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Gas chromatography-mass spectrometric analysis of clenbuterol from urine

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Abstract

Clenbuterol which is mostly used as an anabolic agent. It is also used for treatment of asthma. Clenbuterol was analysed from urine by using gas chromatography-mass spectrometry. GC-MS parameters were determined. Timolol was used as an internal standard. Extraction and derivatisation procedure of clenbuterol from urine were developed. Clenbuterol was extracted by using diethylether/ter-butanol (4:1; v:v) and pH 12 $K_2CO_3/KHCO_3$ (3:2; w:w) buffer. MSTFA/NH_4I (1 ml/10 mg) mixture was used for derivatization of clenbuterol. Selected ions of clenbuterol-bis-TMS were m/z: 405, 337, 336, 335, 300, and 227. Extraction yield and minimum detection limit of clenbuterol from urine were identified. Extraction yield was 94.30% and minimum detection was found 0.02 ng ml^{-1} urine. It has been concluded that the GC-MS method is sensitive, accurate, precise, and reproducible for analysing of clenbuterol from urine.

Keywords: Clenbuterol; GC-MS analysis; urine

1. Introduction

Clenbuterol (4-amino-3,5-dichloro-α-{((1,1-dimethylethyl)amino)methyl}benzenemethanol) is a β-2 agonist that is used as an antiasthmatic and tocolytic agent [1]. It is also an anabolic agent [2]. It promotes muscle growth and reduces body fat and increases muscle mass [3]. It has been used to produce meat in farm animals [4]. Some sportsman (body builders) began to use of clenbuterol and it appeared that the administration of this substance could be widespread among athletes seeking for muscle mass enhancement and strength. So, this drug was banned by the Medical Commission of the International Olympic Committie (IOC) [5]. The active dose of clenbuterol is $20-40$ μg, the resulting urine concentration of it following oral administration of the drug is very low [6].

Some analytical methods available for the identification of clenbuterol. Clenbuterol can be deter-

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mined by electrochemical [2], spectrophotometric [7], chromatographic [8,9], mass spectrometric [6,10,11], immunoassay [12] methods in biological materials.

We describe here a sensitive GC-MS method and extraction procedure suitable for the determination of clenbuterol from urine. The proposed method has lower detection limit to find clenbuterol from urine after administration of 20–40 μg drug.

2. Experimental

2.1. Chemicals and materials

Clenbuterol-HCL and timolol were obtained from Sigma (St. Louis, MO). *N*-methyl-*N*-trimethylsilyltriflouroacetamide (MSTFA) was purchased from Fluka (Buchs, Switzerland). All solvents and other chemicals were analytical

Table 1
Precision and accuracy of the method

Theoretical Concentration (ng ml^{-1})	*n*	% Extraction yield	Standard deviation	% Coefficient of variation	% Error
0.08	6	78.07	10.98	14.07	4.48
0.1	6	88.73	4.91	5.54	2.00
1.0	6	94.30	1.17	1.24	0.47
10.0	6	90.86	5.76	6.34	2.35

