An Improved Method for Clenbuterol Screening using High Resolution Selected Ion Recording.

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Introduction

Clenbuterol, *i.e.* 4-amino-α[(tertbutylamino)methyl]-3,5-dichlorobenzyl alcohol, a β₂-agonist and a stimulant of the nervous system, is used as an anti-asthmatic and tocolytic drug in the treatment of both humans and animals.

Clenbuterol and other β-agonists exhibit growth stimulatory effects also, which lead to enhanced muscle and decreased fat deposition.

A consequence of this is the use of clenbuterol as a growth stimulant in farm animals, and by athletes seeking enhanced performance from the protein anabolic response to the drug. It is this abuse of clenbuterol in sport which is attracting the attention of various sporting authorities, and which requires investigation and monitoring with precision.

The dosage for this malpractice is typically in the µg/kg range of human body weight, and hence a sensitive but specific method is required to check for the presence of clenbuterol in body fluids, most commonly urine.

The analysis of clenbuterol in biological fluids using chemical derivatisation techniques followed by low resolution GC-MS is documented in the literature. This application note describes methodology which extends the use of GC-MS by improving the specificity of the technique using the high resolution capabilities of a magnetic sector mass spectrometer. The procedure developed is sufficiently specific to be used for the analysis of heavily contaminated urine samples which have undergone enzymatic hydrolysis.
Experimental

The standard samples of clenbuterol were analysed at the appropriate concentrations after derivatisation using N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) to produce bistrimethylsilylclenbuterol (figure 1). MSTFA is a volatile silylating agent which forms the trimethylsilyl derivatives of hydroxy and primary amine functional groups.

The first urine sample was prepared according to the procedure outlined in figure 2, whereby a measured aliquot of centrifuged urine was spiked with a known concentration of clenbuterol, extracted, evaporated to dryness and derivatised using MSTFA.

The second urine sample was prepared according to the method illustrated in figure 3 i.e. the urine was spiked with a known amount of clenbuterol, hydrolysed enzymatically with β-glucuronidase to regenerate free hydroxyl groups from any glucuronide metabolites, extracted, evaporated to dryness and derivatised with MSTFA.

The samples were injected splitless (1µL injection, injector temperature 250°C) onto a DB1 gas chromatography column of 15m length and 0.25mm internal diameter (J&W Scientific, USA) which was held at an initial temperature of 150°C. After 1 minute the oven temperature was ramped at a rate of 15°C per minute to a final temperature of 300°C, which was maintained for 10 minutes.

The gas chromatograph (CE Series GC) was interfaced directly to the mass spectrometer, a Micromass AutoSpec Ultima (Micromass UK Ltd., Manchester, UK), comprising a double focusing analyser of EBE geometry operated in conjunction with a VaxStation 3100 (DEC, USA) data system. The mass spectrometer was equipped with a dedicated electron impact source operating in positive ionisation mode at a temperature of 240°C, an electron energy of 35eV and a trap current of 800µA.

For full scanning experiments, the m/z range 500-50 was scanned exponentially at 0.5 seconds per mass decade. A resolution of 5000 (10% valley definition) was used to verify the exact mass measurements of bistrimethylsilylclenbuterol. For selected ion recording (SIR) experiments, the ions m/z 335.0695 and 337.0666 (see table 1) were monitored with a 0.54 second cycle using various resolutions, as stated in the text, depending on the constitution of the matrix.
Discussion

Establishing a Method for the Detection of Clenbuterol.

The derivatised standard sample, bistrimethylsilylc clenbuterol was analysed in accordance with the specified gas chromatographic conditions at a concentration of 1ng/µL with the mass spectrometer operating in full scan mode at a resolution of 5000 (10% valley definition).

The chromatogram showed a component of retention time 6 minutes 10 seconds, the spectrum of which (figure 4) shows a significant ion at m/z 335 corresponding predominantly to loss of C_5 H_11 N from the molecular ion. The accurate mass measurements of this ion and its Cl^{37} isotope (table 1), and study of the observed fragment ions together indicate a composition consistent with the proposed structure and fragment ions of bistrimethylsilylc clenbuterol (figure 5).

