Reprint from

RECENT ADVANCES IN DOPING ANALYSIS (11)

W. Schänzer
H. Geyer
A. Gotzmann
U. Mareck
(Editors)

Sport und Buch Strauß, Köln, 2003

SHEN LI, CUI KAIRONG, WU MOUTIAN:
Screening and Confirmatory Analysis of Fenoterol, Bambuterol and its Metabolites in Human Urine by GC-MS
In: W. Schänzer, H. Geyer, A. Gotzmann, U. Mareck (eds.) Recent advances in doping analysis (11). Sport und Buch Strauß, Köln, (2003) 101-107

Shen Li, Cui Kairong and Wu Moutian

Screening and confirmatory analysis of Fenoterol, Bambuterol and its metabolites in human urine by GC-MS

China Doping Control Center, Beijing 100029, China

INTRODUCTION

Recent reports [1,2,3,4] on the misuse of β_2-agonists, both as stimulants and as "anabolic agents" in sports, highlight the importance of screen and confirmation methods for these compounds in anti-doping control procedures. Previously, most of the work in this field has been focus on clenbuterol, salbutamol and terbutaline by GC-MS or HPLC-MS.

In our laboratory, GC-MS method after derivatization, although requiring laborious and time-consuming sample preparation, is still the method of choice for the analysis of β_2-agonists. Bambuterol (BAM) and fenoterol (FEN) are two of these drugs banned by IOC/WADA .A practical and reliable methods for the screening and confirmation analysis for these two drugs is needed for doping control.

Due to the lack of volatility of these compounds, the derivatization is a critical step in GC-MS analysis to improve the poor gas chromatographic performance.

Because these drugs are excreted, both conjugated and unconjugated, or changed to their metabolites, a hydrolysis procedure is necessary in order to dissociate the compounds. As a result the search for metabolites is also very important.

A highly sensitive method for the screening and confirmation analysis of fenoterol, bambuterol and its metabolites in human urine is described. Following acid hydrolysis, the drugs and metabolites are extracted with ether/isopropanol (9/1) and derivatized with MSTFA/MBTFA. The derivatives are analyzed by capillary gas chromatography-mass spectrometry. The limits of detection for fenoterol and bambuterol are 0.25ng and 0.50ng. This method has been used for routine analysis of urine samples in our laboratory.

Excretion curves of these two drugs after oral administration are also presented in this paper.

EXPERIMENTAL

Instruments and Reagents

HP5972/HP5890II GC/MSD

Column: 25m×0.2mm×0.33μm HP-5 cross-linked capillary column.

Injector temperature: 250°C Transfer line temperature: 290 °C

Oven temperature program: 180°C-10°C/min→220°C -5°C/min →260°C-10°C/min →280°C

Fenoterol HBr standard and Bambec, containing bambuterol hydrochloride (Astra. Sweden) after adjusting PH to 9.6, both were extracted then used as standard solution

OUYA-RP18 (Unicorn, Switzerland) was made into a slurry with water and poured onto a column of 10 cm in length with diameter of 10 mm to make a bed of 30mm in height. The column was washed with methanol and distilled water successively. This column was used for solid phase extraction.

MSTFA, MBTFA, TMSI and Dithioerythritol (Sigma, USA); L-Cysteine (Merck, Germany); β-Glucuronidase (E.Coli, Sigma, USA)

Sample preparation

Collection of Positive Urine

All urine samples of healthy male volunteers within 48 hours were collected periodically after oral administration with a single dose and stored at -20°C. The dosage of fenoterol HBr was 7.5mg and that of Bambec was 20mg.

