PERIÓDICO TCHÊ QUÍMICA
ARTIGO ORIGINAL

# CARACTERIZAÇÃO POR ESPECTROMETRIA DE MASSA DE METABÓLITOS DE MEBEVERINA PLASMÁTICA E SUA SÍNTESE

# MASS SPECTROMETRIC CHARACTERIZATION OF PLASMA MEBEVERINE METABOLITES AND ITS SYNTHESIS

# СИНТЕЗ И МАСС-СПЕКТРОМЕТРИЧЕСКАЯ ХАРАКТЕРИСТИКА МЕТАБОЛИТОВ МЕБЕВЕРИНА

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## RESUMO

A mebeverina é um medicamento antiespasmódico musculotrópico amplamente utilizado no tratamento da síndrome do intestino irritável. Como um éster de álcool de mebeverina e ácido verátrico, a mebeverina é rapidamente metabolizada e é praticamente indetectável no plasma sanguíneo. O principal objetivo deste trabalho foi estabelecer a estrutura dos principais metabólitos da mebeverina no plasma sanguíneo humano. O estudo foi realizado por *time-of-flight mass spectrometry* (LC-IT-TOF MS), os metabólitos da mebeverina foram extraídos do plasma com acetonitrila. Quando comparados os cromatogramas de plasma sanguíneo obtidos antes e após a administração da droga, quatro picos principais de metabólitos foram detectados. Para estabelecer a estrutura dos compostos, foram obtidos espectros de massa da primeira e segunda ordem. Os espectros de primeira ordem foram usados para calcular a fórmula do metabólito e a estrutura foi determinada a partir dos espectros de fragmentação, bem como comparando os espectros de fragmentação da mebeverina e seus metabólitos propostos. Os compostos propostos foram sintetizados e sua estrutura foi confirmada usando espectrometria de RMN e cromatografia de massa. Quatro metabólitos principais foram encontrados neste estudo: ácido desmetilmebeverina (DMAC), glicuronídeo do DMAC (DMAC-Glu), ácido de mebeverina (MAC) e álcool de mebeverina (MAL). Os resultados complementam os dados disponíveis na literatura sobre o metabolismo do ácido verátrico, urina e estudos microssomais. De acordo com os dados obtidos, o principal metabolito da mebeverina no sangue é o DMAC. A concentração de MAC após a administração de mebeverina é quase dez vezes menor do que a DMAC, o conteúdo de MAL e DMAC-Glu é insignificante e provavelmente não afeta o efeito farmacológico da mebeverina. Portanto, a concentração de DMAC é o principal parâmetro a ser monitorado em estudos farmacocinéticos.

**Palavras-chave**: *Metabolismo, Mebeverina, UHPLC-IT-TOF, Síntese*.

## ABSTRACT

Mebeverine is a musculotropic antispasmodic drug that is widely used in the treatment of irritable bowel syndrome. As an ester of mebeverine alcohol and veratric acid, mebeverin is quickly metabolized and is practically undetectable in blood plasma. The main goal of this work was establishing the structure of the main metabolites of mebeverin in human blood plasma. The study was conducted by time-of-flight mass spectrometry (LC-IT-TOF MS), metabolites of mebeverine were extracted from plasma with acetonitrile. When comparing chromatograms of blood plasma obtained before and after drug administration, four main peaks of metabolites were detected. To establish the structure of the compounds, mass spectra of the first and second order were taken. The first-order spectra were used to calculate the metabolite formula and the structure was determined from the fragmentation spectra, as well as by comparing the fragmentation spectra of mebeverine and its proposed metabolites. The proposed compounds were synthesized, and their structure was confirmed using NMR and chromatography-mass spectrometry. Four main metabolites were found in this study: desmethylmebeverine acid (DMAC), glucuronide product of DMAC (DMAC-Glu), mebeverine acid (MAC) and mebeverine alcohol (MAL). The results complement the available literature data about the veratric acid





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metabolism, urine, and microsomal studies. According to the data obtained, the main metabolite of mebeverine in the blood is DMAC. The concentration of MAC after mebeverine administration is almost ten times less than DMAC, the content of MAL and DMAC-Glu is insignificant, and probably does not affect the pharmacological effect of mebeverine. Therefore, the concentration of DMAC is the main parameter to be monitored in pharmacokinetics studies.

