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Determination of clenbuterol in bovine urine using gas chromatography–mass spectrometry following clean-up on an ion-exchange resin

Lee X. Whaites, E. John Murby*

Australian Government Analytical Laboratories, P.O. Box 385, Pymble, NSW 2073, Australia

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Abstract

A gas chromatographic–mass spectrometric method was developed for the determination of residues of clenbuterol in bovine urine. The method involves a simple cation-exchange clean-up and concentration of clenbuterol in the acidified urine, followed by ethyl acetate extraction. The analyte is determined as the di-trimethylsilyl derivative and quantitated against an internal standard of penbutolol. Using a 5-ml sample of urine, a detection limit of 0.07 ng/ml can be achieved with recoveries close to 100% for fortification levels of 0.2 and 1 ng/ml. By increasing the sample volume to 50 ml, a detection limit below 0.01 ng/ml was achievable with recovery averaging 70%. The coefficient of variation of the assay ranged from 15% at 0.01 ng/ml (50-ml sample) to 6% at 1 ng/ml (5-ml sample). It was demonstrated that the method can detect the presence of clenbuterol in bovine urine at sub-ppb (ng/ml) levels using low resolution GC–MS with electron impact (EI) ionization.

Keywords: Urine; Cation-exchange; Clenbuterol

1. Introduction

Clenbuterol is a synthetic β_2-adrenoreceptor agonist compound. It has legitimate uses as a bronchodilator or tocolytic agent in veterinary medicine. When administered to farm animals at multiples of the therapeutic dose, a shift in the flow of nutrients away from adipose tissue towards muscle tissue occurs, in a process known as repartitioning. The net result is the production of a leaner carcass. Reports of toxic effects related to this usage has led to clenbuterol being banned as a growth-promotant in many countries, particularly in Europe [1].

For reasons of public health and safety, many countries have established their own control levels of clenbuterol residues [2]. The limit of reporting required for methods used in the Australian National Residue Survey is 1 ppb in urine [3]. Apart from its use as a growth promotant in food producing animals, clenbuterol is also illegally used as a doping agent in racehorses and human athletes. It can be used by athletes to improve their performance and as a result, the use of clenbuterol has been banned by the International Olympic Committee.

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*Corresponding author. Fax: +61-2-9449-1254.

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promotant, urine is frequently chosen as the test matrix. A number of methods for testing urine have been previously reported. These may depend on enzyme immunoassay as a first screening step [4–7] with confirmation by gas chromatography–mass spectrometry (GC–MS).

Such confirmation methods have involved a variety of different extraction, clean-up, derivatisation and ionisation techniques. Recently a number of methods using liquid–liquid partition or SPE cartridge extraction for clenbuterol confirmation have been reported [2,8]. Detection limits of 0.05–0.1 ppb, and 1 pg of minimum detectable mass were reported. However, the methods need sensitive and specialized equipment such as GC–MS–MS or GC–high-resolution MS to achieve such low detection levels.

The most common GC–MS equipment in analytical laboratories, which comprises a capillary gas chromatograph coupled to a low resolution mass spectrometer, is capable of high selectivity and sensitivity. If a method can achieve low detection levels with a commonly used GC–MS, it will be more acceptable for routine use. A variety of derivatisation reagents have been employed in the GC analysis of clenbuterol [1]. Of these, reagents producing TMS derivatives have given the fewest problems in our hands with respect to stability and GC column degradation. Unfortunately, the silylating reagent which has been most commonly reported in clenbuterol analysis, BSTFA, produces a mono-TMS derivative which gives a single major fragment ion at m/z 86 in a region of the mass spectrum which is subject to matrix interference. Confirmation of the identity of this derivative using a protocol such as that used by the EU [9] is difficult due to the low intensity of other fragment ions. The present work involved use of a more active silylating reagent [10,11] to produce a di-TMS derivative of clenbuterol which has a fragmentation pattern more suitable for confirmation, coupled with an efficient clean-up procedure to reduce matrix interferences and improve method sensitivity.