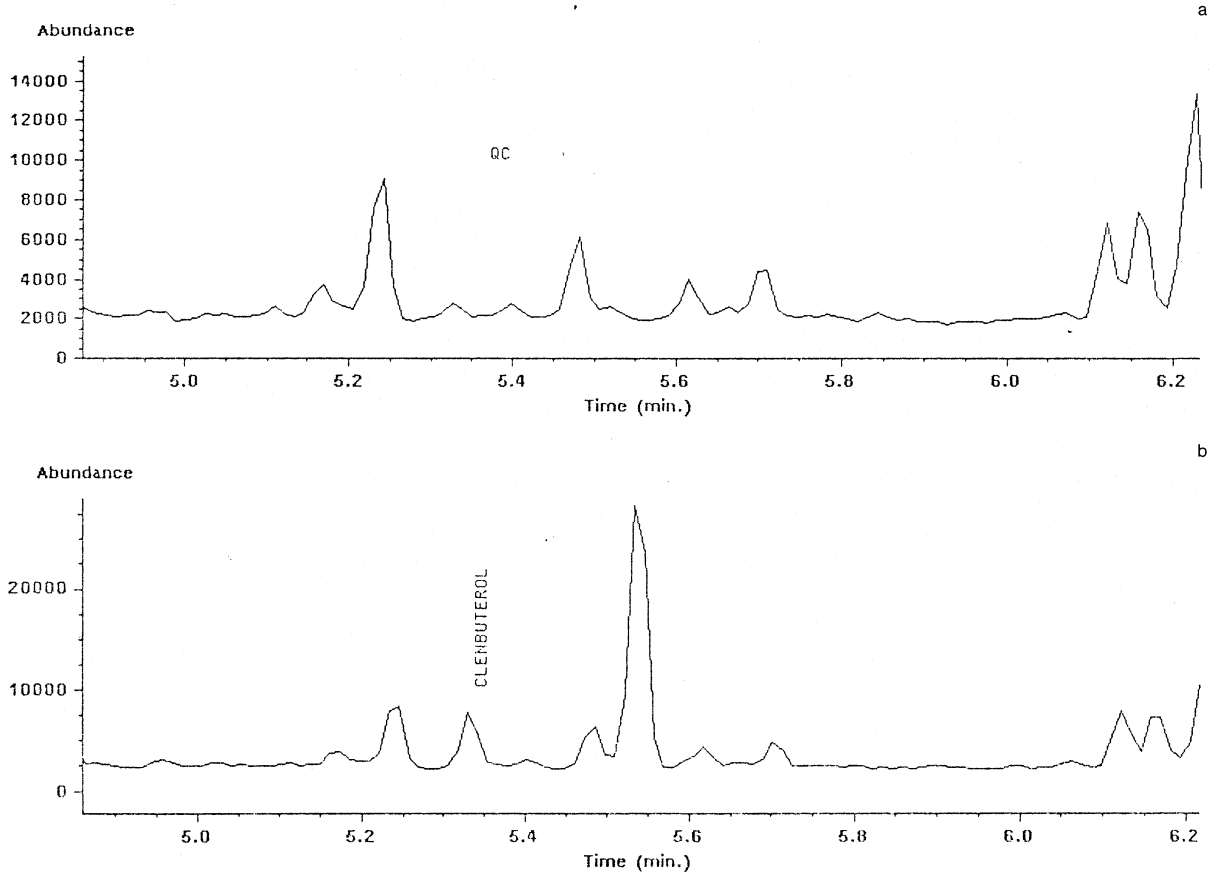

Fig. 1. Chromatogram of Clenbuterol (a) blank urine; (b) 0.02 ng ml^{-1} spiked urine.

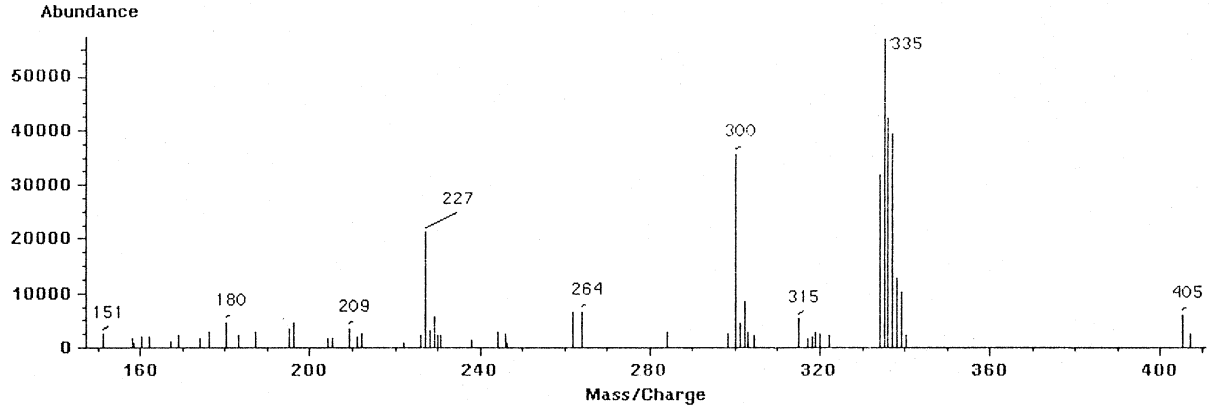

Fig. 2. The mass spectrum of Clenbuterol (scan value in between m/z: 150–650).

reagent grade. A derivatisation solution of MSTFA/NH_4I (1 ml/10 mg; v/w) was stored in the dark.

