Determination of β_2-Agonists in Bovine Urine: Comparison of Two Extraction/Clean-Up Procedures for High-Resolution Gas Chromatography–Mass Spectrometry Analysis

Bernardete F. Spisso[1], Cláudio C. Lopes[2], Marlice A.S. Marques[2], and **Francisco R.A. Neto[3]**

[1]Instituto Nacional de Controle de Qualidade em Saúde (INCQS), Fundação Oswaldo Cruz, Av. Brasil, 4365, Rio de Janeiro, Brazil; [2]Departamento de Química Analítica; and [3]Departamento de Química Orgânica, Instituto de Química, Ilha do Fundão, CT, Bl. A, 21949-900, Universidade Federal do Rio de Janeiro (UFRJ), Rio de Janeiro, Brazil

Abstract

Two extraction/clean-up analytical procedures were investigated and compared regarding their recovery and matrix-purification efficiency for screening β_2-agonist residues in fortified bovine urine by high-resolution gas chromatography–mass spectrometry (GC–MS). The first procedure, based on an analytical method originally developed for detecting anabolic steroids, consists of the employment of the nonionic resin, Amberlite® XAD-2, a styrene-divinylbenzene copolymer for solid-phase extraction (SPE), followed by liquid–liquid extraction with diethyl ether. The second focuses on the use of a mixed SPE cartridge (reversed-phase and ion-exchange sorbent, Bond Elut Certify®). In both cases, the trimethylsilylated derivatives were analyzed by GC–MS with an ion-trap detector. Clenbuterol, salbutamol, and terbutaline were used to spike urine samples during the comparison experimental phase. Afterwards, tulobuterol, mabuterol, mapenterol, cimbuterol, and brombuterol were included in the evaluation of the second procedure (the Bond Elut Certify procedure). At this stage, the detection was accomplished by GC–MS (quadrupole mass analyzer) with selective ion monitoring acquisition. The isotopic dilution method with the hexadeuterated analogues of clenbuterol and salbutamol was applied to prepare calibration curves and calculate recovery percentages. With XAD-2 resin, terbutaline and salbutamol (resorcinol and phenol-type β_2-agonists, respectively) could not be detected at 20 ng/mL or at 40 ng/mL. In spite of clenbuterol having been detected at 20 ng/mL, the results obtained were not reproducible. The use of the reversed-phase and ion-exchange sorbent Bond Elut Certify allowed multiresidue detection and showed several advantages for the screening of clenbuterol such as higher recoveries, cleaner final extracts, reduced sample preparation time, less labor intensive, and easier solvent consumption and disposal. Recoveries over 88% (concentrations ranging from 0.5 to 10 ppb) and limits of detection equal to 0.5 ppb were met for all the β_2-agonists studied with the last method.

Introduction

The development of multiresidual analytical strategies to control the illegal use of several growth-promoting agents, among them the β_2-adrenergic agonists, has drawn much attention due to evidence of mixtures being used in animal production, including new substances developed and distributed in the black market such as mabuterol, mapenterol, cimaterol, and others (1,2).

The general chemical structure of most of these compounds is shown in Figure 1, and the substituent groups of the β_2-agonists included in this work are presented in Table I.

The control of nontherapeutic use of these phenylethanolamines became essential to maintaining consumer's protection, because there have already been several reported cases of food poisoning after the consumption of bovine meat and liver with clenbuterol residue (3,4). Urine, although not a matrix that presents the highest accumulation of these residues, is most commonly used to detect drugs in live animals. This allows for a possible risk evaluation at the beginning of the food chain.

The inclusion of β_2-agonists in the group of residues monitored by one of the procedures employed in the investigation of

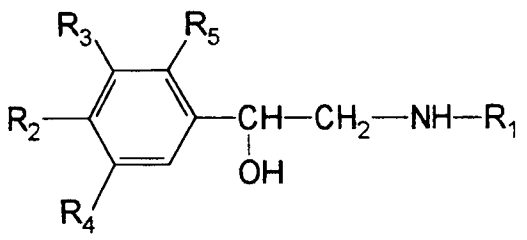

Figure 1. General structure of β_2-agonists (see Table I for substituents structures).

anabolics in the Brazilian National Program for the Control of Residues in Meat could make feasible the analysis of many such substances at the same time, thus shortening the number of methods to be validated by the control laboratory and reducing screening costs per residue. As new growth promoters have been frequently detected and identified, these advantages are becoming very attractive as well as the use of techniques that provide information about the structure of the substances, such as mass spectrometry (MS). Among the main physicochemical multiresidue methods mentioned in previous literature for the determination of β_2-agonists, those based on the use of mixed phases (sorbents containing both ion exchange and nonpolar functionalities) appear to be the most suitable ones (5–10).