A distinctive spectrum with diagnostic ions has been produced, and the elemental composition of the major ions has been verified by medium resolution accurate mass measurements.

In order to develop the method further into a procedure for low level monitoring of clenbuterol and other related drugs in urine or alternative biological matrices, an analysis is required which selectively monitors the important, diagnostic ions rather than scanning the whole mass range.

This type of analysis (Selective Ion Recording or SIR), when used in conjunction with the GC retention time of the compounds under scrutiny, provides a highly specific method of detecting unambiguously such drugs of abuse.

2.5mL of centrifuged urine
add solution of clenbuterol at known concentrate

treat with 20 µL β–glucuronidase from E.coli enzymatic hydrolysis, 1hr/50°C

add 100mg NaHCO_3/K_2 CO_3 1:1 (w/w)

add 5mL diethylether

add 1g Na_2 SO_4

evaporate organic phase
desicate (P_2 O_5)
derivatise: 40µL MSTFA/NH_4 1, 15min/ 60°C

GC/MS

Figure 3: Sample Preparation B.

Figure 4. Electron Impact spectrum of bistrimethylsilylclenbuterol.

<table>
<thead>
<tr>
<th>Measured Mass</th>
<th>ppm</th>
<th>Calculated mass</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>337.066147</td>
<td>1.3</td>
<td>337.066577</td>
<td>C 13</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>H 23</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>N 1</td>
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<td></td>
<td>O 1</td>
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<td></td>
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<td>Si 2</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>Cl 1</td>
</tr>
<tr>
<td>335.070267</td>
<td>-2.2</td>
<td>335.069527</td>
<td>C 13</td>
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<td>H 23</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Cl 2</td>
</tr>
</tbody>
</table>

Table 1: Accurate mass measurements of the major bistrimethylsilylclenbuterol fragment ions.
To obtain an estimate of the sensitivity of this method for the detection of bistrimethylsilylclenbuterol, the standard was analysed by Selective Ion Recording of the two isotopic ions m/z 335.0695 and 337.0666, which differ by the presence of a Cl\(^{37}\) rather than a Cl\(^{35}\) atom in the latter. A resolution of 10,000 (10% valley) was chosen to avoid co-monitoring interfering ions from any matrices which may be present in future analyses.

The theoretical intensity ratio of these ions i.e. m/z 337.0666:335.0695, calculated from their formulae (table 1 and figure 5), is 0.74 assuming no interference from other ions. Although other ions do exist in the vicinity, the accuracy of this ratio is little altered by their presence and can be used as a further check in the identification of this sample.

The unsmoothed ion chromatograms from m/z 335.0695 and 337.0666 (figure 6) demonstrate a signal:noise ratio of at least 40:1 from the chromatographic peak of retention time 6:08 minutes. These signals were produced from a 1µL injection of 1pg of derivatised clenbuterol (i.e. the equivalent of 1ppb), and clearly indicate that lower levels still could be detected.

The reproducibility which may be expected from this type of analysis, in terms of the precision of the GC retention times and of the benefit in comparing the isotope ratios of the two pertinent ions, is excellent, even at very low concentration levels. Information from three analyses detecting 1pg, 10pg and 100pg of bistrimethylsilylclenbuterol is presented in table 2.
Detection of Clenbuterol in Urine

Having established suitably sensitive and reproducible conditions for monitoring clenbuterol, the method was extended to its detection in urine.

Using the same conditions specified above, the ion chromatograms produced from SIR monitoring of a urine sample spiked with clenbuterol to produce a final concentration of 10pg/μL (10ppb) of the drug in the matrix (see Sample Preparation A, figure 2), indicated the presence of numerous components which exhibited ions at either \(m/z\) 335 or 337. Figure 7 shows the SIR chromatograms obtained with the mass spectrometric resolution set at 10,000 (10% valley). Although both give evidence for the presence of the expected compound with a retention time of 6:12 minutes, in both cases it is one of the minor components, and detection at any level less than 10pg would not be reliable.

