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Identification of the Metabolites of Salmeterol in Urine of Mice by LC/MS/MS and GC/MS

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ABSTRACT

Salmeterol is one of the long acting β_2 –adrenoceptor agonists, the molecular structure of salmeterol is shown in Figure 1. It is normally used in the treatment of asthma and chronic bronchitis. Due to the stimulation on the central nervous system and certain anabolic-like effects obtained when higher doses of these compounds are administrated. The use of mostβ_2 –agonists has been prohibited by the international Olympic Committee since 1995. Small doses are required for administration because of its high efficacy. The concentration of the drug in urine is quite low. Identification of the drug and its metabolites became very difficult. High doses were administrated by ig to mice in this paper, higher concentration of salmeterol and its metabolites were obtained. The urine was analyzed by using LC/MS/MS and also GC/MS. Four metabolites were identified and would be helpful for the further detection of the drug in human urine.

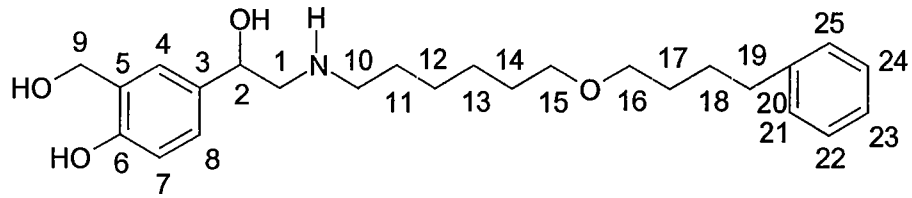


Figure 1 the structure of salmeterol

Experimental

1 Chemicals and regents

1.1 1090 liquid Chromatogram was from HP company USA; Finnigan TSQ-7000MS/MS

system. equipped with atomic pressure chemical ionization(APCI) and ICIS data system; 6890 Gas Chromatograph and 5973 MS detector were also purchased from HP Company USA.

1.2 Regents Salmeterol hydroxynaphthoate was supplied by Glaxo Wellcome Company, acetonitrile, methanol and methyl t-butyl ether are of chromatography grade. β-glucuronidase and MSTFA were purchased from Sigma Co. USA.

2 LC/MS/MS analysis

2.1 LC column: Agilent Zorbax SB-C_{18} column (2.1mm×150mm×5μm); elution gradient: 0.01mol·L^{-1} of ammonium formate (A, pH 3.5), acetonitrile(B), 0.01min A(90%), to 10.00min (60%), to 18.00min A(30%), and kept for 6min; flow rate: 0.25ml·min^{-1}; injection volume: 5μl。

2.2 MS : APCI (positive mode); evaporating temp: 450°C; capillary temp: 250°C; Spray voltage: 4500v; EM voltage: 1300v; sheath gas(N_2): 40psi; collision gas: Argon(Ar):1.9mT.

3 GC/MS conditions

3.1 GC column: HP-1 (0.2mm×17m×0.11μm); Temp program: 180°C→3.3°C/min→231°C→30°C/min→310°C(keep 9min); carrier gas: Helium; flow: 1ml·min^{-1}; Injection port: 280°C; Transfer line: 300°C; injection volume: 1μl; Split ratio: 1:10.

3.2 MS: : EI; 70ev; EM voltage: 1282 V.

4 Sample collections

15 ml of salmeterol suspending solution (1mg/ml) was administrated ig to 10mice (25g each, ICR, purchased from Peking University). 0~24 hours' urine samples were collected and stored at –20°C till analysis.

5 Extractions and Derivatization

A prewash of the solid phase extraction columns was done with 5 ml of methanol and 5ml of water. The samples were applied onto the C18 columns, and were rinsed with 5 ml of water. The columns were eluted with 2 ml of methanol. The alcohol was evaporated to dryness under a nitrogen stream. After that, 1 ml of phosphate buffer(0.2mol/L, pH 6.8～7.1) and 100μl of β-glucuronidase (5000 units) were added. The hydrolysis took 3 hours at 55°C.Once the hydrolysis was completed the basic extraction included the following steps: 100mg of solid buffer(Na2CO3:NaHCO3, 1:10,w/w, pH 8.8～8.9) was added to the hydrolysisate. Then 5 ml of methyl t-butyl ether were added and the mixture was shaken for 10 min. A centrifugation at 3000 rpm for 10 min was done in order to separate both phases. The organic phase was put into a new tube and was evaporated to dryness under a gentle stream of nitrogen. The residue was re-resolved in methanol for LC/MS/MS analysis. Or TMS derivatives were formed for GC/MS analysis. The dried samples were dissolved in 50μl of MSTFA and heated at 70°C for 30 min.