2.2. Sample preparation

The urine sample (5 ml), after addition of the internal standard, timolol (20 ng) was adjusted to pH 12 with the mixture of K_2CO_3: $KHCO_3$ (3:2; w:w). 4 ml diethylether and 1 ml t-butanol were added. After shaking for 5 min, the organic layer was separated, evaporated and dried in a desiccator for at least 20 min before derivatisation. The residue was derivatised by treatment with 50 μl of mixture of MSTFA/NH_4I (1 ml/10 mg; v/w) and heated at 70°C for 15 min. The enzymatic hydrolysis was performed at 50°C for 1 h with the addition of 50 μl of β-glucuronidase from *Escherichia coli* in 0.2 M 1 ml pH 7.0 phosphate buffer, then 2 μl of a mixture was injected to GC-MS with the split ratio: 1/10.

2.3. Gas chromatography-mass spectrometry

GC-MS analysis were carried out with a Hewlett–Packard 5890 Series II gas chromatograph and HP 5970 mass selective dedector. GC was equipped with electronic pressure control unit, autoinjector (HP18593B) and autosampler (HP18596 BX). The system was under computer control with a software of HP 59940C UNIX version A.01.04. A cross linked Ultra-2% 5 phenylmethylsiloxan capillary column (length 17 m, i.d. 0.2 mm, film thickness 0.33 μm) (Hewlett–Packard) was connected into the ion source. Samples were injected in the split mode with the split ratio 1:10. Helium was used as the carrier gas at the flow rate 0.7 ml min^{-1}. Injector and detector temperatures were set at 280 and 290°C, respectively. The column temperature was programmed to start at 150°C, increase at a rate of 15°C min^{-1} up to 280°C. The mass spectrometer conditions were as follows: electron impact ionization voltage 70 eV for both SCAN and selected ion monitoring (SIM) mode.

3. Results and discussion

The increasing use of clenbuterol to enhance the performance of athletes is a cause of concern in urine analysis. The GC-MS method described in this study afforded a sensitive and specific tecnique to measure clenbuterol in urine.

The extraction procedure described here proved reliable in removing the majority of interfering co-extractives and thus allowed accurate determination across the working range of the method. Clenbuterol can be extracted in basic media.

The analysis of underivatised clenbuterol is unsuitable due to the presence of the hydroxy and amino groups. Trimethylsilyl of clenbuterol was stable and suitable for GC-MS analysis.

The precision and accuracy of the method were determined by extracting control urine spiked with clenbuterol at four different concentrations, 0.08, 0.1, 1.0, 10 ng ml^{-1} (Table 1).

A signal to noise ratio of 2.5 ($n = 4$) is the value used for a significant response. The limit of detection (LOD) was defined as the lowest detectable concentration yielding a signal significantly higher than that of the blank control specimens. The limit of detection was calculated as 0.02 ng ml^{-1} from urine with the method developed (Fig. 1).

The seven point calibration curve was obtained using urine spiked with clenbuterol. The samples were extracted and analysed as described above. Calibration curve was plotted over the concentration range 0.08–100 ng ml^{-1} ($r = 0.9985$, $A_{Clen}/A_{ISTD} = 0.27896 + 4.2621\ C_{Clen}/C_{ISTD}$). The slopes of the urine calibration curves (mean $\pm$ SD, 4.28 ± 0.0038, $n = 6$) were reproducable from day to day, with a coefficient of variation of 2.100%. The mean intercept value was (mean $\pm$ SD, 0.327 ± 0.0030, $n = 6$) close to zero. The amount of clenbuterol from spiked urine out of the range of calibration curve was calculated by using internal standard/standard clenbuterol ratio. Foerster et al. [13] used deterio standard of clenbuterol for the determination by ammonia-CI-GC-MS. β-blockers were used as an internal standard for the analysis of clenbuterol in urine [14]. Timolol is the most suitable β-blocker for proposed extraction and GC-MS method. It can be found easily and is cheap.

