The Use of Nonendcapped C18 Columns in the Cleanup of Clenbuterol and a New Adrenergic Agonist from Bovine Liver by Gas Chromatography–Tandem Mass Spectrometry Analysis

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Abstract

More specific official methodology is needed to survey the illegal use of clenbuterol in animal production plus the synthesis of new compounds that currently elude routine analytical methods. The identification of a new adrenergic agonist, *N*1-(2-(4-amino-3,5-dichlorophenyl)-2-hydroxyethyl)-*N*1-isopropyl-propanamide (known as compound A) in animal feed has prompted studies to verify if the existing cleanup procedures developed for clenbuterol are really effective. This study considers the ion-exchange mechanism on cyanopropyl (CN), sulfonic cation exchange (SCX), mixed phase (MPH) (C8 + SCX), and nonendcapped C18 (C18NE) solid-phase extraction (SPE) columns. Results indicate that compound A (by contrast with clenbuterol) is not efficiently retained on the CN, SCX, and MPH SPE columns (recovery < 10%). This finding thus leads to the development of a gas chromatography–tandem mass spectrometry procedure based on C18NE SPE that is able to purify both agonists from bovine livers spiked at 0.5, 1.0, and 2.0 ppb with a mean recovery of 93% for clenbuterol and 92% for compound A.

Introduction

The development of a multiresidue analytical strategy to survey the abuse of adrenergic agonist drugs used as growth promoters in animal production is mainly based on the selectivity of the cleanup procedures and the specificity of the detection systems (1,2). Such requirements have been fundamental steps for the analytical toxicology investigations related to human poisonings described after the ingestion of clenbuterol-contaminated bovine liver and meat (3,4). Most of the analytical procedures reported as effective include an ion-exchange step in the multiresidue cleanup of beta agonists. This procedure involves the interaction between the secondary amino group shared among all beta agonists and the cyanopropyl (CN) (5) and sulfonic cation exchange (SCX) (6,7) functional groups of solid-phase extraction (SPE) columns. The recent characterization of a new clenbuterol-like drug, *N*1-(2-(4-amino-3,5-dichlorophenyl)-2-hydroxyethyl)-*N*1-isopropyl-propanamide (8) (known as compound A), with an amide substitution of the nitrogen atom on the alkylic chain (Figure 1) prompted our group to verify if the cleanup procedures most in use were really selective for such a molecule and explore any possible alternatives. The aim was to limit as much as possible false negative results that could compromise the reliability of the results of forensic investigations, thus exposing consumers to the previously mentioned toxicological risk.

Experimental

Equipment

Cleanup procedures were performed on a Supelco (Milan, Italy) vacuum manifold device. A high-performance liquid chromatography (HPLC) System Gold, a Model 126 pump, a Model 168 diode-array detector (DAD), and a Model 501 autosampler (Beckmann Analytical, S. Ramon, CA) were used to assess the performance of standards on different SPE columns. Chromatographic conditions consisted of a reversed-phase (RP) C18 Lichrosphere Select B column (250 × 4 mm, 5 µm) (Merck, Darmstadt, Germany), a mobile phase of 0.01M sodium acetate (pH 3.0) (A) and acetonitrile (B), and a linear gradient from 10% to 100% B in 20 min. The flow rate was 1.0 mL/min, the DAD was set at 245 and 305 nm, the bandwidth was 4 nm, and the spectra recorded in the range of 220 to 350 nm.

An analytical performance on spiked livers at residue levels

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of 0.5, 1.0, and 2.0 ng/g were carried out on a GCQ ion trap detector (ThermoQuest Italia, Milan, Italy) with a CP-SIL 8CB-MS FS 30X.25(.25) capillary column (Chrompack Italia, Milan, Italy). The injector temperature was set at 250°C and was in splitless mode. The constant velocity of the carrier gas (He) was 40 cm/s. The oven temperature program raised from 70°C to 230°C in 11 min (20°C/min), then raised to 280°C in 10 min (5°C/min), and was held for 5 min at 280°C. The GCQ acquisition was in electron-impact mode (70 eV), the multiplier was set at 1300 V, and the resolution 0.5 amu. For clenbuterol the precursor ion was *m/z* 262; the width was 4; the excitation volts 1.1; and the product ions were *m/z* 188–192, 225–229, and 262–264. For compound A the precursor ion was *m/z* 262; the width was 4; the excitation volts 1.2; and the product ions were *m/z* 73–77, 188–192, and 262–264.