The object of the present study was to develop an economical, simple and highly efficient extraction method, employing an ion-exchange resin clean-up, coupled with a modified TMS derivatization reagent to allow the determination of clenbuterol in urine in the sub-ppb range using a commonly available bench top GC–MS.

2. Experimental

2.1. Reagents and supplies

AR-grade hydrochloric acid was purchased from Ajax Chemicals (Sydney, Australia). AR-grade potassium hydrogen phthalate and sodium hydroxide were obtained from BDH Chemicals (Poole, UK). AR-grade ethyl acetate and HPLC-grade methanol were supplied by Mallinckrodt Australia (Clayton, Australia). *N*-Methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) was supplied by Macherey-nagel (Düren, Germany). Dithioerythritol (dithio) and ammonium iodide were supplied by Sigma (Sydney, Australia) and analytical-grade 200–400 mesh AG-MP 50 resin was purchased from Bio-Rad Labs (Sydney, Australia). Clenbuterol hydrochloride and penbutolol were provided by the Curator of Standards, National Analytical Reference Laboratory, AGAL, Pymble, Australia. The three batches of bovine urine used in this work were supplied by DPS Abattoirs (Sydney, Australia) between 1994 and 1998.

2.2. Extraction and derivatization

Internal standard solution (20 μl of 50 ng/ml penbutolol in methanol) was added to 5 ml of sample or clenbuterol-spiked blank urine, followed by addition of 5 ml of pH 2.2 potassium hydrogen phthalate buffer solution. The urine solution was then transferred to a 3-ml polypropylene SPE cartridge which contained a 0.5-cm deep bed of AG-MP 50 resin. After allowing the sample to pass through to waste under gravity, the resin was washed with 1 ml of water and 1 ml of 2 N sodium hydroxide solution. Finally the analyte was eluted into a capped test tube with 2 ml of 10% methanol in ethyl acetate. One ml of water and 7 ml of ethyl acetate were added to the tube and it was shaken for 30 min. The upper layer

was transferred into a Quickfit test tube and evaporated to dryness under nitrogen at 40°C. Silylation was performed by adding 50 μl of MSTFA/dithio reagent (0.4% dithioerythritol, 0.2% ammonium iodide in MSTFA) [10,11] to the test tube sealing it with a glass stopper, and heating for 15 min at 60°C. The solution was transferred to a low-volume autosampler vial for GC–MS analysis.

2.3. Instrumentation

GC–MS measurements were performed on a Hewlett-Packard 5890 gas chromatograph equipped with a HP 5971 mass selective detector and a SGE (Melbourne, Australia) BP5 capillary column (cross-linked 5% Ph–Me silicone) with dimensions of 12.5 m×0.22 mm I.D. and film thickness of 0.33 μm. The carrier gas was helium at a flow rate of 1 ml/min and the injection volume was 3 μl. The sample was introduced into the gas chromatograph in split mode with a split ratio of 10:1. The injector and detector were maintained at 280°C. The column initial temperature was 150°C and was increased by 10°C/min to 220°C, then increased to 300°C at 40°C/min. The mass selective detector was operated in the selected-ion mode (SIM), monitoring the fragment ions at m/z 86 (clenbuterol and penbutolol); and m/z 335 (clenbuterol). The ions at m/z 300, 336, 337 and 405 were also monitored to allow for clenbuterol confirmation. The dwell time for each ion was 50 ms.

