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The detection of the administration of salmeterol in urine after inhalation

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Introduction

The discussion regarding the use of β 2-adrenoceptor agonists in sports has regained new interests in doping analysis after the discovery of the abuse of clenbuterol in 1993 and the apparent increase of therapeutic use of salbutamol. Because of this, the IOC list of doping classes and methods underwent several changes (Table 1) and as the discussion is still going on future adaptations are still not unlikely. The introduction of long acting β 2-adrenoceptor agonists in the nineties and its gaining importance in the treatment of asthma in general and exercise-induced asthma in particular, led to the 1996 adaptation.

Table 1 Adaptions in the IOC list of doping classes and methods regarding β 2-adrenoceptor agonists since 1989

Year	Adaption
1989	The use of only the following β 2-agonists is permitted in the aerosol form: <i>bitolterol</i> , <i>orciprenaline</i> , <i>rimiterol</i> , <i>salbutamol</i> , <i>terbutaline</i> .
1993	The use of <i>bitolterol</i> , <i>orciprenaline</i> and <i>rimiterol</i> is withdrawn and they are therefore banned. <i>Salbutamol</i> and <i>terbutaline</i> remain the permitted β 2-agonists by inhalation only. β 2-Agonists are also classified as anabolic agents (e.g. <i>clenbuterol</i>).
1995	Of the β 2-agonists only <i>salbutamol</i> and <i>terbutaline</i> are permitted and only by inhalation. Any physician wishing to administer β 2-agonists by inhalation must give written notification to the relevant medical authority. When given systemically, may have powerful anabolic effects, and their use is therefore banned or restricted (e.g. <i>clenbuterol</i>).
1996	<i>Salbutamol</i> , <i>salmeterol</i> and <i>terbutaline</i> are permitted by inhalation only and must be declared in writing, prior to competition, to the relevant medical authority.

Salmeterol is one of the long acting β 2-adrenoceptor agonists [1], which are available commercially. The structure of salmeterol is similar to salbutamol and consists, compared to the salbutamol structure, of an additional extended side-chain. It is manufactured by GlaxoWellcome and is administered by means of an aerosol in the form of salmeterol xinafoate at 50 μ g dosages. The molecular structures of salmeterol and as well as of xinafoate (also known as 1-hydroxy-2-naphtoic acid) are shown in Figure 1.

