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Gas and liquid chromatography coupled to tandem mass spectrometry for the multiresidue analysis of β-agonists in biological matrices

G. Van Vyncht[a,b,*], S. Preece[c], P. Gaspar[b], G. Maghuin-Rogister[b], E. DePauw[a]

[a]*Mass Spectrometry Center, Chemistry Department, University of Liège, B-6, Sart Tilman, 4000 Liège, Belgium*

[b]*Laboratory of Analysis of Foodstuffs from Animal Origin, Faculty of Veterinary Medicine, University of Liège, B-43-b, Sart Tilman, 4000 Liège, Belgium*

[c]*VG Organic (VG-Biotech), Altrincham, WA14 5RZ, UK*

Abstract

β-Agonists are substances used in veterinary and human medicine for the treatment of pulmonary disorders. They have found a use as growth promoters to improve meat-to-fat ratios in cattle but they are not authorized for use in the European Union. Due to their presence in trace levels (usually less than 1 μg/kg), to the diversity of the illegally used compounds and to the complexity of the biological matrices analysed, the detection of these residues requires a very sensitive and specific method of determination. This work describes the strategy of analysis we developed for five β-agonists in urine and liver. The combination of improved solid- or liquid-phase extraction methods and LC or GC–MS–MS (in the multiple reaction monitoring mode) has shown to provide a system suitable for the control of these substances. The efficiency of extraction and the sensitivity and selectivity allow this multiresidue detection down to, and below, the UK regulatory level of 0.5 μg/kg. Moreover, the use of LC removes the need for the derivatisation step (cyclic methylboronate derivatives) which is required prior to GC–MS–MS analysis.

Keywords: Beta-Agonists; Clenbuterol; Salbutamol; Cimaterol; Mabuterol; Terbutaline

1. Introduction

β-Agonists, like clenbuterol (Fig. 1), are substances used in veterinary and human medicine for the treatment of pulmonary disorders. However, when these compounds are administered at levels higher than the therapeutic dose, they are active in the repartition metabolism and favor the protein accretion in muscle tissue at the expense of fat [1]. They have therefore found a use as growth promoters in cattle (a very flourishing black market based on veterinary drugs is now providing farmers with these prohibited substances) to improve meat-to-fat ratios. At high concentrations in liver or in meat, residues of these compounds are toxic to humans, leading to sickness and possible heart complications [2]. In the European Union (EU), they are not authorised for use as growth promoters and a maximum residue level of 0.5 μg/kg is proposed for clenbuterol in animal tissue [3].

All over the world and particularly in the EU, attention of consumers is focused on chemical compounds generating residues in foodstuffs from animal origin. Public health authorities and agrifood industries are faced with many difficulties in satisfying consumers and exportation market demands

*Corresponding author.

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about quality of meat and the safety and control of such residues. The detection of artificial repartitioning agents illegally used as growth promoters in industrial farming is considered a priority [3].

The diversity of the substances illegally used in meat production, the presence of these compounds in trace levels and the complexity of the biological matrices (urine or liver) analysed, made indispensable the development of a multiresidue strategy of analysis, involving a quick and easy sample pretreatment, followed by a specific and sensitive determination of several residues within the same determination run.

The official techniques used until now for these controls present many drawbacks.

The immunochemical screening methods, radio and enzyme immunoassays [4,5], are very sensitive but the cross-reactivity properties of these tests with structural analogs of the controlled residues preclude a non ambiguous identification of the compounds in the complex matrix of a biological sample. As a consequence, the positive results have to be confirmed by other more reliable analytical methods.

The confirmatory analytical technique most often used is mass spectrometry coupled to gas chromatography [6,7]. It requires, to reach acceptable detection limits (μg/kg levels), an extensive purification of the sample by HPLC or immunoaffinity chromatography before the analytical determination itself [8,9]. Moreover, these efficient purifications are rather selective and do not allow a real multiresidue analysis within the same chromatographic run.

The technique of determination that appears to be the most powerful for multiresidue trace analysis is the collisionally activated dissociation tandem mass spectrometry (CAD-MS–MS) in multiple reaction monitoring mode (MRM), coupled to gas or liquid chromatography [10–12].

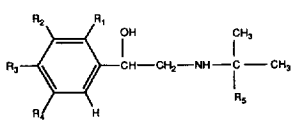

Fig. 1. Structures of the target β-agonists. Clenbuterol: R_1=H; $R_{2,4}$=Cl; R_3=NH_2; R_5=CH_3. Mabuterol: R_1=H; R_2=Cl; R_3=NH_2; R_4=CF_3; R_5=CH_3. Cimaterol: $R_{1,4,5}$=H; R_2=CN; R_3=NH_2. Salbutamol: $R_{1,4}$=H; R_2=CH_2OH; R_3=OH; R_5=CH_3. Terbutaline: $R_{1,3}$=H; $R_{2,4}$=OH; R_5=CH_3.