**Keywords**: *Metabolism, Mebeverine, UHPLC-IT-TOF, Synthesis*.

## АННОТАЦИЯ

Мебеверин – противоспазматическое лекарственное средство, которое широко применяется при лечении заболеваний кишечника и желчевыводящих путей. При попадании в организм, мебеверин, представляющий собой эфир мебеверинового спирта и вератровой кислоты, быстро метаболизирует и практически не обнаруживается в плазме крови и моче. Целью данной работы был поиск и установление структуры основных метаболитов мебеверина в плазме крови человека. Исследование проводилось методом времяпролетной масс-спектрометрии (LC-IT-TOF MS), метаболиты мебеверина извлекали из плазмы крови используя метод осаждения белков ацетонитрилом. При сопоставлении хроматограмм плазмы крови, полученным до и после приема препарата выявлены четыре основных пика метаболитов, структура которых была определена по масс-спектрам высокого разрешения. Для установления структуры соединений метаболитов мебеверина снимались масс-спектры первого и второго порядка. Спектры первого порядка были использованы для расчета брутто-формулы метаболита, структура была определена по спектрам фрагментации, а также при сопоставлении спектров фрагментации мебеверина и предполагаемых его метаболитов. В качестве подтверждения обнаруженные соединения были синтезированы и их структура подтверждена при помощи ЯМР и по хромато-масс-спектрометрическим характеристикам. Согласно полученным результатам была установлена структура четырех основных метаболитов мебеверина в плазме крови человека: дезметил-мебевериновая кислота (DMAC), глюкуронид дезметил-мебевериновой кислоты (DMAC-Glu), мебевериновая кислота (MAC) и мебевериновый спирт (MAL). Результаты дополняют имеющиеся литературные данные по метаболитам мебеверина, обнаруженным в моче и при исследованиях на микросомах и описывающие, в основном, метаболизм вератровой кислоты. Согласно полученным в работе данным, основным метаболитом мебеверина является DMAC. Концентрация MAC после введения мебеверина почти в десять раз ниже, чем DMAC, содержание MAL и DMAC-Glu незначительно и, вероятно, не влияет на фармакологический эффект мебеверина. Таким образом, концентрация DMAC является основным параметром, который необходимо отслеживать в фармакокинетических исследованиях мебеверина и при установлении биоэквивалентности.

**Ключевые слова:** *метаболизм, мебеверин, времяпролетная масс-спектрометрия, синтез*.

## 1. INTRODUCTION

Mebeverine is a musculotropic antispasmodic drug that is widely used in the treatment of the irritable bowel syndrome (Baume, 1972; van Outryve *et al*., 1995; Chapman *et al.,* 1990; Evans *et al.,* 1996). According to the articles (Dickinson *et al*., 1991; Kristinsson *et al*., 1994; Tulich *et al*., 1996), mebeverine was not detected in blood after the oral administration. Since mebeverine is an ester of veratric acid and 4-{ethyl-[2-(4-methoxyphenyl)-l-methylethyl1amino) butan-1-01 (MB-alcohol, MB-OH), these compounds might be expected to be formed upon hydrolysis of the drug *in vivo.* The metabolic fate of veratric acid was studied in more detail: *O*-demethylation gave vanillic acid and isovanillic acid, which subsequently led to protocatechuic acid on *O*-demethylation (Kristinsson *et al*., 1994). Tulich *et al*., (1996) identified mebeverine alcohol and O-dimethyl-mebeverine alcohol in human plasma and urine. Recently (Kraemer *et al.,* 2000), a detailed mass spectrometric analysis of hepatic microsomal incubation extracts and of human urine samples allowed the identification of additional seven biotransformation products of mebeverine alcohol resulting from *N*-deethylation, *N*-dehydroxybutylation, ring hydroxylation, and conjugation. However, these metabolites were not quantified and were assumed to be part of the missing mebeverine alcohol metabolites. Lately, investigations revealed the presence of mebeverine acid as an oxidation product of mebeverine alcohol (Stockis *et al.,* 2002) but noticed, that the possibility that other metabolic products display some antispasmodic activity cannot be ruled out. Bergerone *et al*., (2013) developed an LC-MS method for measuring of mebeverine and desmethylmebeverine acid, but their concentrations in biological samples were

not published.