3. Results and discussion

3.1. Derivatisation of clenbuterol

The mono-TMS derivative of clenbuterol prepared using BSTFA has been reported to give a minimum detectable mass of 0.2 ng of the analyte [12] on a similar benchtop mass spectrometer to that used here. Unfortunately in electron impact MS this derivative gives a single major fragment ion which, at m/z 86, is subject to matrix interference. Other ions of higher m/z are at least 10 times less intense than the base peak for this derivative [13], making confirmation by EI alone impractical at the required detection levels. MSTFA produces a di-TMS derivative of clenbuterol which has a variety of ions above m/z 300 with intensity >20% of the base peak at m/z 86 (Fig. 1), which would allow effective confirmation by protocols requiring measurement of the ratios of four characteristic ions [9]. Catalysis of the MSTFA derivatisation reaction using dithioerythritol and ammonium iodide [10,11] produced a significant increase in yield relative to MSTFA alone and allowed full confirmation of clenbuterol by EI at levels where the mono-TMS derivative produced by BSTFA is barely detectable. Using the described GC conditions, the clenbuterol di-TMS derivative could be detected at an injected concentration of 0.02 μg/ml (0.006 μg detected). Given that the extraction procedure described has a concentration factor of 100 for a 5-ml sample concentrated to 50 μl, this corresponds to 0.2 ng/ml in the urine sample (Fig. 2).

3.2. Extraction and clean-up

The extraction procedure described concentrates clenbuterol from urine onto an ion-exchange resin. Possible matrix interferences are eliminated using both the cation binding properties of the functional groups on the resin and the hydrophobic retention characteristics of the polymer resin backbone. In the initial stage of the clean-up the resin's sulfonic acid groups retain protonated clenbuterol from the acidified urine along with other positively charged compounds. The sodium hydroxide wash then releases the clenbuterol in its uncharged basic form, which is retained by hydrophobic interaction with the styrene–divinylbenzene polymer of the resin. Other more water-soluble protonated compounds, including zwitterionic β-agonists like salbutamol, are discarded in this wash. Clenbuterol is finally released from the resin in a small volume of ethyl acetate containing 10% methanol. Any salts carried through into the final eluent from the hydroxide wash are eliminated by partitioning between ethyl acetate and water.

The clean-up afforded by consecutive application of the ionic and hydrophobic interaction properties of the cation-exchange resin produced chromatograms with very little chemical background interference. This allowed the detection limit of the method to

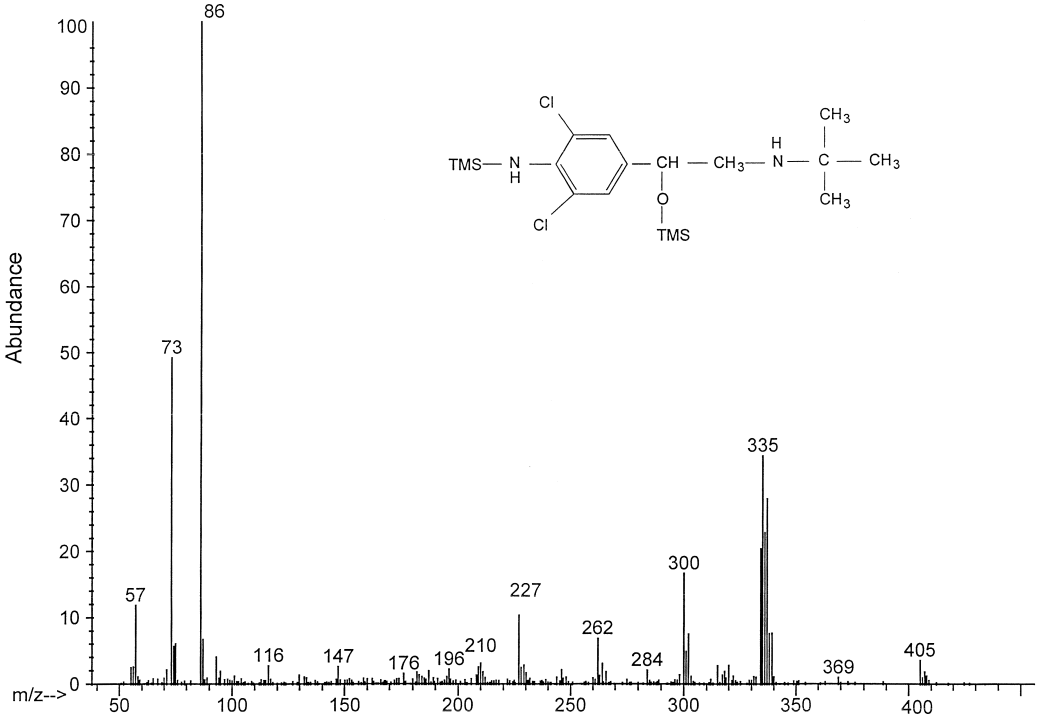

Fig. 1. Mass spectrum of clenbuterol di-TMS derivative.