These are very selective methods that can be used with simple pretreatment of purification of the biosample. They are sensitive enough to reach the μg/kg range of detection and they also enable the determination of several residues within the same chromatographic run [13].

The aim of this study was to develop a general strategy for the screening and confirmatory analysis of β-agonists in urine and liver samples from animal origin. Urine analysis is needed so that cattle may be monitored in farms and slaughterhouses for the abuse of β-agonists by farmers, and liver is monitored to check the presence of β-agonist residues in meat for human consumption (liver is the center of the metabolic activity in the body, it usually contains higher levels of residues than muscle).

This paper describes the development of a rapid procedure for the detection of these residues. It involves solid-phase extractions on SCX cation-exchange disposable columns as sample pretreatment of urine, or liquid extraction followed by the same solid-phase extraction for the purification of β-agonists from liver samples [14,15]. Tandem mass spectrometry (in MRM mode) coupled to GC or LC was used as determination step.

The selected target compounds were β-agonists commonly, but illegally used, in industrial farming (clenbuterol, salbutamol, terbutaline, cimaterol and mabuterol) [16].

2. Experimental

2.1. Material and reagents

All the solvents used were analytical grade from Merck (Darmstadt, Germany). β-Glucuronidase/arylsulphatase digestive juice of *Helix pomatia* was from Boehringer (Mannheim, Germany). Methylboronic acid (MBA) was from Aldrich (Steinheim, Germany). N,N-Bis-trimethylsilyl-trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) were from Macherey-Nagel (Düren, Germany). Clenbuterol, clenbuterol·HCl, salbutamol, mabuterol, cimaterol and terbutaline were from Sigma (Deisenhofen, Germany). Deuterated standards ([$^2H_6$]clenbuterol, [$^2H_6$]salbutamol) for quan-

titative analysis by isotope dilution were a gift from RIVM (Bilthoven, Netherlands). [$^2H_6$]Terbutaline was from Astra Draco (Lund, Sweden). Isolute SCX solid-phase extraction disposable columns (500 mg, 3 ml) were from IST International (Glanorgan, UK). The DB-5 fused-silica capillary gas chromatography column (Permabond SE-52, 25 m×0.32 mm of I.D. and 0.50 μm film thickness) was from Macherey-Nagel. The α-chrom HPLC column (C_{18}, 25 cm×3 mm I.D., 5 μm particle diameter) was from Upchurch Scientfic (Oak Arbor, CA, USA).

2.2. Apparatus

The extraction apparatus was a Baker Vac Elut for disposable columns (Deventer, Netherlands). The GC–MS–MS experiments were performed using a HP-5890-series II gas chromatograph and a HP-7673 automatic injector from Hewlett–Packard (Palo Alto, CA, USA), coupled to a VG-AutoSpec-Q hybrid tandem mass spectrometer from Fisons (VG Analytical, Manchester, UK). The LC–MS–MS experiments used a HP-1050-series quaternary solvent delivery system and an automatic injector from Hewlett–Packard, coupled to a VG-Quattro-II quadrupolar tandem mass spectrometer from Fisons (VG Biotech, Manchester, UK).

2.3. Treatment of the calves' incurred samples

Clenbuterol incurred urine and liver samples were obtained by oral administration of 1 μg/kg body mass and per day of clenbuterol·HCl (14 days treatment) before slaughtering. Salbutamol incurred samples were obtained using the same treatment, but with a daily salbutamol intake of 10 μg/kg body mass.

2.4. Extraction of β-agonists from urine

The urine sample (5 ml) was adjusted to pH 3.0 with 6 *M* HCl prior to addition of 5 ml of 0.01 *M* KH_2PO_4 (pH 3.0). β-Glucuronidase/arylsulphatase of *H. pomatia* (50 μl) were then added to perform the hydrolysis of the conjugated β-agonists residues, for 2 h at 50°C. The hydrolysed urine sample was centrifuged at 2000 *g* for 10 min at room temperature.

The supernatant was extracted on a SCX cation-exchange disposable column under a depression of 0.4 bar.

The solid-phase extraction column was preconditioned with 5 ml of methanol, 5 ml of Milli-Q water and finally with 5 ml of 0.01 *M* KH_2PO_4 (pH 3.0). The sample was then applied to the extraction column. The washing sequence involved 2 ml of 0.01 *M* KH_2PO_4 (pH 3.0) and 2 ml of methanol. The column was dried under depression for 5 min and the β-agonists were eluted with 2 ml of a methanol–ammonia solution (95:5, v/v).