Results

1 Identification of metabolites of salmeterol by LC/MS/MS

Comparing various chromatograms in scan mode, it appears that there were not many interfering compounds in urine, but from the mass spectra recorded the presence of salmeterol and of it's by the corresponding peaks, metabolites were clearly identified (Fig 3). Their quasi-molecular ions were m/z416, m/z432, m/z414, m/z430 and m/z462. On the basis of metabolic theory and the interpretation of the MS/MS spectra, the structures of the metabolites were proposed. They are 19-hydroxy-salmeterol (M_1), 2-carbonyl-salmeterol (M_2), 19-carbonyl-salmeterol (M_3) and 19-hydroxy-8-methoxy-salmeterol (M_4).

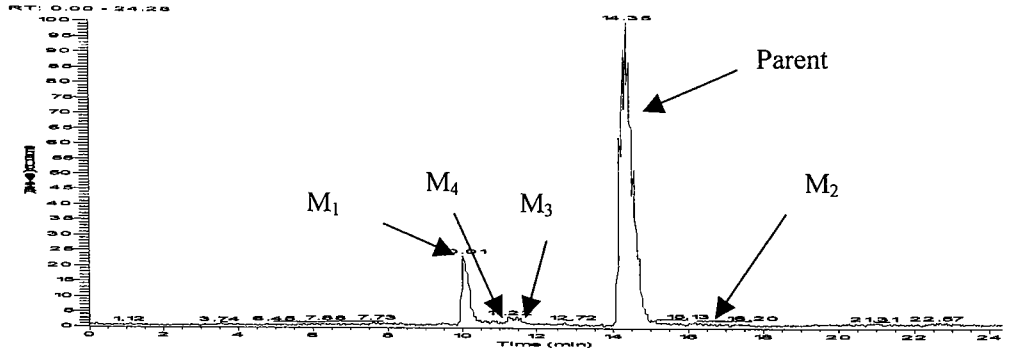


Figure 2 Total ion chromatogram of LC/MS for urine of mice after ig administration of salmeterol