approach that achievable by the GC–MS instrument itself, as can be seen in Fig. 2. In fact the cleanliness of the extracts suggested the possibility of achieving significantly lower detection limits if required, by use of a larger sample volume. The potential for this was demonstrated by analysing samples of bovine

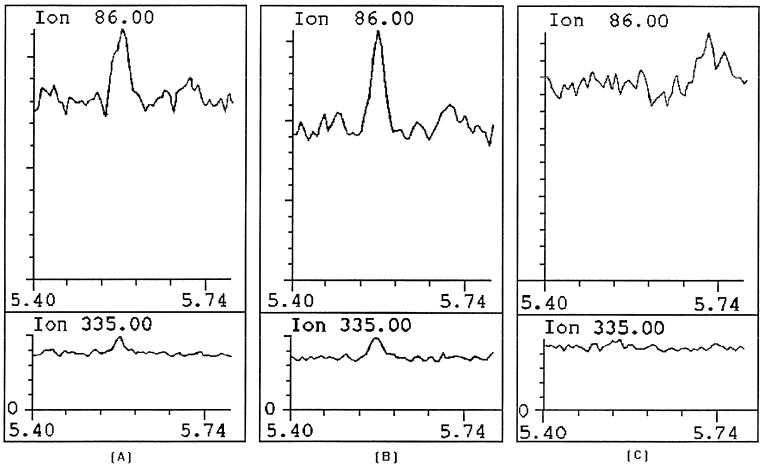

Fig. 2. Ion chromatograms after clean-up procedure: (A) 0.02 ng/μl clenbuterol standard; (B) 5-ml bovine urine spike at 0.2 ppb (A and B are equivalent); (C) 5-ml bovine urine blank.

urine spiked with clenbuterol at 0.01 ppb with no modification to the procedure apart from increasing the sample volume used to 50 ml. As the final volume of the extract is 50 ml, a sample concentration factor of 1000 is obtained. The extract gave a relatively clean chromatogram (Fig. 3), still with very little matrix interference at m/z 335.

The clean-up method could be expected to be applicable for other non-phenolic β-agonists such as cimaterol, mabuterol or similar compounds with a weak basic group but no acidic group.

3.3. Linearity

To evaluate the linearity of the method, blank urine was spiked in duplicate at four concentrations in the range 0.2–2 ng/ml. After analysis by this method the ratios of the peaks heights of the fragment ions at m/z 86 for clenbuterol and penbutolol (internal standard) were plotted against the clenbuterol concentration. Good linearity was observed in this range with a calibration line of slope 0.5696 ml/ng (standard error, 0.0077) and intercept

