridge was rinsed with 0.5 ml of PBS followed by 1 ml of 20% (v/v) ethanol in water. The rinsings were discarded. Clenbuterol was eluted from the cartridge with 2 ml of 80% (v/v) ethanol in water and collected in a 5-ml graduated centrifuge tube. The eluate was evaporated to 0.1 ml under a stream of nitrogen at 30°C and then diluted to 0.4 ml with mobile phase containing no methanol and mixed. A 150-μl aliquot of this solution was analysed by LC. The IA cartridge was rinsed with 5 ml of water followed by 5 ml of PBS (pH 7.4) and stored at 4°C until further use.

3. Results and discussion

3.1. Liquid chromatography

Initial attempts to use standard reversed-phase C_{18} columns for clenbuterol analysis as reported by others [19–21] were not successful. Poor peak shape and inconsistent retention times were observed even though various mobile phase additives were tried. Similar problems were encountered by Hooijerink et al. [22] who found that a base deactivated C_{18} column [23] was preferred for their work using ion-pair chromatography. We also found that a base deactivated column worked very well, even without the use of ion pairing agents. Since detector sensitivity was a limiting factor in our work, gradient elution with a fairly steep rate (30–70% methanol in 5 min) was employed to elute clenbuterol at an acceptable retention time and in a minimum volume of mobile phase. This resulted in a rising baseline during each chromatographic run at high detector sensitivity (see Fig. 1 Fig. 2). However, it did not create a problem with detecting low ng g^{-1} concentrations of clenbuterol in beef tissues. Under these conditions, the linearity of response ranged from about 2 ng per injection to greater than 40 ng per injection.

3.2. Immunoaffinity chromatography

The capacity of each IA cartridge was verified before use. The actual capacity was always found to be in the range of 40–45 ng. However for practical studies, no more than 30 ng of clenbuterol (as standard or in a sample extract) were applied to the cartridges. Although this capacity was small, it was sufficient for LC analysis which required a minimum of about 1 ng of clenbuterol per injection to be detected (3:1 signal-to-noise ratio). For accurate quantitation, about 5–15 ng of clenbuterol were usually injected. The negative aspect of the low capacity was that the elution fraction containing the clenbuterol had to be reduced to a small volume so that enough could be injected into the LC system. This required extra time and led to some loss of clenbuterol (see below).

It was found that the IA cartridges could be used repeatedly without significant loss of antibody activity, as long as the washing, regeneration and storage conditions described in the experimental section were followed. Before actual sample analysis, 3 ml of an aqueous solution of clenbuterol standard were passed through a cartridge according to the experimental procedure and analysed by LC to ensure that the cartridge functioned properly. Recoveries of pure standard through the IA cartridges ($n=6$) were always greater than 90% when 30 ng or less of clenbuterol were applied. Some loss of clenbuterol was observed during the concentration of the ethanol–water elution fraction to about 0.1 ml. It was found to be essential that the evaporation temperature be kept to 30°C or less. Under these conditions, losses of clenbuterol were <5%. However, losses of 30% or more of clenbuterol were observed when temperatures of 40–50°C were employed.

Once the cartridge was successfully tested, it was regenerated and used for sample analysis. After each

