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Excretion study of the β_2-agonist reproterol in human urine

C.G. Georgakopoulos*, C. Tsitsimpikou, M.-H.E. Spyridaki

Doping Control Laboratory of Athens, Olympic Athletic Center of Athens, Kifissias 37, 15123 Maroussi, Greece

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Abstract

An excretion study of the β_2-agonist 7-[3-[(β-3,5-trihydroxyphenethyl)amino]-propyl]theophylline (reproterol) in human urine, which is reportedly misused by athletes and horses as a doping agent, is presented. The study was performed after an oral administration of 20 mg of reproterol hydrochloride. The collected urine samples were prepared using the standard anabolic steroid extraction procedure and analyzed by gas chromatography coupled with quadrupole mass spectrometry and, also, with high-resolution mass spectrometry (HRMS). The main reproterol metabolite was found, whereas unchanged reproterol was not detected. The structure of the main metabolite was confirmed by an accurate HRMS measurement of diagnostic ions. Finally, an excretion urine profile of the main metabolite is presented. The mass spectrum of another possible unidentified reproterol metabolite is also reported.

Keywords: Reproterol

1. Introduction

β_2-Agonists are a group of drugs designed for the treatment of pulmonary diseases. However, administration of such agents at high doses produces side effects on protein synthesis and lipolysis resulting in an anabolic effect [1]. The results from the doping controls conducted during sport events or in out of competition tests, revealed an increasing use by the athletes of the β_2-adrenoceptor agonists for apparent therapeutic treatment [2]. Therefore, the International Olympic Committee (IOC) has introduced several β_2-agonists in the list of prohibited classes of substances. Of the β_2-agonists, only salbutamol, salmeterol and terbutaline are allowed and only by inhalation, after prescription by a physician and declaration to the relevant medical authority prior to competition [3].

The detection of β_2-agonists in biological fluids is a difficult analytical problem due to the low detection limits at ng/ml levels. Many different methods have been described for their determination, but the approach of choice is gas or liquid chromatography with mass spectrometry (MS) [1,4–7].

Reproterol (7-[3-{(β-3,5-trihydroxyphenethyl)-amino}-propyl]theophylline), Fig. 1 (I), is a β_2-agonist with a similar to salbutamol effectiveness in controlling exercised-induced asthma [8], introduced this year in the list of IOC. Its molecule combines the structure of a β_2-agonist with a xanthine group. Its action as a xanthine has not yet been confirmed and only traces of theophylline have been detected in blood of subjects after administration [9]. Biotransformation of reproterol in animals and in man leads

*Corresponding author. Tel.: +30-1-686-8549; fax: +30-1-686-8549.
E-mail address: oaka@compulink.gr (C.G. Georgakopoulos)

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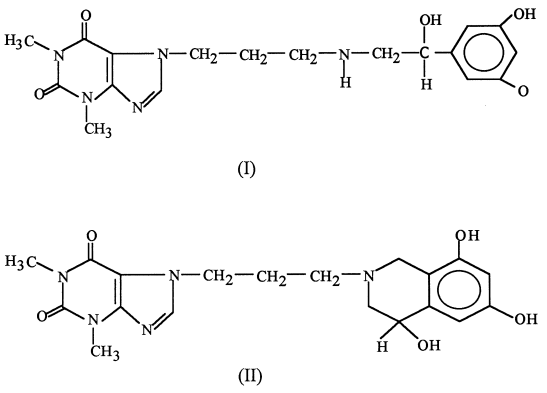

Fig. 1. Structures of reproterol (I) and reproterol metabolite (II).

to the same main metabolite, Fig. 1 (II), which has been shown to be a tetrahydroisoquinoline derivative, produced by uptake of an additional carbon atom with concomitant cyclisation [10]. At present, there is limited number of reports available on the metabolism and disposition of reproterol in human body [11,12]. In man, reproterol is rapidly distributed and eliminated. The parent compound is detected in plasma after oral administration of one tablet containing 20 mg reproterol·HCl and the highest concentration is reached within 2 h after oral administration and ranges between 2 and 6 ng/ml, depending on the subject [12]. Renal elimination in the dog and rabbit seems to be the main route of excretion, while in the rat there is 58% fecal excretion [11].