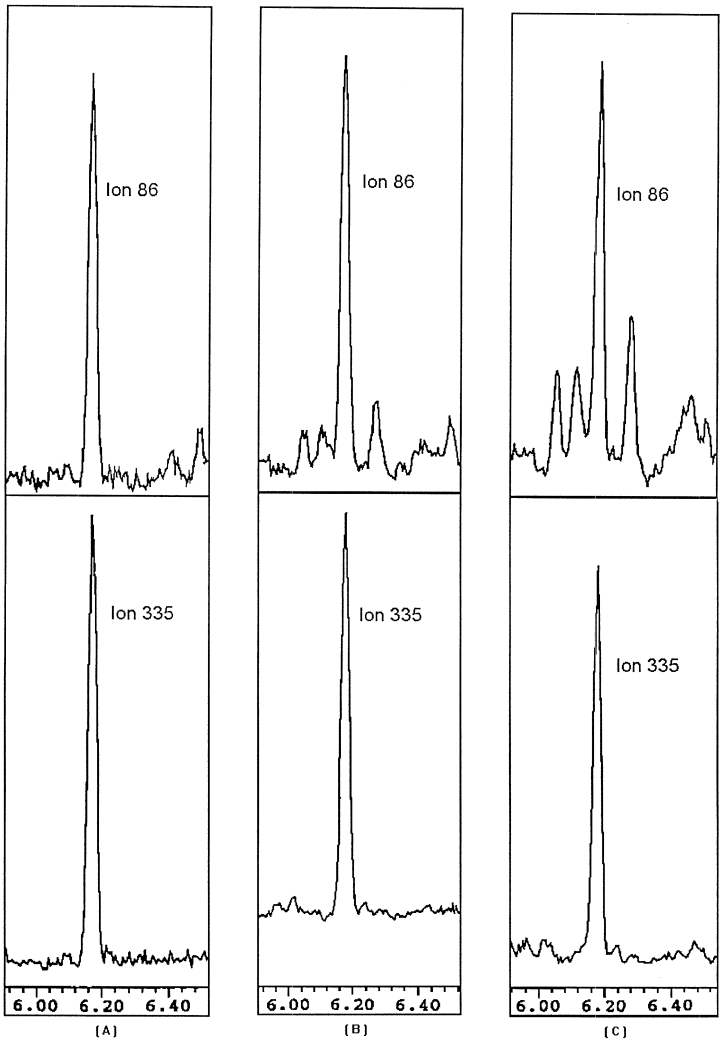

Fig. 3. Ion chromatograms after clean-up procedure: (A) 0.1-ng/μl clenbuterol standard; (B) 5-ml bovine urine spike at 1 ppb; (C) 50-ml bovine urine spike at 0.1 ppb (A, B and C are equivalent).

Table 1
Precision and recovery of the described assay procedure for analysis of bovine urine spiked with clenbuterol

Concentration (ng/ml)	Volume (ml)	*n*	Mean (ng/ml)	SD (ng/ml)	C.V. (%)	Recovery (%)
1	5	9	1.03	0.064	6.2	103
0.2	5	8	0.21	0.023	11	106
0.01	50	8	0.0070	0.0011	15	70

0.01069 (standard error, 0.00671) having a correlation coefficient (r^2) of 0.9992 to the data.

3.4. Recovery, precision and limit of detection

The precision and recovery of the method were determined by analysing 5-ml samples of bovine urine spiked with clenbuterol at concentrations of 0.2 and 1.0 ng/ml. The results are shown in Table 1. Recovery was calculated using ratios of the peak height of the analyte to that of the internal standard. Complete recovery of the analytes through the extraction procedure was achieved at both concentrations with very good coefficients of variation. Recoveries ranged from 97 to 114% at 1 ng/ml and from 93 to 125% at 0.2 ng/ml. The limit of detection for the method was calculated as three times the standard deviation of analytical results from eight replicate 0.2-ng/ml spikes (a level close to the detection limit) [14]. The calculated limit of detection is 0.07 ng/ml clenbuterol in a 5-ml urine sample.

When eight 50-ml urine samples were spiked at 0.01 ng/ml a recovery of 70% with a coefficient of variation of 15% was achieved (Table 1). The data indicates that a detection limit of 0.003 ng/ml is possible using this technique on a 50-ml urine sample.

4. Conclusions

The determination of clenbuterol in bovine urine using GC–MS followed by clean-up on cation ion-exchange resin is a rapid, sensitive, specific and inexpensive assay. This method is capable of detecting clenbuterol in 5-ml samples of bovine urine at the 0.1 ng/ml level using a widely available low resolution benchtop mass selective detector. The detection limit can readily be improved by a factor of 10 by increasing the volume of sample used for analysis.

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