In order to develop the method further into a procedure for low level monitoring of clenbuterol

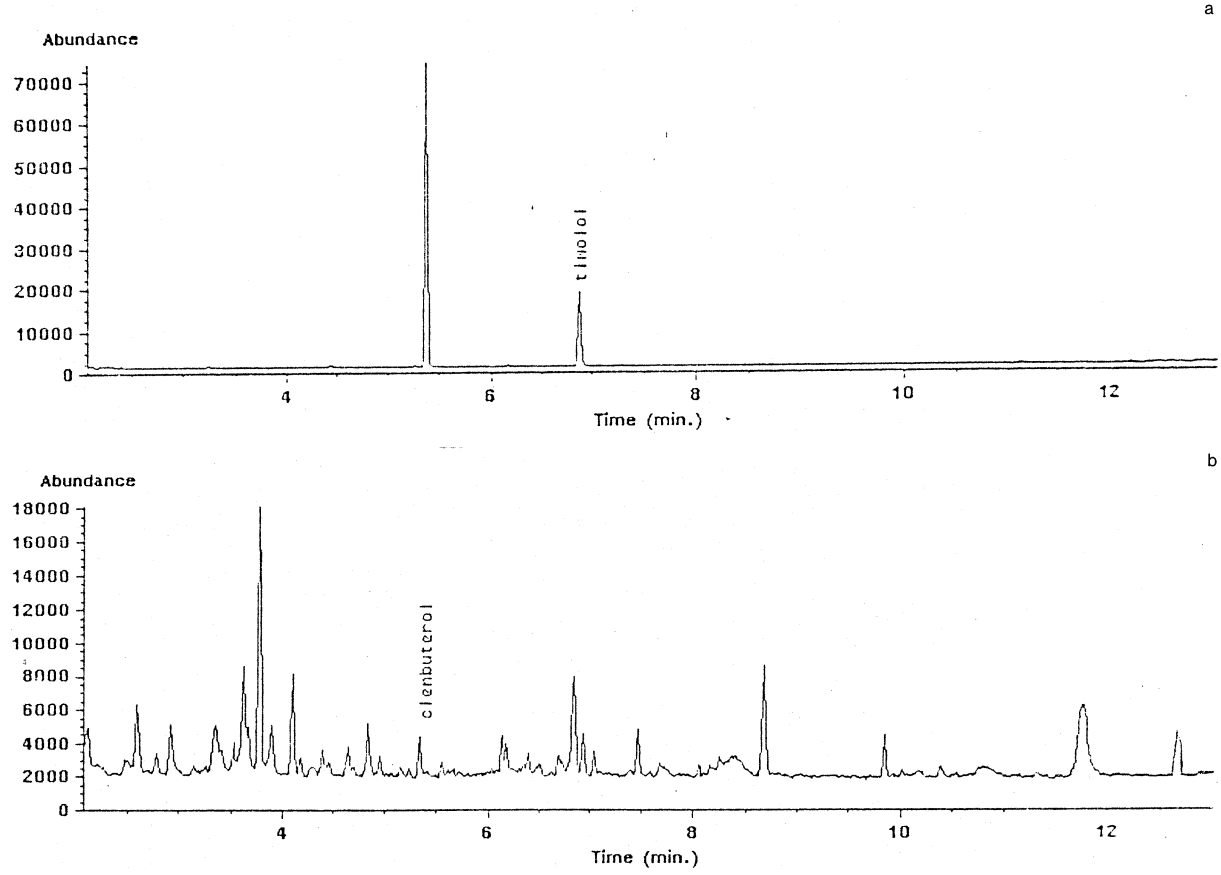

Fig. 3. The chromatogram of Clenbuterol (a) standard (100 ng ml^{-1} Clenbuterol, 20 ng ml^{-1} Timolol); (b) spiked urine (20 ng ml^{-1} Clenbuterol, 20 ng ml^{-1} Timolol).

