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CLENBUTEROL DETECTION AT THE FARM: FAECES AS MATRIX FOR GC-MS ANALYSIS.

PETER BATJOENS[1], DIRK COURTHEYN[2], HUBERT F. DE BRABANDER[1], JAN VERCAMMEN[2] and JAN VAN HOOF[1].
1 Dept. Veterinary Food Inspection, Faculty of Veterinary Medicine, University of Ghent, Salisburylaan 133, 9820 Merelbeke, Belgium.
2 State Laboratory (ROL), Braemkasteelstraat 59, 9050 Gentbrugge, Belgium.

Introduction

In veterinary medicine, the beta-adrenergic agonist (BAA) clenbuterol is used as bronchodilator for the treatment of respiratory diseases and to control parturition.
Clenbuterol and other substances of this family are also known for their growth promoting characteristics when administered at higher doses (1). They decrease the deposition of fat and increase the nitrogen retention in muscular proteins(2).
In all E.U.-member states the use in livestock farming of certain substances having a hormonal action is prohibited (3,4). Illegal administration of clenbuterol at these higher concentrations results in accumulations of residues in the liver (5).The potential hazard for human health is already proven by several cases of intoxications reported in Spain and France(6,7).
For current regulatory control of beta-agonists in Belgium, our laboratory uses enzyme immunoassays for screening purposes and GC-MS techniques for confirmation of the screening results.
Since we are receiving samples from both the slaughterhouses (Ministry of Public Health) as well as from the farms (Ministry of Agriculture) the matrixes of choice vary from liver, urine to faeces.
On the farms only urine and/or faeces can be taken. Sampling of urine from bulls or steers can cause some problems. That 's why faeces is taken more and more as matrix for clenbuterol analysis.
The objective of this paper is to describe the extraction and clean-up method for GC-MS analysis of clenbuterol in faeces.

Experimental

Apparatus

The following apparatus were used: extraction flasks (10 - 20 ml), home made extraction flask tumbler, nitrogen evaporator, reacti-term heating module, autosampler vials (e.g. Chromacol 07-CPV (A)).
Magnum Ion Trap System (Finnigan Mat., San Jose , CA., U.S.A) 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.

Reagents and reference components

Clenbuterol (CB) was obtained from Sigma-Aldrich N.V.(Bornem Belgium). Deuterated clenbuterol (CB-D6) (internal standard BAA's) was purchased from the European Communities Reference Laboratory (National Institute of Public Health and Environmental Protection, Laboratory for Residue Analysis, Bilthoven, The Netherlands).
MSTFA (N-Methyl N-trimethylsilyl-trifluoroacetamide) is from Macherey-Nagel (Düren, G.F.R.) and TMSI (Iodotrimethylsilane) from Janssen Chimica (Geel, Belgium).
$MSTFA^+$ is prepared by dissolving 1‰ TMSI in MSTFA (8).
Stock solutions of CB and the internal standard (CB-D6) were prepared at 10 ng/ul in 0.01 M HCl.

Sample preparation

Based on the method described by Courtheyn et al. (9) a slightly modified extraction and clean-up procedure was worked out.
Eleven grams of faeces were weighed out in a nalgene tube. Twenty-two ml of 0.5 M hydrochloric acid, saturated with ethyl acetate, were added and 5 ppb internal standard (CB-D6) was spiked. The tubes with contents were then tumbled for 30 minutes and centrifuged afterwards during 20 minutes at 10000 rpm. The supernatant was set to at least pH = 12 with sodium hydroxide 33 %. After a second centrifugation during 5 minutes at 10000 rpm the supernatant was brought onto an extrelut (Varian, Chem Elut CE 1020) column and allowed to stand for 10 minutes. The sample was eluted with 2 x 25 ml tolueen/dichloromethane (80/20) into a 50 ml conical-bottomed glass sovirel tube. Two hundred and fifty ul of 0.1 M hydrochloric acid solution were added and shaken vigorously by hand for 1 minute. Prior to centrifugation the tube was placed in an ultrasonic bath for 5 minutes. A complete separation of both layers was achieved in 10 minutes at 2500 rpm.
The droplet in the tip of the tube was transferred to an autosampler vial, evaporated in a heated centrifuge in vacuo and derivatized for GC-MS purposes.

Derivatization and GC-MS conditions

Clenbuterol is derivatized with $MSTFA^+$: the sample or 5 ul standard solution (100 ng) is evaporated under a gentle N_2-stream (autosampler vial) , 25 ul of $MSTFA^+$ is added, the vial is heated at 60 °C during 30 min. One ul (4 ng) is injected splitless (autosampler).
GC-MS conditions : initial: 100 °C, to 200 °C at 33.3 °C/min., to 250 °C at 7 °C/min, 250 °C to 300 °C at 50 °C/min., 300 °C 6 min.(total run ca 17 min).Temperatures of injector: 260°C, transfer-line: 300°C. Column: SGE BPX-5-0.25 (25 m X 0.22 mm ID., film thickness 0.25 um).
Acquisition method : 1 scan/sec during 15 min (mass range 80-600 amu., filament-multiplier delay 200 sec), positive CI parameters : reagent gas : isobutane, max. ionisation time : 1500 usec, max. reaction time : 80 msec., ionisation level : 20 amu, reaction level : 40 amu.

