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Determination of clenbuterol in human urine by GC–MS–MS–MS: confirmation analysis in antidoping control

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Abstract

This work presents a GC–MS–MS–MS method for the direct determination of clenbuterol in human urine. The method comprises a pretreatment procedure and the instrumental analysis of the derivatives performed by GC–MS^3 (ion trap) with electron impact ionization. The GC–MS^3 analysis allows isolation and characterization of specific fragments from the original (MS^1) molecular structure, and in particular, those fragments originating from the precursor ion cluster ($m/z=335$–337) characteristic of clenbuterol. The MS^2 product fragment $m/z=300$ is in turn used as a further precursor fragment giving rise to a MS^3 spectrum specific for clenbuterol. MS^4 fragmentation spectra were also investigated. However, further fragmentation of MS^3 product ions does not lead to functional MS^4 spectra nor to any significant increase in the signal-to-noise ratio. The sensitivity limit of the MS^3 technique is lower than 0.2 μg/l, with a linear range between 0.5 and 5 μg/l, thus matching the basic requirements for antidoping analysis according to the guidelines of the International Olympic Committee. Due to its overall analytical performance, the method is presently being evaluated as a confirmation protocol to be followed to detect illicit clenbuterol administration to the athletes, and compared with reference GC–MS and GC–MS–MS techniques.

Keywords: Antidoping control; Clenbuterol

1. Introduction

Clenbuterol (Fig. 1) is a beta-2-adrenergic receptor agonist administered as a bronchodilator for the treatment of respiratory diseases, primarily bronchial asthma and as a tocolytic agent [1]. Apart from its official pharmaceutical formulation, it is also available as a veterinary drug [2,3] and, often illegally, through the internet and other parallel markets.

Clenbuterol is supposed to be illicitly used by some athletes due to its ''repartitioning effects'', defined as the increase of lean muscle mass and the concomitant decrease of fat deposition [4]. The repartitioning effects are in turn achieved through both an increase of muscle protein deposition and an

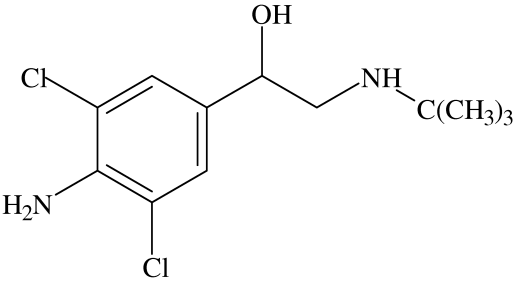

Fig. 1. Molecular structure of clenbuterol (4-amino-α-[(tert.-butylamino)methyl]-3,5-dichlorobenzyl alcohol).

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augmented lipolysis [4,5]. For both its potential positive effects on sport performance and the associated toxicological risks, clenbuterol and other β_2-agonists have been recently banned by the Sport Authorities, and are presently included in the list of doping substances and methods of the Olympic Movement Antidoping Code [6].

Clenbuterol is a low polar β_2-agonist characterized by a long plasma half-life and relatively slow urinary excretion rate. The fraction of drug that is excreted unmodified in the urine oscillates between 35 and 85%, thus making suitable the detection of its administration by urine analysis of the unchanged drug. The average urinary level of clenbuterol following oral administration of therapeutic doses is generally higher than 10 μg/l. Nonetheless, in the case of antidoping analysis, and especially in the case of ''in competition'' testing, it may be necessary to reach lower sensitivities, since the administration of clenbuterol can take place, and still be effective, in the training period and therefore relatively far from the actual collection of the urine sample. The decisional threshold stated by the present International Olympic Committee Medical Commission guidelines is indeed fixed at 2 μg/l, but several sport federations may require informal notification for concentration values below this limit also.

Detection and quantitative determination of clenbuterol in routine analysis of biological matrices is being accomplished by a variety of methods, including immunological, electrochemical and chromatographic–spectrometric (HPLC–UV, HPLC–MS, GC–MS) techniques [7–12]. In forensic toxicology applied to sport medicine, and especially in the search for clenbuterol and other β_2-agonists in the urine of athletes by the antidoping laboratories, the screening analysis is carried out by different methods, while the confirmation analysis is performed exclusively by GC–MS, using as detector a single quadrupole MS, either in full scan or in selected ion monitoring (SIM) acquisition mode, a magnetic high resolution mass spectrometer (HRMS), as well as hyphenated techniques.

This work presents a novel approach for the confirmation analysis of clenbuterol in human urine. The method consists of a pre-treatment stage (prepurification on preactivated cartridges, enzymatic hydrolysis, liquid–liquid extraction and derivatization) and the subsequent chromatographic–spectrometric analysis of the bis-TMS derivative by GC–MS[3].