Experimental

Equipment

The Waters Vacuum Manifold device (Waters, Milford, MA) was employed in solid-phase extraction (SPE) with Bond Elut Certify columns. For evaporation with N_2 and the incubation of derivatized samples, the Pierce Reacti-Therm (Pierce, Rockford, IL) was used.

Two high-resolution gas chromatography–mass spectrometry (HRGC–MS) systems were used in this study. The first was a model 3400 GC with a model 8100 autosampler, and an MS Saturn II ion-trap mass detector (Varian Instruments, Sunnyvale, CA). The other was an HP 5890 series II GC with a 6890 autosampler and an HP 5972 mass selective detector (Hewlett Packard, Palo Alto, CA). Analytical operating conditions and parameters were as follows: (a) chromatography: an HP-5 fused-silica capillary column (24-m × 0.2-mm i.d., 0.11-µm film thickness); temperature programming, 120 to 300°C at 11°C/min and then held for 5 min; carrier gas, He; linear velocity, 38 cm/s; injector temperature, 250°C; splitless injection; and (b) mass spectrometry, ionization by electron impact (70eV); transfer line temperature, 285°C (Saturn) and 300°C (HP); ion source temperature, 220°C (Saturn) and 170°C (HP); quadrupole temperature, 120°C (HP); autotune calibration.

Table I. Substituent Groups of β_2-Agonists Employed in the Studies Described in this Paper*

Substance	R_1	R_2	R_3	R_4	R_5
Tulobuterol (TULO)	$C(CH_3)_3$	H	H	H	Cl
Clenbuterol (CLEM)	$C(CH_3)_3$	NH_2	Cl	Cl	H
Brombuterol (BROM)	$C(CH_3)_3$	NH_2	Br	Br	H
Mabuterol (MABU)	$C(CH_3)_3$	NH_2	Cl	CF_3	H
Mapenterol (MAPE)	$C(C_2H_5)(CH_3)_2$	NH_2	Cl	CF_3	H
Cimbuterol (CIMB)	$C(CH_3)_3$	NH_2	CN	H	H
Terbutaline (TERB)	$C(CH_3)_3$	H	OH	OH	H
Salbutamol (SALB)	$C(CH_3)_3$	OH	CH_2OH	H	H

* See Figure 1 for structural features.

One to two microliters of the derivatized samples were injected in the GC–MS systems.

Materials

All reagents and solvents employed were of analytical-reagent or chromatographic grade. Analytical-reagent-grade diethylether was distilled. Salbutamol sulfate and terbutaline sulfate were a generous gift from Glaxo of Brazil S.A. (Rio de Janeiro, RJ, Brazil) and Merrell Lepetit (Santo Amaro, SP, Brazil), respectively. Clenbuterol hydrochloride, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), Amberlite resin XAD-2, and an enzyme mixture from *Helix pomatia* (100,000 U/mL β-glucuronidase and 5000 U/mL sulfatase) were purchased from Sigma Chemical Co. (St. Louis, MO).

Ampoules containing 0.10 mg of hexadeuterated clenbuterol and salbutamol (internal standards), tulobuterol, mabuterol, mapenterol, cimbuterol, and brombuterol were kindly provided by Dr. R.W. Stephany (National Institute of Public Health and Environment/Laboratory for Residue Analysis—ARO/RIVM, Bilthoven, The Netherlands). Calwer 2.2 software (11) was donated by the same institution.

Stock solutions of the test solutes were prepared in methanol (100 µg/mL) and diluted to the required concentrations to prepare mixed intermediate and working solutions. $[^2H_6]$-Clenbuterol and $[^2H_6]$-salbutamol solutions were prepared individually in concentrations of 10.0 and 1.0 µg/mL. Working and stock solutions were kept at –8°C and –20°C, respectively, and were protected from light. For the comparison experiments, urine samples were spiked at 40 and 20 ng/mL with clenbuterol, salbutamol, and terbutaline. For the purpose of calculating the Bond Elut Certify method's recoveries and limits of detection (LOD) urine samples were fortified at 10, 5, 2, 1, and 0.5 ppb, with all available analytes (see Table I).

