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Determination of clenbuterol and salbutamol in urine by capillary gas chromatography with capillary columns of 100 μm

J.A. García Regueiro*, B. Pérez and G. Casademont

IRTA, Unitat Tecnologia Analítica, Centre de Tecnologia de la Carn, Granja Camps i Armet, 17121 Monells, Girona (Spain)

ABSTRACT

A method to determine clenbuterol and salbutamol in calf urine is described. Two independent extraction procedures using Extrelut (clenbuterol) and octadecylsilica (salbutamol) were used; the extracts obtained were mixed and purified over a cyanopropyl minicolumn. Trimethylsilyl derivatives were prepared and analysed by GC–MS in selected-ion monitoring mode using a fused-silica open tubular capillary column, 10 m × 100 μm coated with 5% phenylmethylsilicone. Splitless injection was optimized to achieve low percentage residual standard deviation of absolute areas. The best conditions were: injection volume 0.5 μl, column head pressure 22 p.s.i. (1 p.s.i. = 6894.76 Pa), inlet temperature 250°C and glass liner volume 250 μl. The recoveries of the complete procedure were in the range 50–60% for both compounds.

INTRODUCTION

β-Agonists can be used as growth promotors in animal production because they increase protein deposition and lipid degradation [1–3]. However β-agonist residues can affect the health of meat consumers, and their use in animal production is forbidden. Clenbuterol application in animal production has been intensively studied [3,4]. This compound is one of the most used illegal products and has been associated with toxic episodes in meat eaters [5].

Different analytical methods have been used to determine clenbuterol: high-performance thin-layer chromatography (HPTLC) [6,7], high-performance liquid chromatography (HPLC) [8–10] and gas chromatography–mass spectrometry (GC–MS) [11–14]. As other β-agonists can be used (*e.g.* salbutamol), methods for the simultaneous determination of β-agonists have been developed: clenbuterol and cimaterol are determined by HPTLC and HPLC [7], while a more general procedure described by Van Ginkel *et al.* [14] allows the analysis of four β-agonists by GC–MS using an immunoaffinity column to purify the extract. This procedure offers a high level of selectivity, but the purification step is slow. Capillary gas chromatography (cGC) coupled to mass spectrometry offers the most selective methodology, while HPTLC also allows a selective analysis but with lower sensitivity. Enzyme-linked immunosorbent assay is an alternative method of screening a large number of samples, but its lack of selectivity means that it is necessary to confirm positive results by other techniques (*e.g.* cGC–MS). The injection mode has a strong influence on the recovery of clenbuterol and salbutamol when cGC–MS is used, the best choice being on-column, with low residual standard deviation (R.S.D.) and highest recoveries [15]; splitless injection needs to be optimized and can be used taking care that inlet temperature is maintained below 250°C. The use

* Corresponding author.

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of capillary columns of 100 μm diameter in cGC–MS can improve the vacuum intensity and reduce the column bleed when a direct interface is used; but the injection in splitless mode becomes critical because it is difficult to transfer the solutes from the injector to a narrow column. In this work a method to determine clenbuterol and salbutamol by cGC–MS with columns of 100 μm diameter is presented, the injection conditions being optimized to allow the use of such capillary columns.

MATERIAL AND METHODS

Chemicals

Clenbuterol and salbutamol were from Promochem (Germany). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was from Supelco (USA). Triethylamine was from Fluka (Switzerland). β-Glucuronidase arylsulphatase (*Helix promatia*) and isooctane, acetonitrile and methanol of analytical grade were from Merck (Darmstadt, Germany). The fused-silica open tubular capillary column (10 m × 100 μm, 0.17 μm film thickness) was from Hewlett-Packard (USA).

Analytical procedure

Salbutamol solid phase extraction. The method was based on that described by Fürst *et al.* [12], but using an octadecylsilica column. A 20-ml volume of calf urine sample was enzymatically hydrolysed at pH 5.0 with 25 μl of β-glucuronidase arylsulphatase; the mixture was incubated overnight at 37°C. After the hydrolysis the pH was brought to 7.6 with 0.1 *M* sodium hydroxide and acetate buffer; the solution obtained was applied to an octadecylsilica minicolumn (500 mg, Waters–Millipore) previously activated with 6 ml of acetate buffer at pH 7.6. The column was rinsed with 4 ml of water and 2 ml of methanol–water (2:8); afterwards, salbutamol was recovered with 6 ml of methanol, this fraction was evaporated to dryness and redissolved in 0.5 ml of methanol.

Clenbuterol solid phase extraction. A 20-ml volume of calf urine sample was brought to pH 12 with 1 *M* sodium hydroxide and hydrolysed for 1 h at 45°C. The solution obtained was applied to an Extrelut column (Merck) and allowed to soak for 15 min; clenbuterol was recovered with 40 ml of dichloromethane, which was evaporated to dryness in a rotatory evaporator and redissolved in 0.5 ml of methanol.