2. Experimental

2.1. Materials and reagents

All reagents (analytical grade) were supplied by Carlo Erba (Milano, Italy). β-Glucuronidase from *E. coli* was supplied by Boehringer Mannheim (Germany). *N*-methyl-*N*(trimethylsilyl)-trifluoroacetamide (MSTFA) was supplied by Macherey-Nachel. Ammonium iodide (NH_4I) and dithioerythritol (DTE) were supplied by by Sigma (St. Louis, MO, USA) and by Serva, respectively. The derivatizing agent was a mixture of MSTFA–NH_4I–DTE (1000:4:2, w/w) and darkly stored in screwed cap vials at $T=4$ °C.

Methyltestosterone (used as internal standard, I.S.) and clenbuterol were supplied by Sigma (St. Louis, MO, USA). Stock standard solutions were prepared dissolving the standard in methanol (200 μg/l); all stock solutions were darkly stored in screwed cap vials at $T=4$ °C. Working standard solutions were daily prepared, at the appropriate dilution, from the correspondent stock solution. Twice distilled water was used for the preparation of all reagents and solutions. C_{18} cartridges (Sep-Pak) were supplied by Waters (Waters, Milano, Italy).

Spiked urine samples were prepared by diluting the corresponding methanol standard solution with blank reference urine to a final concentration between 0.1 and 5 μg/l.

2.2. Urine pre-treatment

Five ml urine, added with I.S. (methyltestosterone 4 μg/l) were passed across a C_{18} Sep-Pak (previously activated by 3 ml of MeOH and washed twice with 3 ml H_2O and 3 ml MeOH) and then eluted with 3 ml of methanol. The resulting solution was evaporated to dryness under N_2 stream at $T=40$ °C.

The enzymatic treatment is carried out by β-glucuronidase: 2.0 ml of phosphate buffer (0.2 *M*, pH 7.4), and 25 μl of β-glucoronidase from *E. coli*

Table 1
Gas chromatographic conditions of the GC–MS^n assays

Carrier gas	He
Column	HP5 (cross-linked phenylmethylsilicone, 30 m)
Injector temperature (°C)	240
Transfer line temperature (°C)	260
Constant flow-rate (ml/min)	0.6
Injection type	Splitless
Injected volume (μl)	2
Oven temperature program:	
Initial *T* (°C)	125
Initial time (min)	2.00
Rate 1 (°C/min)	20
Final *T*1 (°C)	225
Hold time 1 (min)	1
Rate 2 (°C/min)	10
Final *T*2 (°C)	320
Hold time 2 (min)	10

are added to the sample, which is then incubated at $T=50$ °C for 1 h.

After correction of pH to 9.2 with carbonate buffer, organic fraction is extracted by 10 ml of Et_2O and again brought to dryness; the product is derivatized by 25 μl MTSFA–NH_4I–DTE (1000:2:4, w/w) (30 min, $T=70$ °C). Following this procedure a concentration factor of approximately 200-fold is achieved.

Table 2
Mass spectrometric conditions of the GC–MS^n assays

Source temperature (°C)	180
Segment 1 (clenbuterol)	
Start time (min)	10.00
Scan mode	MS–MS–MS; $\theta=0.25$
Precursor 1 (m/z)	336
Width (m/z)	3
Collision energy (V)	0.75
Precursor 2 (m/z)	300
Width (m/z)	1.5
Collision energy (V)	0.75
Product ions (m/z)	200–340
Segment 2 (I.S.)	
Start time (min)	16.00
Scan mode	MS–MS; $\theta=0.25$
Precursor (m/z)	446
Width (m/z)	1
Collision energy (V)	0.8
Product ions (m/z)	245–360

2.3. Instrumentation and GC–MS parameters

All GC–MS^n assays were performed on a ThermoQuest GCQ Ion Trap MS^n system (ThermoQuest Italia, Rodano, MI, Italy); the reference GC–MS assays were performed on a Hewlett-Packard 5973 GC–MS system (Agilent Technologies, Cernusco sul Naviglio, MI, Italy).

GC conditions and MS conditions are summarized in Tables 1 and 2, respectively.

3. Results

Sequential MS^n experiments of clenbuterol–2TMS were performed to select both the optimal fragmentation stage and the most suitable precursor ions. Fig. 2 shows the MS^2 (A), MS^3 (B) and MS^4 (C) spectra obtained starting from a standard solution of clenbuterol bis-TMS 4 mg/l.

