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Gas chromatographic–tandem mass spectrometric analysis of clenbuterol residues in faeces

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Abstract

In all EU member states, the use in livestock farming of certain substances having a hormonal action is prohibited. Clenbuterol, the β-adrenergic agonist, has some growth promoting characteristics. Screening for clenbuterol can be carried out by an immunoassay. Gas chromatography–mass spectrometry (GC–MS) is very valuable for confirmatory purposes. In full scan MS it is impossible to fulfil the EU criteria of four diagnostic ions with one single ionisation mode. Some alternative possibilities are: (1) the use of two different ionisation modes, (2) the use of different derivatization methods or (3) the use of tandem MS. Each derivatisation or ionisation mode on its own did not give a sufficient number of ions. By combining these different possibilities we were able to obtain four ions, fulfilling the EU criteria.

Keywords: Clenbuterol; Beta-agonists

1. Introduction

Clenbuterol is licensed in most EU member states for use in the relief of bovine and equine respiratory diseases and in the management of parturition. Given at 5 to 10 times the therapeutic dose it has a positive effect on the growth of slaughter animals. Also with other β-agonists a shift in carcass composition of poultry, pigs, cattle and sheep is obtained. The fat content is dramatically reduced in favour of a higher percentage of muscle. Therefore they are called repartitioning agents [1–3].

In all EU member states the use in livestock farming of certain substances having a hormonal action is prohibited [4,5]. This illegal use in meat production has prompted the development of analytical techniques capable of detecting residues of those substances. Illegal administration of clenbuterol at these higher concentrations results in accumulation of residues in the liver [6]. The potential hazard for human health is already proved by several cases of intoxications reported in Spain and France [7,8].

Multi-analyte screening methods for β-agonists based on enzyme immunoassay have been developed in numerous laboratories. For confirmatory purposes thin-layer chromatography, high performance liquid chromatography and gas chromatography are described. Among the chromatographic methods, those using mass spectrometric detection should be preferred because of their high specificity.

The gas chromatographic–mass spectrometric criteria of the EU demand that at least four diagnostic ions must be measured and that the relative

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intensities must be the same of those for the standard analyte within a margin of ±10% [electron impact (EI) mode] or ±20% [chemical ionization (CI) mode]. As the common derivatives show low abundances for the most specific ions, in many cases identification of residues of β-agonists has to be based on the results of two independent gas chromatography–low-resolution mass spectrometry (GC–LRMS) methods with different derivatives and/or ionization techniques, each producing two or three diagnostic ions.

The objective of this paper is to evaluate the possibilities of tandem MS in order to obtain sufficient evidence of positive identification in only one run. This has been done by means of the determination of clenbuterol at residue level in faecal material.

2. Experimental

2.1. Apparatus

Magnum Ion Trap System (Finnigan MAT, San Jose, CA, USA) consisting of: Finnigan MAT A200S GC Autosampler, Varian 3400 GC with 1077 capillary split/splitless injector, Finnigan MAT Magnum Ion Trap Mass Spectrometer with electron impact and advanced positive chemical ionisation. Column: SGE BPX-5-0.25 (25 m×0.22 mm I.D., film thickness 0.25 μm).

GCQ (Finnigan MAT) with Finnigan MAT A200S GC Autosampler (for the butylboronate derivatives and MS–MS measurements) and Hewlett-Packard Model 5989 A MS Engine with 5890 Series II gas chromatograph and Hewlett-Packard 7673 Autosampler (for the TMS-derivatives). In both cases a Hewlett-Packard HP-5 MS fused-silica capillary column (25 m×0.25 mm, 0.25 μm film thickness) was used.

2.2. Standards and reagents

Clenbuterol·HCl [2-*tert.*-butylamino-1-(4-amino-3,5-dichlorophenyl)ethanol hydrochloride] was obtained from Sigma (Eupen, Belgium). Deuterated [$^2H_6$]clenbuterol (clenbuterol-d_6) was a gift from the State Institute for Quality Control of Agricultural Products (RIKILT, Wageningen, Netherlands). Standards were dissolved in methanol at concentrations of 1 ng/μl. These solutions were stored in the dark at 4°C.