Bond Elut Certify disposable cartridges (LRC 10 mL, 200 mg) employed in SPE were purchased from the manufacturer (Varian Sample Preparation Products, Harbor City, CA).

Samples

A pool of urine samples obtained from five different states of Brazil (Minas Gerais, São Paulo, Mato Grosso do Sul, Goiás, and Mato Grosso) and previously individually tested to verify the possibility for use as blanks was employed. All the samples were negative for the studied β_2-agonists and interferences at the same retention times and were kept frozen at –20°C until used.

Analytical procedures with nonhydrolyzed urines

Procedure using Bond Elut Certify cartridges. This procedure followed the method described by Montrade et al. (5), with minor modifications. In brief, 5 mL of pooled urine was buffered to pH 5.2 with a 2M sodium acetate buffer (pH 5.2) and spiked with the aforementioned analytes and internal standards. Soon afterwards, the pH was adjusted to pH 6.0 with a 0.1M potassium phosphate buffer (pH 6.0), and the samples were loaded onto the Bond Elut Certify columns that were previously conditioned with methanol and phosphate buffer. The cartridges were then rinsed with 1 mL of acetic acid 1.0M and dried under vacuum. The columns were washed

with 6 mL of methanol, and then the drying procedure was repeated. The compounds of interest were finally eluted with 6 mL ethyl acetate/ammonium hydroxide solution (97:3, v/v), and the solvent was concentrated with N_2 until dryness (45–50°C).

Procedure using Amberlite XAD-2 developed for anabolic steroids and modified for β_2-agonist analysis. This procedure is an adaptation of the method of reference 12 that was used in a Brazilian reference laboratory for the control of anabolics in cattle urine. Five milliliters of blank urine (pooled) without pH correction (pH range of samples: 8.1–8.3) was fortified at the desired level with the reference solution at suitable concentrations (methanolic mixtures). After addition of the internal standards and homogenization, the extraction on Amberlite XAD-2 in-house-prepared glass columns was performed. The resin had been previously purified and conditioned with acetone and water before use (12). Urine eluate was discarded and each column was washed with 5 mL of distilled water. The target compounds were eluted with 15 mL of methanol, and the eluate was reduced in rotavapor to approximately 2 mL. This material was then transferred to a glass, conical-bottom centrifuge tube with 3 × 1 mL of methanol, and the volume was again reduced, but in this instance to nearly 100 µL under a gentle stream of N_2 (45–50°C). Five milliliters of 1M sodium carbonate buffer (pH 10.0) was added to adjust the pH to 10.0, and the material was extracted with 3 × 5 mL of distilled diethyl ether in an orbital shaker. The combined ether phases were evaporated to approximately 200 µL at 45°C with N_2, and each tube was mixed in a vortex after the addition of 1 mL of diethylether to wash the internal surfaces of the tube. The solvent was completely evaporated.

Derivatization

The dried residues were kept dried in a vacuum desiccator over P_2O_5 and KOH for at least 1 h until derivatization with 50 µL of BSTFA (60°C/60 min).

Enzymatic hydrolysis of the conjugated metabolites

This procedure was incorporated only to the Bond Elut Certify method (before pH adjustment with potassium phosphate buffer to load the samples on the cartridges), because the XAD-2 method was abandoned because the results obtained with nonhydrolyzed urines were unsatisfactory. Fifty microliters of *Helix pomatia* was added to the fortified samples and incubated for 37°C/18 h.

Calculations

Peak-area ratios (analyte/internal standard) were used to construct calibration curves with 0.5, 1, 2, 5, and 10 ng/mL. Urine samples for the recovery studies were fortified at the same concentrations (four replicates each). The recovery and the limits of detection and determination were calculated from the curve obtained by regression analysis, plotting the added concentration as the x variable and the measured concentration as the y variable.

The calculations were accomplished with Saturn and Calwer 2.2 software (11).

Results and Discussion

General considerations regarding extraction mechanisms

The diversity of functional groups existing in β_2-agonist molecules represents a problem in the development of multiresidual control strategies. At pH values ~12, which is reported as ideal for extracting aniline-type β_2-agonists (13–15) such as clenbuterol, salbutamol and terbutaline are in the ionic form, making liquid–liquid extraction or the use of reversed-phase sorbents difficult.