The molecular ion cluster 335–337 has been selected as the precursor group for MS^2 experiments; the MS^2 product fragment $m/z=300$ has in turn been used as a further precursor fragment giving rise to the MS^3 spectrum specific for clenbuterol (characteristic product ions: $m/z=284$, 226 and 210). The most abundant MS^3 product ion ($m/z=284$) has also been selected as the precursor ion for MS^4 fragmentation experiments. As it can be seen from Fig. 2C, this further fragmentation does not lead to functional MS^4 spectra, nor to a significant increase in the signal-to-noise ratio.

The proposed fragmentation pattern, as obtained by MS, MS^2, MS^3 and MS^4 assays, is shown in Fig. 3. The MS^3 spectrum, as obtained by precursor ions 335–337→300, is characteristic of clenbuterol and it has therefore been considered for the confirmation analysis of clenbuterol in urine.

Figs. 4 and 5 show the GC–MS^3 chromatograms (total ion: A; extracted ions: B–D) of two spiked urines (clenbuterol concentration: 2 and 0.1 μg/l, respectively); while Figs. 6 and 7 report the MS^3 spectra, from precursor ions 335–337→300, obtained on the same spiked urines. Finally, Fig. 8 shows the GC–MS^3 analysis of a real positive sample (IOC positive control urine).

The range of linearity has been verified on spiked urines (clenbuterol concentration: 0.5, 1.0, 3.0, 5.0

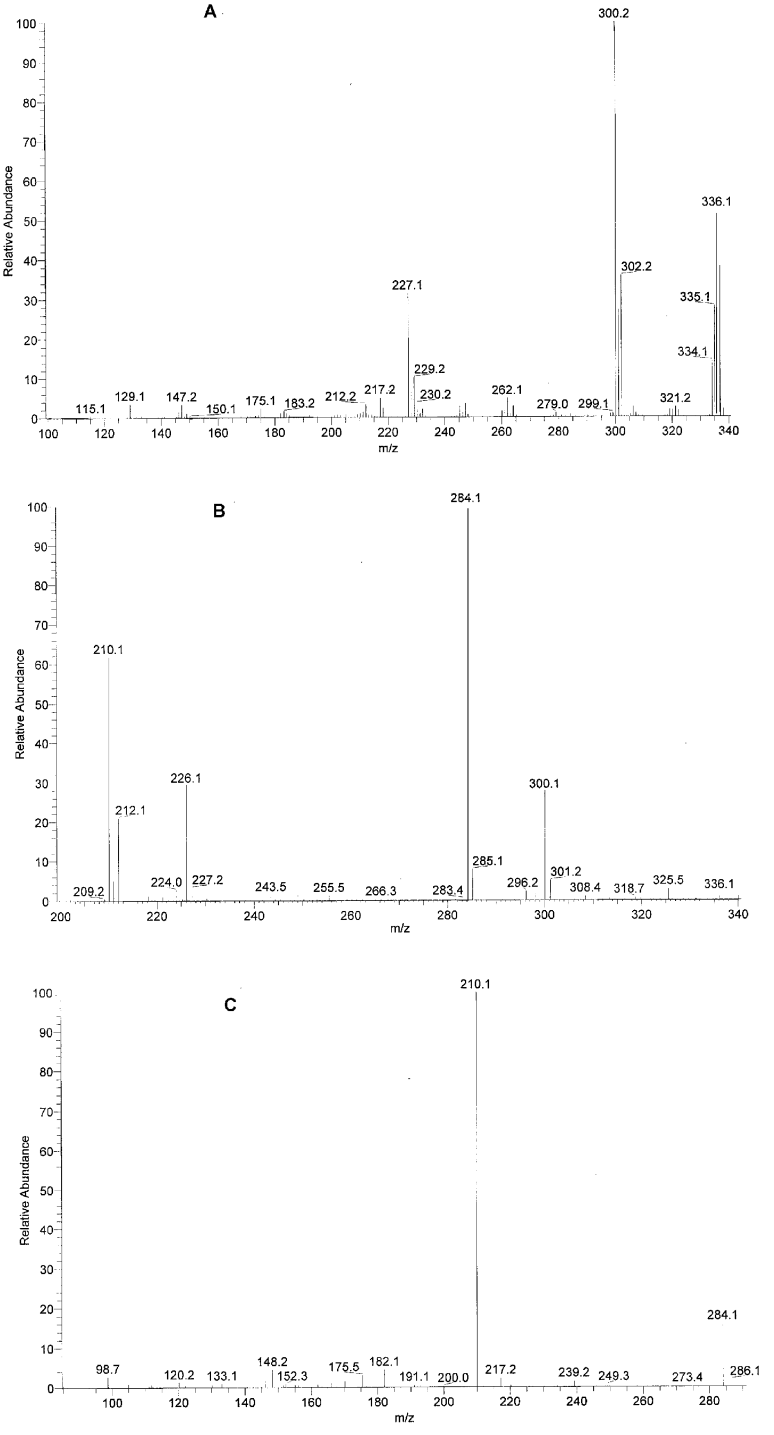

Fig. 2. (A) MS^2, precursor ion cluster $m/z = 335–337$; (B) MS^3, precursor ion $m/z = 300$; and (C) MS^4, precursor ion $m/z = 284$ spectra, obtained starting from clenbuterol bis-TMS.