Sample preparation procedures

We have compared four sample preparation procedures. 1. An aliquot of 5ml urine sample, add 2g of basic solid buffer (sodium bicarbonate: potassium bicarbonate 3:2, pH 9.6), 3ml mixture of ether/isopropanol（9：1）, shake for 10min, centrifuge for 5min, evaporate the organic phase to dryness under nitrogen at 60 °C, add 100μl of MSTFA, heat at 70°C for 10 min then, add 30μl of MBTFA, heat at 70°C for 10min(F/M method). 2. An aliquot of 5ml urine sample, add 100mg of cysteine, 0.5ml of concentrated HCl, heat at 100°C for 30 min, wash with 3ml of diethyl ether, then extracted and derivatized as the same procedure of above (C/M method). 3. An aliquot of 5ml urine sample, apply to the RP18 column, wash with 5ml of purified water, elute with 2ml of methanol, rotary evaporate to dryness, redissolve in 1ml of buffer solution (35.8g of $Na_2HPO_4 \cdot 12H_2O$ and 15.6g of $NaH_2PO_4 \cdot 2H_2O$ are added to 1 liter of distilled water, the pH value of the solution is 6.8), add 100mg of solid buffer (sodium bicarbonate: potassium bicarbonate 9:1 pH 8.8) and 5ml of ter-butylmethyl-ether, shake for 10min, centrifuge for 5min, collect the organic phase and evaporate to dryness under nitrogen at 60 °C, add 100μl of MSTFA/TMSI/ Dithioerythritol (1000/3/1), heat at 70°C for 10min (F/I method). 4. An aliquot of 5ml urine sample, apply to the RP18 column, wash with 5ml of purified water, elute with 2ml of methanol, rotary evaporate to dryness, redissolve in 1ml of buffer solution (pH 6.8), add 100μl of β-Glucuronidase (4000 unit), incubate at 55°C for 3hours, add 100mg of solid buffer (pH8.8) and 5ml of ter-butyl-methyl-ether, shake for 10min, centrifuge for 5min, collect the organic phase and evaporate to dryness under nitrogen at 60 °C, add 100μl of MSTFA/TMSI, heat at 70°C for 10min. (C/I method).

Excretion Studies

All-positive urine samples were prepared using the acid hydrolysis liquid-liquid extraction combined mixed derivatization (C/M method). We obtained the excretion curves of fenoterol, bambuterol and its two metabolites. Using the same method, we also obtained the limits of detection and the recoveries.

RESULTS AND DISCUSSION

The comparison of different derivatizations

The structures of fenoterol and bambuterol are shown in figure 1. On the basis of their molecular structures, the EI mass spectra of their TMS or TMS-TFA-derivatives should show a base peak formed by α-cleavage as shown in the figures below.

Fenoterol: Two peaks were found when it was derivatized with MSTFA/TMSI. One is the tetra-O-TMS derivative. Its mass spectrum and possible cleavage pathway is shown in figure 2. The other is tetra-O-TMS-N-TMS derivative. Fig.3 is shown as the spectrum and its proposed cleavage pathway. Only one derivative was detected when MSTFA/MBTFA method was used. The mass spectrum of tetra-O-TMS-N-TFA derivative and its proposed cleavage pathway are shown in figure 4.

The sensitivity of the mixed derivatization was better than that of TMS derivative (Table 1) due to

a single derivative being obtained.

Bambuterol: This only gave the mono-O-TMS derivative. The peak area is about two times higher when MSTFA/TMSI/ Dithioerythritol was used (Table 1).

The mass spectrum and the possible cleavage pathway are shown in figure 5.

The comparison of preparing procedures and metabolite studies

No compound was found when we used direct extraction and enzymatic hydrolysis for the fenoterol positive urine. The peak of fenoterol was detected only when the acid hydrolysis procedure (C/M method) was adopted. This means that it must be a sulphate not a glucuronide. No metabolite was detected.

Bambuterol is described in the literature [5] as a prodrug of terbutaline (TER). There are two dimethyl-amino-formoxyl groups in the structure instead of the bis hydroxyl groups in the molecular of terbutaline. About 20% of an oral dose of bambuterol is absorbed. After absorption, bambuterol is slowly metabolized via hydrolysis (plasma cholinesterase) and oxidation to active terbutaline. About 10% of administered dose is converted to terbutaline. The parent drug and its metabolites are mainly excreted via the kidneys.

The metabolism of bambec is shown in figure 6. BAM and its two main metabolites MI and MII (TER) could be derivatized with mono-TMS, bis-TMS and tri-TMS respectively. BAM and TER could be easily identified via the relative retention times and the mass spectra. The mass spectrum and the possible cleavage pathway of MI are shown in figure 7. The mass spectrum and the possible cleavage pathway of MII (terbutaline) are shown in figure 8.

As shown in table 2, we found:

(1) Bambuterol, MI and TER were all detected by every procedure; and after enzymatic hydrolysis, the concentrations of these three substances increased. Bambuterol, MI and TER are excreted both by conjugated and unconjugated form. Using the method of liquid-liquid extraction after acid hydrolysis, the abundance of bambuterol was decreased. It appears the strong acid hydrolysis destroys bambuterol by converted it to MI and MII in vitro. Another experiment was performed to study whether or not this conversion should occur and how much the conversion ratio would be.