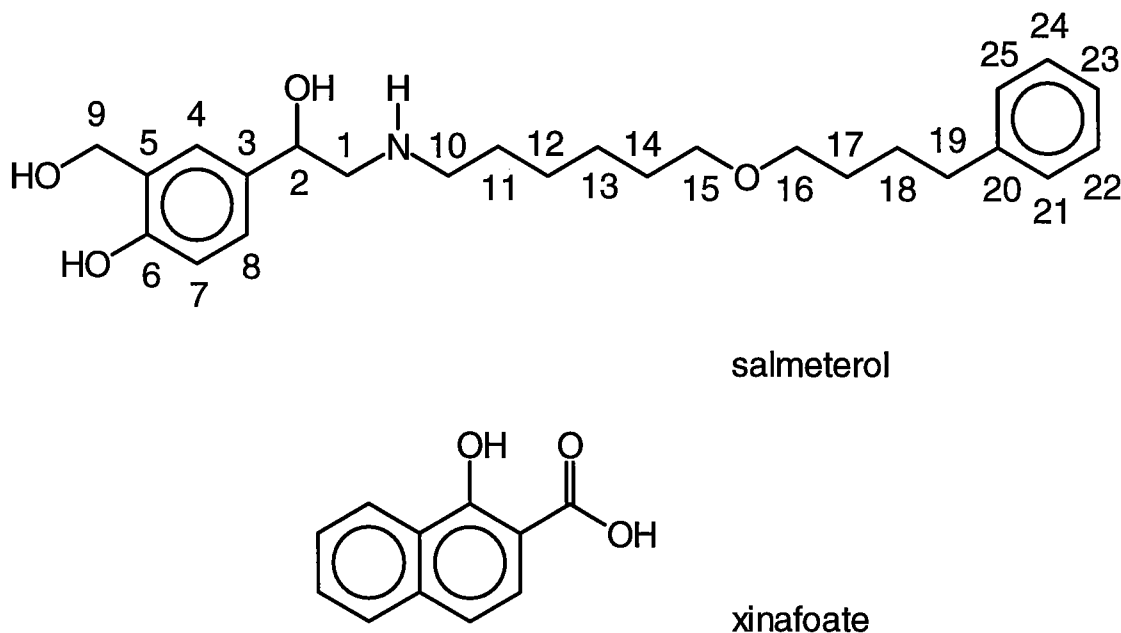


Figure 1: Molecular structures of salmeterol and xinafoate

At present there is a limited number of reports available in literature about the metabolism and disposition of salmeterol xinafoate in the human body [2]. The metabolism of salmeterol xinafoate in humans (Figure 2) has been described by Manchee *et al.* [2]. Biliary excretion of radioactive material accounted for 57% of a dosage, whereas in urine 23% was recovered. Unchanged salmeterol in urine and in bile accounts for <5% of the excreted dose. The main metabolite in feces is an alcohol metabolite, which is formed after aliphatic oxidation at 19-C-position on the phenylbutoxy side chain (see also Figure 1). In urine the presence of non-conjugated and/or glucuronic conjugate of the *O*-dealkylated salmeterol, the acidic metabolite, was reported.

Even less reports are describing the analysis of the respective metabolites in biological specimens [2,3]. The administration of salmeterol xinafoate can also be detected indirectly in urine by measuring xinafoate in plasma [4]. This study evaluates the

possibility of the direct and indirect detection of the administration of salmeterol in urine after inhalation using common applied doping analytical procedures.

Materials and methods

Materials

Salmeterol (1-[4-hydroxy-3-hydroxymethylphenyl]-2-[6-phenylbutoxy-hexylamino]-ethanol), the alcohol metabolite of salmeterol (1-[4-hydroxy-3-hydroxymethylphenyl]-2-[6-[2-hydroxy-1-phenyl-butoxy]-hexylamino]-ethanol) and the acidic metabolite of salmeterol (1-[4-hydroxy-3-hydroxymethylphenyl]-2-[hexanoic]-ethanol) were a generous gift of GlaxoWellcome. All reagents and other chemical are the usual products as used in common applied doping analytical procedures.

Collection of urine

A healthy male volunteer inhaled one dosage of 50 µg of salmeterol xinafoate using the Servent® diskhaler (GlaxoWellcome). Besides a blank urine sample, urine samples were collected at several time intervals during 18 h.

Isolation of compounds of interest and derivatization

All used glass ware was silanized. To five ml of urine bevantolol was added as internal standard. The urine (not corrected for pH) was applied on a XAD-2 column in order to remove anorganic ions and to isolate all compounds of interest. Enzymatic hydrolysis was performed with a crude mixture of arylsulphatase and glucuronidase form *Helix pomatia* in acetate buffer at pH 5.2. A second XAD-2 extraction (no pH correction) was then again applied to isolate the non-conjugated and deconjugated compounds of interest. The compounds were eluted with methanol. The methanol was removed at 55°C under a flow of nitrogen. The residue was dried under reduced pressure in the presence of phosphorous pentoxide and potassium hydroxide for at least a hour. The dry residue was derivatized at 80°C for 40 min using a mixture of MSTFA/NH₄/ethanethiol (1000:2:3; v/w/v).

GC/MS analysis

Electron Impact GC/MS analysis was performed by a 5790 GC coupled to a 5970 MSD of

Hewlett Packard. The full scan mode was operated in the range of m/z 50 - 800 and the SIM mode according groups of ions as displayed in Table 2.

Compound	Group	Ions measured
xinafoate	1	243.0/ 317.1/ 332.2
clenbuterol		300.1/ 335.0/ 405.2
salbutamol		294.1/ 369.1/ 440.3
acidic metabolite	2	288.1/ 369.1/ 642.4
bevantolol	3	266.1/ 338.2/ 474.3
salmeterol	4	262.2/ 334.1/ 369.1/ 613.3
alcohol metabolite	5	260.1/ 350.2/ 369.1/ 422.2

Results and discussion

The isolation procedure in this study was designed in such a way that salmeterol, its acidic and alcohol metabolite, and xinafoate were extracted in one step and that in principal the obtained residue contained all compounds of interest. The first XAD-2 extraction is essential to remove anorganic ions which may inhibited the applied sulfatase. The second XAD-2 extraction was chosen, because it extracted at the respective pH all the compounds of interest in one step.