High-performance liquid chromatography (HPLC) coupled to high-resolution MS, specifically using hybrid ion-trap time-of-flight (IT-TOF) MS, is one of the most attractive techniques for the open detection of unknown metabolites (Appolonova *et al*., 2004; Boix *et al*., 2014; Bijlsma *et al*., 2011; Boix *et al.,* 2013; Appolonova *et al*., 2011; Pozo *et al*., 2015). This configuration allows for operating in the so-called MSE mode (i.e., two accurate mass full spectra are acquired sequentially). The first one is acquired without applying collision energy, providing information about the intact molecules [commonly the (de)protonated molecule] present in the serum sample. The second, applying a collision energy ramp, promotes fragmentation obtaining accurate mass fragment ions useful for metabolite identification. This approach has been successfully applied for the detection of metabolites/transformation products of several xenobiotics.

The goals of the present work were to enhance our knowledge of the metabolism of mebeverine by using mass spectrometric techniques and to reveal the main biomarker for human pharmacokinetics studies of mebeverine. According to the articles (Dickinson *et al.*, 1991; Kristinsson *et al.*, 1994; Tulich *et al.*, 1996), mebeverine is not detected in blood after the oral administration as it quickly decomposes to mebeverine alcohol and veratric acid. While veratric acid metabolic pathway is well researched (Kristinsson *et al.*, 1994), major metabolites of mebeverine alcohol are not investigated enough (Tulich *et al.*, 1996).

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and reagents

Mebeverine hydrochloride was purchased from Sigma (USA). Mebeverine alcohol (MAL), mebeverine acid Li-salt (MAC) and desmethylmebeverine acid (DMAC) were synthesized in our laboratory. Ultrapure water for LC-MS/MS was purchased from Biosolve BV (Netherlands), and acetonitrile (LC-MS grade) was purchased from AppliChem (Panreac, Germany). Methanol (HPLC gradient grade) was obtained from Fisher Scientific (UK). Formic acid and ammonium formate (Sigma-Aldrich, Germany) were used as additives for the mobile phase.

Stock solutions of mebeverine hydrochloride, mebeverine alcohol, mebeverine acid, and desmethylmebeverine acid were 1 mg $mL^{-1}$ (calculated to base compounds) in 50% methanol and were stable for the at least 1 month when stored at -20°C. Standard working solutions were prepared by diluting each stock solutions with the mobile phase.

Pooled blank human plasma was obtained from the hospital blood bank and thawed at room temperature before use.

### 2.2. Synthesis of desmethylmebeverine acid HBr (5, DMAC)

The scheme of synthesis of desmethylmebeverine acid HBr (5, DMAC) was shown in Figure 1A. 4-Methoxyphenylacetic acid **1** (2g, 12 mmol) was dissolved in acetic anhydride (6 ml, 6.5 g, 63mmol), at room temperature, and the solution was stirred and purged with $N_2$ for several minutes. The reaction was initiated by the addition of catalyst (0.5 ml, 0.51 g, 6 mmol), and the reaction was continuously purged with a slow flow of $N_2$ over the course of the reaction. After completion (48 h), water (10 mL) was added to the reaction flask to hydrolyze acetic anhydride. The reaction mixture was extracted with ethyl acetate (3 per 50 mL), and the extracts were combined and washed with saturated potassium bicarbonate (2 per 50 mL) followed by water (2 per 50 mL), then dried over magnesium sulfate and filtered. Removing the solvent by rotary evaporation gave the 1-(4- methoxyphenyl) propan-2- one (product 2). Yield 1.6 g (80 %). 1H NMR ($CDCl_3$): 2.17 (s, 3H, $CH_3$); 3.66 (s, 2H, $CH_2$); 3.83 (s, 3H, $CH_3O$), 6.90 (d, J = 8.55 Hz, 2H, CH); 7.14 (d, J = 8.55 Hz, 2H, CH).

To an ice-cold stirred solution of 1-(4-methoxyphenyl) propan-2- one 2 (1.6 g, 9.7 mmol), methyl 4-aminobutanoate hydrochloride (2.24 g, 14.5 mmol) and sodium acetate (2 g, 24.3 mmol) in methanol (25 ml), sodium cyanoborohydride (1g, 15.9 mmol) were added. The mixture was stirred at ambient temperature overnight, acidified with diluted aqueous HCl and evaporated at reduced pressure. The residue was partitioned between dichloromethane and water. Organic layer was discarded; aqueous layer was basified with solid sodium carbonate and the product was extracted with dichloromethane (3 per 10 ml). Combined organic solutions were dried over $MgSO_4$ and concentrated to afford 2 g (77 %) of the product 3. 1H NMR ($CDCl_3$): 1.12 (d, J = 7.23 Hz, 3H,