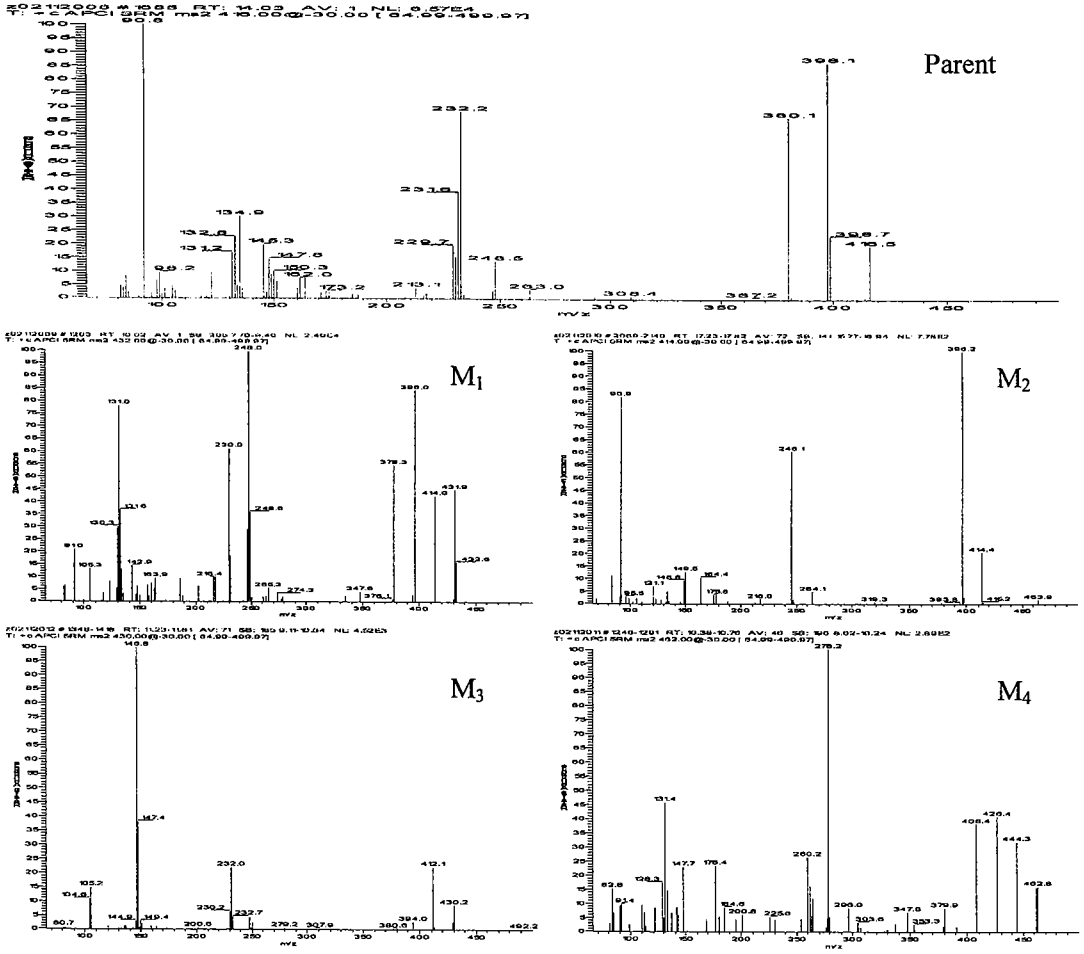


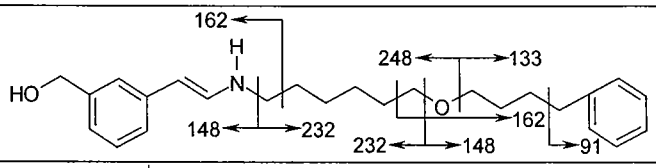
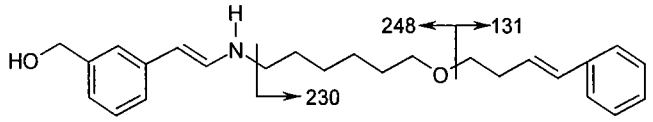
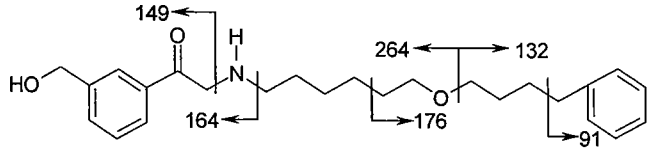
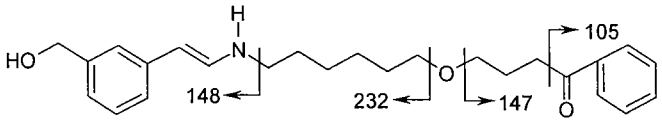
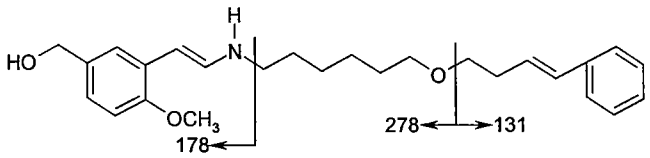
Figure 3 LC/MS/MS mass spectrum of salmeterol and its metabolites (M_1~M_4) in urine of mice

2 Identification of metabolites of salmeterol by GC/MS

To improve the identification capacity of the metabolites with more informative mass spectra, the samples were derivatized for GC/MS analysis. The peaks with corresponding metabolites were detected. The mass spectra were shown in Figure 5. Interpretation of the GC/MS spectra provided further evidences for the structures of the metabolites. On the basis of the data of LC/MS/MS and GC/MS, possible metabolic pathways of salmeterol were proposed in Figure 6.

Table 1 LC/MS/MS mass analysis of salmeterol and it's metabolites in urine of mice

$[M+1]^+$ion (m/z)	Characteristic ion (m/z)	Proposed assignment
416(SAL)	398 380	$[M+1-H_2O]^+$ $[398-H_2O]^+$

		
432(M_1)	414 396 378	$[M+1-H_2O]^+$ $[414-H_2O]^+$ $[396-H_2O]^+$ 
414(M_2)	396 246	$[M+1-H_2O]^+$  $[264-H_2O]^+$
430(M_3)	412 394	$[M+1-H_2O]^+$ $[412-H_2O]^+$ 
462(M_4)	444 426 408 260 147	$[M+1-H_2O]^+$ $[444-H_2O]^+$ $[426-H_2O]^+$  $[278-H_2O]^+$ $[178-OCH_3]^+$