N-methyl N-trimethylsilyl-trifluoroacetamide (MSTFA) is from Macherey–Nagel (Düren, Germany) and iodotrimethylsilane (TMSI) from Janssen Chimica (Geel, Belgium). MSTFA$^+$ is prepared by dissolving 1‰ TMSI in MSTFA [9]. N,O-bis-(trimethylsilyl)trifluoroacetamide (BSTFA)+1% trimethylchlorosilane (TMCS) was from Pierce (Rockford, IL, USA). The reagent was freshly prepared by adding 1 ml of pyridine (dried over KOH) to 1 ml BSTFA+1% TMCS and vortexing.

1-Butaneboronic acid (BBA) was obtained from Janssen Chimica (Beerse, Belgium). The BBA solution was prepared by dissolving the 1-butaneboronic acid in ethyl acetate (1 mg/1 ml), and drying it over anhydrous sodium sulphate (UCB, Leuven, Belgium). Some molecular sieves (0.3 nm, beads about 2 mm, Merck) were added to the solution. The solution was stored in the dark at −24°C.

2.3. Sample preparation

The earlier described extraction and clean-up procedure [10] was slightly modified. Eleven grams of faeces were weighed out in a disposable polypropylene tube and spiked with 2 ppb of clenbuterol-d_6 (22 μl of clenbuterol-d_6 in methanol at 1 ng/μl). Thirty ml of 0.5 *M* hydrochloric acid, saturated with ethyl acetate, were added and the mixture was shaken vigorously during 15 min by means of a mechanical shaker. After centrifugation during 15 min at 4000 *g*, the supernatant was decanted and the pH was adjusted to 10 with 32% sodium hydroxide. The alkaline solution obtained was centrifuged for 5 min at 4000 *g* and transferred onto a Chem Elut CE 1020 column (Varian). After 10 min elution was carried out with three 20-ml portions of toluene. The eluate was collected into a screw-topped 50-ml conical-bottomed centrifuge tube. A volume of 250 μl of 0.1 *M* hydrochloric acid was added to the tube, which was then shaken vigorously by hand for 1 min and placed in an ultrasonic bath for 5 min. A complete separation of both layers was achieved by centrifugation at 2000 *g* for 10 min. The lower acidic layer was then transferred to an autosampler vial, evaporated at 50°C under nitrogen and derivatized for GC–MS purposes.

2.4. TMS derivatisation and GC–MS conditions

2.4.1. Formation of di-TMS derivatives

Derivatisation: the sample or 5 μl standard solution (100 ng) is evaporated under a gentle N_2-stream (autosampler vial), 25 μl of $MSTFA^+$ is added, the vial is heated at 60°C during 30 min. A 1 μl volume (4 ng) is injected splitless (autosampler).

GC–MS conditions: initial: 100°C, to 200°C at 33.3°C min^{-1}, to 250°C at 7°C min^{-1}, 250°C to 300°C at 50°C min^{-1}, 300°C 6 min. (total run ca. 17 min). Temperature of injector: 260°C, temperature of transfer-line: 300°C.

Acquisition method: 1 scan/s during 15 min (mass range 80–600 a.m.u., filament-multiplier delay 200 s), positive CI parameters: reagent gas: isobutane, max. ionisation time: 1500 μs, max. reaction time: 80 ms, ionisation level: 20 u, reaction level: 40 u.

2.4.2. Formation of the mono-TMS derivative

Derivatisation: The sample or standard solution is evaporated under nitrogen, 100 μl of BSTFA+1% TMCS is added and vortexed. The vial is heated for 30 min at 60°C. The reagent is evaporated, and the residue redissolved in toluene with 1% BSTFA.

GC–MS conditions: Oven temperature was initially held at 100°C for 1 min, then raised to 216°C at a rate of 20°C min^{-1}, then kept constant for 6 min and raised again at a rate of 20°C min^{-1} to 300°C. The final oven temperature was held for 3 min.