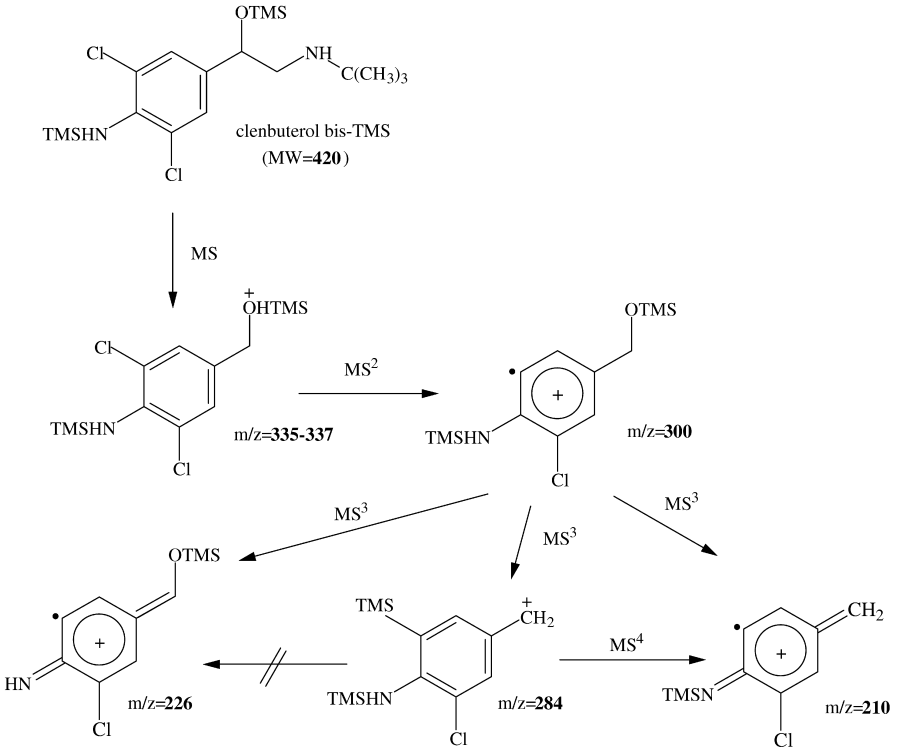

Fig. 3. Proposed fragmentation pattern of clenbuterol–2TMS as obtained by the MS^2, MS^3 and MS^4 spectra reported in Fig. 2.

μg/l), analyzed in triplicate, by comparison between the area of the ion m/z 284 (clenbuterol, obtained by GC–MS^3 chromatograms from the precursor ions 335–337→300) and the area of the ion m/z 301 (I.S. methyltestosterone, obtained by GC–MS^2 chromatograms from the precursor ion m/z 446). The response is linear in the above mentioned concentration range (0.5–5.0 μg/l in urine), with a correlation factor $R^2 = 0.9838$.

The value of the limit of detection (LOD), corresponding to a value of the signal-to-noise ratio = 3, were determined by GC–MS^3 assays carried out on standard solutions at different dilution factors (final clenbuterol concentration: 0.5, 0.2, 0.1 μg/l). In the optimal tuning condition and with the source and trap as clean as possible, a LOD of 0.1 μg/l can be achieved (see again Figs. 5 and 7). This value is lower than the LOD reached in the same experimental conditions by a single quadrupole GC–MS system in SIM acquisition mode (0.5 μg/l).

4. Discussion

Many methods have been presented for the identification and quantitative determination of clenbuterol in human urine by GC–MS technique, either in electron ionization and in chemical ionization mode. Sequential mass fragmentation experiments of clenbuterol have recently been described in both GC–MS^n and LC–MS^n studies [13,14], thus showing that the portion of the molecular structure comprising the aromatic ring can be selected as the key diagnostic fragment in MS^n experiments.