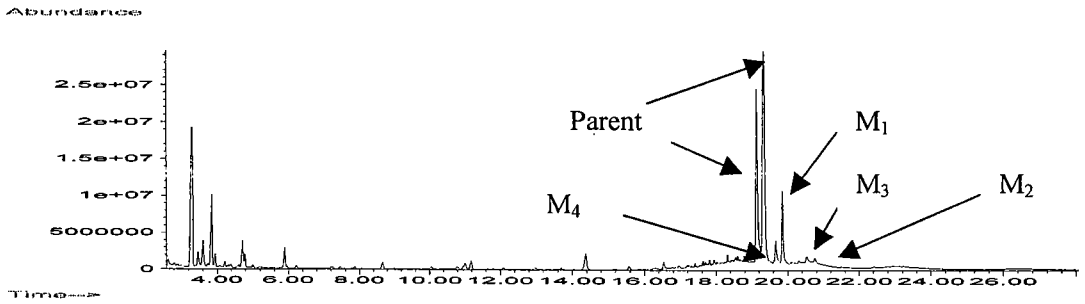


Figure 4 Total ion chromatogram of GC/MS for urine of mice after ig administration of salmeterol

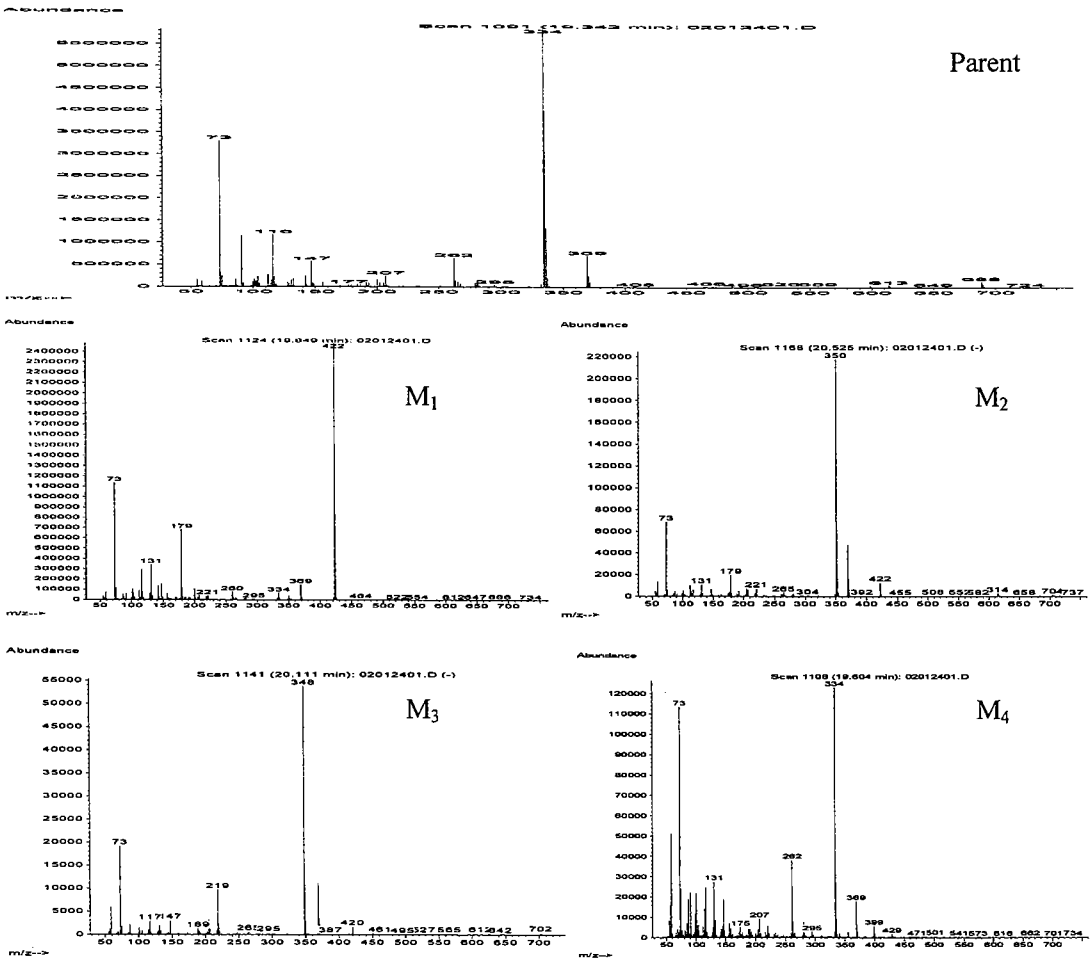
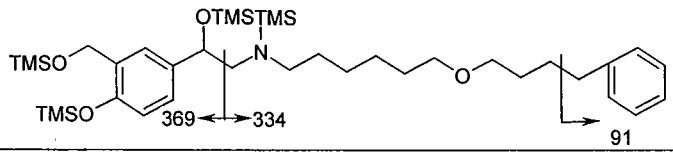
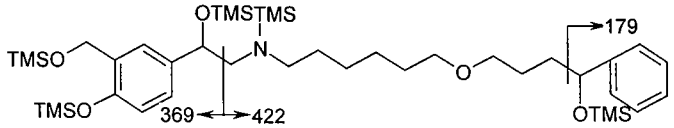
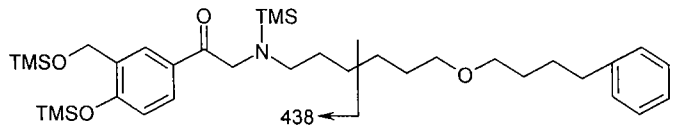
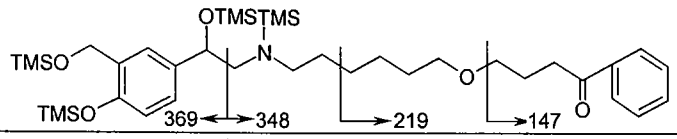
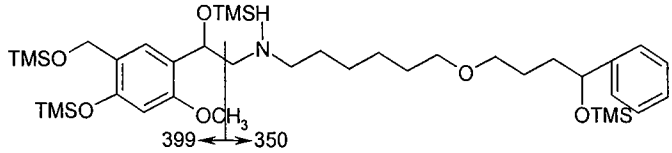