$CH_3$); 1.85 (dd, J = 6.35 Hz, J = 12.56 Hz, 2H, $CH_2$); 2.37 (t, J = 7.30 Hz, J = 7.30 Hz, 2H, $CH_2$); 2.60-2.69 (m, 2H, $CH_2$); 2.74-2.81 (m, 2H, $CH_2$); 2.94 (dd, J = 6.35 Hz, J = 12.56 Hz, 1H, CH); 3.68 (s, 3H, $CH_3OC$), 3.82 (s, 3H, $CH_3O$); 6.86 (d, J = 8.55 Hz, 2H, CH); 7.12 (d, J = 8.55 Hz, 2H, CH).

To an ice-cold stirred solution of methyl 4-((1- (4-methoxyphenyl) propan-2-yl)amino)butanoate 3 (2 g 7.5 mmol) and freshly distilled acetaldehyde (1 ml, 0.78 g, 17.8 mmol) in methanol (25 ml), sodium cyanoborohydride (1g, 15.9 mmol) was added. The mixture was stirred at ambient temperature overnight, acidified with diluted aqueous HCl and evaporated at reduced pressure. The residue was partitioned between dichloromethane and water. The organic layer was discarded; the aqueous layer was basified with solid sodium carbonate, and the product was extracted with dichloromethane (3 per 10 ml). Combined organic solutions were dried over MgSO4 and concentrated to afford the residual oil that was subjected to flash chromatography using EtOAc/hexane as the eluent. The pure product 4 was isolated in 68% yield (1.3 g). 1H NMR ($CDCl_3$): 1.26 (dd, J = 6.75 Hz, J = 13.35 Hz, 3H, $CH_3$); 1.33 (d, J = 6.50 Hz, 3H, $CH_3$); 1.47 (dd, J = 7.00 Hz, J = 14.20 Hz, 2H, $CH_2$); 2.79 (d, J = 6.50 Hz, 2H, $CH_2$); 3.00-3.18 (m, 2H, $CH_2$); 3.31-3.38 (m, 3H, $CH_2$+CH); 3.76 (dd, J = 7.00 Hz, J = 14.20 Hz, 2H, $CH_2$); 3.81 (s, 6H, $CH_3O$ + $CH_3OC$); 6.86 (d, J = 8.55 Hz, 2H, CH); 7.12 (d, J = 8.55 Hz, 2H, CH).

Methyl 4-(ethyl(1- (4-hydroxyphenyl) propan- 2-yl)amino)butanoate 4 (0.5 g, 1.7 mmol) was dissolved in 48 % aqueous HBr (10 ml) and the resulted solution was refluxed for 8 hours. The reaction solution was concentrated dryness, dissolved in acetic acid and evaporated again to remove an excess of HBr. Residual solid was dissolved in water (1 ml), boiled with activated carbon and after filtration the solution was evaporated to dryness. The product 5 was dried in vacuum at 0.1 mm overnight. Yield 0.2 g (32%). 1H NMR (DMSO-d6): 0.84 (d, J = 6.49 Hz, 3H, $CH_3$); 0.94 (dd, J = 5.96 Hz, J = 12.7 Hz, 3H, $CH_3$); 1.17 (dd, J = 7.07 Hz, J = 12.62 Hz, 2H, $CH_2$); 2.18 (t, J = 7.20 Hz, 2H, $CH_2$); 2.27 (t, J = 7.16 Hz, 2H, $CH_2$); 2.33-2.41 (m, 4H, $2CH_2$); 2.85 (d, J = 12.74 Hz, 1H, CH); 6.82 (d, J = 8.45 Hz, 2H, CH); 7.07 (d, J = 8.45 Hz, 2H, CH); 7.07 (s, 1H, OH); 12.00 (bs, 1H, CO2H).