Analysis of reproterol and its metabolites in biological specimens has been performed by high-performance liquid chromatography (HPLC) in combination with UV absorbance [10], fluorescence [10], amperometric detection [13] and using radiometric methods [10], while a HPLC–atmospheric pressure chemical ionization (APCI)–tandem MS technique for the determination of reproterol in human plasma has been recently reported [14].

The possible use of reproterol as anabolic agent renders its detection in urine, during doping control-screening analysis, a necessary task. The aim of the present work was to study the excretion of reproterol in human urine after oral administration of a single dose of 20 mg reproterol·HCl and to report on the detection of the parent compound and its metabolites using gas chromatography–quadrupole MS (GC–MS) analysis and GC–high-resolution mass spectrometry (HRMS).

2. Experimental

2.1. Materials

Reproterol Bronchospasmin was a ASTA Medica (Frankfurt, Germany) product. A reproterol standard solution was prepared from the above formulation. All reagents and organic solvents used in the extraction procedures were of analytical grade. β-Glucuronidase from *Escherichia coli* (Boehringer, Mannheim, Germany) and β-glucuronidase/arylsulphatase from *Helix pomatia* (Sigma–Aldrich, Steinheim, Germany) were used for enzymatic hydrolysis. Derivatization reagents, *N*-methyl-*N*-trimethylsilyltrifluoro-acetamide (MSTFA), *N*-methylbis(trifluoroacetamide) (MBTFA), ammonium iodide (NH_4I) and dithioerithritol (DTE), were purchased from Macherey-Nagel (Düren, Germany). Perfluorokerosene (PFK) mass spectrometric grade (Apollon, Manchester, UK) was used as calibration standard in HRMS analysis.

2.2. Instrumentation

A Hewlett-Packard 6890 gas chromatograph coupled with a 5973 quadropole MS detection system with a crosslinked methylsilicone gum capillary column (17 m×0.200 mm I.D., 0.11 μm, HP Ultra1) was used. Helium, at a flow-rate of 0.8 ml/min, was used as carrier gas. A 3-μl volume of sample was injected in the split mode (1:10). Temperatures of the injector and the transfer line were set at 250 and 310°C, respectively. Initial oven temperature was 180°C, then ramped at 3°C/min to 235°C and at 30°C/min to 300°C and held for 3.15 min. The MS system was acquiring data in the full scan mode (mass range 80–800) and in the selected ion monitoring (SIM) mode for the screening procedures described in Section 2.6.

HRMS analysis was performed with a three-sector (electrostatic–magnetic–electrostatic) reverse geometry double focusing mass spectrometer (Autospec Micromass) coupled to a HP 6890 gas chromatograph. A HP Ultra1 column (12 m×0.200 mm I.D.,

0.33 μm) was employed with helium carrier gas (flow 1 ml/min). A 1-μl and 2-μl sample volume was injected (split 1:10) in the screening and in the accurate mass measurement analysis, respectively. The GC temperature was ramped as follows: 150°C for 0.5 min, 12.5°C/min to 310°C, 2.5 min final time. The injection port was heated at 250°C and the transfer line at 280°C. In the screening analysis of (I) and (II) the HRMS system was acquiring data in 10 000 resolution in the selected ion recording (SIR) mode. In the accurate mass measurement analysis, the HRMS system was acquiring data in the mass range m/z 450–650 using magnet scan in 7000 resolution.