The derivatization procedure, which normally is applied normally for the TMS-enolTMS derivatization of conjugated or combined fraction of steroids, was the only successful procedure applied. Other derivatization procedures were not successful. Under the conditions studied the derivatization of the compounds of interest was optimized regarding time of derivatization and was almost complete. Small amounts of tri-TMS and tetra-TMS products of salmeterol and its alcohol metabolite still could be observed, respectively. Instrumental limits of detection for the compounds of interest were in the range of 5 ng absolute on the GC-column in the SIM mode.

The mass spectra of the derivatives of the compounds of interest are presented in Figure 3-6. Prominent fragmentation occurs between the 2-C-position and N (see also Figure 1) in the derivatives of both salmeterol and its metabolites. The fragment at m/z

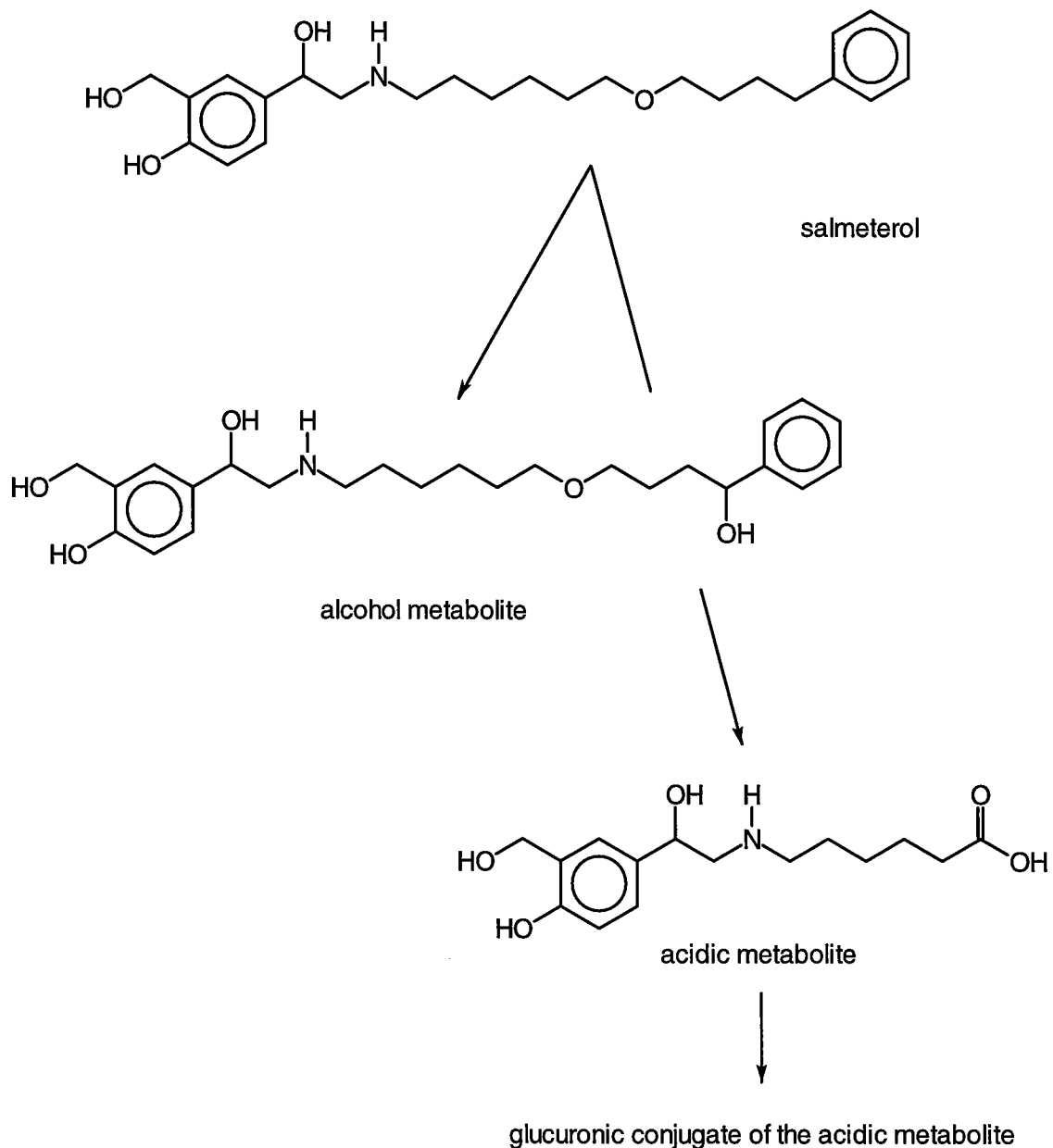


Figure 2: Metabolism of salmeterol in humans [2]