In the case of an ion trap system, ion selection, collisional activation and product ion analysis can be performed sequentially within the same chamber. It is therefore relatively easy to perform progressive fragmentation experiments. In the analysis of complex matrices and in the presence of interferences useful information can be obtained by the serial fragmentation of a suitable precursor fragment, since

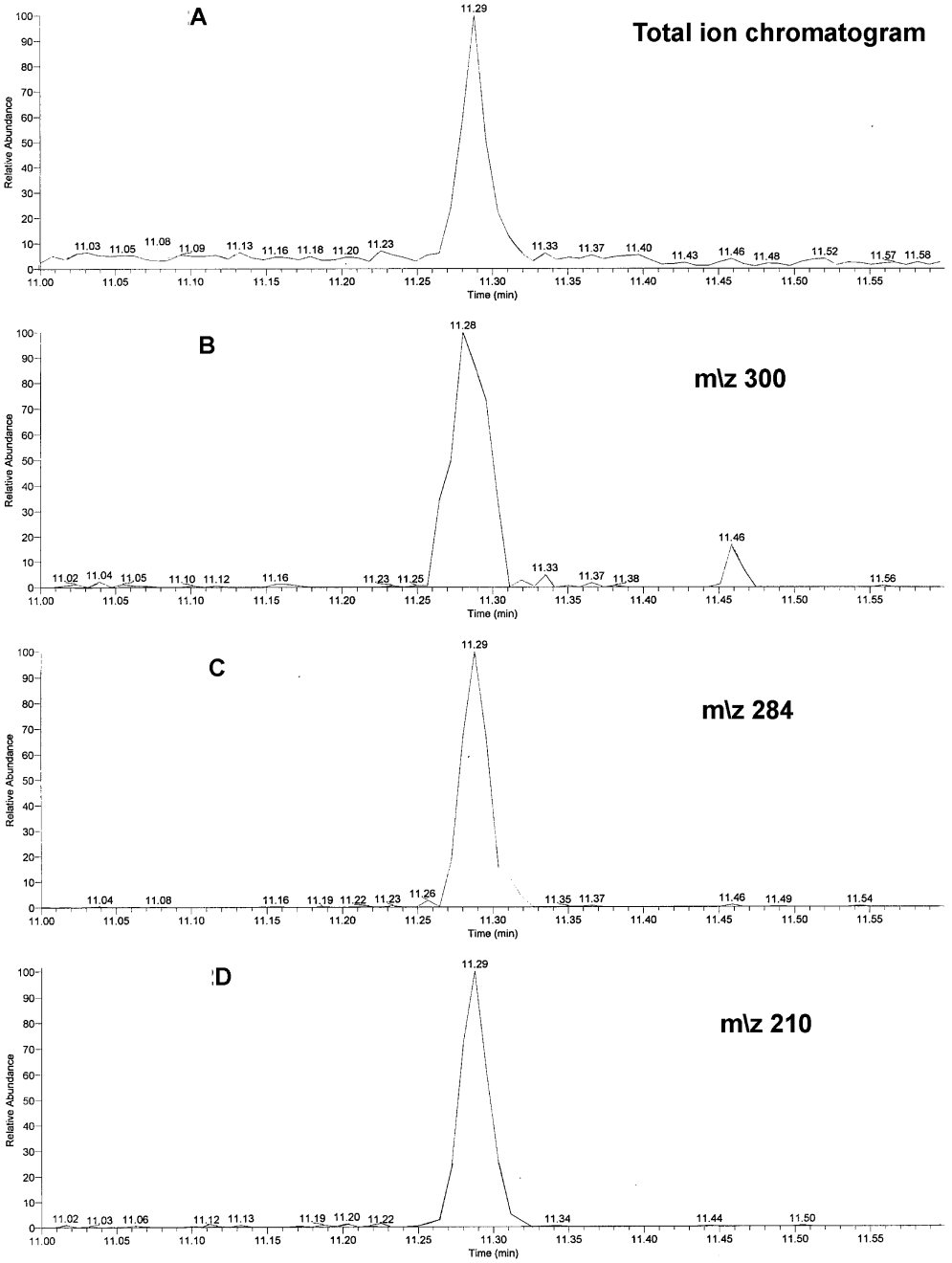

Fig. 4. GC–MS^3 chromatograms of a spiked urine containing clenbuterol 2.0 μg/l. (A) Total ion; (B) extracted ion m/z 300; (C) extracted ion m/z 284; (D) extracted ion m/z 210.