2.3. Accurate mass measurement

The accurate mass measurement experiment was performed with the HRMS instrument in order to calculate the elemental composition of the diagnostic ions of (II). The calculations were based on the adjacent ions of the calibration reference substance PFK, which was simultaneously eluted in the ion source. The experiment comprised the following steps: (a) magnet scan acquisition in 1000 resolution of the sample with (II), together with the acquisition of PFK in order to adjust their abundances, (b) repetition of the (a) experiment in 7000–8000 resolution, in order to acquire the data to be used for the calculation, (c) searching of PFK ions in a chromatographic time range close to the elution time of (II) and creation of the calibration scale for the particular acquisition, (d) selection of a representative mass spectrum of (II), which had its ions accurately calculated and, finally, (e) determination of the elemental composition of the diagnostic ions.

2.4. Detection criteria

The minimum criteria necessary for the identification of compounds by MS techniques, as determined by the IOC, used in the present study are listed below.

(1) Chromatography. The retention time (t_R) or relative retention time (t_{RR}) of the analyte should not differ by more than 1% from that of the standard solution.

(2) Mass spectrometry. (a) For scan mode (low resolution) evaluation of a scan must include consideration of a minimum of three diagnostic ions. The signal-to-noise ratio of the diagnostic ions must be greater than three to one. The relative abundance of any of the ions shall not differ by more than 5% (absolute) or 20% (relative), whichever is greater, from that of the standard solution. (b) For SIM or SIR a minimum of three diagnostic ions must be monitored in a single mass spectrum. The signal-to-noise ratio of the diagnostic ions must be greater than three to one. The relative abundance of any of the ions shall not differ by more than 5% (absolute) or 20% (relative), whichever is greater, from that of the standard solution.

2.5. Drug administration

Twenty milligrams of reproterol hydrochloride (one tablet, Bronchospasmin) was orally administered to a healthy male volunteer. Urine samples were collected up to 72 h post-dose and stored at 4°C.

2.6. Sample preparation

For the screening procedure of anabolic steroids, excreted as free compounds and conjugated esters (combined fraction, procedure IV, [2,15]), 2.5 ml of urine were extracted with diethyl ether at pH 9.6, after enzymatic hydrolysis with β-glucuronidase from *E. coli* at pH 7.0. The dry extract was derivatised as described below.

In order to elucidate the conjugation of reproterol and its metabolites in human, detected after urine excretion, 5.0 ml of urine were extracted with diethylether prime hydrolysis, at the pH of urine and consecutively at alkaline pH. The pH was set to 9.6–10 using a 30% (w/v) K_2CO_3 solution. In the remaining urine, the pH was set to 7.0 using phosphate buffer and enzymatic hydrolysis with *E. coli* β-glucuronidase was performed, followed by alkaline extraction, as described above. Hydrolysis with β-glucuronidase/aryl sulphatase from *H. pomatia* at pH 5.0, using acetate buffer, was successively conducted on the urine sample, which had been previously extracted for unconjugated compounds and hydrolysed with *E. coli* for glucuronides. The hydrolysed sulphates were extracted under al-

kaline conditions as described above. All organic extracts were separately dried, derivatised and analysed.

Acidic hydrolysis [15] of urine sample was also performed using 6.0 *M* HCl at 100°C for 30 min and the diethylether alkaline extract was dried and derivatised as follows.

Derivatisation was accomplished using 100 μl of MSTFA–NH_4I–DTE (1000:2:4) and incubating at 60°C for 30 min. After heating, the *O*- and *N*-trimethylsilyl (-OTMS, -NTMS) derivatives of the compounds of interest were analysed by GC–MS.

3. Results and discussion

3.1. Analytical consideration

Reproterol (I) from a reproterol standard solution and its main metabolite (II) from an excretion study sample, analysed with procedure IV, were detected at retention times relative to methyltestosterone of 1.50 and 1.53, respectively. They eluted after stanozolol metabolite, 3′-OH-stanozolol tris-TMS.