### 2.3. Synthesis of mebeverine acid Li-salt (6, MAC)

The scheme of synthesis of mebeverine acid Li-salt (6, MAC) was shown in Figure 1A. Methyl-4-(ethyl (1- (4-hydroxyphenyl) propan- 2-yl)amino)butanoate 4 (0.25 g, 0.8 mmol) was dissolved in THF (10 ml) and an aqueous solution (0.5 ml) of LiOH (0.04 g, 1.6 mmol) was added and the reaction mixture was stirred at ambient temperature for overnight. Then the solvent was evaporated under reduced pressure and the solid was dried under vacuum at 0.1 mm overnight. Yield 0.2 g (97%). 1H NMR (DMSO-d6): 0.84 (d, J = 6.49 Hz, 3H, $CH_3$); 0.94 (dd, J = 5.96 Hz, J = 12.7 Hz, 3H, $CH_3$); 1.17 (dd, J = 7.07 Hz, J = 12.62 Hz, 2H, $CH_2$); 2.18 (t, J = 7.20 Hz, 2H, $CH_2$); 2.27 (t, J = 7.16 Hz, 2H, $CH_2$); 2.33-2.41 (m, 6H, $3CH_2$); 2.85 (d, J = 12.74 Hz, 1H, CH); 3.81 (s, 3H, $CH_3O$); 6.82 (d, J = 8.45 Hz, 2H, CH); 7.07 (d, J = 8.45 Hz, 2H, CH).

### 2.4. Synthesis of mebeverine alcohol Li-salt (2, MAL)

The scheme of synthesis of mebeverine acid Li-salt (2, MAL) was shown in Figure 1B. Mebeverin 1 (0.1 g, 0.23 mmol) was dissolved in THF (5 ml) and an aqueous solution (0.5 ml) of LiOH (0.03 g, 1.25 mmol) was added and the resulting mixture was stirred at ambient temperature for overnight. The solvent was stripped off and the residue was partitioned between dichloromethane (5 ml) and water (5 ml). Organic phase was separated and aqueous layer was extracted with dichloromethane (5 ml). Combined organic phases were dried over MgSO4 and concentrated. Product 2 was dried in vacuum and weighted 0.05 g (80 %). 1H NMR ($CDCl_3$): 0.92 (d, J = 6.50 Hz, 3H, $CH_3$); 1.04 (t, J = 14.10 Hz, 3H, $CH_3$); 1.27 (dd, J = 7.00 Hz, J = 13.54 Hz, 2H, $CH_2$); 1.64 (t, J = 7.33 Hz, OH); 1.71 (dd, J = 7.00 Hz, J = 13.32 Hz, 2H, $CH_2$); 2.29 (t, J = 7.33 Hz, 2H, $CH_2$); 2.34 (t, J = 7.39 Hz, 2H, $CH_2$); 2.43-2.51 (m, 2H, $CH_2$); 2.82 (d, J = 13.24 Hz, 1H, CH); 3.51 (dd, J = 7.00 Hz, J = 14.20 Hz, 2H, $CH_2$); 3.81 (s, 3H, $CH_3O$); 6.86 (d, J = 8.55 Hz, 2H, CH); 7.12 (d, J = 8.55 Hz, 2H, CH).

### 2.5. LC-IT-TOF-MS

The LCMS-IT-TOF analysis was performed using a NexeraXR LC system (Shimadzu Corp.) equipped with two LC-20AD pumps, a DGU-$20A5_R$ degasser, a SIL-$20AC_{XR}$ autosampler, a CTO-20AC column oven, and a CBM – 20A control module, coupled to a LCMS-IT-TOF that combines QIT (ion trap) and TOF (time-of-flight) mass spectrometer (Shimadzu

Corp.). IT-TOF-MS was equipped with an electrospray ionization (ESI) source, and analysis was performed in positive-ion mode with a mass resolution of 40,000 FWHM. Accurate masses were corrected by calibration using the trifluoroacetic acid sodium solution (2.5 mM from 50 Da to 1000 Da as an external reference). The scan range was 100–500 m/z. The voltage of the ESI source and detector was 1.50 kV and 1.65 kV, respectively. The ion source temperature was maintained at 230 ºC. The skimmer voltage was 8.5 V. The ion accumulation time was 10 ms. The selected width of precursor ion was 1.0 m/z; the selected time and the collision-induced dissociation (CID) collision time were 20 ms and 50 ms, respectively. The collision energy was 50%. Peak and spectra picking were performed using the LabSolutions software (Shimadzu Corp.)

The LC separation was carried out using ACQUITY UPLC BEN C8 column 1.7um 2.1x50mm (Waters, USA). The mobile phase consisted of the following: (A) 5mM ammonium formate aqueous solution with formic acid 0.1% and (B) acetonitrile. The column temperature was 40ºC, and the injection volume of the samples was 5 µl. Elution was carried out at a flow rate of 0.5 ml/ min. The linear gradient was formed as follows: 0 min (1% B), 1.5 min (99% B), 2.5 min (99% B), 2.6 min (1% B).