Liver extraction was performed by an ultraturrax apparatus and a rotary evaporator (Buchii, Zurich, Switzerland).

Evaporation under a nitrogen stream and trimethylsilyl derivatization of the extracts were carried out on a Heat Block (Pierce Italia, Milan, Italy).

Materials

Clenbuterol HCl (Sigma, Milan, Italy) and compound A (courtesy of Prof. G. Boatto, Dept. of Pharmacology University of Sassari, Sassari, Italy) were used as pure standards. Methanol, acetonitrile, and all other reagents and solvents were of analytical grade. Bakerbond CN propyl SPE (100 mg) (CN), aromatic sulfonic acid (100 mg) (SCX), light-load (500 mg) C18 nonendcapped (C18NE) (J.T. Baker Italia, Milan, Italy), Bond Elut certify columns (200 mg) (mixed phase, MPH) (Varian Italia, Milan, Italy), and Extrelut 20 columns (Merck) were also used.

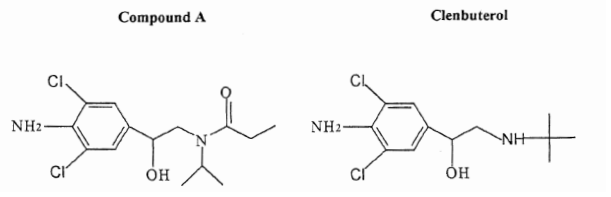

Figure 1. Structures of compound A and clenbuterol.

A standards phosphate buffer (PBS) (0.1M, pH 6.0) and 100% and 20% methanol stock solutions were prepared at 1 mg/mL and stored at +4°C. Working solutions were freshly obtained by dilutions in the appropriate solvents in the range of 100.0 to 0.1 µg/mL.

Samples

Seven different incurred beef livers (previously tested as negative for beta agonists) were spiked at levels of 0.5, 1.0, and 2.0 ng/g each by the addition of 25, 50, and 100 µL of the 0.1-µg/mL PBS working solutions, respectively. Such spiking levels have been chosen according to the pharmacokinetics data of clenbuterol in calves (9). Analyses were repeated on three different sessions, and recoveries and reproducibility were calculated according to Gowick et al. (10).

Analytical procedure

Different protocols were followed depending on the SPE sorbent tested. From fractions of 1 mL from the applications, washing and elution steps were collected separately, brought to dryness, and resuspended in a 200-µL HPLC mobile phase to assess recovery. For each SPE procedure considered, reproducibility was assessed on 12 replicates on two different days.

The CN procedure was done according to Musch and Massart (5). Columns were conditioned with 2 mL of MeOH followed by 1 mL H_2O, not allowing the column to dry. Then, 1 mL of 20% of a 100-µg/mL MeOH standard working solution was applied at a flow rate of 0.5 mL/min. The column was washed with 3 mL of MeOH and eluted by another 3 mL of MeOH, which had 1% triethylamine (TEA) as the counter ion.

The SCX procedure was as follows. Columns were conditioned with 2 mL MeOH and 1 mL 0.1M acetic acid. Then, 1 mL of the standard working solution (20% MeOH) was applicated at a flow rate of 0.5 mL/min. After washing with 3 mL MeOH, elution was performed with 3 mL MeOH (1% TEA).

The MPH columns were performed as follows. According to the procedure described by Montrade et al. (6), columns were conditioned with 2 mL MeOH and 0.1M PBS (pH 6.0). Application of the standard working solution (100 µg/mL) in PBS (0.1M, pH 6.0) was performed at a flow rate of 0.5 mL/min. Rinsing with 1 mL 1.0M acetic acid, washing with 3 mL MeOH,

Table I. Recovery Expressed as the Percentage of Compound A and Clenbuterol* on CN, SCX, MPH, and C18NE SPE Columns†

	CN		SCX		MPH		C18NE	
Fraction	Compound A	Clenbuterol	Compound A	Clenbuterol	Compound A	Clenbuterol	Compound A	Clenbuterol
Application	22 ± 3	n.d.‡	78 ± 4	n.d.	n.d.	n.d.	n.d.	n.d.
First washing	64 ± 5	2 ± 2	22 ± 3	n.d.	56 ± 81	2 ± 1	n.d.	n.d.
Second washing	13 ± 3	n.d.	n.d.	n.d.	27 ± 5	n.d.	n.d.	n.d.
Third washing	3 ± 4	n.d.	n.d.	n.d.	12 ± 3	n.d.	18 ± 5	n.d.
First elution	n.d.	71 ± 4	n.d.	82 ± 3	n.d.	78 ± 4	29 ± 6	66 ± 3
Second elution	n.d.	28 ± 3	n.d.	24 ± 1	n.d.	21 ± 2	49 ± 3	29 ± 2
Third elution	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	4 ± 2	1 ± 2

* Mean ± standard deviation, $n = 12$.
† 100 µg of each compound loaded.
‡ n.d., not determined.

and eluting by 3 mL MeOH (1% TEA) was then performed.

