IMPROVED GAS CHROMATOGRAPHY-MASS SPECTROMETRY METHOD FOR THE DETERMINATION OF CLENBUTEROL AND SALBUTAMOL IN ANIMAL URINE

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Abstract

An improved gas chromatography-mass spectrometry method, prepared for the confirmatory purposes, included solid phase extraction, derivatization, and analysis of clenbuterol and salbutamol derivatives. The whole procedure was validated in terms of linearity, decision limit detection capability precision, recovery, and uncertainty. The results of validation indicate that the prepared procedure is suitable for the confirmatory purposes. The decision limit for clenbuterol and salbutamol was established at the level of 0.022 ng/ml and 0.024 ng/ml, respectively, and the recoveries were about 70%.

Key words: clenbuterol, salbutamol, residues, urine, gas chromatography.

The β-agonists, such as clenbuterol and salbutamol, are substances used in veterinary and human medicine for the treatment of pulmonary disorders. When the dosage is 5 to 10 times higher than that used for therapeutic treatment of bronchial diseases, β-agonists act like, anabolic steroids which help developing muscle tissue at the expense of fat (1, 6, 7, 9). The high concentration residues of clenbuterol and some others β-agonist in animal liver or in meat are toxic to humans, leading to sickness and possible heart complication. In the European Union, the β-agonists are not authorized for the use as growth promoters, and bovine and swine urine samples are used for routine control of the β-agonists by instrumental techniques (1-3, 10-13). With regard to control of unapproved use of β-agonists, the identification and determination of analytes in biological matrices should be performed at levels as low as 0.1 ng/ml of samples. The working at so low levels is a very complicated task that requires the proper sample preparation by the use of the effective isolation and separation of analytes from biological matrices followed by sensitive and selective detection methodologies (15, 16).

Because of new EC analytical criteria (4) our previously described GS-MS procedure prepared for clenbuterol (14) should be modified and improved. For this reason, we used more selective NARC-2 SPE columns, additionally the new procedure was developed for salbutamol, another β-agonist compound. The whole procedure was validated according to quality of EC analytical criteria in residue analysis (4).

Material and Methods

Materials. Clenbuterol and salbutamol were obtained from Sigma Co, BSTFA (N,N-bis(trimethylsilyl trifluoroacetamide)) + 1% TMCS (trimethylchlorosilane) was from Fluka. Water was deionized (> 14MΩ x cm); hexane, methanol and acetic acid supplied by J.T.Baker. NARC-2 columns were from Merck. Potassium dihydrogenphosphate was purchased from Standart. Disodium hydrogenphosphate and sodium hydroxide were purchased from P.O.Ch. Gliwice.

Stock solution and standard. The individual stock solutions of clenbuterol and salbutamol at concentration of 1mg/ml were prepared in methanol and stored in the dark at < -16°C (stable for six months). The working solutions 100 ng/ml and 10 ng/ml were prepared in methanol and stored in the dark at < 6°C (stable no longer than three months).

Gas chromatography-mass spectrometry. Trace GC 2000 (Thermo Quest). Gas carrier: helium. Chromatographic separation was performed in capillary column DB-1MS, 30 m x 0.25 mm; 0.25 μm (J&W Scientific). The injection and transfer line were kept at 250°C and 280°C, respectively. The gas chromatography oven was programmed from 70 to 200°C at the rate of 18°C/min and subsequently to 250°C at 5°C/min and finally to 300°C at 20°C/min and stay for 8 min.

The gas chromatography device was coupled to Finnigan Polaris MS detector (Thermo Quest), operating in MS/MS mode at m/z: 262 (precursor ion) and ions at m/z: 188, 225, 227 as transition products of clenbuterol, and operating in single ion mode (SIM) with the selection ions at m/z: 369, 350, 294, 207, 86 for salbutamol.
**Extraction of urine.** A 10 ml portion of urine sample was diluted with 10 ml of phosphate buffer (pH = 7) and centrifuged at 4000 g for 10 min at room temperature. The whole solution was cleaned up by solid phase extraction (SPE) technique.

**Cleanup.** SPE NARC-2 cartridges were preconditioned with 2 ml of methanol, 2 ml water and finally with 2 ml of phosphate buffer. After percolation of the whole solutions, the columns were washed with 1 ml of acetic acid, 6 ml of methanol and dried under depression for 5 min. Samples were eluted with 5 ml of acetyl acetate with ammonia (4%).

**Derivatization.** The eluate was dried under gentle nitrogen stream at 45°C. The dry residue was dissolved in 300 µl of methanol and shaken vigorously with a vortex to retrieve the whole residue. Then the solution was transferred to a reagent vial and dried under gentle nitrogen stream. Twenty microlitres of BSTFA, containing 1% of TMCS, was added to the reagent vial and heated at 65°C for 1.5 h. The contents of the vial was analysed by GC-MS.

**Validation of β-agonists.** Blank sample of urine was spiked with clenbuterol and salbutamol, at level of 0.25, 0.5, 2.0 ng/ml, respectively. The spiked samples were processed through the whole procedure. Each concentration of β-agonists were analysed six times. The external standard method was used for quantitation. The recovery of β-agonists was evaluated by comparing the concentration found in the standard solution. The precision of the method was measured using the same samples.