Extract purification. This procedure was similar to that described by Brambilla *et al.* [9]. Salbutamol and clenbuterol extracts were mixed and applied to a cyanopropyl minicolumn (500 mg, Waters-Millipore) previously activated with 10 ml of methanol. The column was rinsed with 1 ml of methanol, and salbutamol and clenbuterol were eluted with 2 ml of 1% triethylamine in methanol. This solution was evaporated to dryness in a stream of nitrogen. The residue was redissolved with 200 μl of acetonitrile, and 50 μl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) were added. The mixture was heated at 80°C for 1 h to obtain trimethylsilyl (TMS) derivatives; after this step the solution was evaporated to dryness in a stream of nitrogen and redissolved in 100 μl of isooctane. Blank samples of urine were spiked with clenbuterol and salbutamol to obtain 10, 5 and 2.0 ng/ml concentrations to study the recovery of the complete procedure.

Chromatographic analysis

The injection mode was splitless: the inlet temperature was 250°C, and the split valve was closed for 1 min using a deactivated glass liner of 250 μl volume. Carrier gas helium at a head column pressure of 22 p.s.i. (1 p.s.i. = 6894.76 Pa) was used. Column temperature was held at 80°C for 1 min, increased by 15°C/min to 150°C, then increased to 250°C at 5°C/min.

Samples and standards were analysed by cGC–MS using an HP 5890 gas chromatograph coupled to an HP 5970 mass-selective detector (Hewlett-Packard) by a direct interface at 280°C, under the following conditions: spectra were obtained in the electron-impact mode (70 eV), selected ions monitored were: *m/z* 86, dwell time 50 ms (salbutamol and clenbuterol); *m/z* 262, dwell time 100 ms (clenbuterol); and *m/z* 369, dwell time 50 ms (salbutamol). Methane positive chemical ionization was used to confirm clenbuterol identification by monitoring ions at *m/z* 349 and 333 and salbutamol at *m/z* 440 and

366, the dwell times being 100 ms; the instrument used was an HP 5971 A mass selective detector (Hewlett-Packard).

Standard solutions of clenbuterol and salbutamol (2.0, 1.6, 1.0 and 0.8 ng/μl) were prepared to evaluate the linear range and repeatability. A standard solution of clenbuterol and salbutamol (1.0 ng/μl) was used to evaluate splitless conditions: column head pressure (range 15–25 p.s.i.), glass liner volume (1000, 800 and 250 μl), temperature (240, 250, 260 and 280°C), sample volume (0.5–1.5 μl) and split valve closed time (45, 60 and 120 s). To establish the optimal conditions the peak width, resolution and analysis time were determined.

RESULTS AND DISCUSSION

Urine samples

The extraction of clenbuterol and salbutamol was carried out by two different procedures because it was not possible to recover both compounds with a single extraction. Extrelut columns were not able to recover salbutamol at basic or acid pH: the values obtained were lower than 20%. Solid phase extraction with octadecylsilica columns showed a low recovery for clenbuterol at pH 7.6, a value that was optimal for retaining salbutamol because at higher pH the phenolic group of this β-agonist remains ionized and it is impossible to retain it effectively. The purification step was performed in a single operation for both compounds. The recovery of this step was higher for clenbuterol (83%) than for salbutamol (70%). The recoveries of the complete procedure were 57.2% for clenbuterol (7% R.S.D.) and 47.8% for salbutamol (15.9% R.S.D.). These values agree with others reported previously [7].

GC–MS analysis

The use of a direct interface in GC–MS analysis requires the carrier gas flow to be as low as possible. One of the ways of achieving this is to use capillary columns of narrow diameter, for example 100 μm. A five-fold reduction in vacuum pressure compared with that obtained with capillary columns of 200 μm diameter was observed. The decrease of analysis time was not possible because high temperature ramps (>20°C/min) showed band broadening and lower resolution. Splitless injection is critical because it is difficult to transfer the solutes to a narrow column from the injector. It was observed that glass liners with volumes greater than 0.5 ml are not appropriate as they produce an increase in peak width; the optimal injection volume was 0.5 μl and long periods with the split valve closed did not improve the mass transfer. Column head pressures below 20 p.s.i. produced a slow introduction. An alternative procedure, increasing the inlet pressure only during the injection and reducing it afterwards during the separation, did not improve the resolution between clenbuterol and salbutamol peaks. Salbutamol showed a lower percentage R.S.D. (2.9%) than clenbuterol (7.8%), but in the case of spiked urine samples the repeatibility was poorer for both compounds amounting to 15% R.S.D. The calibration curve (areas *versus* concentration) of salbutamol was linear in the range 0.4–1.2 ng with $y = 148420 + 145343x$ ($r = 0.992$), and clenbuterol data in the same range of