Results

In the European Union, the use of beta-adrenergic products is increasing in comparison with the classically abused steroidal anabolic agents (10). In Belgium, the Royal Decree of july, 15, 1985 provides to the inspectors of the Institute of Veterinary Food Inspection the possibility to take samples at the farm and not only at the slaughterhouses. This procedure allows the Inspection Services to locate at an early stage suspect or positive animals.
Many different matrixes are described in literature. Liver is the most cited matrix whereas there is an accumulation of clenbuterol residues in that organ (5). Other tissues and body fluids, such as eyes (choroid/pigmented retinal epithelium), pigmented hair, bile and urine, were proposed as matrixes (1, 11, 12, 13, 14). From all these matrices only urine and pigmented hair were the only available at the farm. Both of them can cause some problems. Urine sampling in bulls is not always that easy. Therefore some screening tests were described on faeces as a matrix for clenbuterol and analogues analysis (15, 16).
Screening of clenbuterol can be carried out by an immunoassay. For confirmatory purposes a physico-chemical method is required. With the proper extraction method GC-MS is capable of detecting both the aniline-derivatives (i.e. clenbuterol) as the phenol-derivatives (i.e. salbutamol)(17). The possibility of generating a full scan spectrum is a very valuable tool in the identification of residues of abused drugs.
To fulfil the gas chromatographic-mass spectrometric confirmation criteria of the E.U.(18) it is necessary to measure 4 independent mass fragments in the mass spectrum. In full scan MS it is impossible to fulfil these criteria with one single ionisation mode. Some possibilities are: 1) the use of two different ionisation modes, 2) following 3 diagnostic ions in full scan, taking into account their respective intensities or 3) the use of GC-MS-MS. Identification with GC-MS is based on the presence of 3 diagnostic ions in the right ratio and at the correct retention time compared with the standard.
Under these ionisation conditions, the spectrum of clenbuterol shows the 3 expected diagnostic ions of the CI (chemical ionisation) spectrum (fig. 1a), as well as a fourth very abundant ion, i.e. 335. This ion is a diagnostic peak in the EI (electron impact) spectrum of clenbuterol (fig. 1b). Typical of these spectra is also the presence of isotope peaks of the chloride ion, i.e. Cl^{37}, producing a typical isotope cluster for every

fragment containing a Cl ion. The presence of this interfering EI ion can be used as a fourth identification ion to fulfil E.U. criteria. The ions 86 (14), 331 (100), 405 (41), 421 (79) are used as diagnostic ions (+their relative intensities). In Belgium, limit of decision in routine analysis is set at 2 ppb. This method can meet the decision limit in Belgium (Fig. 2).
To obtain an even higher sensitivity and specificity the buying of the GCQ, a bench-top GC-MS-MS, is planned in the near future. This should allow us to get more specific spectra of daughter ions, decreasing the interference of the background and thus increasing sensitivity. Results of this new technique will be compared with the conventional GC-MS data. Exact results will than be presented at the poster of this congress.

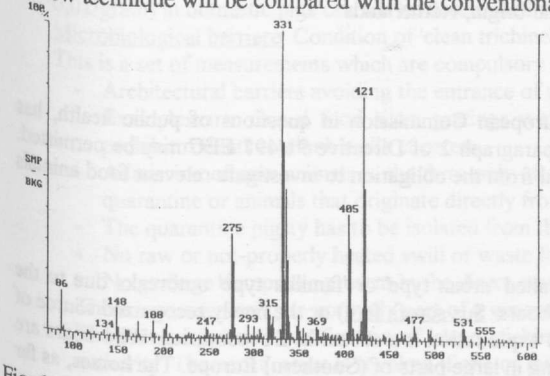

Fig. 1a: CI spectrum CB (4 ng)

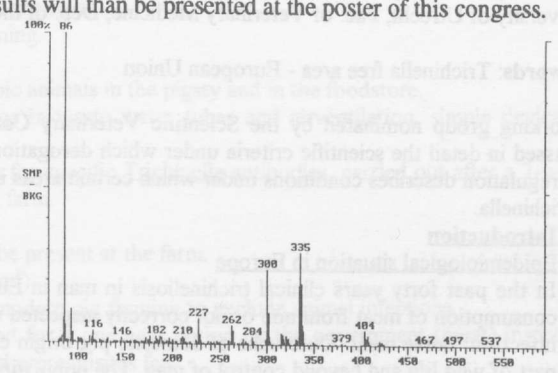

Fig. 1b: EI spectrum CB (4 ng)

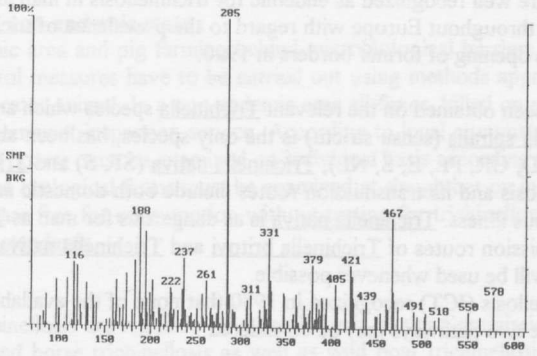

Fig. 2: CI spectrum spiked faeces sample (1 ppb)

Conclusions

Despite the many analogue beta-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. , developed for the aniline-group of beta-agonists, such as clenbuterol, is found suitable for GC-MS.
The use of GC-MS is preferred because of the specificity of the detection technique (specific full scan spectrum). The technique performed as described here, allows us to screen and confirm real faeces samples at the lower ppb level.(limit of decision: 2 ppb).
Finnigan-MAT brought out the GCQ at Pittcon conference 1995. This new apparatus is a modified version of the Magnum GC-MS and is able to perform tandem MS. Our laboratory has already bought such an apparatus. This should result in an even greater sensitivity and specificity for the detection of clenbuterol at sub-ppb level.
The results of a comparison between the 2 apparatus will be presented at the poster session of the congress.

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