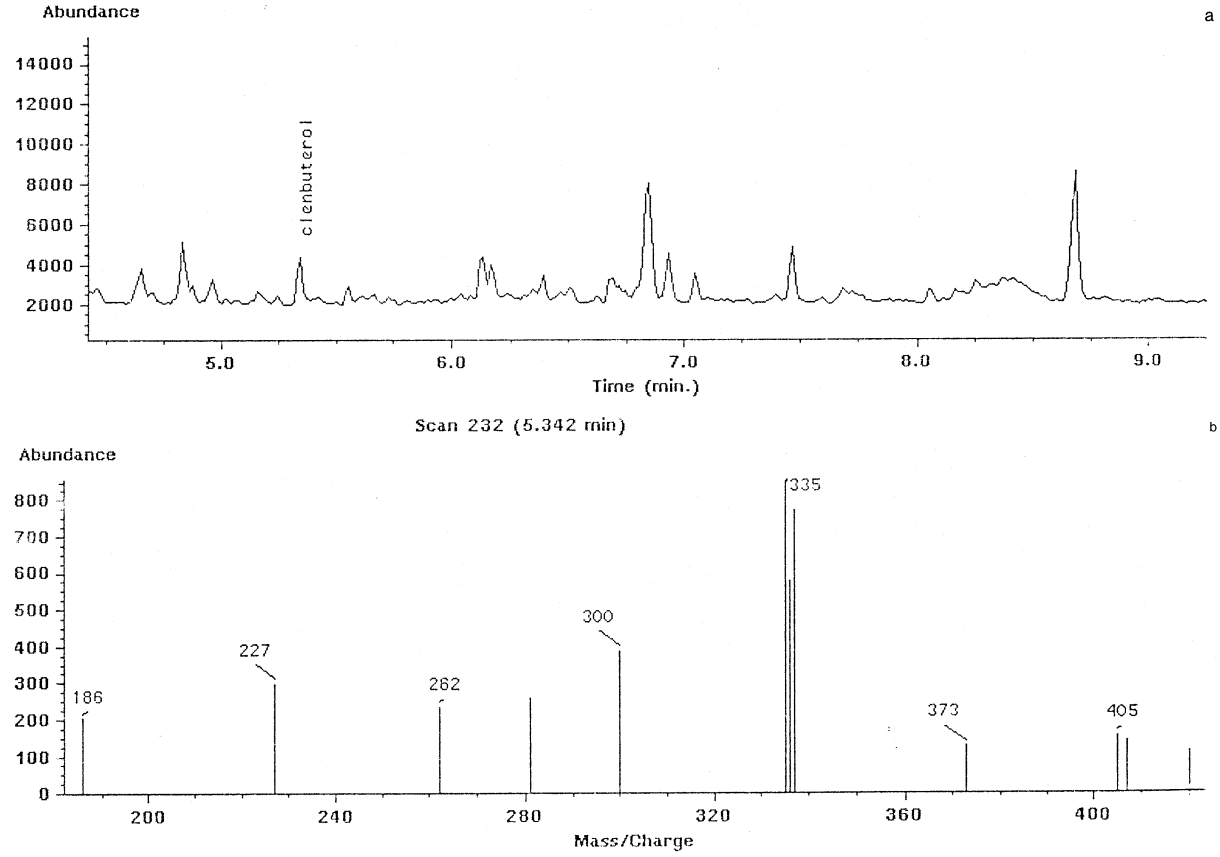

Fig. 4. (a) The chromatogram of urine sample containing Clenbuterol; (b) selected ion chromatogram of the peak related to Clenbuterol.

and other related drugs in urine or alternative biological matrix, an analysis is required which selectively monitors the important diagnostic ions rather than scanning the whole mass range. SIM tecnique was used to determine clenbuterol from urine. SIM, when used in conjuction with the GC retention time of the compounds, provides a highly specific method of detecting drugs of abuse. Retention time of clenbuterol is 5.342 min. Clenbuterol shows characteristic ions at m/z: 405 (M^+-15), 335 (M^+-CH-NH-C(CH3)3, 300 (M^+-CH2-NH-C(CH3)3-Cl) (Fig. 2). GC chromatogram of clenbuterol spiked urine and standard of clenbuterol-bis-TMS were given in Fig. 3.

The method developed was applied to the real urine samples which contain clenbuterol (Fig. 4). The analytical procedure was done with and without enzymatic hydrolysis. The results of these analyses were given in Table 2. All samples were analysed in dublicate. The proposed mechanism of the enzymatic hydrolysis procedure is to minimise the effect, if any, of side products and impurities on the sensitivity and accuracy of the method. There is no significance difference be-

Table 2
The amount of Clenbuterol in real urine samples (ng ml^{-1})*

	Sample 1	Sample 2	Sample 3
With hydrolysis	7.05	7.14	15.09
Without hyrolysis	8.67	7.49	13.40

* $P > 0.05$ no significance found between two analysis procedures.

tween the results of with and without hydrolysis procedure ($P > 0.05$, $t_{cal} < t_{table}$; $4.341 < 7.453$). The results show that clenbuterol seems to be excreted free in urine rather than conjugates.

The precision and accuracy of the method were found to be suitable for routine analysis of clenbuterol. The method was successfully applied to the analysis of urine samples after administration of clenbuterol. The limit of detection is good enough to find clenbuterol in urine.

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