### 2.6. Excretion study

All experiments were performed in accordance with the guidelines and regulations of the I.M. Sechenov First Moscow State Medical University. Blank human plasma was obtained from the Blood Bank at the I.M. Sechenov First Moscow State Medical University, Moscow, Russia. Written informed consent was received from all participants before inclusion in the study. Studies were carried out according to “The code of ethics of the World Medical Association (Declaration of Helsinki)” and all experimental protocols were approved by the Human Investigation Ethical Committee at the I.M. Sechenov First Moscow State Medical University, Moscow, Russia.

One oral dose of 200 mg of mebeverine hydrochloride was administered to ten healthy male volunteers under fasting conditions. Five milliliters of venous blood samples were withdrawn from each volunteer and transferred into heparinized tubes according to the following time schedule: prior to dosing (0) and at 0,5 (30 min) h, 1,0 h, 1,50 (1 h 30 min), 2,0 h, 2,25 (2 h 15 min), 2,5 h (2 h 30 min), 2,75 (2 h 45 min), 3,0 h, 3,25 (3 h 15 min), 3,5 (3 h 30 min), 3,75 (3 h 45 min), 4,0 h, 5,0 h, 6,0 h, 8,0 h, 10,0 ч, 12,0 h and 24,0 h post-dose. All blood samples were centrifuged immediately at 5000 rpm for 10 min to obtain plasma. The plasma samples were labeled and kept frozen at -30 ºC until analysis.

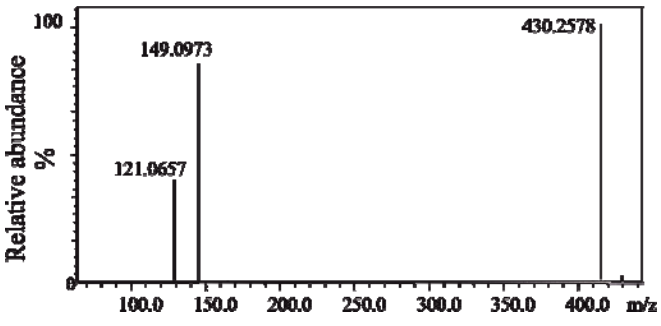
### 2.7. Sample preparation

A 200 µL aliquot of human plasma samples was added with 800 µL of acetonitrile. The mixtures were vortex mixed for 3 min and centrifuged at 14,000 g for 5 min. The supernatants were evaporated at 37°C, diluted with 200 µL 50% MeOH and transferred into autosampler vials. An aliquot of 5 µL of each sample was injected for LC-MS-MS analyses.

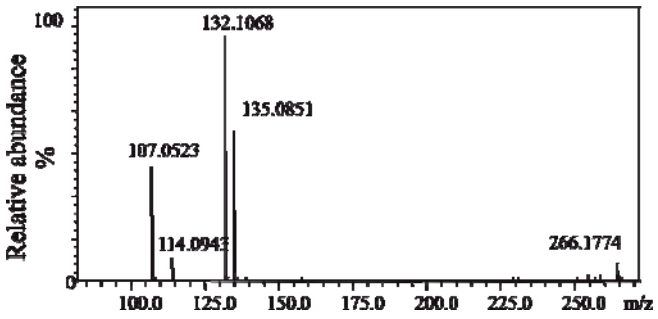
## 3. RESULT AND DISCUSSION

Using these approaches, several peaks were detected in mebeverine post-administration plasma. After combining the information from the MS and MS/MS high-resolution methods, and contrasting this with the results from pre-administration samples, the list of possible metabolites was reduced to 4 potential mebeverine metabolites candidates (Table 1).

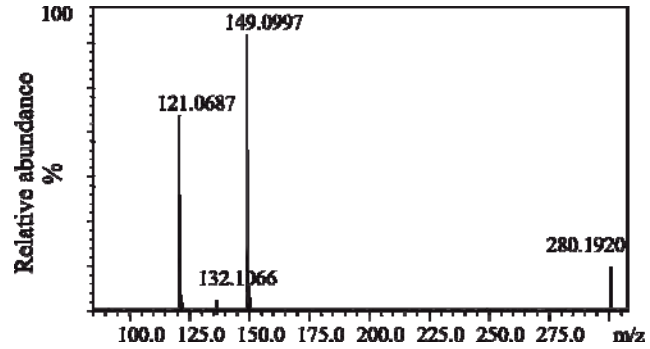
Parent drug mebeverine was not detected in plasma after drug administration. Fragmentation spectrum of mebeverine standard is shown in Figure 2 (a) ([M+H]$^+$, *m/z* 430.2578. The most intense fragments are the ions with *m/z* 121.0685 and 149.0993, belonging to the mebeverine alcohol moiety. Product ion structure and the proposed fragmentation pathways of the molecular ion of mebeverine are shown in Figure 5 (pathways a and b).