Similar results were obtained with the resolution of the mass spectrometer set to 20,000 and 30,000; only when the resolution was raised to 40,000 (10% valley) were the interfering chromatographic peaks excluded sufficiently to permit accurate calculations of the height and area of the peak of interest (retention time 6:12 minutes) to be made, figure 8. the signal:noise ratio from the ions monitored was calculated to be at least 30:1 for both \(m/z\) 335.0695 and 337.0666, using unsmoothed data.

The signal from a sample of extracted urine without the addition of clenbuterol was monitored also to verify the origin of the interfering chromatographic peaks.

<table>
<thead>
<tr>
<th>Quantity (pg)</th>
<th>Retention time (minutes:seconds)</th>
<th>Ratio 337:335</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6:08</td>
<td>0.73</td>
</tr>
<tr>
<td>10</td>
<td>6:07</td>
<td>0.72</td>
</tr>
<tr>
<td>100</td>
<td>6:08</td>
<td>0.73</td>
</tr>
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</table>

Table 2: Data comparison from the analyses of 1pg, 10pg and 100pg of bistrimethylsilylclenbuterol.

![Figure 7: SIR chromatograms at 10,000 resolution produced from 10pg of clenbuterol spiked in urine.](image)

![Figure 8: SIR chromatograms at 40,000 resolution produced from 10pg of clenbuterol spiked in urine.](image)
Detection of Clenbuterol in Enzymatically Hydrolysed Urine

A urine sample prepared according to the method Sample Preparation B, detailed in figure 3, was analysed also. This sample preparation involves an extra step of treating clenbuterol-spiked urine with the enzyme β-glucuronidase. This enzyme hydrolyses glucuronide derivatives produced by drug metabolism, with the effect of regenerating any hydroxy functionalities. Although clenbuterol itself is not habitually metabolised to form glucuronides, other drugs, including steroidal ones which may be under simultaneous investigation, do so regularly. The purpose of this extra step is to establish the effect, if any, of the enzymatic hydrolysis procedure and any of its resulting side-products and impurities, on the sensitivity and veritability of this method.

The urine sample prepared by the method incorporating this extra step was analysed by SIR at 10,000 resolution monitoring the same two significant ions and found to be far more complex than the previous urine sample. Once more, in order to obtain ion chromatograms in which the signals from bistrimethylsilylclenbuterol could be integrated accurately, a resolution of 40,000 had to be used.

Figure 9 shows a comparison of the SIR chromatographic traces from a urine sample spiked to produce a concentration of 125pg/µL of clenbuterol in which the ion m/z 335.0695 has been monitored with resolutions of 10,000 and 40,000. With 10,000 resolution the ion chromatogram is complex and the peak of interest flanked by a more dominant peak of retention time 6:05 minutes. Although this interfering peak does not exhibit a corresponding ion at m/z 337.0666 and hence cannot be misidentified as the clenbuterol derivative, its presence would present difficulties when calculating the peak area of the component of interest, particularly at lower drug concentrations.

With 40,000 resolution the bistrimethylsilylclenbuterol peak (retention time 6:17) is clearly the most significant peak, and lower levels of the drug could be detected confidently under these conditions.
Summary

The increasing use of clenbuterol and similar drugs to enhance the performance of athletes is a cause of concern to sporting authorities. A method has been developed using reliable, chronicled GC derivatisation techniques developed for this type of drug interfaced with a high resolution mass spectrometer. The method has proved successful for the detection of low levels (less than 1ppm) of clenbuterol in complex biological matrices. Selected Ion Recording of two characteristic isotopic fragment ions provides a specific mode of detection by verifying the GC retention time of these ions and also by comparing their relative abundance. Both of these parameters have been shown to be accurate and consistent. Analysis of urine samples demands higher mass spectrometric resolution and 40,000 (10% valley) was found to be a prerequisite for accurate integration of the drug-related chromatographic peaks. The method developed is eminently suitable for adaptation to a completely unattended automated routine incorporating sample injection, storage and retrieval of source tuning parameters, and data processing.

References