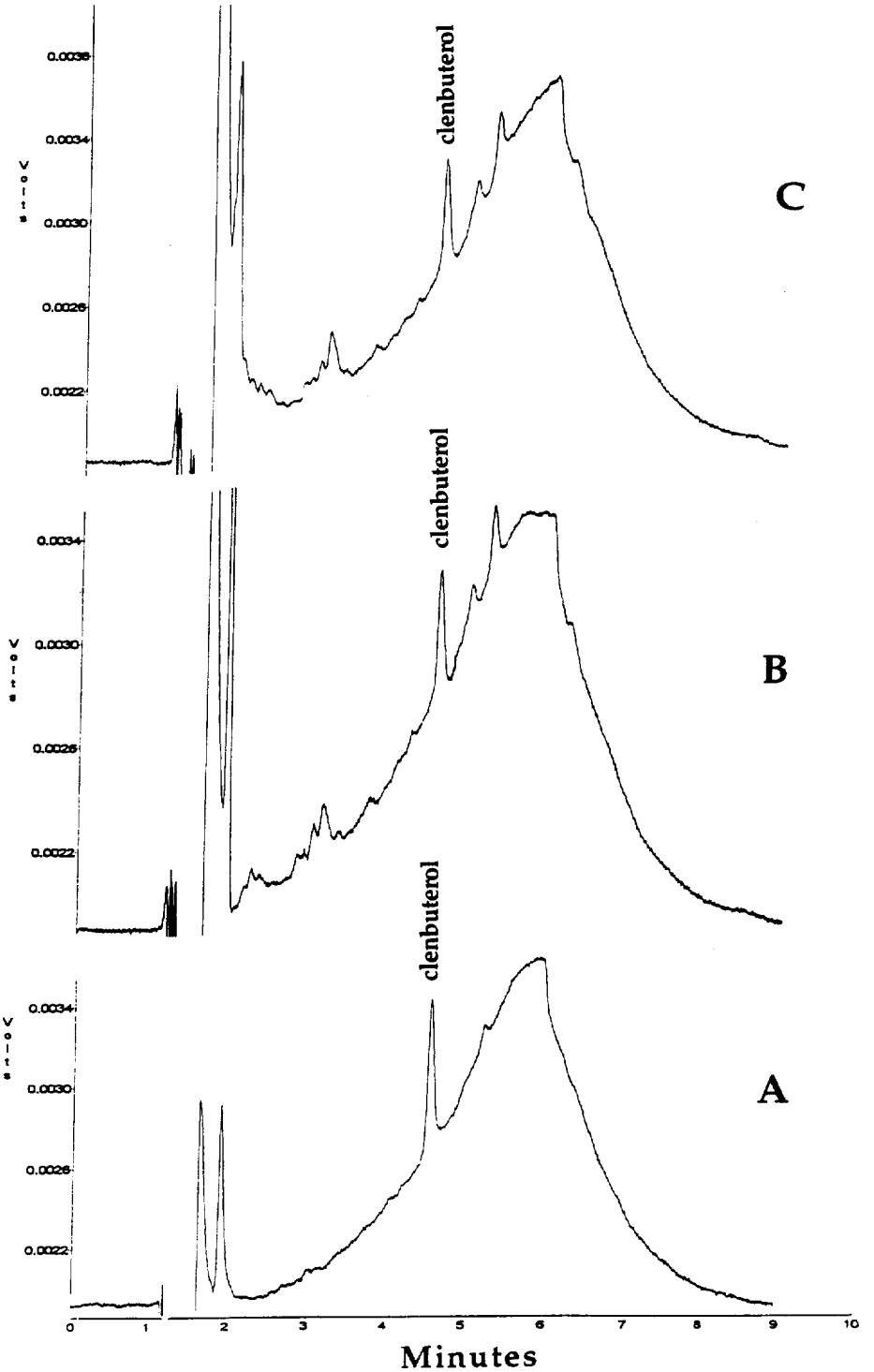

Fig. 1. Chromatograms of: A=standard clenbuterol, 6 ng injected, B=standard clenbuterol (equivalent to 2 ng g^{-1}) through the analytical procedure, C=spiked liver extract (2 ng g^{-1}). Experimental conditions described in Section 2.