Styrene divinylbenzene copolymers without ion-exchange properties, such as XAD-2 resin, had been employed in the extraction and concentration of nonpolar molecules (or containing a nonpolar portion) from aqueous matrices, including the β_2-agonist salbutamol (16,17). The retention mechanism is mainly based on interactions by van der Waals forces between the analytes and the polymeric hydrophobic surface (18). It is known that sorption of ionic or ionizable substances on resins like this one is favored by pH values in which these substances are in the neutral, nonionic form. Therefore, pH values near the isoelectric point, where the number of molecules without net charge (zwitterionic form) is maximum, are the most suitable for liquid–liquid extraction as well as for SPE with nonpolar sorbents. Terbutaline and salbutamol will always be in the ionic form independent from the pH because of the presence of both acidic groups (phenol) and basic groups (amino terminal).

For this reason, basic pH was chosen to test β_2-agonist sorption on XAD-2 columns, because dissociation constants of aromatic hydroxyl groups are higher than 8 (19). Studies with clenbuterol, salbutamol, and terbutaline at a high level, 500 ng, were done regarding the recovery at the sorption step. The results indicated that the recovery at the 500 ng level from XAD-2 resin at pH 10.0 (nearby salbutamol and terbutaline isoelectric point) was almost complete (> 96%) for the three substances. Nevertheless, the experiments done to calculate the final recovery of the XAD-2 + diethylether extraction method were discouraging, because really poor recoveries for salbutamol and terbutaline were obtained. Thus, the extraction with diethylether may be responsible for not detecting salbutamol and terbutaline in the final extract. As a matter of fact, those two substances are not easily extracted from aqueous solutions, because they exhibit a significant hydrophilic character. These results met those obtained by Courtheyn et al. (20) that related low salbutamol and terbutaline recoveries with *tert*-butylmethylether.

Other experiments done, including changing pH sorption from 10 to 8, resulted in no observed significant differences. Then, β_2-agonists were extracted with XAD-2 columns at pH 8 (average urine pH).

Bond Elut Certify cartridges have been widely employed with the aim of enhancing the recovery of more polar β_2-agonists, such as those of phenol and resorcinol type, together with that of the aniline type (6–8). Inside the same extraction cartridge, there are two chemically bonded silica phases: octylsilane (n-C_8) and benzenesulfonylpropylsilane, which makes possible the exploration of two retention mechanisms, the nonpolar and the

cationic exchange, respectively. Sample pH is adjusted to 6.0 with the purpose of minimizing the number of compounds that could be coextracted with the target analytes by a nonpolar retention mechanism. In spite of the fact that at pH 6.0 all substances studied are in the ionic form, the octyl chain is able to retain the analytes by interactions with the *tert*-butyl groups in the aliphatic termination. The octyl chain is said to supply superior selectivity compared to the most widely used reversed-phase, octadecylsilane, in the concentration of medium polarity molecules. The slight rise in polarity of the octyl chain, compared to the octadecyl chain, allows many contaminants that would probably be retained on the C_{18} sorbent to break through on the C_8 sorbent. It seems that at this pH, both mechanisms, nonpolar interactions and cation exchange, act simultaneously, because at pH 6.0 all the target analytes are positively charged and are able to be retained at the sorbent by the exchange cationic retention mechanism. Rinsing with acetic acid solution favors the exchange cationic retention mechanism, increasing the process selectivity even more, as only positively charged analytes will interact by electrostatic attraction with the negatively charged benzenesulfonic group. Methanol washing makes it possible to remove neutral interference components, but is not able to disrupt the ionic interactions. Ethyl acetate with ammonium hydroxide disrupts the ionic exchange interactions (the main interactions) and also the hydrophobic interactions (secondary interactions) through the neutraliza-

Table II. Recovery Data of Terbutaline, Clenbuterol, and Salbutamol Extracted from 5 mL Nonhydrolyzed Urine Samples Spiked at 20 ng/mL and Obtained from XAD-2 and Bond Elut Certify Procedures

	Recovery (%)*	
β_2-Agonist	Bond Elut Certify procedure	XAD-2 + diethylether extraction procedure
Terbutaline	67.9 (23.0)	n.d.†
Clenbuterol	85.9 (1.49)	78.4‡
Salbutamol	87.6 (0.35)	n.d.