Measurements were made in selected ion monitoring (SIM) mode. The dwell time was 40 ms. The source temperature was 250°C.

2.5. Butylboronate derivatisation and GC–MS conditions

Derivatisation [11]: The sample or standard solution is evaporated under nitrogen, 50 μl ethyl acetate is added and evaporated again. A 100 μl volume of the derivatisation solution is added. The vial is vortexed and heated for 15 min at 45°C. The reagent is evaporated. The residue is redissolved in the reagent and injected.

GC–MS conditions: The oven temperature was initially held at 100°C for 2 min, raised to 250°C at a rate of 20°C min^{-1}, then raised to 300°C at a rate of 5°C min^{-1} and maintained at this temperature during 3 min. Measurements were made in full scan mode, scanning from 100–400 u with 0.75 s per scan. The source temperature was 200°C.

3. Results and discussion

3.1. Faecal material as matrix for clenbuterol detection

Many different matrixes are described in literature. Liver is the most cited matrix because there is an accumulation of clenbuterol residues in that organ [6]. Other tissues and body fluids, such as eyes (choroid/pigmented retinal epithelium), pigmented hair, bile and urine, were proposed as matrixes [2,12–15]. From all these matrixes, only urine and pigmented hair were available at the farm. Meyer and Rinke [16] found that the clenbuterol elimination has a bi-phasic character. A first rapid decline was noted with a half-life of about 10 h and a half-life of approximately 2.7 days was noted for the second phase. Residue concentrations in blood plasma quickly reach the detection limit. Therefore only a half-life of ca. 18 h could be determined for the first phase only.

Urine sampling in bulls is not usually easy. Therefore some screening tests were described on faeces as a matrix for analysis of clenbuterol and its analogues [17,18]. Delahaut et al. observed that the elimination and concentration of clenbuterol in the different matrixes were dose-dependent. The higher the dose, the higher the residue levels. So levels of about 421 ng/ml and 9 ng/ml were found at the highest dose for urine and faeces, respectively [18]. Because of these low concentrations in faeces, a more specific and sensitive analytical technique is required.

Data from a study of Elliott et al. prove that at therapeutic or illegal growth stimulating levels of clenbuterol, liver samples contained high residues for up to 15 days after withdrawal [19]. The fact is that animals presented for slaughter should be free of residues of any kind at levels beyond an acceptable “tolerance level” (0.08 ppb) [16]. Since we are using a limit of decision of 2 ppb (i.e. 2 ng/g faeces) for declaring a sample as positive for containing residues of clenbuterol, there is still a wide range of certainty. At this point there are two possibilities: (1) the withdrawal time after therapeutic use was not implemented or (2) there has been an illegal administration which resulted in higher residue amounts for a longer period. Either way, it is not free of residues and therefore harmful for human health.

3.2. GC–MS(–MS) confirmation of clenbuterol residues in faecal material

Screening of clenbuterol can be carried out by an immunoassay. For confirmatory purposes a physico-chemical method is required. High-performance liquid chromatography with post-column derivatisation as described by Courtheyn et al. [10] is one possibility. GC–MS is an alternative which has some advantages. With the proper extraction method, GC–MS is capable of detecting both the aniline derivatives (i.e. clenbuterol) and the phenol derivatives (i.e. salbutamol) [20]. The possibility of generating a full scan spectrum is a very valuable tool in the identification of residues of drugs which are abused. No difference in performance and sensitivity was observed in comparison with HPLC post-column derivatisation.

To fulfil the gas chromatographic–mass spectrometric confirmation criteria of the EU [21] it is necessary to measure four independent mass fragments in the mass spectrum. In full scan MS it is impossible to fulfil these criteria with one single ionisation mode. Some possible alternatives are: (1) the use of two different ionisation modes, (2) the use of different derivatisations or (3) the use of GC–MS–MS.