Figure 5 GC/MS mass spectrum of salmeterol and its metabolites (M_1~M_4) in urine of mice

Table 2 GC/MS mass analysis of salmeterol and it's metabolites in urine of mice

Metabolite	Characteristic ion (m/z)	Proposed assignment
salmeterol	688	$[M+4TMS-CH_3]^+$ 
M_1		
M_2	614	$[M+3TMS-CH_3]^+$ 
	350	$[438-OTMS+H]^+$
M_3	702	$[M+4TMS-CH_3]^+$

		
M_4	734	$[M+4TMS-CH_3]^+$ 
	334	$[350-CH_4]^+$
	262	$[350-OTMS+H]^+$

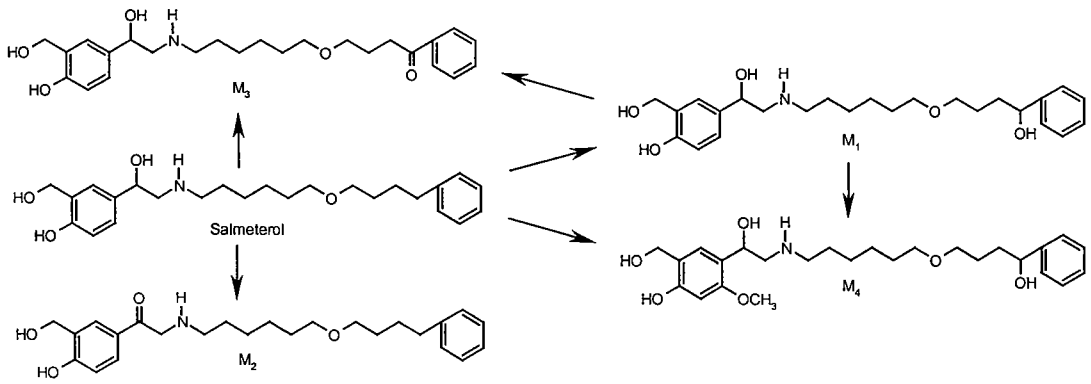


Figure 6 Proposed metabolic pathways of salmeterol in mouse

Discussion

Parent drugs and four metabolites of salmeterol in urine of mice were identified by using LC/MS/MS and GC/MS. They are 19-hydroxy-salmeterol (M_1), 2-carbonyl-salmeterol (M_2), 19-carbonyl-salmeterol (M_3) and 19-hydroxy-8-methoxy-salmeterol (M_4). The concentration of 19-hydroxy-salmeterol was higher than that of other metabolites.

References

[1] International Olympic Committee, Prohibited classes of substances and prohibited methods. In: IOC Medical Code and Explanatory Document, IOC, Lausanne, 1995.

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[3] Lho DS, Hong JK, Paek HK, et al. Determination of phenolalkylamines, narcotic analgesics, and beta-blockers by gas chromatography/mass spectrometry. J Anal Toxicol [J], 1990,14(2):77-83.