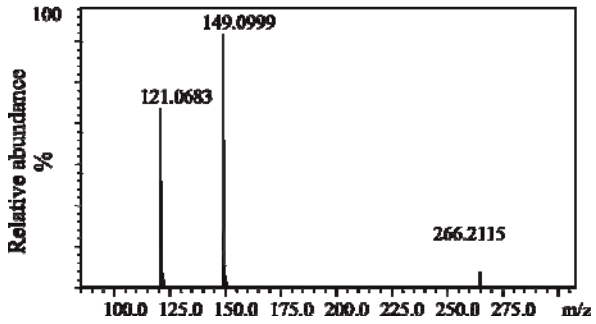

(A)




(B)



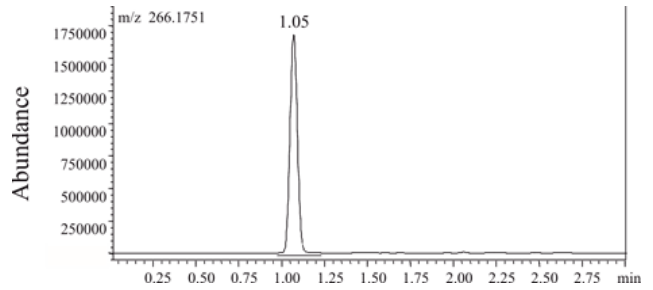

(C)



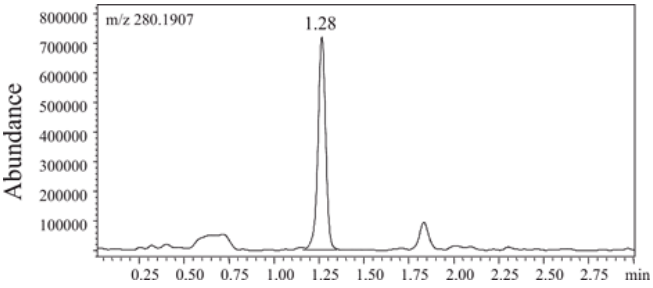

(D)

***Figure 2.*** *Product ion mass spectra of [M+H]+ of mebeverine (A), DMAC: desmethylmebeverine acid (B), MAC: mebeverine acid (C) and MAL: mebeverine alcohol (D).*

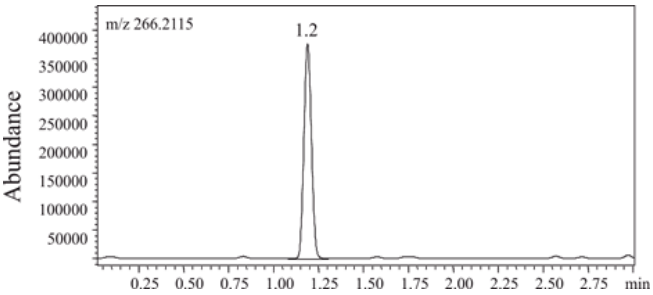
The primary metabolite is mebeverine alcohol (MAL), which is found on the chromatogram as a peak with a retention time of 1.2 min (chromatogram is shown in Figure 3 (c)). According to the data obtained by IT-TOF scanning, MAL ionized in positive mode with the formation of $[M+H]^+$ ion (*m/z* 266.2136, 2.1 mDa) (Table 1). The product ion spectrum of MAL showed the same fragmentation pattern as parent drug (ions with *m/z* 121 and 149, Figure 2(d) and fragmentation pathways *a* and *b* are shown in Figure 5) indicating that mebeverine moiety remained unaltered.