The bambuterol standard with the same concentration were added to blank urine and processed two different procedures. Using liquid-liquid direct extraction, only bambuterol itself was detected; whereas bambuterol and MI were both found after acid hydrolysis. Comparing the peak areas between direct extraction and extraction after acid hydrolysis, we calculated that 24% of bambuterol was destroyed by acid and 35% was hydrolyzed to metabolite one (Table 3).

The result of solid phase extractions with the RP C18 column in our work was not satisfactory.

(2) MI should also be converted to terbutaline by acid hydrolysis in vitro. As we do not have a standard of MI, this assumption could not be proved.

(3) As described above, of the administrated dose of bambuterol, about 10% is converted to terbutaline. But when the urine sample was processed using acid hydrolysis liquid-liquid extraction, the concentration of terbutaline was much higher than this ratio.

The excretion curves

The positive urine samples of fenoterol were prepared using the acid hydrolysis procedure, monitoring m/z ion 355, the excretion curve was plotted using peak area verse time (Fig 9). We found the metabolism of fenoterol was fast. The largest concentration of fenoterol in urine was at two hours after oral administration. Using the procedure mentioned above, fenoterol could be detected within 48 hours.

Using the same method, we obtained the excretion curves of bambuterol and its two metabolites at m/z 86 (Fig 10). The highest concentrations of bambuterol, MI and terbutaline in urine appeared at 3 hours, 8.5 hours and 3 hours after oral administration. Because of the in vitro hydrolysis of bambuterol and metabolite one, these curves may be not reflecting the actual concentrations of these substances in urine. The parent drug and its two metabolites could be detected within 48 hours by this method.

The recoveries of the method of liquid-liquid extraction after acid hydrolysis were 73.15% for fenoterol and 73.52% for bambuterol. Limit of detection using 5ml urine was 1.65ng/mL and 3.2ng/mL respectively.

CONCLUSION

The procedure using acid hydrolysis and MSTFA/MBTFA derivatization is a better method for the screening and confirmation analysis of fenoterol, bambuterol and its metabolites.