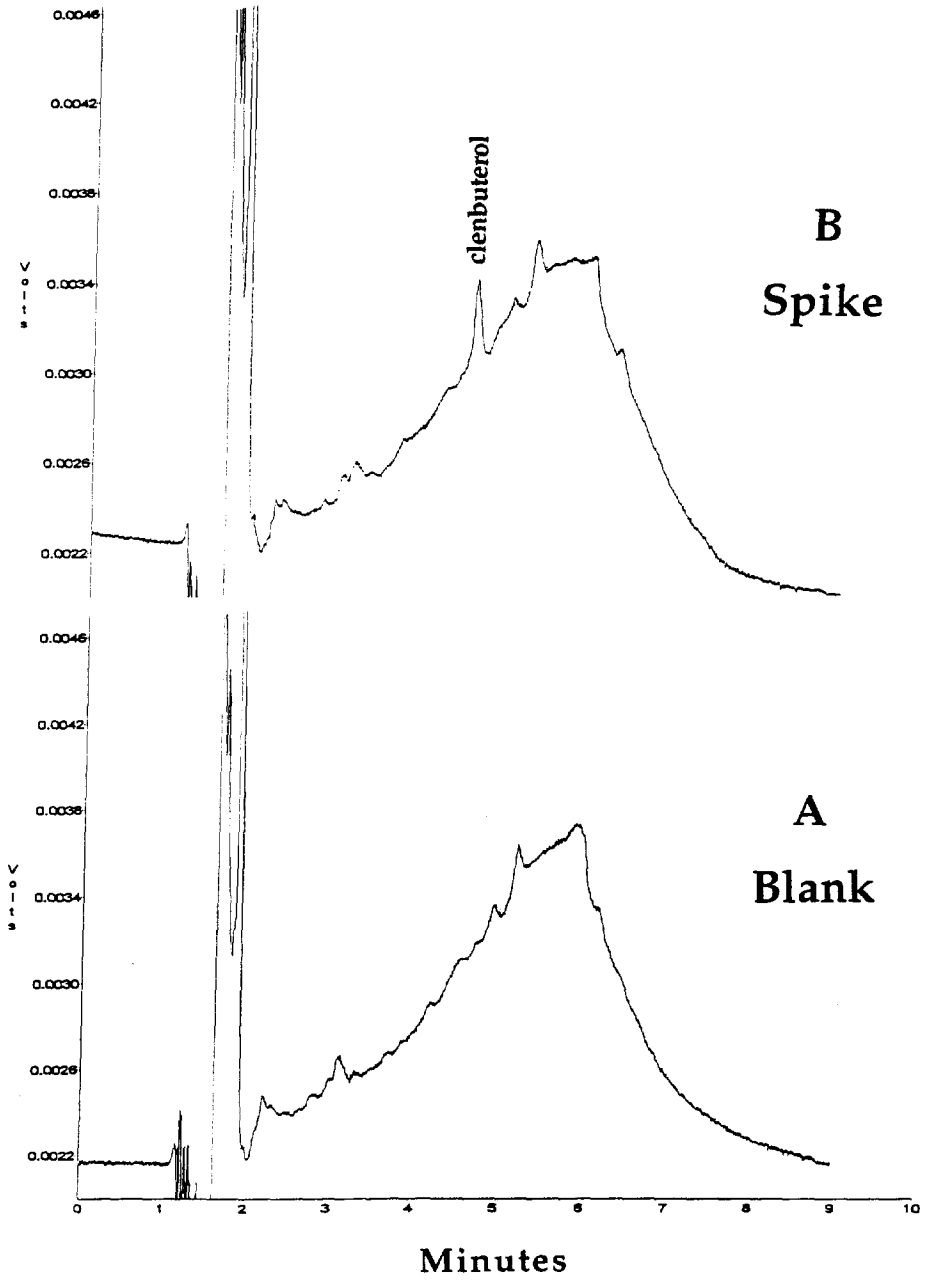

Fig. 2. Chromatograms of liver extracts (3 g liver). A=blank sample, B=spiked sample (5 ng g^{-1}). Experimental conditions described in Section 2.

application the cartridge was immediately regenerated for further use or storage. We have found that a single cartridge could be used for at least 18 (the largest number tried) analysis–regeneration cycles (including 10 liver samples) with no observable loss in effectiveness.