Under the previously described derivatisation conditions, reproterol gives both the tri- (*m*/*z* 605, M^+)

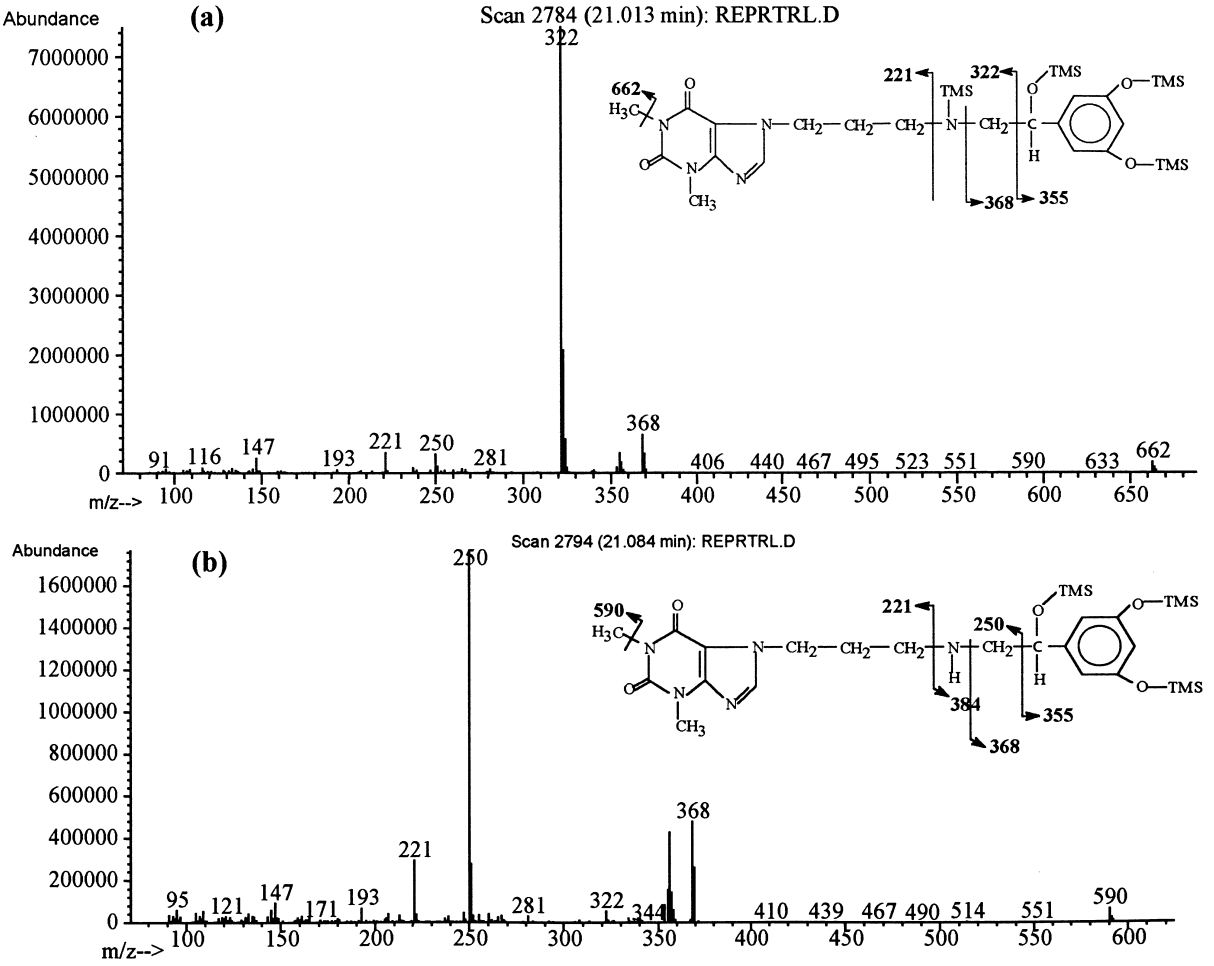

Fig. 2. Mass spectra and fragmentation pattern of tetra-TMS (a) and tri-TMS (b) derivatives of reproterol. Derivatisation was carried out as described in Section 2.6.