* Average (standard deviation), $n = 2$.
† n.d. = not detected.
‡ $n = 1$.

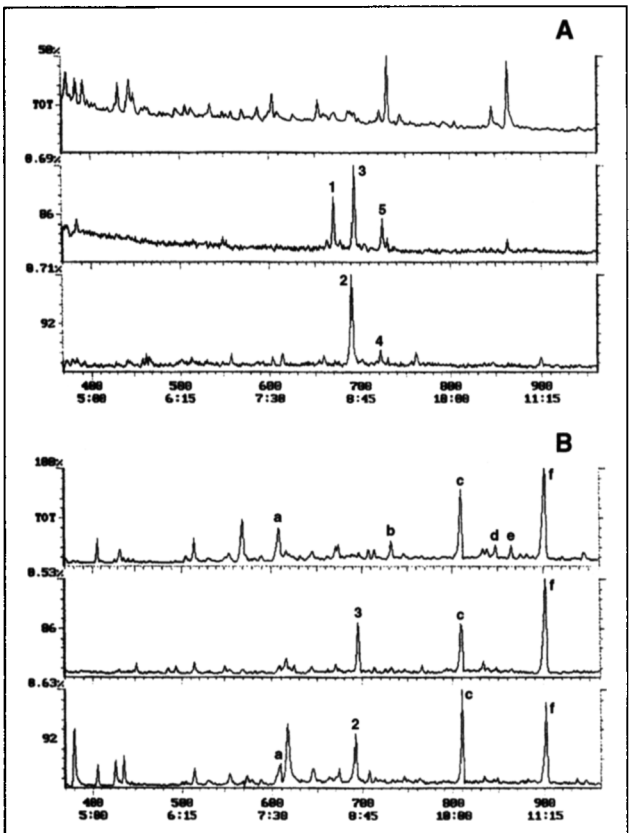

Figure 2. Comparison of extraction procedure with Bond Elut Certify cartridges (A) and extraction procedure with Amberlite XAD-2 resin followed by liquid–liquid extraction with diethylether (B). Urine samples without enzymatic hydrolysis. Total ion chromatograms and selected ion chromatograms corresponding to analytes base peak (m/z 86) and internal standards (m/z 92) from urine samples fortified at 20 ng/mL (20 ppb) with terbutaline (1), clenbuterol (3), and salbutamol (5). Internal standards: clenbuterol-d_6 (2) and salbutamol-d_6 (4). TMS derivatives: Phthalate (a); fatty acid TMS-ester derivatives (b), (d), (e); nonidentified (c), (f). For conditions, see Equipment (Experimental).