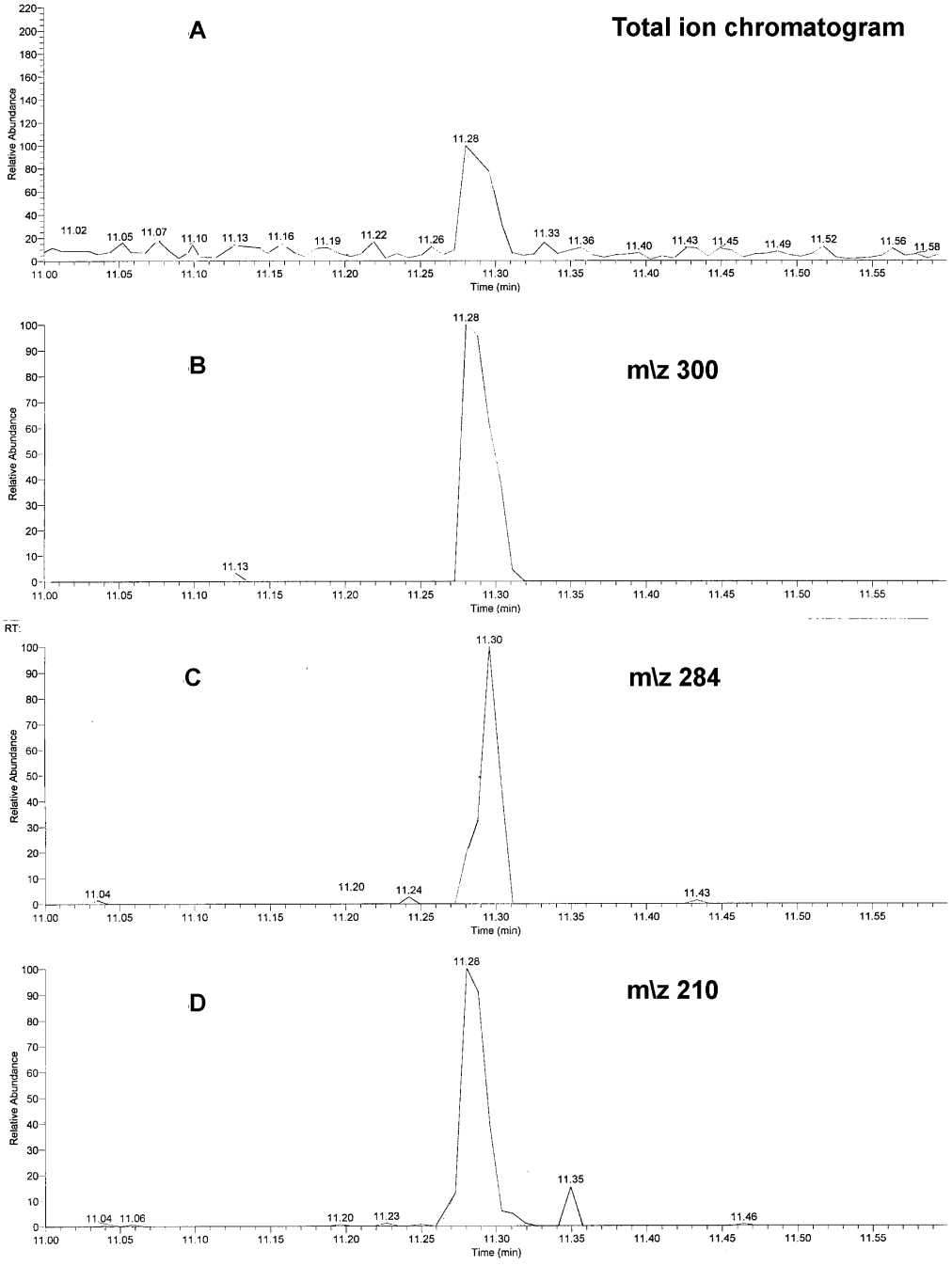

Fig. 5. GC–MS^3 chromatograms of a spiked urine containing clenbuterol 0.1 μg/l. (A) Total ion; (B) extracted ion m/z 300; (C) extracted ion m/z 284; (D) extracted ion m/z 210.