and tetra-TMS derivative (m/z 677, M^+) (Fig. 2). The fragmentation pattern of derivatised reproterol is similar to the fragmentation pattern of the underivatised molecule [10]. The most abundant ion in tri-TMS (m/z 250) and tetra-TMS derivative (m/z 322) is originated from cleavage of the hydroxy-methylene-dihydroxyphenyl group.

Reproterol main metabolite was identified as 2-[3-theophyllinyl(7)-propyl]-4, 6, 8-trihydroxy-1, 2, 3,4-tetrahydroisoquinoline from the mass spectrum of its tris-TMS derivative (m/z 617, M^+) (Fig. 3). Exact mass measurement of the rerpoterol metabolite (II) was carried out using the GC–HRMS and structural proposals were checked in order to confirm the

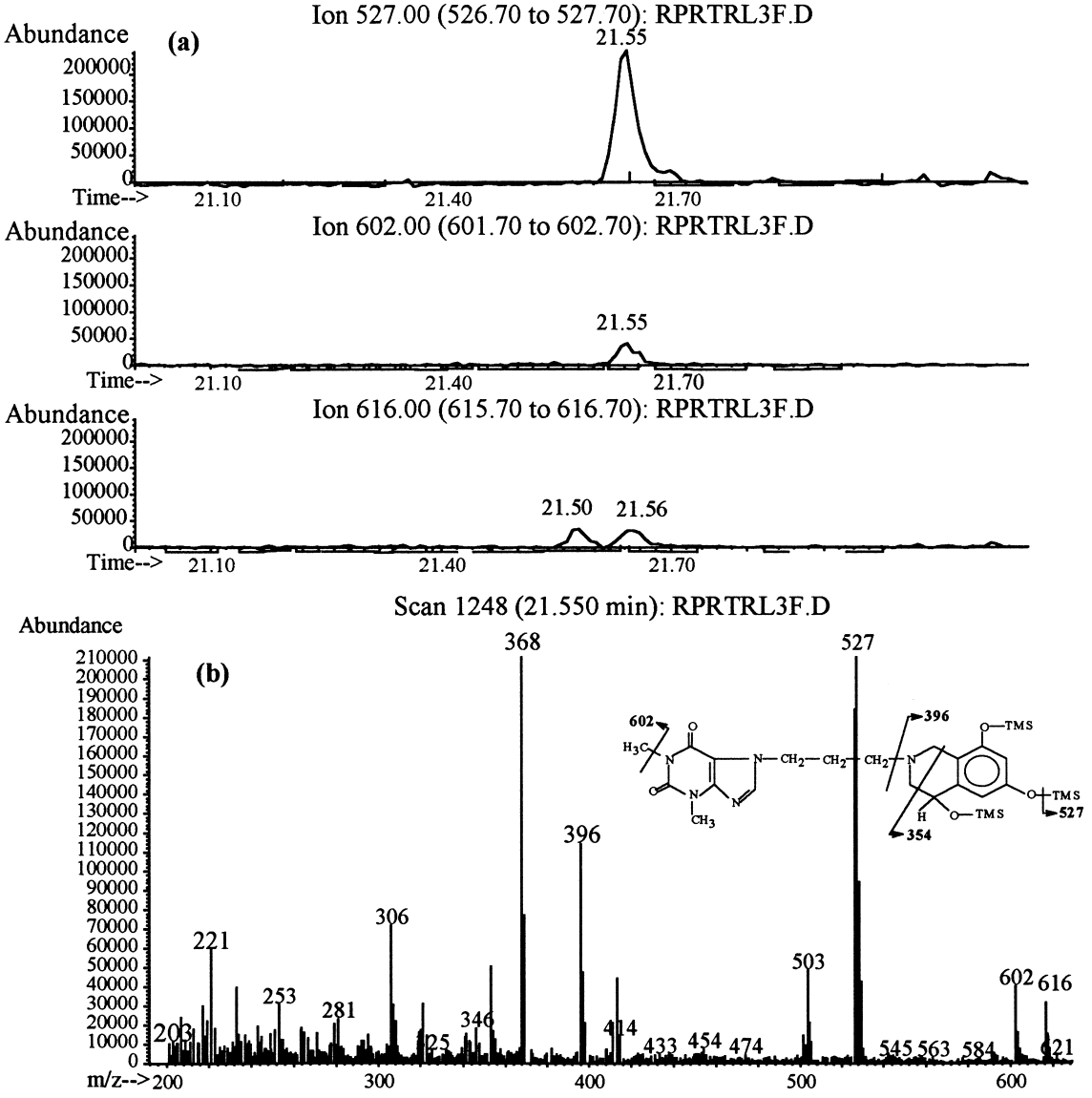

Fig. 3. Ion chromatograms obtained by scan mode (a) and mass spectrum (b) of tri-TMS derivative of reproterol metabolite (II) in urine sample taken from a healthy volunteer 3.8 h after oral administration of 20 mg reproterol·HCl. The sample had been prepared according to procedure IV – anabolic steroids combined fraction. Internal standard methyltestosterone. Relative retention time 1.53 min.

Table 1
Calculated data of the exact mass measurements of the reproterol metabolite (II) (experimental conditions are detailed in Section 2.3)

Calculated mass	Real mass	$\Delta m/m$[a] (ppm)	mDa	Structure				
				C	H	N	O	Si
617.2903	617.2885	−2.8	−1.8	28	47	5	5	3
616.2814	616.2807	−1.2	−0.7	28	46	5	5	3
602.2672	602.2650	−3.6	−2.2	27	44	5	5	3
527.2357	527.2384	5.1	2.4	25	37	5	4	2

[a] $\Delta m/m = \frac{\text{Real mss} - \text{calculated mass}}{\text{Real mass}} \times 10^6$

suggested elemental composition of (II). The comparison of experimental with proposed mass spectrum demonstrates differences below 10 ppm (ppm= $\Delta m/m$). The results are summarised in Table 1. In Fig. 4, the full mass spectrum (m/z 450–650) of (II) in the accurate mass measurement experiment is shown, after subtraction of the PFK calibration reference peaks.

In the m/z 616 ion chromatogram shown in Fig. 3, a peak with retention time 21.50 min was observed having a mass spectrum shown in Fig. 5. Accurate mass measurement in the spectrum of the above