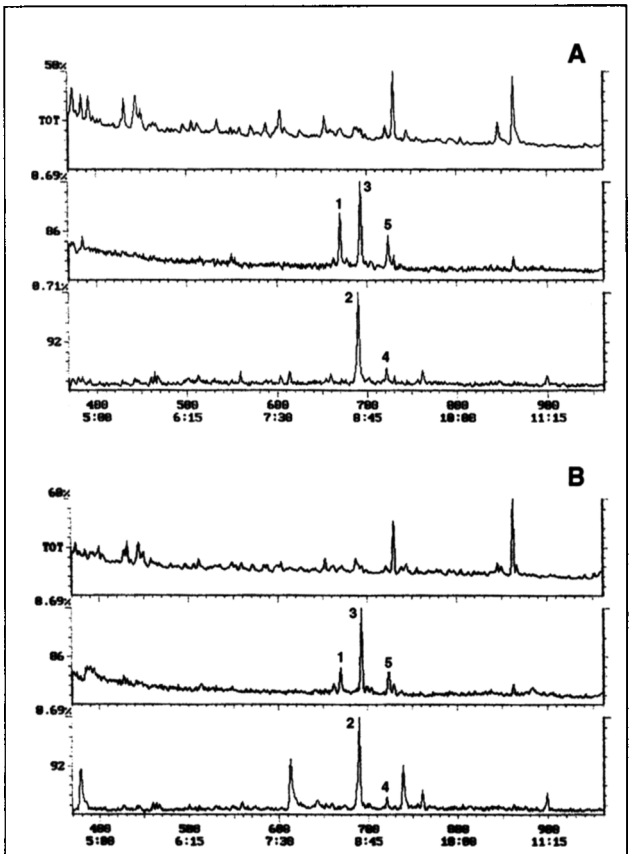

Figure 3. Comparison of extraction procedure with Bond Elut Certify cartridges, nonhydrolyzed urine (A); extraction procedure with Bond Elut Certify cartridges, hydrolyzed urine (with *Helix pomatia*, incubated for 37°C/18 h) (B). Total ion chromatograms and selected ion chromatograms, corresponding to analytes base peak (m/z 86) and internal standards (m/z 92) from urine samples fortified at 20 ng/mL (20 ppb) with terbutaline (1), clenbuterol (3), and salbutamol (5). Internal standards: clenbuterol-d_6 (2) and salbutamol-d_6 (4); TMS derivatives. For conditions, see Equipment (Experimental).

tion of β_2-agonists amino groups that are otherwise positively charged in acidic pH.

Comparison experiments using ion trap detection

Although β_2-agonist trimethylsilylated derivatives do not provide MS spectra with enough ions in number and intensity to meet the European Union requirements to confirm the presence of these drugs (21), selection of ions *m/z* 86, 92, and 100 obtained by cleavage at the alpha position made possible the screening of all substances studied with *N-tert*-butyl or *N-tert*-pentyl groups.

Table II and Figure 2 show for comparison the recovery, the total ion chromatograms, and the selected ion chromatograms of TMS derivatives (clenbuterol, salbutamol, and terbutaline) extracted from 5-mL urine samples spiked at 20 ng/mL and obtained from both procedures proving clearly the superiority of the recovery and clean-up achieved by the mixed-phase procedure and its multiresidual potential. It can be noted in Figure 2B that the final extract obtained by the sequence XAD-2 + diethylether extraction showed more impurities. Some of them could be identified by their mass spectra. Rigorous clean-up procedures are required before using synthetic polymers in residue analysis, because these resins exhibit significant amounts of preservatives and monomers resulting from manufacturing as well as other contaminants (18). Nevertheless, resin-purifying processes can many times result in increasing contaminants in concentration instead of decreasing its amount.

Employing bonded-phase ready-for-use sorbents decreases the probability of introducing contaminants by cleaning procedures and of course contributes to diminishing total analysis time, thus eliminating this step. Long periods to evaporate the relatively big volumes of methanol used to elute XAD-2

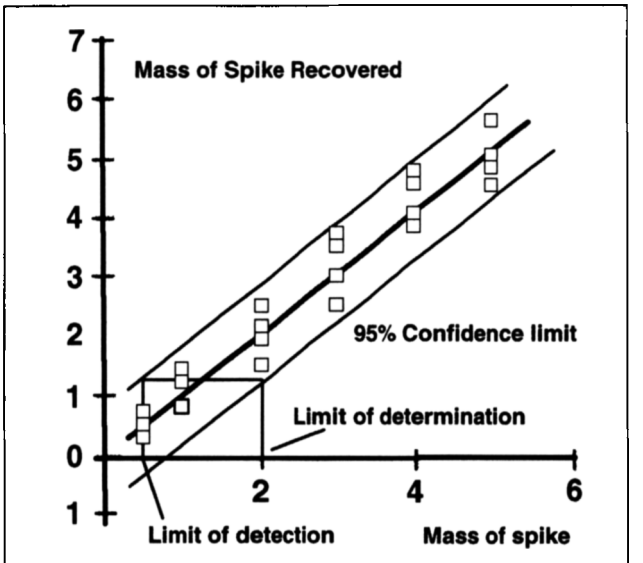

Figure 4. Graphical method for the calculation of limits using recoveries according to the Heitzman approach (1).

Table III. Statistical Data for the Determination of β_2-Agonists in Urine Samples Fortified from 0.5 to 10 ppb Obtained from Procedure Using Bond Elut Certify Cartridges and a Quadrupole HRGC–MS System