This extract was dried under nitrogen stream in a water bath at 40°C. The dry residue was dissolved in 500 μl of methanol and stored at 4°C before analysis.

2.5. Extraction of β-agonists from liver

The liquid extraction of β-agonists from the liver sample (5 ml) was performed by addition of 15 ml of 0.01 *M* KH_2PO_4 (pH 3.0) and 3 ml of Milli-Q water, prior to the Turrax disruption of the tissue (for 2 min). The homogenate was then centrifuged at 2000 *g* for 10 min at room temperature and the supernatant was collected. This liquid extraction using a Turrax disruption followed by a centrifugation was repeated twice.

The two collected supernatants were pooled and 50 μl of *H. pomatia* digestive juice were added to perform the hydrolysis of the conjugated β-agonists residues, for 2 h at 50°C and the hydrolysate was centrifuged at 2000 *g* for 10 min at room temperature.

To avoid saturation of the column phase, the extract was divided in two fractions and simultaneous solid-phase extractions of the β-agonists were performed on SCX disposable cartridges, using the same protocol as for urine samples. The final extracts were pooled before storage at 4°C.

2.6. Derivatization procedure [6,17]

When the determination was performed using GC coupled to MS–MS, a derivatization of the β-agonists was necessary to avoid degradation and/or peak tailing during chromatography. Moreover, the quality of the full scan spectrum (less fragmentation) of

these residues was enhanced when cyclic methylboronate derivatives were formed (see later).

The sample extract was evaporated under a nitrogen stream, at 40°C. 50 μl of the derivatization mixture (methylboronic acid in ethyl acetate, 10 mg/ml) were then added to the dry residue and the reaction was performed at 50°C for 15 min.

2.7. Gas chromatography

The initial temperature of the GC program was 120°C for 1 min. The temperature increased at a rate of 10°C/min, up to 310°C. This final temperature was held for 5 min. The injector and transfer line temperature was 300°C. The carrier gas was helium (N_{60}) with a head column pressure of 100 mbar and a flow-rate of 1 ml/min. 1 μl of the derivatization mixture was injected in splitless mode.

2.8. Liquid chromatography

A 50-ml sample of the methanol extract was injected and the flow-rate was 0.5 ml/min. The column head pressure was about 100 bars. The mobile phase composition was: (A) acetonitrile–10 m*M* ammonium acetate (pH 7.0)–water (77:12:11, v/v/v) and (B) acetonitrile–25 m*M* ammonium acetate (pH 3.5) (65:35, v/v). The gradient used 100% of (A) from 0 to 5.5 min and 100% of (B) from 5.6 to 15 min.

2.9. Tandem mass spectrometry

2.9.1. MS–MS strategy of analysis

One of the mass spectrometers used was a VG-AutoSpecQ ("EBEQQ" hybrid instrument), coupled to a gas chromatograph and interfaced with an electron impact ionization source, performs the collisionally activated dissociation mode of tandem mass spectrometry. It involves the selection of a precursor ion (formed in the ion source) in the sector part (EBE) of the mass spectrometer, its fragmentation in the quadrupolar collision cell (energetic collisions with a gas like air or argon) followed by the mass-to-charge ratio (m/z) analysis of the product ions by the last quadrupolar analyser.

This configuration allows the monitoring of transitions from selected precursor ions to particular product ions (MRM mode), the sector part of the instrument being thus considered as the first analyser that will avoid a major part of the biological matrix interferences to be analysed by the second analyser [11].

A triple stage quadrupole instrument (VG-Quattro-II), coupled to liquid chromatography and using an atmospheric pressure ionization interface [12] was also used, allowing the same MS–MS mode of analysis.

2.9.2. VG-AutoSpecQ

The positive electron impact mode was used (EI^+), with an electron energy of 70 eV and a trap current of 200 μA. The source temperature was 190°C. The collision gas (CAD) was air, at a pressure of 10^{-6} mbar and the collision energy was 20 eV. The resolution (10% valley) was set to 1.500 in the sector part of the instrument (EBE) while the last quadrupole was tuned to the mass unit resolution. The dwell time for each transition was 80 ms.

2.9.3. VG-QuattroII

The positive atmospheric pressure chemical ionization mode was used ($APCI^+$), with a cone voltage of 20 V. The source temperature was 140°C and the APCI probe was set to 550°C. The drying and sheath gas flow-rates were 300 and 50 l/h, respectively. The collision cell (CAD) was filled with argon, at a pressure of $2 \cdot 10^{-3}$ mbar and the collision energy was 15 eV. The mass unit resolution was used on both quadrupoles. The dwell time for each transition was 200 ms.