369 is also characteristic for the tri-TMS derivative of salbutamol. The side chain also produces in the derivatives of salmeterol and its metabolites a prominent fragment also after fragmentation between the 2-C-position and N (see also Figure 1). The tetra-TMS derivative of salmeterol shows ions at m/z 613 and 598 with low abundances, being the $[M - \text{TMSOH}]^+$ and $[M - \text{CH}_3 - \text{TMSOH}]^+$ ions, respectively. The penta-TMS derivative of the acidic metabolite also shows an ion at m/z 642 with a low abundance, being the $[M$

- CH₃]⁺ ion. In the mass spectrum of the di-TMS derivative of xinafoate the [M - CH₃]⁺ ion at *m/z* 317 is dominant, whereas the molecular ion at *m/z* 332 has a low abundance.

Analyzing the collected urine samples in the SIM mode, only in the sample collected between 9 and 18 h (overnight) after inhalation a trace of the acidic metabolite at the correct relative retention time was observed (Figure 8). In the blank urine no such peak was seen (Figure 7). However, correct identification of the acidic metabolite was not possible when based on at least 4 ions, because of the fact that only the major ion of the derivative of the acidic metabolite was visible in the chromatogram. None of the other compounds analyzed for were found. The results in this study are therefore, in agreement with literature [2], although the excretion in urine and metabolism of xinafoate is still unknown.

Conclusions

1. The acidic metabolite can be detected in urine after inhalation of 50 µg dosage of salmeterol xinafoate.
2. Using a low resolution mass spectrometer in the Electron Impact SIM mode under the studied circumstances identification of the acidic metabolite according to current criteria was impossible.

Acknowledgement

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References

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Figure 3: Mass spectrum of the tetra-TMS derivative of salmeterol