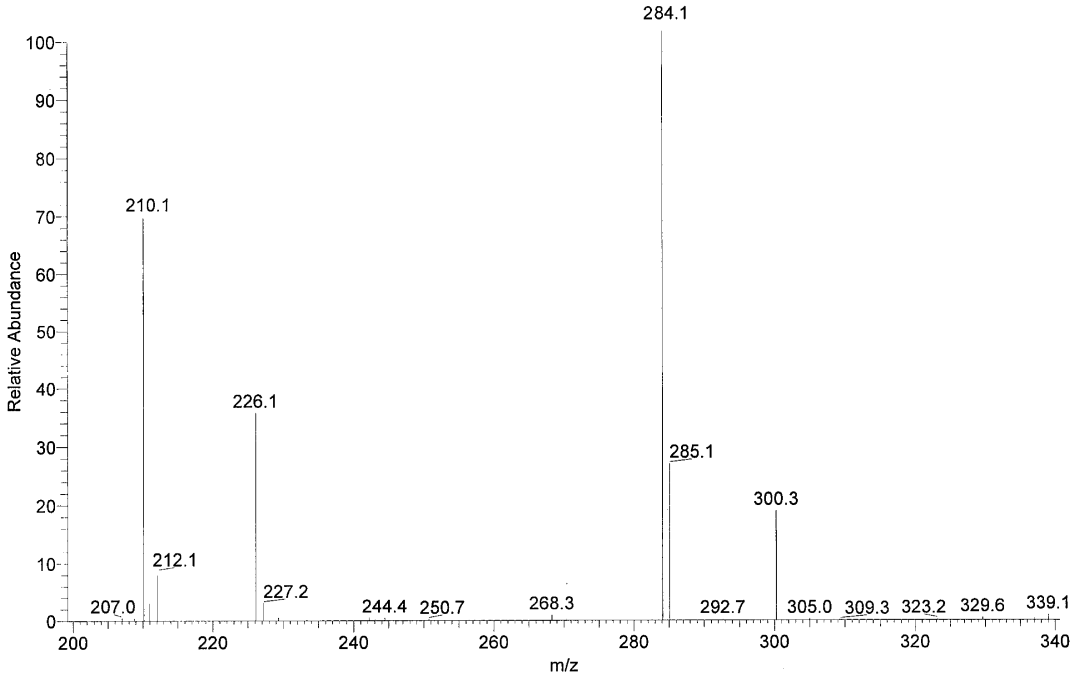

Fig. 6. MS^3 spectrum (precursor ions: 335–337→300) of a spiked urine containing clenbuterol 2.0 μg/l.

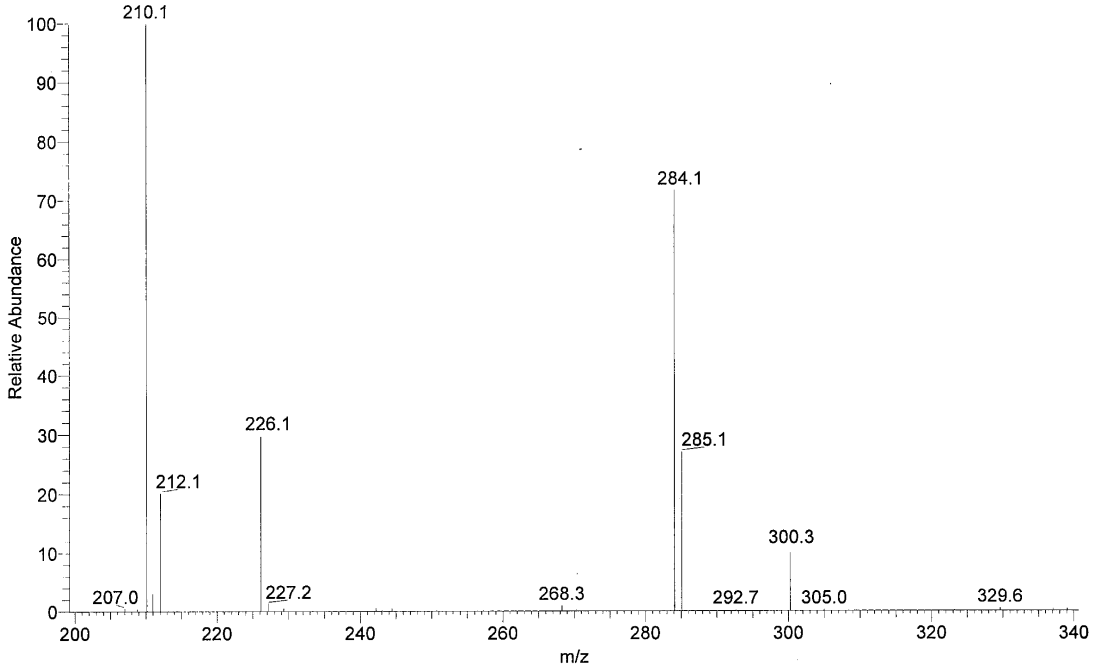

Fig. 7. MS^3 spectrum (precursor ions: 335–337→300) of a spiked urine containing clenbuterol 0.1 μg/l.