C18NE columns were conditioned with 3 mL MeOH and 1 mL H_2O. Application of the working standard solution (20% MeOH) involved washing with 3 mL of 50% MeOH and elution with 4 mL MeOH (1% TEA).

Blank and spiked liver samples

From each liver 5 g was sampled with the addition of 200 µL of the appropriate internal standard. A 0.1-µg/mL working solution in PBS (0.1M, pH 6.0) (clenbuterol for compound A analysis and vice-versa) corresponded with 4 ng/g. The liver samples were minced in 50-mL polypropylene tubes by ultra-turrax in 20 mL HCl (0.5 N). After sonication (RT = 15 min), the samples were allowed to hydrolyze overnight at room temperature under shaking. Supernatants were recovered by centrifugation (3000 g, 30 min, and 4°C), thus removing the upper solid lipid layer. After the addition of 200 µL NaOH (10 N) under vortexing, the liquid was adsorbed on Extrelut 20

Table II. Recovery Study* by GC–MS–MS from 20 Livers Spiked at Residue Levels of 0.5, 1.0, and 2.0 ng/g

Drug	Retention time	Ions (*m/z*)	Spiked level (ppb)	*N*	Mean recovery	CVR	CC alpha	CC beta
Clenbuterol	14.37	262	0.5	21	93.9	4.2	0.55	0.58
		225	1.0	21	93.0	1.6		
		188	2.0	21	94.1	1.8		
Compound A	20.47	262	0.5	21	93.2	3.1	0.53	0.56
		188	1.0	21	93.4	2.4		
		73	2.0	21	91.4	2.5		

* Mean ± standard deviation.