3. Results

3.1. Solid-phase extraction recoveries

The recoveries of the extraction procedures were estimated, for the different target residues, using a multiresidue enzyme immunoassay developed by Degand et al. [5]. The samples (urine and liver) were spiked with the reference compounds and the complete extraction procedures were performed for each compound separately.

For urine samples, the observed extraction re-

coveries ranged from 80% for clenbuterol to 60% for salbutamol. In liver samples, they ranged from 45% for clenbuterol to 55% for salbutamol.

The relatively poor extraction recoveries can be explained by the very different physical and chemical properties of β-agonists [16]. The unique compromise we found to elaborate a multiresidue extraction technique was to acidify the pH of the aqueous solution to 3.0, so that all the β-agonists get protonated. In this way, it was possible to extract them using a SCX cation-exchange column.

3.2. Derivatization procedure

The quickest and most sensitive technique was methylboronic acid derivatization, to form cyclic derivatives [6]. Using this method, the structure of the derivatised residue is stabilized. It avoids high fragmentation in electron impact mode, generating abundant ions at high mass-to-charge ratios and so allowing the tandem mass spectrometric analysis of these ions.

Unfortunately, terbutaline did not form a cyclic derivative and it was not possible to detect this compound using this procedure. A solution to this problem was to form trimethysilyl derivatives [7], but due to the very high fragmentation of these derivatives in electron impact mode, chemical ionization (with ammonia as reactant gas) had to be used to generate an abundant MH^+ ion prior to its collisionally activated fragmentation in the quad collision cell. Using this procedure, the detection limit reached for terbutaline was approximately 1 μg/kg.

3.3. MS–MS determination

For both GC and LC–MS–MS, different tandem mass spectrometric modes of analysis were tested (daughter and parent ion spectra, constant neutral loss scanning and multiple reaction monitoring) in the first free field region (linked scans using the EBE sector part of the VG-AutoSpecQ) and in the collision quadrupole cell. Only the multiple reaction monitoring (MRM) mode using the quadrupole collision cell allowed to reach detection limits below the 0.5 μg/kg decision level in multiresidue analysis, since other modes required residue levels over 5 μg/kg.

Under the above conditions, GC–MS–MS analyses in the MRM mode were performed by the selection of an abundant ion in the sector part of the instrument, its fragmentation in the quad collision cell and finally the detection of the most abundant daughter ion produced. The most sensitive transitions were observed from the M-15 to the M-57 ions, corresponding to the loss of the boronic part of the molecule.

The MRM mode performed in LC–MS–MS used the same procedure: the transition from the MH^+ ion to the most abundant daughter ion (represented by the loss of the side chain of the β-agonists following the loss of water) [12] allowed us to reach very low detection limits.

Some examples of the chromatograms obtained in both GC and LC–MS–MS are shown in Figs. 2 and 3 respectively.

The detection limits reached under these conditions are summarized in Table 1, for each compound and relative to the matrix and the determination technique used. All of them range from 0.05 to 0.2 μg/kg, allowing the control of β-agonists in urine and liver samples below the decision level of 0.5 μg/kg, while monitoring the 5 transitions (and so the 5 target residues) simultaneously.

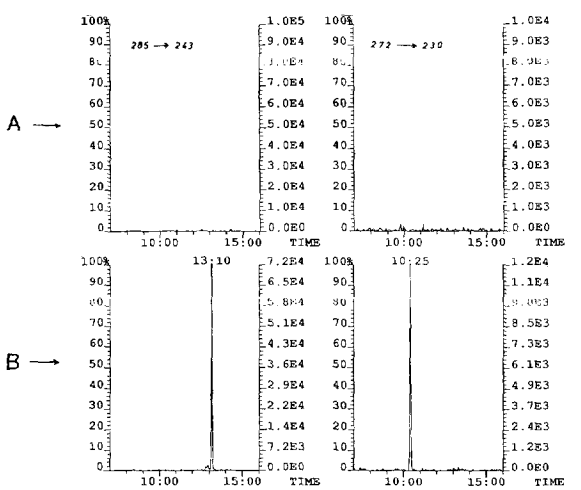

Fig. 2. MRM mode chromatograms for the monitoring of a blank (A) and of a 2 μg/kg spiked (B) urine sample (Transitions: 285>243 for clenbuterol; 272>230 for salbutamol).