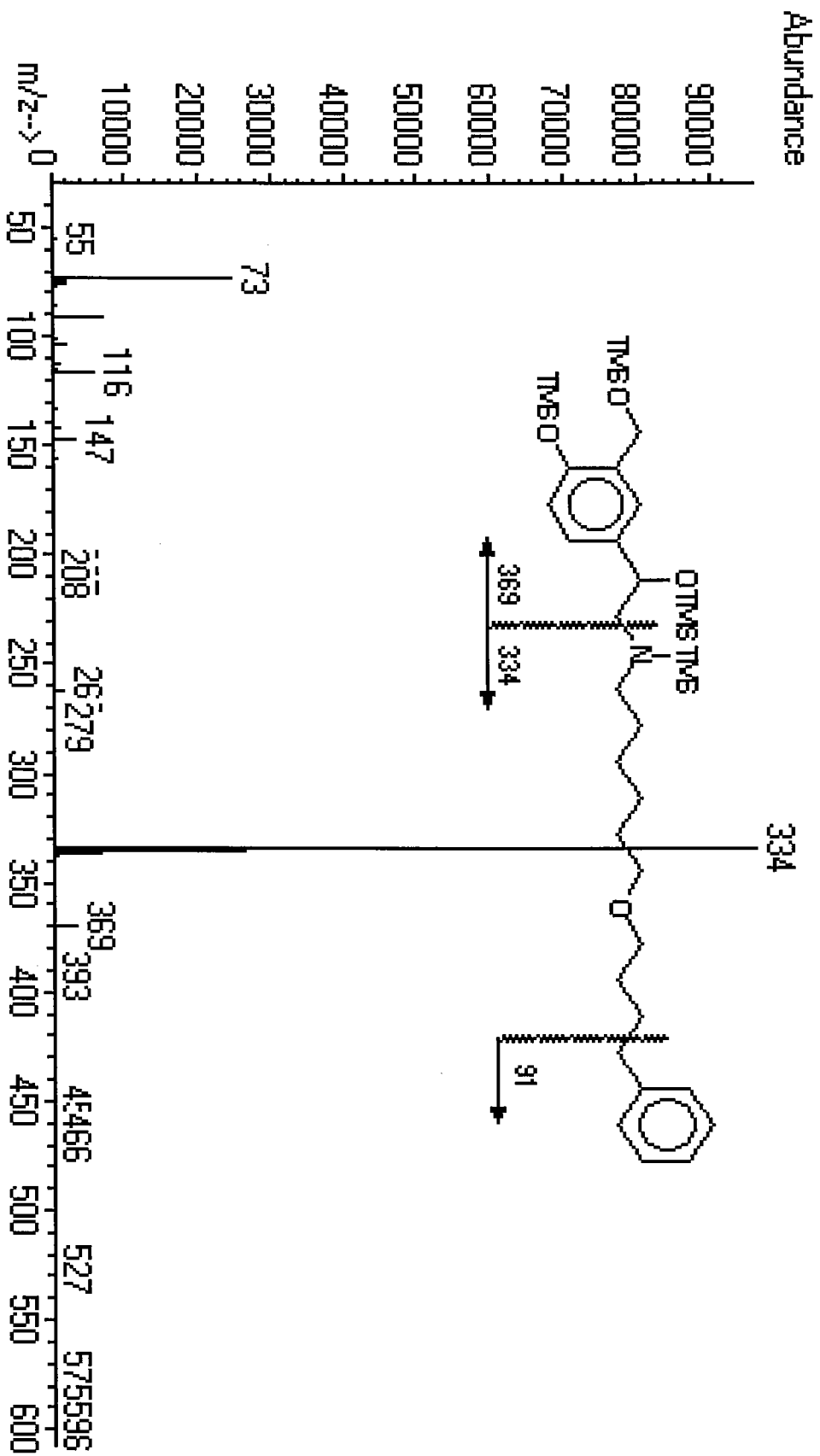


Figure 4: Mass spectrum of the penta-TMS derivative of the acidic metabolite of salmeterol