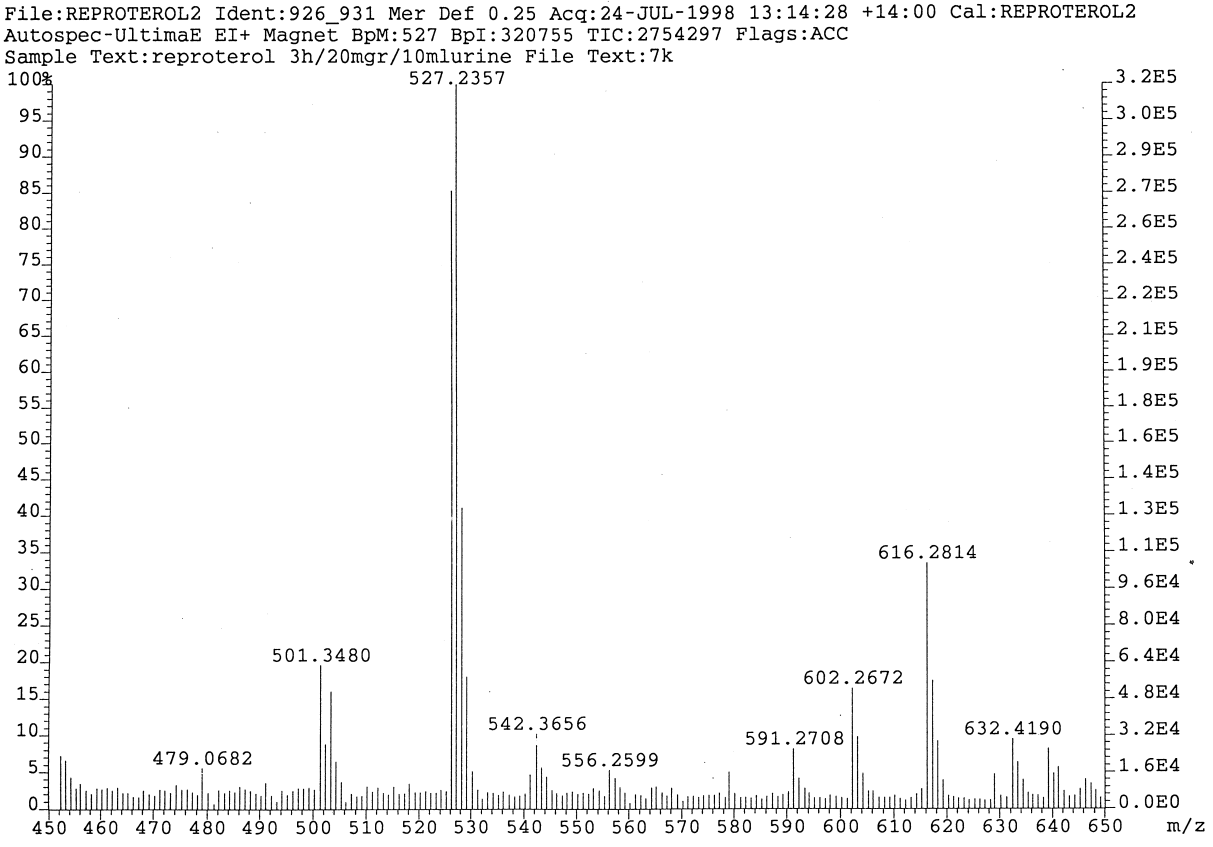

Fig. 4. Full mass spectrum of reproterol metabolite (II) in the accurate mass measurement experiment after substraction of the PFK calibration reference peaks.