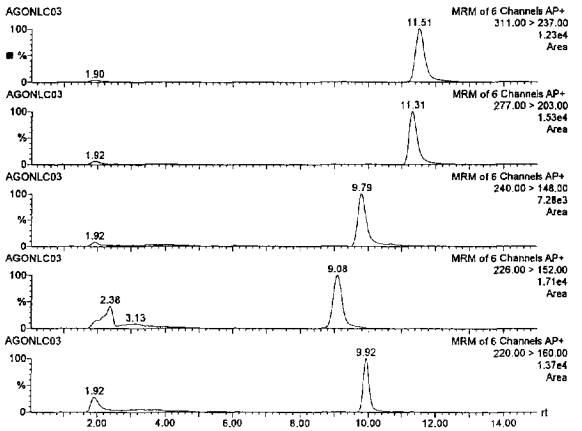

Fig. 3. MRM mode chromatograms for the monitoring of a 2.5 μg/kg spiked liver sample. (Transitions monitored are listed in Table 1.)

3.4. Quantification tests

We also performed quantification tests for β-agonists, using LC–MS–MS in the multiple reaction monitoring mode. The relationship between concentration and response was shown linear for each compound in the tested range of 0.5 to 25 μg/kg.

Quantitative estimations for clenbuterol and salbutamol in liver were performed on incurred samples, by isotope dilution using hexadeuterated standards of β-agonists. As shown in Table 2, the results obtained showed good correlations with the same determinations using EIA and isotope dilution single mass spectrometry (GC–MS in the single ion monitoring mode, with purification of the biosample by immunoaffinity chromatography).

Table 1
Detection limits reached for each target β-agonist, relative to the matrix analyzed and to the determination technique used

β-Agonists	Determination techniques	Transitions monitored	Detection limit (μg/kg)	
			Urine	Liver
Clenbuterol	GC–MS–MS	285–243	0.05	0.1
	LC–MS–MS	277–203	0.05	0.05
Salbutamol	GC–MS–MS	272–230	0.1	0.2
	LC–MS–MS	240–148	0.05	0.05
Cimaterol	GC–MS–MS	228–186	0.05	0.1
	LC–MS–MS	220–160	0.05	0.05
Mabuterol	GC–MS–MS	319–277	0.05	0.1
	LC–MS–MS	311–237	0.05	0.05
Terbutaline	GC–MS–MS	No derivative		
	LC–MS–MS	226–152	0.05	0.1

Table 2
Quantification of clenbuterol and salbutamol contents in incurred liver samples by different analytical techniques

β-agonists	Analytical techniques	Quantifications (μg/kg±S.D.; $n=3$)
Clenbuterol	EIA	6.1±0.5
	GC–MS	5.2±0.7
	LC–MS–MS	5.0±0.4
Salbutamol	EIA	8.6±0.6
	GC–MS	7.0±0.6
	LC–MS–MS	7.3±0.5

4. Conclusions

The combination of tandem mass spectrometry in the multiple reaction monitoring mode (coupled to GC or LC) and improved rapid extraction methods (solid-phase extraction or/and liquid extraction) has shown to provide a system suitable for the multiresidue analysis of β-agonists in urine and liver.

The efficiency of extraction and the sensitivity and selectivity of MS–MS allowed the detection of these compounds below the regulatory level of 0.5 μg/kg. Moreover, the use of LC (APCI ionisation) negates the need for the derivatization of these residues which is required prior to GC–MS–MS analysis and so enables the monitoring of β-agonists like terbutaline that does not form methylboronic derivative.

We also performed further experiments to determine how far the detection level could be reduced. The volume of the final methanol extract was decreased and set to 100 μl instead of 500 μl. The detection of all the β-agonists was enhanced except for terbutaline where the chromatographic resolution was impaired, probably due to the too high concentration of matrix components.

The "chemical background" observed in these low resolution MS–MS analyses is greatly reduced (or nearly absent) compared to the chromatograms obtained in the low or middle resolutions single ion recording mode (SIR), in which it is impossible to

interpret data in the μg/kg range without further purification of the complex biosample.

A parallel approach using high resolution (>10 000) mass spectrometry in single ion recording (SIR), coupled to GC, provides approximately the same detection limits for individual compounds. Unfortunately, the detection of several β-agonists within the same chromatographic run would imply an acquisition based on voltage SIR and the monitoring of 3 to 4 ions per compound. So, the detection limits of the different residues would rise, due to the fact that numerous ions should be controlled simultaneously.

A last remark about future developments of these analytical techniques. It should be possible to monitor other families of residues, like anabolizing steroids or corticosteroids in complex biological matrices with LC–MS–MS strategies when electrospray/APCI sources develop to produce a better ionization efficiency.

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