In electron impact mode the spectrum of the mono-TMS derivative of clenbuterol, obtained by derivatisation with BSTFA (see conditions above), shows the following five diagnostic ions: 86 (100.0), 243 (3.0), 262 (7.0), 264 (4.7) and 277 (1.3) (see Figs. 1 and 2). The ion 264 is an ion of the typical isotope cluster from the double chlorinated fragment. At residue-levels however it is clear that the relative low intensities of the ions, except for 86, are easily interfered with by matrix components. This phenomenon is illustrated in Fig. 2 with the full scan spectrum of the clenbuterol peak in a faecal sample, spiked at the level of 5 ppb.

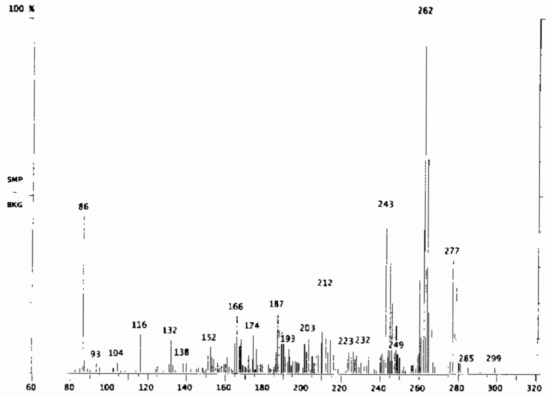

Fig. 1. EI spectrum of clenbuterol-mono-TMS (full scan).

In order to obtain the necessary evidence it is therefore often necessary to complete this first run with a second run in chemical ionisation mode or with another derivative. In the chemical ionisation mode the diagnostic ions with higher masses are much more abundant than in electron impact mode. The stability of the spectra however is much less, again causing problems for the meeting of criteria in one single run (see Figs. 3 and 4).

Of the common reagents used for derivatisation of β-agonists, boronic acids often are preferred as they produce derivatives with abundant ions in the high-mass range. At residue-level again however, obtaining four ions is a problem (see Fig. 5).

After formation of the di-TMS derivatives, the

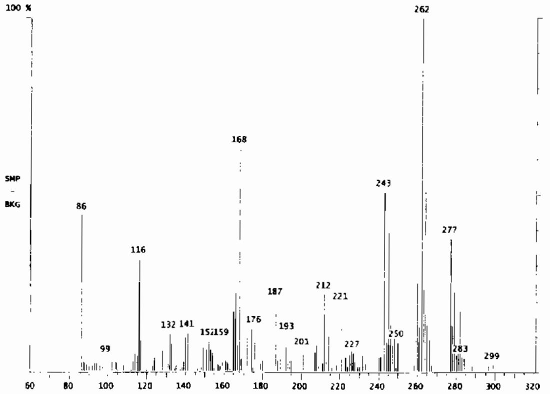

Fig. 2. EI spectrum of clenbuterol-mono-TMS (full scan) in a spiked faecal sample (5 ppb).

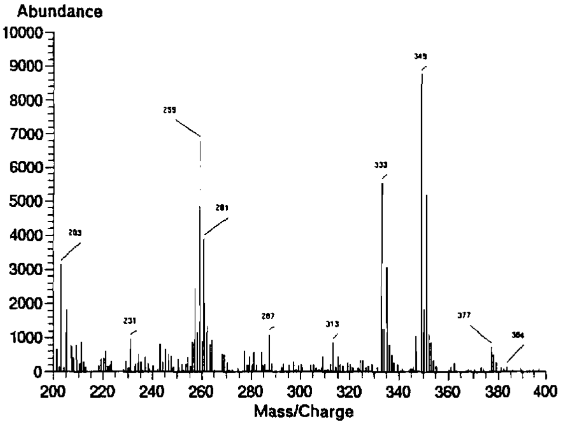

Fig. 3. CI spectrum of clenbuterol-mono-TMS (full scan).

spectrum of clenbuterol shows three diagnostic ions in the CI spectrum. Typical for these spectra is also the presence of isotope peaks of the chloride ion, i.e. ^{37}Cl, producing a typical isotope cluster for every fragment containing a Cl ion. The ions 86 (14), 331 (100), 405 (41), 421 (79) are used as diagnostic ions (relative intensity). Figs. 6 and 7 present the typical CI and EI spectrum of 4 ng of clenbuterol-di-TMS standard.