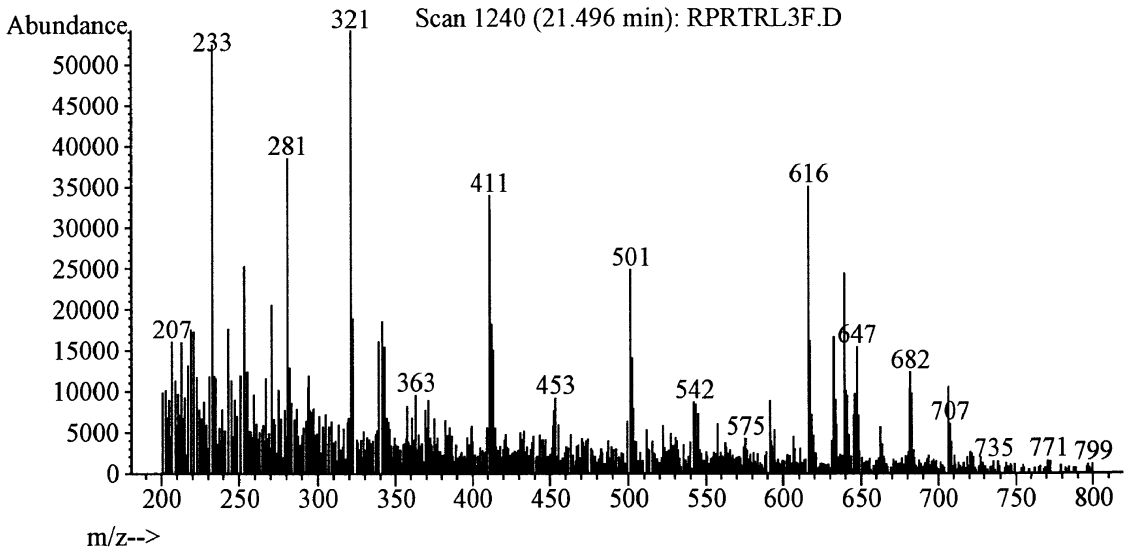

Fig. 5. Full mass spectrum of a *n*-TMS derivative of a possible unidentified metabolite of reproterol. Retention time 21.50 min. The sample had been prepared according to procedure IV – anabolic steroids combined fraction.

mentioned chromatographic peak resulted in the same atomic composition for m/z 616.2807 as (II). Therefore, it is concluded that this mass spectrum corresponds to another, yet unidentified, metabolite of reproterol, which displays an excretion pattern similar to (II).

3.2. Excretion in human urine

Reproterol was secreted in human urine as metabolite (II), mainly conjugated as glucuronide (70%) and sulphate ester (30%). Trace amounts of unconjugated metabolite (II) (4%) were detected after extraction at the urinary pH, while no traces of (II) could be detected after acidic hydrolysis. Compound (I) was not detected in any fraction in the GC–MS and GC–HRMS screening.

The excretion profile after oral administration of 20 mg reproterol·HCl is shown in Fig. 6, after analysis of the excretion study urine samples according to procedure IV and detection by GC–MS. The % secreted reproterol metabolite (II) was determined based on the maximum secreted amount of (II), as calculated from chromatographic data. Metabolite (II) was detected by GC–HRMS for more than 60 h post-administration. With regard to (II) pharmacokinetic features, reproterol could be classified as a ''highly polar'' β_2-agonist characterised by high therapeutical oral doses and short urine half-life [1].

Finally, when the standard reproterol solution was analysed by GC–HRMS, traces of (II) were detected, probably existing as an impurity.

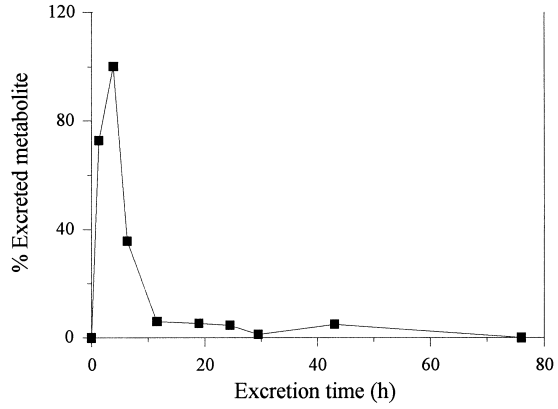

Fig. 6. Percent secreted reproterol metabolite (II)–time profile after oral administration of a single dose of 20 mg reproterol hydrochloride to a healthy volunteer. Analysis of the excretion study urine samples was performed according to procedure IV and detection by GC–MS. The % secreted reproterol metabolite (II) was determined based on the maximum secreted amount of (II), as calculated by chromatographic data.

4. Conclusions

In this paper, the excretion profile of reproterol after oral administration of 20 mg of reproterol·HCl is presented. The main reproterol metabolite was found, whereas unchanged reproterol was not detected. The structure of the main metabolite was confirmed with an accurate HRMS measurement of diagnostic ions. Reproterol main metabolite was secreted both as glucuronide and sulphate esters and can be detected after analysis with the screening procedure of anabolic steroids – combined fraction by GC–MS for 45 h and by GC–HRMS for more than 60 h post-administration. The mass spectrum of another possible unidentified reproterol metabolite is also reported.

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