**Results**

Typical chromatograms of extracts obtained from standard solution, blank urine, β-agonists-fortified urine samples and spectra are shown in Figs 1 and 2. As it was shown in Figs 1B and 2B retention time was 10.90 min and 11.68 min for salbutamol and clenbuterol, respectively. Chromatograms of salbutamol and clenbuterol urine sample spiked with at the level 0.25 ng/ml are shown in Figs 1B and 2B, respectively. The single peak seen in both chromatograms indicates that there were no side products and no multiple derivatives. Recoveries were determined by comparing the peak areas of the urine samples spiked with corresponding amounts of β-agonist before and after extraction. The whole procedure was validated according to the quality standard ISO/IEC 17025 and 2002/657/EC Commission Decision (4, 5). As it was shown in Table 1, the following parameters were established: linear regression equation, correlation coefficient, linearity (working range), decision limit CCα, detection capability CCβ, recovery, repeatability and uncertainty.

**Discussion**

This work presents a suitable method for the sample preparation and detection of two β-agonists (clenbuterol, salbutamol), representatives of the most important groups of these substances.

### Table 1

**Validation report**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Results</th>
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<tbody>
<tr>
<td></td>
<td>Clenbuterol</td>
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<tr>
<td>Linear regression equation (y = ax + b)</td>
<td>y = 2271.02x + 277,426</td>
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<tr>
<td>Correlation coefficient</td>
<td>0.9883</td>
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<tr>
<td>Linearity (working range), ng/ml</td>
<td>0.1-2.0</td>
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<tr>
<td>Decision limit CCα, ng/ml</td>
<td>0.022</td>
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<tr>
<td>Detection capability CCβ, ng/ml</td>
<td>0.037</td>
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<td>Level in spiked urine samples, ng/ml</td>
<td>0.25</td>
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<tr>
<td>Recovery, %</td>
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<td>Repeatability (r)</td>
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<tr>
<td>x, ng/ml</td>
<td>0.172</td>
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<tr>
<td>s, ng/ml</td>
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<tr>
<td>RSD, %</td>
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<tr>
<td>Uncertainty</td>
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<td>0.0093</td>
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<tr>
<td>expanded (U)</td>
<td>2</td>
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<tr>
<td>coverage factor (k)</td>
<td>0.25 ± 0.0186</td>
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</tbody>
</table>
Fig. 1. Chromatograms of salbutamol: A - blank sample, B - spiked sample at 0.25 ng/ml, C - standard at 1 ng/ml, D - MS spectra.
Fig. 2. Chromatograms of clenbuterol: A - blank sample, B - spiked sample at 0.25 ng/ml, C - standard at 1 ng/ml, D - MS/MS spectra.
The GC-MS method described in this study afforded a more sensitive and specific technique to measure clenbuterol and salbutamol in urine samples than the procedure previously described (14). The SPE procedure described here proved to be reliable in removing the majority of interfering co-extractive compounds and thus allowed the accurate determination across the working range of the method. The mixed mode working NARC2 columns are more selective than the previously used conventional octadecyl ones. They lead to “clean” solutions and devoid of the most of endogenous impurities. After the use of new SPE columns the matrix noise was so low that it was possible to obtain instrumental detection level below 0.1 ng/ml of samples.

The GC analysis of underivatized clenbuterol or salbutamol is unsuitable due to the presence of the hydroxyl and amino groups in analyte moieties. As it was found in our studies trimethylsilyl derivatives of clenbuterol or salbutamol were stable at GC conditions and the obtained ions were suitable for MS analysis. According to EU criteria for the analysis of veterinary drug residues (4), a system of identification points is adopted to define the numbers of ions and their corresponding ratios that should be measured when using confirmatory techniques. For group A (β-agonist), included in Annex I of Directive 96/23/CE (4), a minimum of 4 identification points are required for the confirmation. In GC-MS techniques, either EI or CI mode, each ion monitored gives 1 point. In GC-MS/MS techniques each parent ion gives 1 point, each daughter ion gives 1.5 point. In this study, for clenbuterol three ions were monitored in GC-MS/MS techniques, for salbutamol four ions were monitored in GC-MS techniques and four points were obtained in each case, that fulfilled EU criteria.

The presented GC-MS method considers the confirmatory purposes because of obtaining of the following criteria: all the selected ions - m/z 262 (parent ion) 188, 225 (daughter ion) for clenbuterol and 369, 350, 294, 207 for salbutamol - are present on the mass spectrum of the chromatographic peak; the relative abundance of these ions correspond to that of the standard, with the acceptable deviations; the ion intensity is more that three times greater than the base noise of the MS detector. As it was found, these criteria allowed to achieve the four points for the identification of banned compounds.

The results of the validation indicate that the analytes were isolated with good recoveries and repeatability. The decision limit (CCα) and detection capability (CCβ) for clenbuterol and salbutamol were below 0.05 ng/ml. For this reason, the procedure is suitable to the confirmatory purposes for the determination of clenbuterol and salbutamol residues in urine samples after the illegal use.

In conclusion, good validation results were obtained taking into account the difficulties of a multi-residue analytical method for the determination of the compounds from the different β-agonists groups. For this reason, the method reported is an interesting tool in the routine laboratories with a lot of samples to be analysed in a short time.

References