Another possibility for providing sufficient evidence of positive identification is tandem MS. Although it has existed for some time on larger machines, this technique only became available for daily routine analysis with the introduction of the bench top MS–MS of Varian in the middle of 1994 and the GCQ from Finnigan MAT in 1995. Application of GC–MS–MS results in an important reduction of the background noise, resulting in completely comparable full scan spectra of standards and the analyte at residue levels. This is illustrated in Figs. 8 and 9, representing the MS–MS spectra of clen-

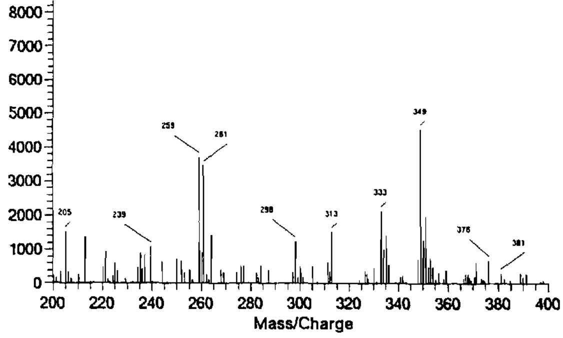

Fig. 4. CI spectrum of clenbuterol-mono-TMS (full scan) in a spiked faecal sample (2 ppb).

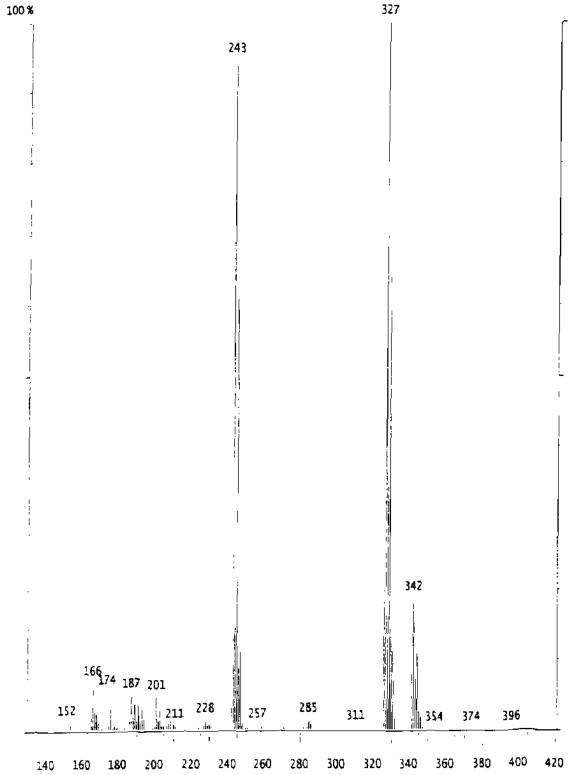

Fig. 5. EI spectrum of clenbuterol butylboronate (full scan).

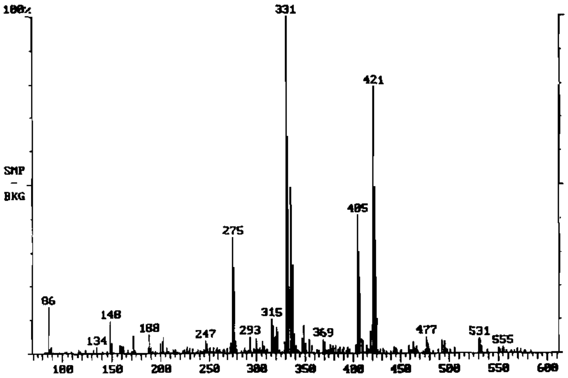

Fig. 6. CI spectrum of clenbuterol-di-TMS (full scan).