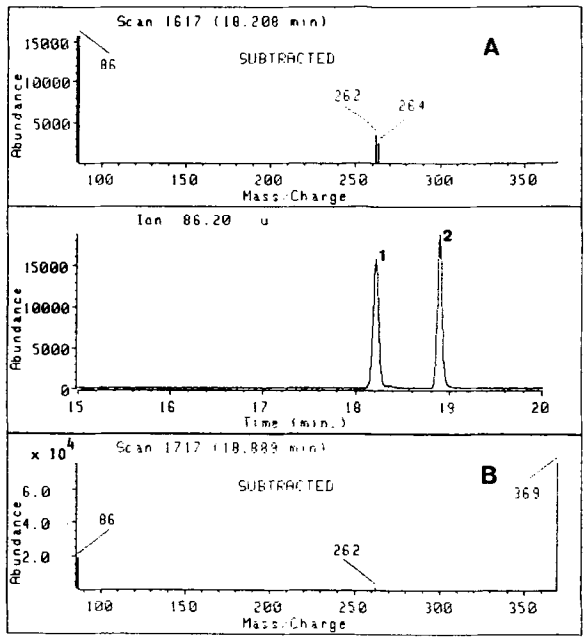

Fig. 1. GC–MS selected-ion monitoring chromatogram of clenbuterol (peak 1) and salbutamol (peak 2). Scan profile of the selected ions: (A) clenbuterol; (B) salbutamol.

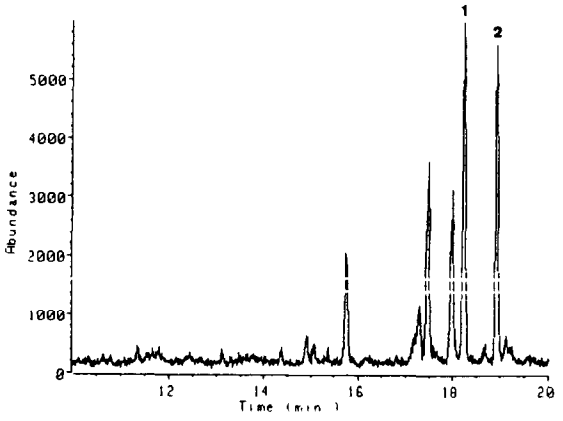

Fig. 2. GC–MS chromatogram of a urine sample spiked with 2 ng/ml clenbuterol (peak 1) and salbutamol (peak 2). For conditions, see text.

concentrations fitted the equation $y = 140631 + 130541x$ ($r = 0.994$). The sensitivity was 0.2 ng for both compounds (m/z 86 clenbuterol; m/z 369 salbutamol), allowing a limit of detection of urine samples of 1.5 ng/ml. Figs. 1 and 2 show chromatograms of standard and urine samples. Selected-ion monitoring in electron-impact mode presented problems for identifying the presence of clenbuterol because of the low mass of the ion with the highest abundance (m/z 86); however, urine blank samples did not show significant interference at the retention times of clenbuterol. In contrast, the identification of salbutamol was favoured in electron impact by the high abundance of the ion m/z at 369. Positive chemical ionization was used to ensure the identification of clenbuterol (Fig. 3), but the sensitivity was lower (0.4 ng, ions m/z 349 and m/z 333), allowing only a limit of detection of 3.0 ng/ml; but when ion at m/z 86 was used the sensitivity was similar to electron impact. Also, salbutamol can be identified by this technique using two m/z values at 440 and 366.

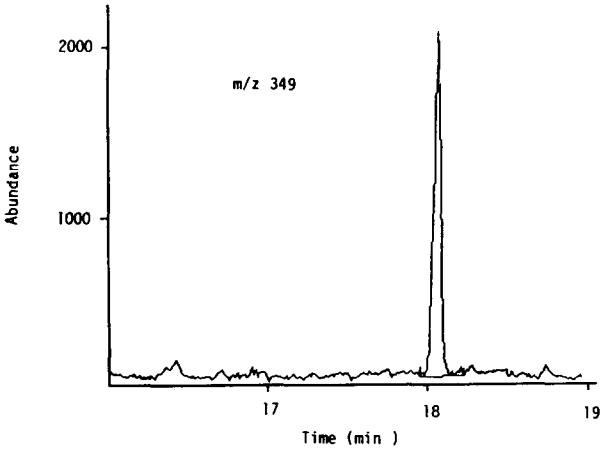

Fig. 3. GC–MS chromatogram of urine sample containing clenbuterol. Methane-positive chemical ionization at m/z 349. For conditions, see text.

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