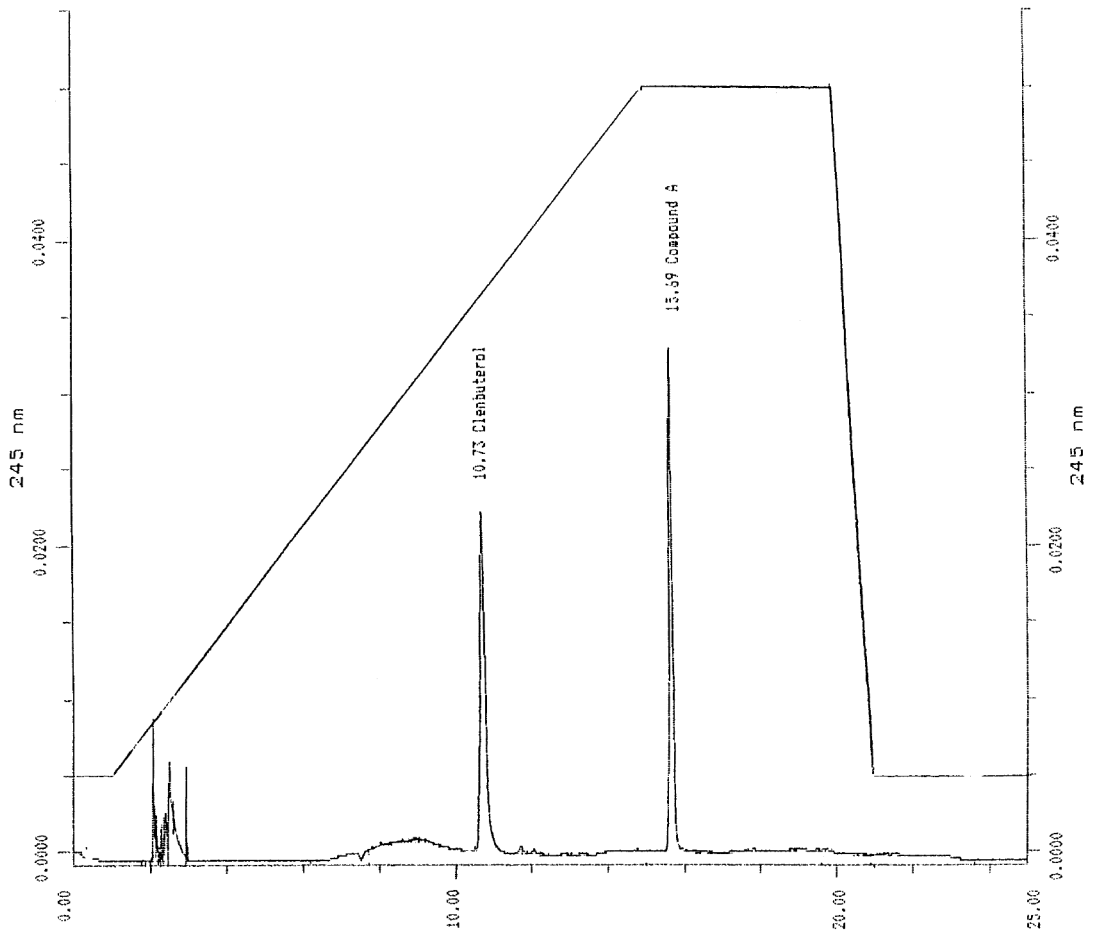

Figure 2. Elution profile of clenbuterol (RT = 10.73) and compound A (RT = 15.69) from RP-HPLC–DAD analysis with a linear gradient of 10% to 100% acetonitrile in 20 min.

columns (1), and the elution was performed by a 60-mL mixture of *n*-hexane–dimethyl chloride (8:2, v/v). Organic phase collected in a 100-mL glass round bottom flask was evaporated to dryness. Residue resuspended in 1 mL 20% MeOH was loaded on C18 SPE columns, according to a 2-mL washing (50% MeOH) and 4-mL MeOH (1% TEA) elution. The eluate was evaporated under a nitrogen stream on the heater block and derivatized with 20 µL *N,O*-bis(trimethylsilyl)trifluoroacetamide (1% trimethylchlorosilane, 60 min, 60°C) (1). We injected 2 µL in the gas chromatography (GC)–tandem mass spectrometry (MS–MS) system. Calibration curves for each analytical session (in the range of 0.20 to 4.00 ng injected) were built for each analyte.

Calculations

For HPLC analysis, calibration curves were built by plotting the peak area of the analyte versus its nominal concentration. For the GC–MS–MS analysis validation study, we considered the sum of the area of the precursor ion and two product ions both for the analyte and the internal standard (clenbuterol for compound A analysis and vice-versa).The peak-area ratio (analyte–internal standard) was plotted against the nominal concentration of the analyte. The calculation of the decision limits for CC alpha (the smallest content of the analyte in liver that may be confirmed with 95% probability) and CC beta (the smallest content of the analyte from which sample is truly violative with a confidence limit of 99%) on livers spiked at residue levels was performed according to the statistical approach of Gowick et al. (10).

Results and Discussion

SPE

The elution profile expressed as the recovery rate of clenbuterol and compound A from different SPE columns is reported in Table I. The coefficient of regression for the calibration curves were r = 0.9976 for clenbuterol and r = 0.9973 for compound A.

Method validation

The results of the recovery study by GC–MS–MS on livers spiked at the residue levels are reported in Table II with method performances. Regression curves over three analytical sessions were r = 0.9985 for compound A and r = 0.9987 for clenbuterol. It is worth noting that the European Commission suggested the acquisition of one parent ion and two product ions for the unambiguous GC–MS–MS identification of the forbidden substances. For this purpose, the chromatograms of a blank liver extract for compound A and a liver extract spiked at 1.0 ng/g (clenbuterol as the internal standard added at 4.0 ng/g) are shown in Figure 2, in which the traces reported on the figure from the top to the bottom refer to the total ion current, the precursor ion, the first product ion, the second product ion, and the sum of the ions (precursor + product ions), respectively.

Discussion

A comparison of the results of the recovery study on SCX and CN columns for clenbuterol and compound A indicates the former bases' binding mainly on the ion-exchange interaction of the secondary amino group. The lack of such a function in compound A greatly affects the recovery, thus demonstrating that the primary amino group shared among both compounds is weakly active charged (its pK_a is lowered) and sterically hindered by the two chlorinated atoms (Figure 1). The behavior of compound A (more retained on propyl CN columns during the application) can be mainly addressed to hydrophobic interactions (Table I). Such hydrophobic interactions that are present in MPH columns as C8 alkylic chains are not sufficient to retain compound A during the methanolic washing, which is a basic step to improve the selectivity of such "cleanup" procedures.