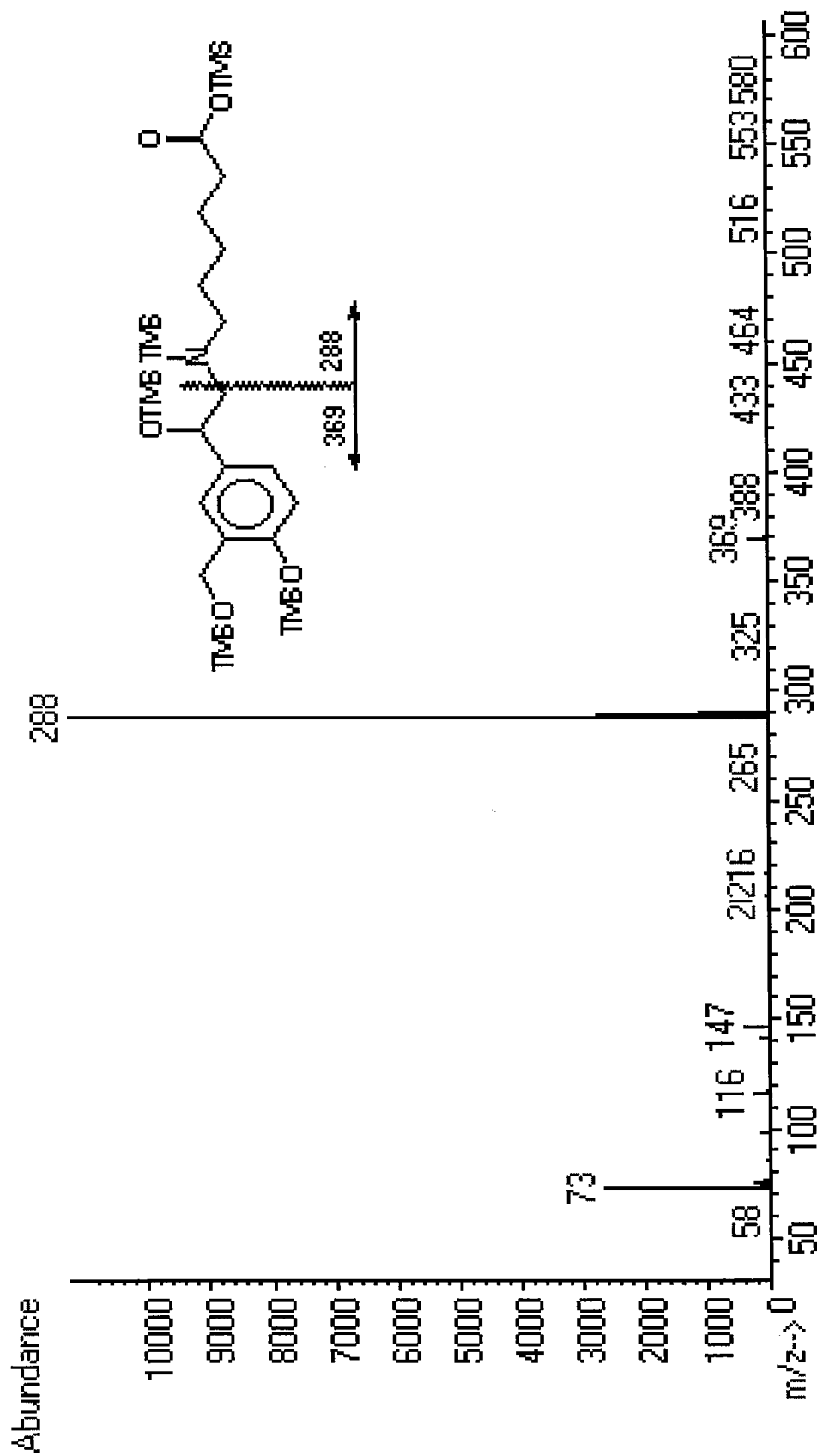


Figure 5: Mass spectrum of the penta-TMS derivative of the alcohol metabolite of salmeterol