β_2-Agonist	t_{Rrel} range (min)	*m/z**	Method calibration line	Sd-res	r†	r²‡	F_{calc}	$F_{tab.}$ (0.05;1,*n* - 3)	Limit of detection§ (ng/mL)	Limit of determination§ (ng/mL)
Tulobuterol	0.610 – 0.612	86	$y = 0.504 + 0.978x$ (nm = 12)‖	0.221	0.949	0.901	0.84	5.12	0.5	1.58
Mabuterol#	0.780 – 0.782	86	$y = 0.256 + 1.208x$ (nm = 16)	0.307	0.984	0.968	2.20	4.75	0.5	1.54
Mapenterol	0.878 – 0.879	100	$y = -0.133 + 1.230x$ (nm = 12)	0.210	0.970	0.941	1.21	4.96	0.5	1.33
Terbutaline**	0.975 – 0.976	356	–	–	–	–	–	–	–	–
Clenbuterol	1.003 – 1.004	86	$y = 0.101 + 0.944x$ (nm = 16)	0.131	0.997	0.994	3.95	4.67	0.5	0.95
Salbutamol††	1.003 – 1.004	86	$y = -0.118 + 1.247x$ (nm = 14)	0.264	0.994	0.988	0.41	4.84	0.5	1.48
Cimbuterol**	1.052 – 1.055 (TMS1)	86	–	–	–	–	–	–	–	–
	1.102 – 1.103 (TMS2)	86	–	–	–	–	–	–	–	–
Brombuterol*	1.151 – 1.152	86	$y = 0.214 + 0.879x$ (nm = 20)	0.186	0.995	0.990	1.09	4.45	0.5	1.25

* Quantitating ion selected.
† Correlation coefficient.
‡ Determination coefficient.
§ According to Heitzmann approach.
‖ nm = total number of determinations, including the number of concentration levels tested and the number of replicates in each level.
Weighted linear regression.
** There was no correlation between variables *x* and *y*.
†† Salbutamol-d_9 was employed as internal standard.

columns is another drawback of this method.

The efficiency of clenbuterol extraction by the XAD-2 method at 20 ng/mL was lower than that obtained with the Bond Elut Certify method (86%, RSD 1.74%), and also the recovery results were not reproducible (Table II).

Enzymatic hydrolysis

Enzymatic hydrolysis is necessary to liberate those phenolic β_2-agonists that conjugate with sulfates and/or glucuronides (22). Thus, a simulation was performed whenever dosified materials (from animals treated with the drugs in question) or isolated β_2-agonist conjugates were not available. The enzymatic hydrolysis introduced in the Bond Elut Certify method did not significantly increase background levels in the final extract obtained (Figure 3).