These considerations suggest the use of stronger hydro-

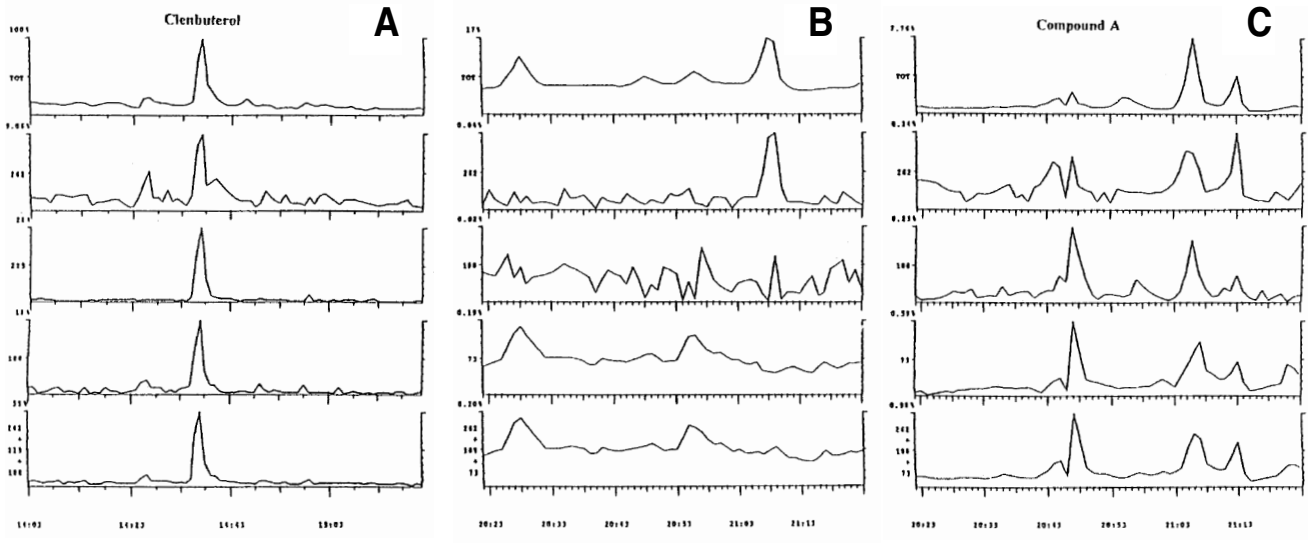

Figure 3. GC–MS–MS analysis of a blank and spiked liver for compound A: (A) clenbuterol used as the internal standard spiked at 4.0 ng/g, (B) the blank liver extract for compound A, and (C) a liver extract spiked with compound A at 1.0 ng/g.

phobic interactions, coupled with the presence of an ion-exchange mechanism for clenbuterol (represented by the free silanols groups in the case of C18NE columns). In order to allow the electrostatic interactions of clenbuterol, the flow rate in the application was reduced to 0.5 mL/min. The information derived from the RP-HPLC analysis of such drugs showed that compound A was eluted only in the presence of 100% acetonitrile (Figure 3). This behavior has been conserved on C18NE SPE columns that allow up to 2 mL 50% MeOH washing without any appreciable loss of compound A.

On this basis, an appropriate SPE procedure was developed on liver extracts. In order to limit as much as possible the presence of interfering substances that could act as counter ions, a preliminary extraction step on Extrelut columns at alkaline pH was carried out, which proved to be effective to concentrate clenbuterol and anilino-like compounds in the organic phase. The limited losses in the recoveries reported in Table II referred to the overall procedure and can be reasonably addressed to the sample handling.

Conclusion

The results encourage the use of C18NE SPE columns, which are able to work at the same time with RP and ion-exchange mechanisms and give satisfactory recoveries for two compounds with quite different chromatographic behavior. The pharmacokinetics evidence that clenbuterol could persist in bovine liver at residue levels above 1 ng/g for at least 400 h from the withdrawal of a growth promoting treatment (9) suggests that this approach could represent a realistic tool for the survey of the illegal use of such a drug and its related substances in animal productions, with an irrelevant probability of having false positive (CC alpha) or false negative (CC beta) results.

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