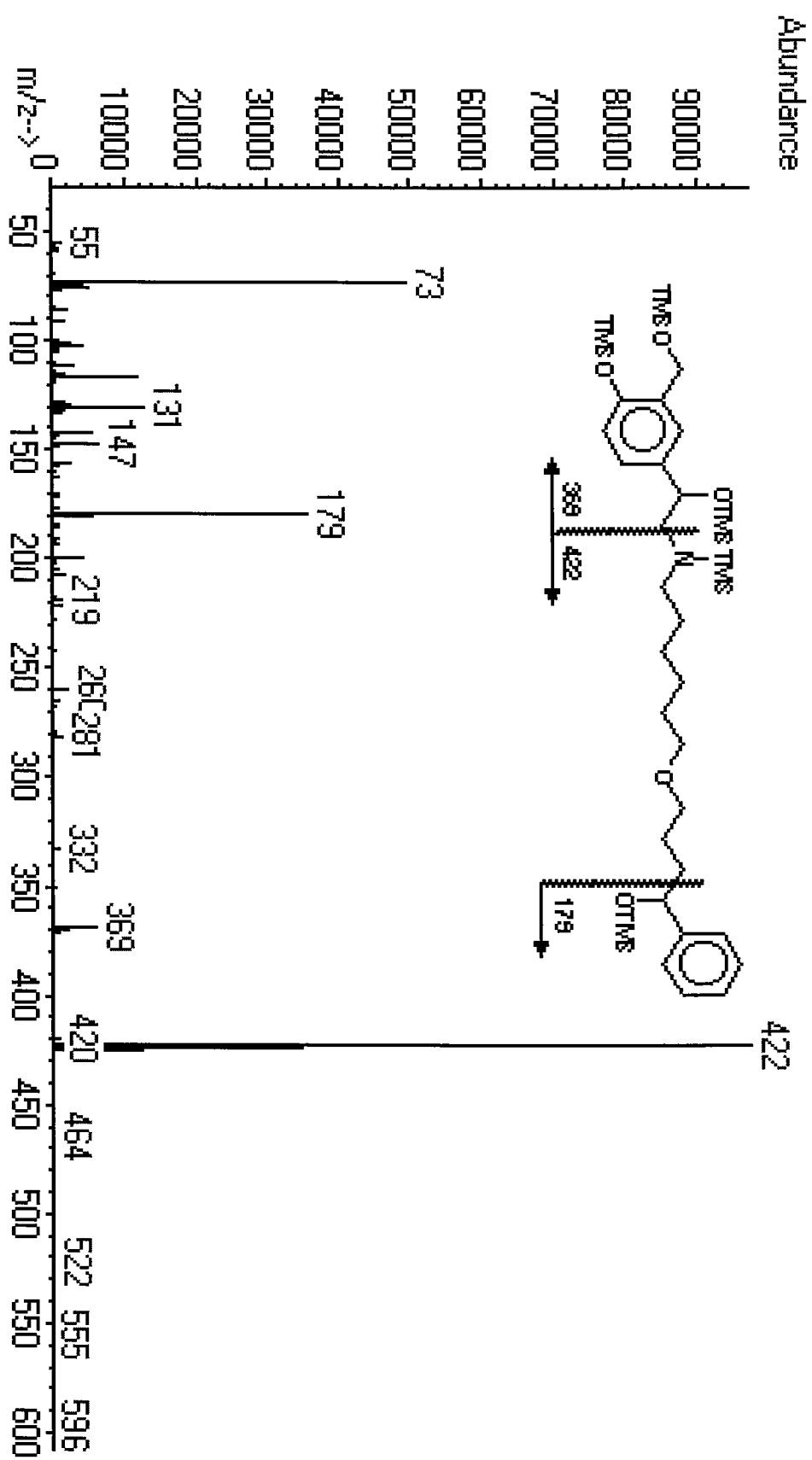


Figure 6: Mass spectrum of the bis-TMS derivative of xinafoate

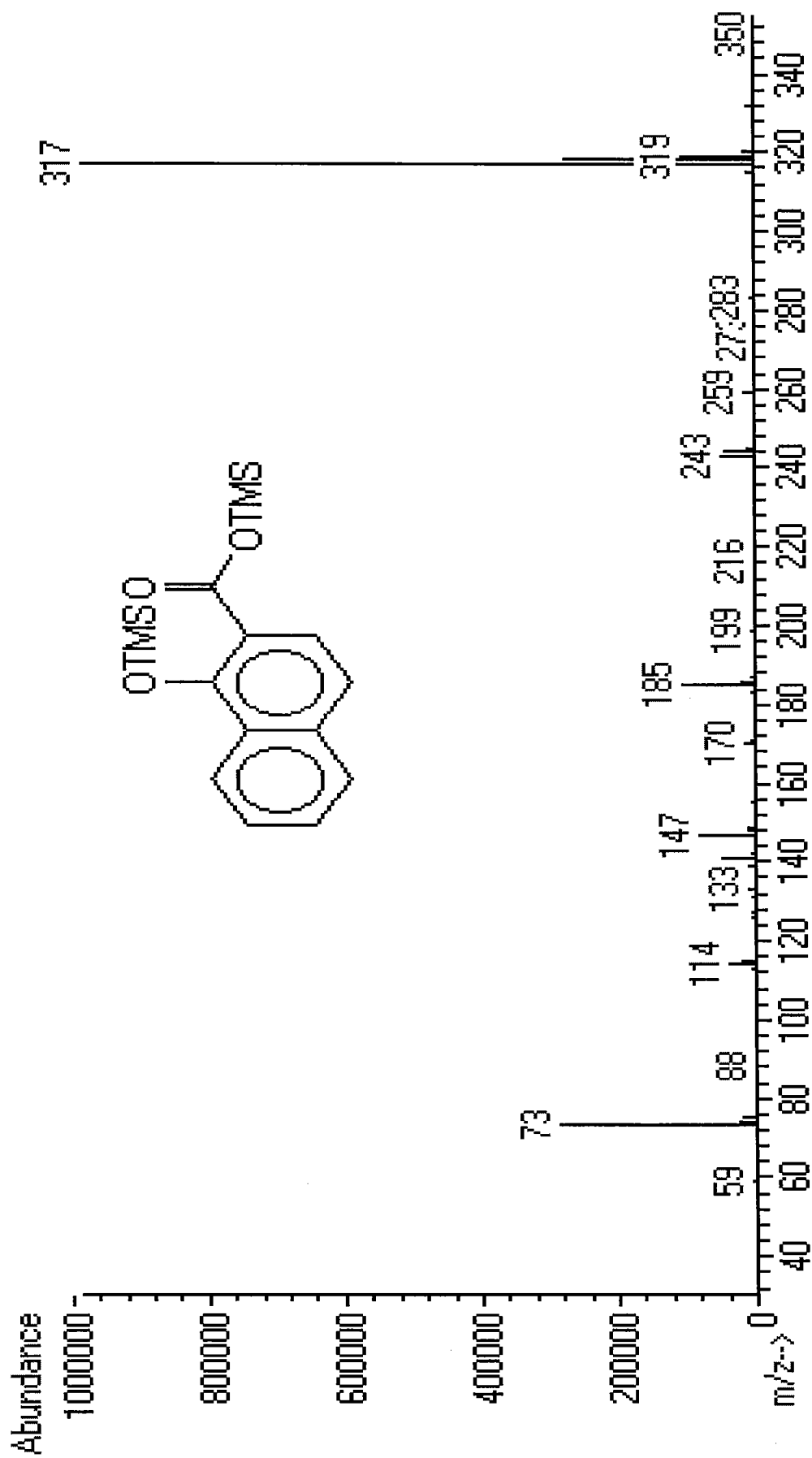


Figure 7: Selected ion chromatogram of the blank urine at m/z 288.1