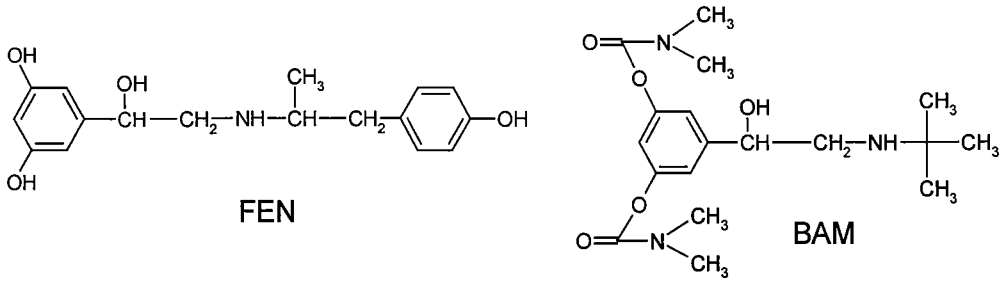

Fig1 The structures of FEN and BAM

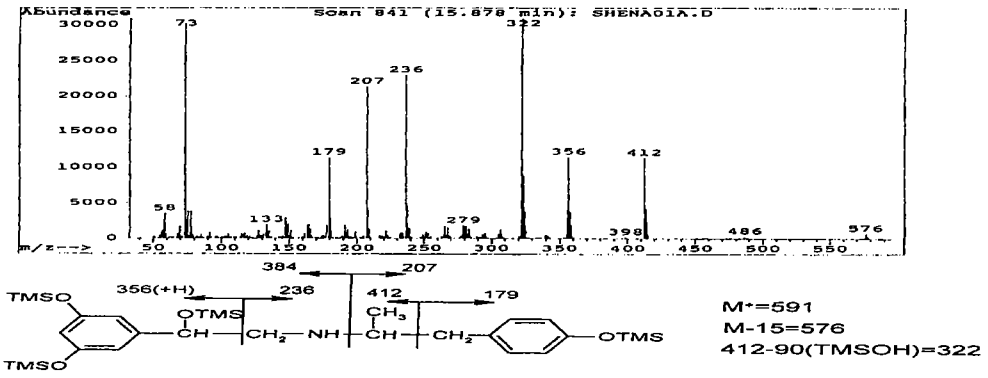

Fig 2 Mass Spectra of tetra-O-TMS-FEN

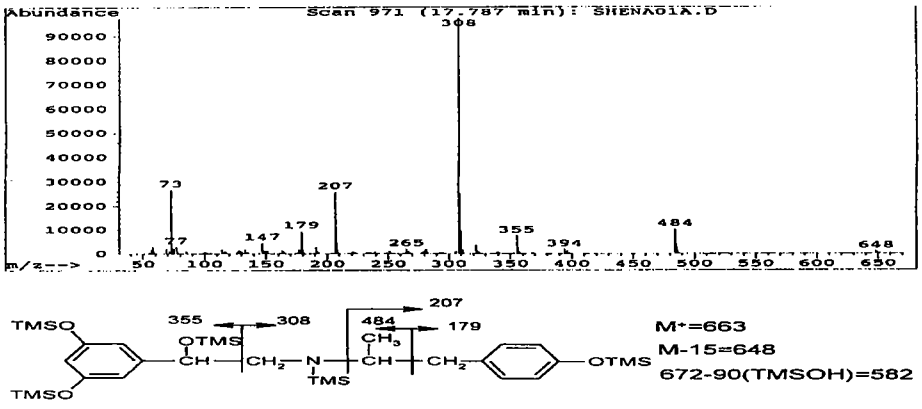

Fig 3 Mass Spectra of tetra-O-TMS,N-TMS-FEN

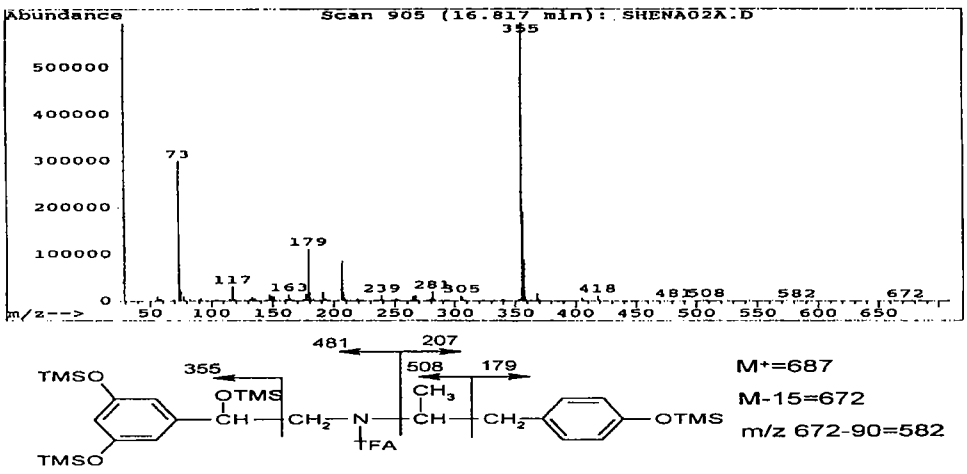

Fig 4 Mass Spectra of tetra-O-TMS,N-TFA-FEN

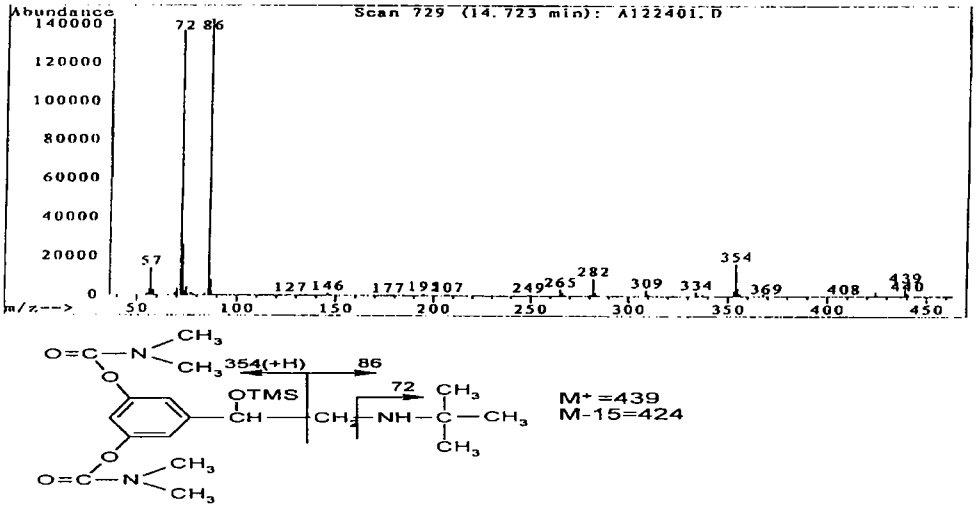

Fig 5 Mass Spectra of O-TMS-BAM

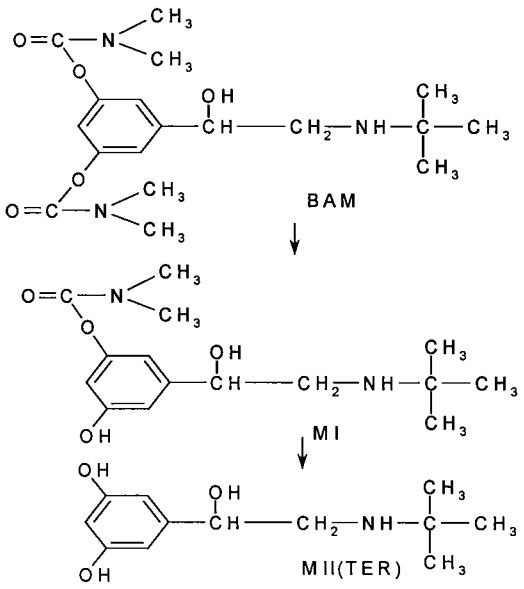

Fig 6 The metabolism of BAM

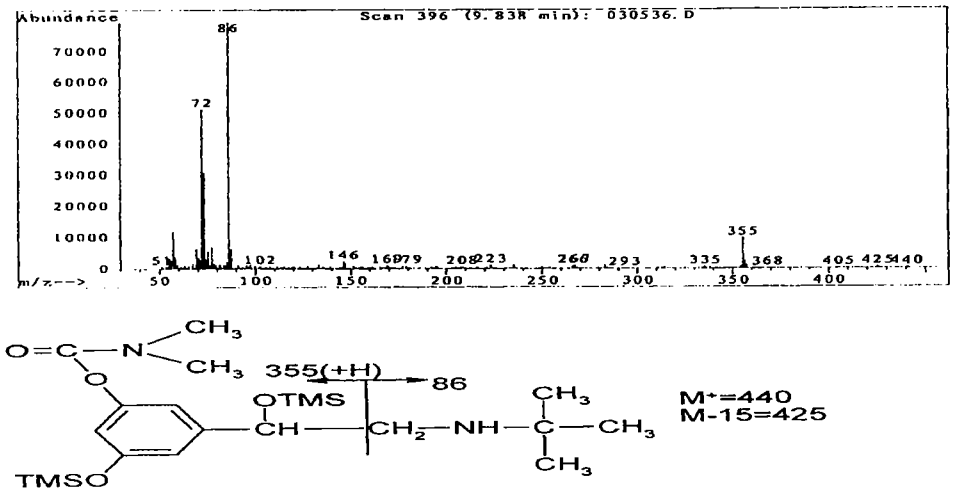

Fig 7 Mass Spectra of BAM-MI-bis-TMS derivative

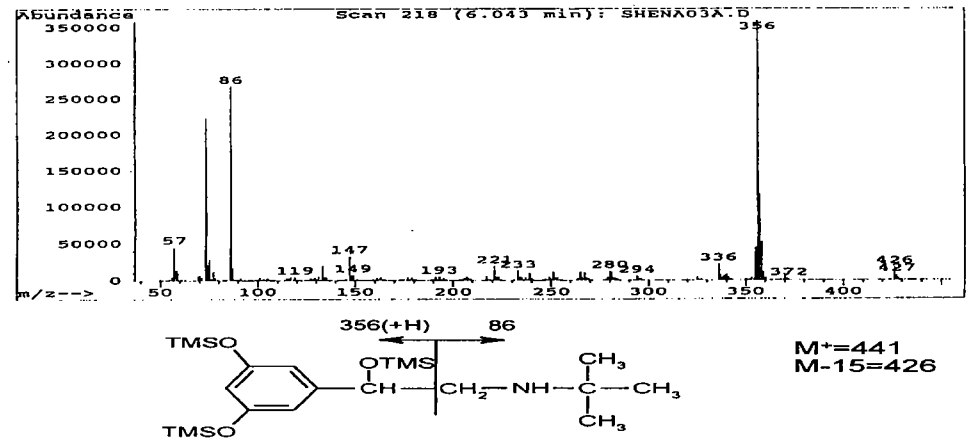

Fig 8 Mass Spectra of BAM-MII (TER)-tri-TMS derivative

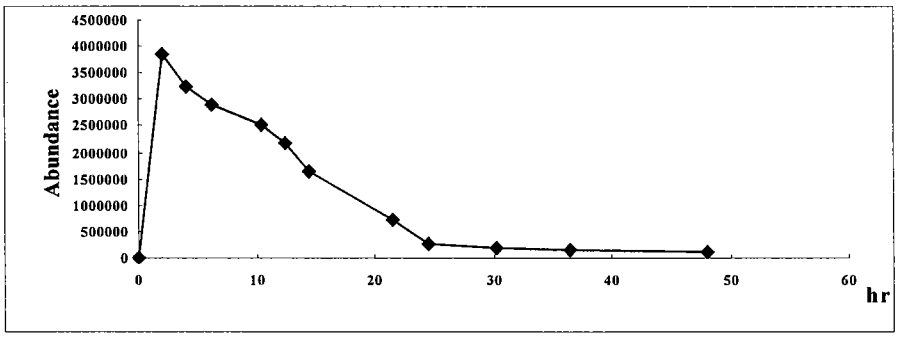

Fig 9 The excretion curve of FEN after oral administration

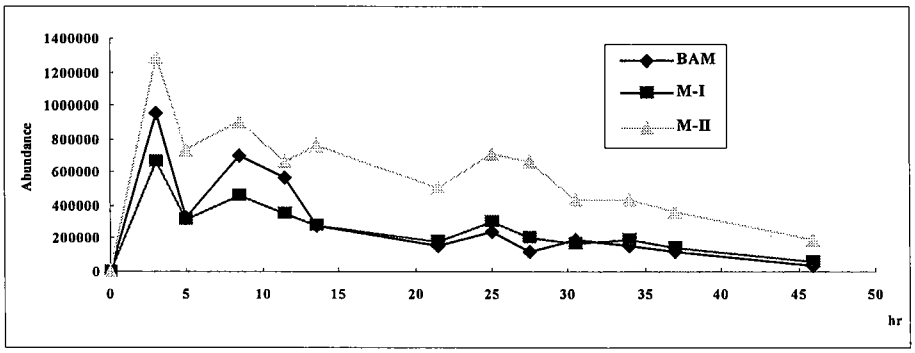

Fig 10 The excretion curve of BAM and its metabolites after oral administration