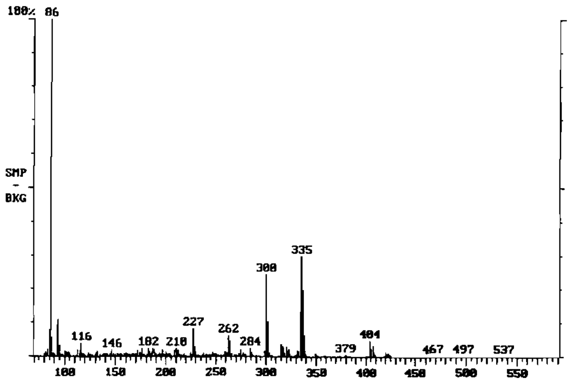

Fig. 7. EI spectrum of clenbuterol-di-TMS (full scan).

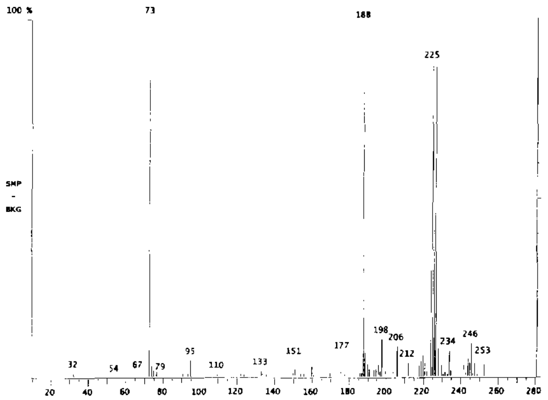

Fig. 8. GC–MS–MS spectrum of clenbuterol-mono-TMS (full scan).

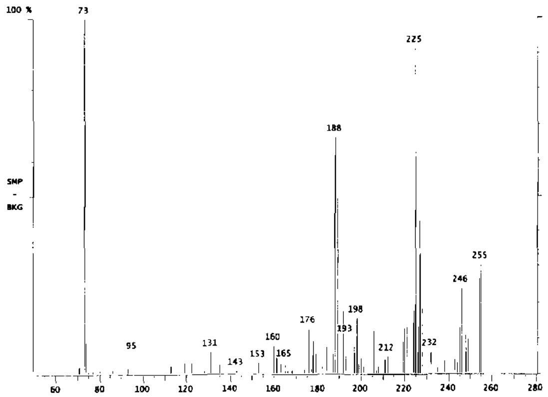

Fig. 9. GC–MS–MS spectrum of clenbuterol-mono-TMS (full scan) in a spiked faecal sample (5 ppb).

buterol-mono-TMS derivative in a standard and a spiked faecal sample at the 5 ppb level, respectively.

4. Conclusions

Despite the many analogue β_2-adrenergic products, clenbuterol is still the most applied component, not only for therapeutical purposes but also illegally in much higher concentrations for promoting growth in cattle.

The sample preparation method of Courtheyn et al. [10], developed for the aniline group of β-agonists, such as clenbuterol, was found suitable for GC–MS.

The use of GC–MS was preferred because of the specificity of the detection technique (specific full scan spectrum) and also for some practical internal advantages for analysing many samples automatically. To obtain four diagnostic ions, as required by the EU criteria, different derivatisations (mono-TMS and butylboronic acid derivatives) and different ionisation methods were combined. Each mode presents at least two to three usable diagnostic ions. By combining the information of the EI and CI spectra of the different derivatisation methods at least eight to ten diagnostic ions are present to help in the identification of a sample as positive for clenbuterol with a great margin of certainty.

The possibility of obtaining positive identification at residue-level in a single run on the one hand and the better availability of MS–MS in control laboratories on the other hand leads to an urgent need for MS–MS criteria. This should result in an even greater sensitivity and specificity for the detection of clenbuterol at sub-ppb level.

As full spectra with low background noise are produced with this apparatus, criteria for this technique will be more reliably based on comparison of the unknown spectrum with a standard spectrum instead of an adaption of the known criteria, based on four ions.

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