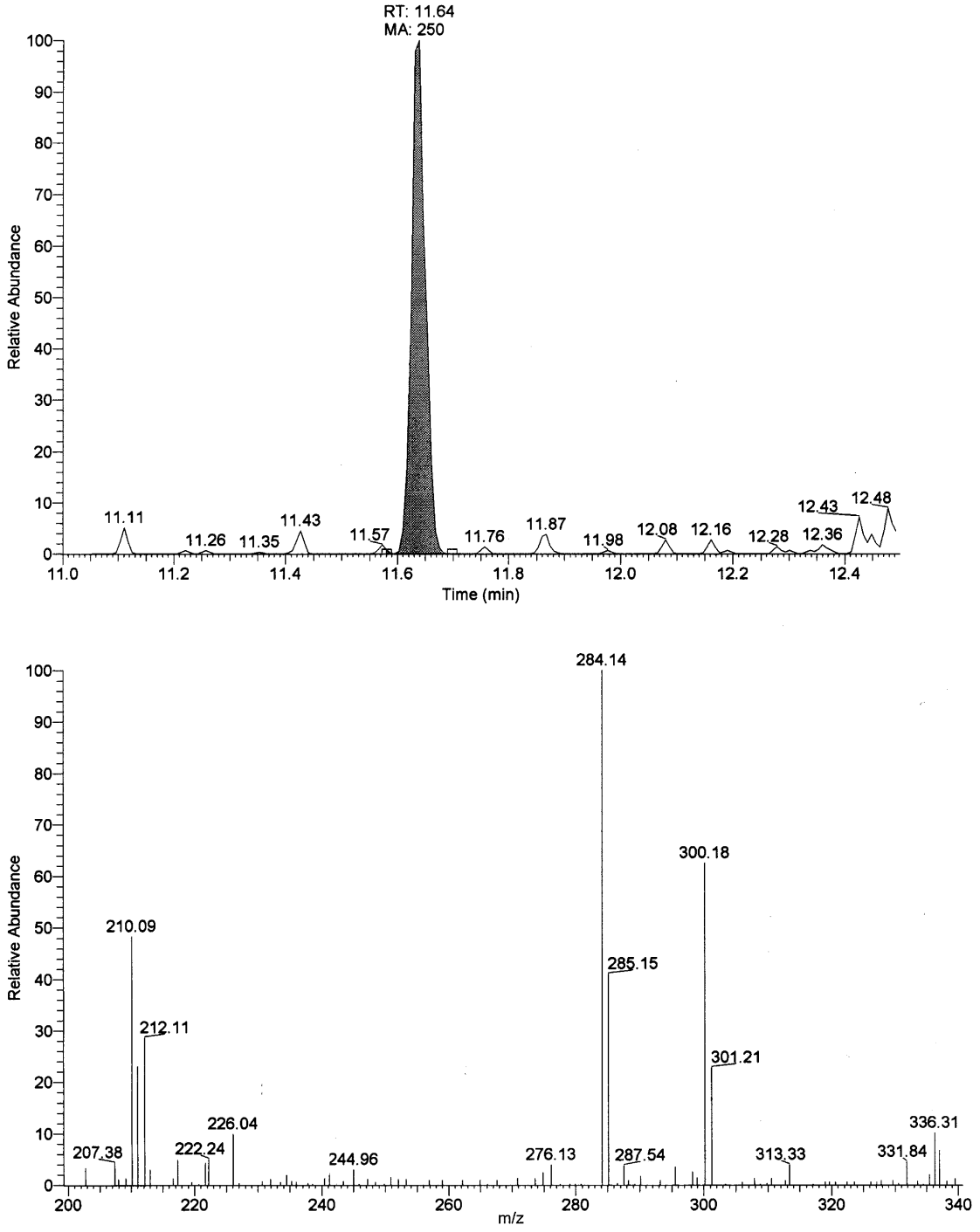

Fig. 8. GC–MS^3 analysis of a real samples (positive control sample supplied by the International Olympic Committee). Top: GC chromatogram (extracted ion m/z 284); bottom: MS^3 spectrum (precursor ions: 335–337→300).

the loss in the absolute intensity of the signal is counterbalanced by the drastic increase of the signal-to-noise ratio. The method here presented gives indeed the advantage to drastically reduce the background noise, thus enhancing the signal-to-noise ratio; at the same time, MS^3 spectra obtained in the experimental conditions here described are still significant enough to give reliable diagnostic evidence for clenbuterol identification.

The procedure here presented is therefore suitable for the rapid confirmation of samples giving doubtful results following the screening analysis. The method here presented could in principle being followed also for the screening analysis of clenbuterol, but in this context the advantages would be less competitive, mainly from a practical point of view, since a constant verification of the performance of the mass spectrometric system, and especially of the conditions of the source and the trap, is necessary to obtain reproducible results.

Although the study was planned to draw practical indication for the activity of an antidoping laboratory, many consideration can be extended to other fields of analytical toxicology, including the analysis of beta-adrenergic residues in food matrices and/or in bioptic materials. Currently in progress are additional studies, aimed to compare the results of the technique here presented with those obtained by GC–MS^n assays in chemical ionization and on different clenbuterol derivatives.

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