3.3. Sample analysis

A number of procedures for the extraction of clenbuterol from liver and muscle tissue have appeared in the literature [8,11,15,18,19]. Of these, ultrasonic extraction appeared the simplest [8,15] and was thus employed in this work. All of the methods mentioned above included at least one purification step using organic solvents such as dichloromethane, ethyl acetate, hexane, acetone or isopropanol in the sample cleanup process. We found that using the extraction and IA chromatographic cleanup procedure described in the experimental section, we could directly quantitate clenbuterol in liver tissue at concentrations as low as 15–20 ng g^{-1} without using organic solvents other than ethanol and methanol. However, at lower concentrations, matrix interferences prevented reliable identification and quantitation. Thus an additional cleanup step was required. Hooijerink et al. [15] and Leyssens et al. [18] used mixed phase SPE cartridges (containing a mixture of cation-exchange and hydrophobic adsorbents) as part of the cleanup procedure in their work. This approach appeared to yield rather clean extracts. Since we did not have this type of SPE cartridge available to us, we evaluated the commonly available weak cation-exchange (WCX–SPE) cartridges using similar eluting conditions as described earlier [15,18]. The results were very positive and it was found that ethanol functioned equally well as ethyl acetate or isopropanol–dichloromethane combinations for elution of clenbuterol. Ethanol was chosen in this work because of its lower toxicity and cost. The recovery of clenbuterol standard (30 ng) through the WCX–SPE under the conditions described in the Section 2 was 70%±5% (n=5). (Most of the losses of clenbuterol in the procedure occurred at this step. Efforts to improve the recovery were unsuccessful). It was important that the pH of the extracts was adjusted to pH 6 to ensure maximum recovery. Also, a maximum of 6 g of homogenized tissue could be applied, otherwise overloading of the WCX–SPE occurred leading to reduced recovery.

The WCX–SPE cleanup step was used before the IA cleanup and served to protect the IA cartridge from much of the sample coextractives. Hooijerink et al. [15] used their mixed-bed SPE after the IA cleanup which facilitated the preparation of trimethylsilyl derivatives required in their procedure for clenbuterol determination by GC–mass spectrometry. However, their cartridges, which had an initial capacity of about 400 ng of clenbuterol, lost the equivalent of 200 ng after the passage of 20 sample extracts. Since the capacity of the cartridges used in this work was only 40 ng, we decided to add the WCX–SPE step before the IA step. This combination worked very well. No noticeable decrease in IA capacity was observed after the passage of 10 liver extracts (the highest number tested) following the procedure described in the Section 2.

Fig. 1 shows chromatograms of a standard and a spiked (2 ng g^{-1}) liver sample carried through the procedure. The spiked sample is very clean and looks virtually like the standard. This is remarkable considering that the detection is by UV absorption at a rather nonselective wavelength (245 nm). Under these conditions, the detection limit for clenbuterol was estimated to be about 0.3 ng g^{-1} (3:1, signal-to-noise). It is possible that this could be improved by carrying out duplicate or triplicate cleanups and combining and concentrating the final extracts before analysis. However, this was not carried out.

Absolute recoveries, through the entire procedure, of clenbuterol from samples spiked at 2 and 5 ng g^{-1} averaged 63±11% (range, 54–74%, n=6). Recoveries were similar for higher concentrations (up to 100 ng g^{-1}) but proportionately less tissue extract was used for the analysis so that the amount of clenbuterol never exceeded the capacity of the IA cartridge. Fig. 2 shows chromatograms of a blank and a spiked (5 ng g^{-1}) liver sample using only 3 g of tissue for the analysis. The method was applied to the analysis of beef muscle tissue spiked at 2 ng/g with similar results.

This same procedure was used successfully for the cleanup of liver tissue spiked at 1 ng g^{-1} clenbuterol for direct confirmation by LC–electrospray MS–MS [24]. Estimated detection limits (3:1, signal-to-noise) were about 0.02–0.04 ng g^{-1}.

4. Conclusions

Immunoaffinity chromatography has been found to be a selective cleanup technique for beef liver and muscle tissue for the determination of clenbuterol at low ng g^{-1} concentrations without the need of

organic solvents other than ethanol and methanol. In addition, no derivatization steps, as required for the GC methods and several LC ones [14,15,19] were employed. The resulting extracts were clean enough to permit direct confirmation by LC–MS–MS without further sample handling.

It was found, however, that IA cleanup alone was not sufficient to determine clenbuterol at concentrations below about 15–20 ng g^{-1}. A WCX–SPE step was incorporated to enable the quantitation of clenbuterol in the 1–5 ng g^{-1} range. With care, the IA cartridges can be used many times without significant loss of cartridge capacity. Although not directly comparable to the present work, Haasnoot et al. [13] were able to use an IA sorbent for clenbuterol in an on-line application more than 200 times for the analysis of urine samples and standard solutions.

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