Table 1 The result of different derivatization methods

Drug	MSTFA/TMSI			MSTFA/MBTFA		
	RRT	Base Ion	Area(CV) n=4	RRT	Base Ion	Area(CV) n=4
FEN	1.681	322	3.11E05(13.15%)	1.791	355	6.14E06(11.11%)
	1.884	308	9.56E05(9.11%)			
BAM	1.537	86	5.32E06(5.59%)	1.537	86	1.96E06(3.68%)

Table 2 The result of four sample prepare methods for BAM

method	F/M	C/M	F/I	C/I
n=4	peak area(CV)	peak area(CV)	peak area(CV)	peak area(CV)
MII(TER)	1.27E06(10.19%)	2.90E06(9.90%)	2.36E05(14.01%)	4.85E05(8.99%)
MI	1.53E06(4.04%)	1.34E06(4.04%)	4.41E05(7.11%)	7.39E05(8.55%)
BAM	3.61E06(9.90%)	2.32E06(4.14%)	1.54E06(4.98%)	3.12E06(2.97%)

Table3 The in vitro converted ratio of BAM

method	F/M	C/M
n=4	peak area(CV)	peak area(CV)
BAM	2.53E07(10.44%)	1.91E07(4.83%)
MI	no peak	1.78E06(9.15%)

REFERENCE

1 L. Leyssens, C. Driessen, A. Jacobs, J. Czech and J. Raus, Determination of β_2-agonists in boving urine and liver by gas chromatography-tandem mass spectrometry, *J. chromatogr.*, 564(1991) 515-527

2 M. C. Dumasia and E. Houghton, Screening and confirmatory analysis of β-agonists, β-antagonists and their metabolites in horse urine by capillary gas chromatography-mass spectrometry, *J. chromatogr.*, 564(1991) 503-513

3 G. Van Vyncht, S. Preece, P. Gaspar, G. Maghuin-Rogister, E. DePauw, Gas and liquid chromatography coupled to tandem mass spectrometry for the multiresidue analysis of β-agonists in biological matrices, *J. chromatogr A.*, 750(1996) 43-49

4 Xu Y., Shen L., Liu X., Zhang C.J., Analytical and metabolic study of clenbuterol in human and rats' urine, Proceedings of the 11Cologne Workshop on DOPE Analysis, 7-12 March 1993, Sport und Buch Strauβ Sport Edition, Cologne, 1993,191-195

5 Astra, The introduction of Bambec, Astra Ltd