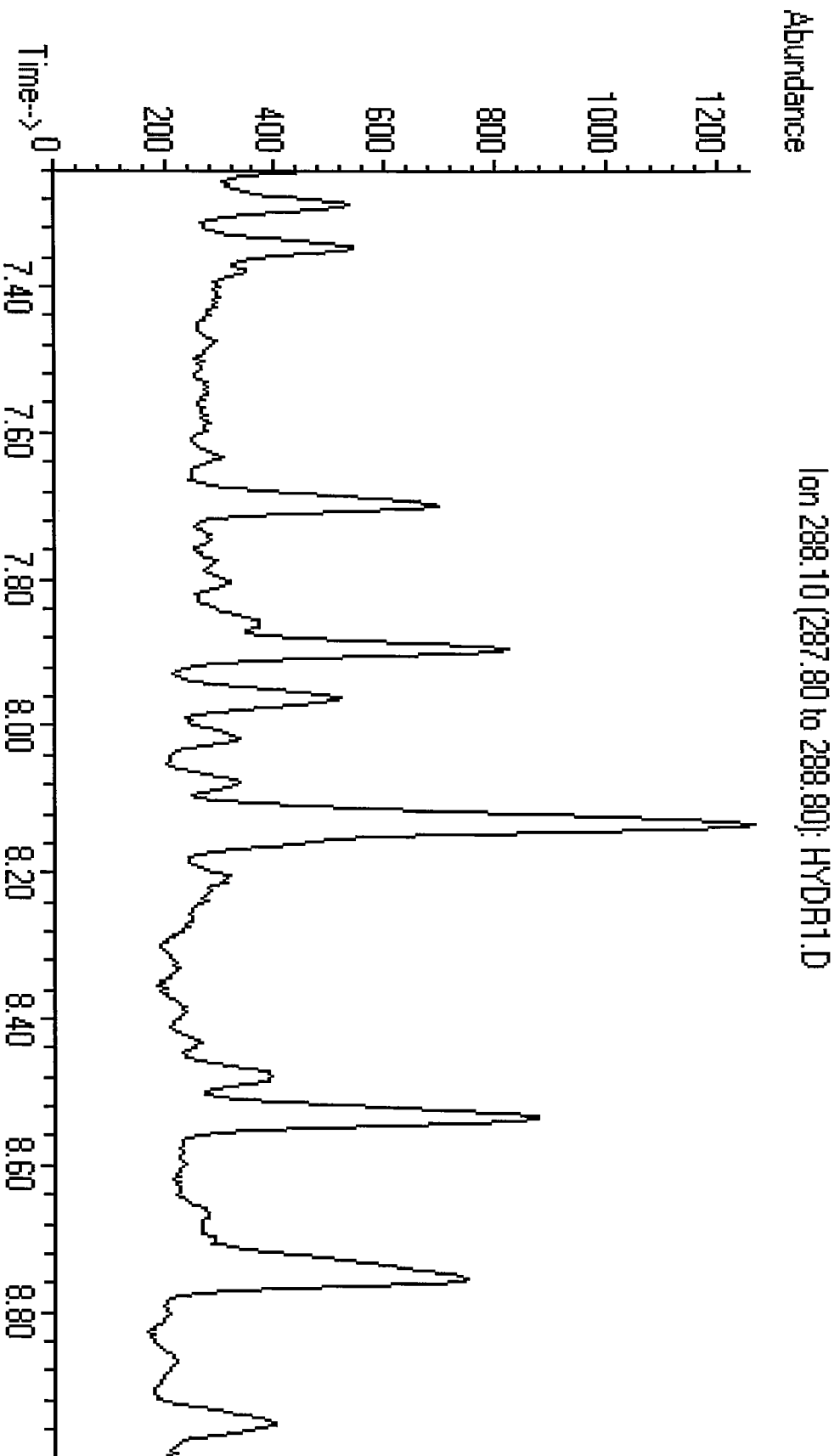


Figure 8: Selected ion chromatogram of the urine sample collected between 9 and 18 h (overnight) after inhalation at m/z 288.1