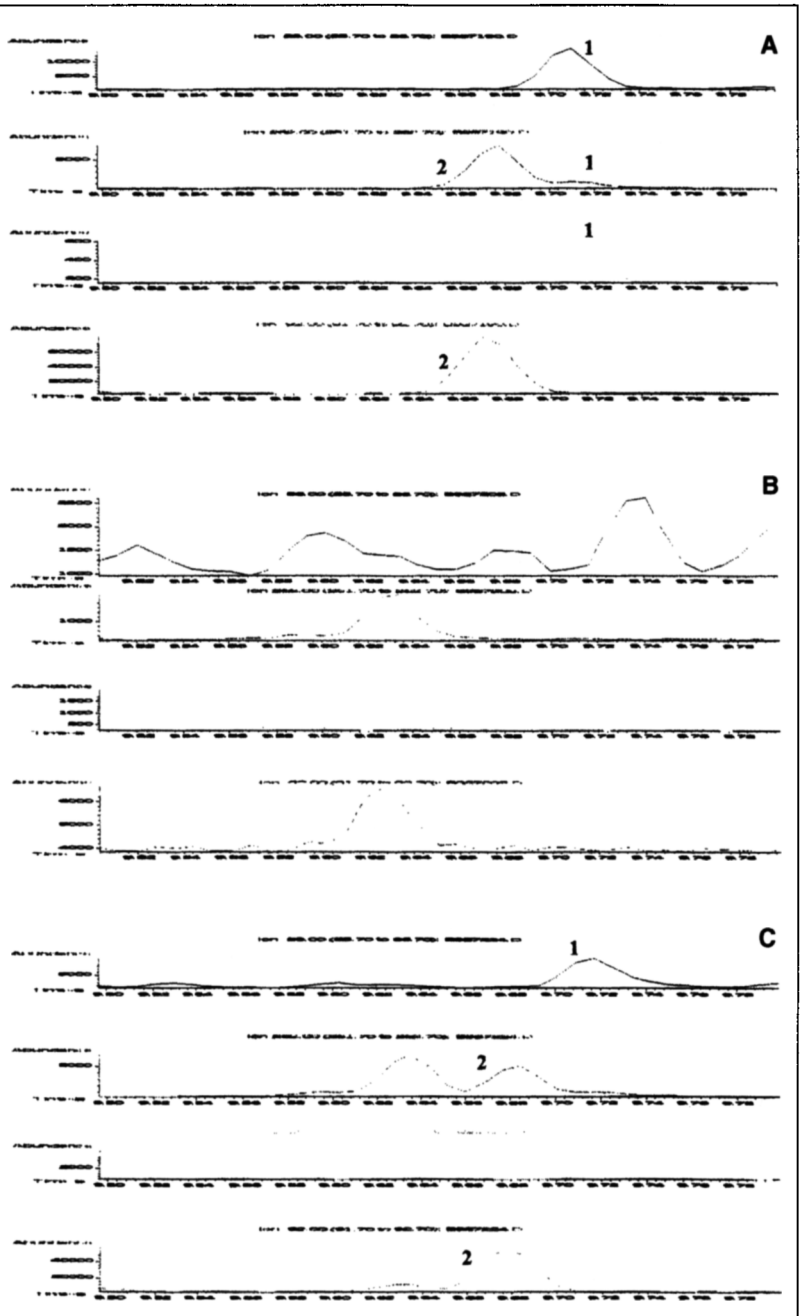

Figure 5. Ion traces of standard solutions, 1 ng/mL clenbuterol (1), and 10 ng/mL clenbuterol-d_6 (2) (A); blank pooled urine (B); and blank pooled urine fortified at 1 ng/mL clenbuterol (1) and 10 ng/mL clenbuterol-d_6 (2) (C). TMS derivatives: clembuterol: *m/z* 86, 243, and 262 and clenbuterol-d_6: *m/z* 92 and 262.

Bond Elut Certify LODs using quadrupole MS

Weighted linear regression analysis (11) was applied to the entire Bond Elut Certify procedure to calculate the LODs when variances were shown not to be constant (heteroscedasticity) in the calibration range tested (0.5 to 10 ppb). LODs, calculated according to the Heitzman approach (1), and other information concerning statistical results are presented in Table III.

The Heitzmann approach (1), in order to calculate the LODs (Figure 4), depends on optimal evaluation of the parameters of calibration as well as the standard residual deviation, though the procedure for the construction and evaluation of the linear model by least squares regression (equations in Table III) includes several statistical parameter evaluations.

Lower correlation coefficients were obtained for tulobuterol, mabuterol, and mapenterol, although these correlation coefficients had proven to be highly significant (by a *t*-test) bearing in mind that the number of determinations were 12, 16, and 12, respectively.

Figure 5 shows ion traces of a fortified sample of pooled urine containing 1 ng/mL clenbuterol, a standard solution at the same concentration, and a blank matrix.

Analytical recovery of the procedure

The mean recoveries of the β_2-agonists evaluated in fortified urine samples were all higher than 88% and are presented in Table IV.

Conclusions

This research has demonstrated that the screening method with XAD-2 resin was not suitable for β_2-agonists. Although clenbuterol had been detected at 20 ng/mL, the results were not reproducible. Bond Elut Certify reversed-phase and ion-exchange sorbent resulted in a lot of advantages besides multiresidual detection, such as better and more easily reproducible recoveries (clenbuterol), cleaner extracts, less time in preparing samples, and less solvent consumption.

The recovery of β_2-agonists in the procedure using Bond Elut Certify cartridges was higher than 88% for all the compounds evaluated.

The LODs calculated by the Heitzmann ap-

Table IV. Mean Recovery from Spiked Pooled Urine at 0.5–10 ppb Range

β_2-Agonist	Method calibration line	Number of determinations*	Mean recovery (%)
Tulobuterol	$y = 0.504 + 0.978x$	12	97.8
Mabuterol†	$y = 0.256 + 1.208x$	16	120.8
Mapenterol	$y = -0.133 + 1.230x$	12	123.0
Terbutaline‡	–	–	–
Clenbuterol	$y = 0.101 + 0.944x$	16	94.4
Salbutamol	$y = -0.118 + 1.247x$	14	124.7
Cimbuterol‡	–	–	–
Brombuterol†	$y = 0.214 + 0.879x$	20	87.9

* Total number of determinations including the number of concentration levels tested and the number of replicates in each level.
† Weighted linear regression.
‡ There was no correlation between variables x and y.

proach (1) were equal to 0.5 ng/mL (0.5 ppb), and the limits of determination were all lower than 1.6 ppb.

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