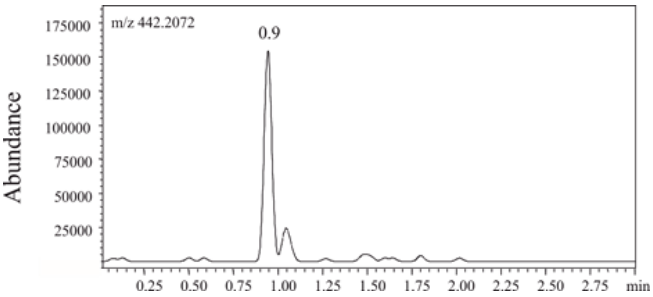

(A)




(B)




(C)




(D)

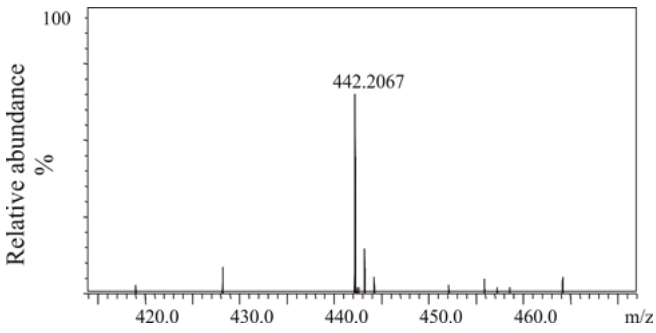
***Figure 3.*** *Chromatograms of mebeverine metabolites – DMAC: desmethylmebeverine acid (A), MAC: mebeverine acid (B) and MAL: mebeverine alcohol (C), DMAC-Glu: desmethylmebeverine acid glucuronic acid (D) in plasma samples*

The more intense peak with retention time 1.28 min and *m/z* 280.1907 was identified as mebeverine acid (MAC) (Figure 3(b)). Since the molecular weight of these metabolites is 14 Da

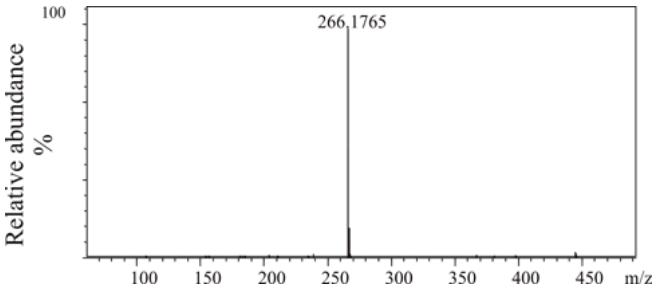
more than that of the MAL, oxidation of the alcohol moiety seems to be the most feasible alternative. The product ion spectra of the $[M+H]^+$ ions at m/z 121 and 149 similar ions to those yielded by the parent drug (Table 1), and ion with *m/z* 132 with amino acid part of the molecule can support the proposed structure (spectrum is shown in Figure 2(c), and fragmentation pathways *a*, *b* and *c* are shown in Figure 5).

Chromatographic peak with RT 1.05 min was identified as the DMAC (desmethylmebeverine acid) ($[M+H]^+$, *m/z* 266.1774, 2.3 mDa). The $[M+H]^+$ ion of DMAC at m/z 266 showed the fragmentation to form product ions at m/z 135 and 175 Da (Table 2, Figure 2(b) and fragmentation pathways *a* and *b* are shown in Figure 5). These ions were equivalent to those obtained for MAC but with a difference of 14 m/z units due to the absence of the methyl group. In addition, the product ion at m/z 132 was associated with the presence of a hydroxy group in the molecule (fragmentation pathway *c* is shown in Figure 5).

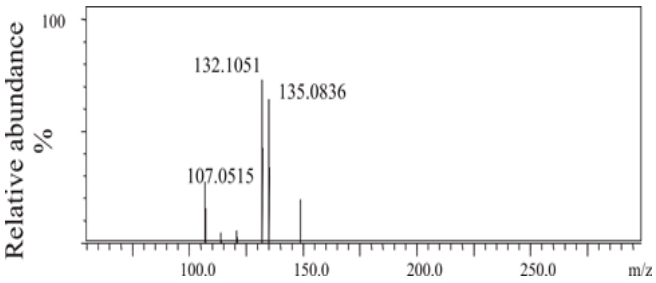
The molecular mass of 441 Da was obtained for metabolite with a retention time of 0.9 min (Table 1, chromatogram shown in Figure 3(d)). The product ion spectrum of protonated ion ($[M+H]^+$, *m/z* 442.2067 Figure 4(a)) exhibited loss of 156 Da (MS\MS spectrum *m/z* 266.1765 Figure 4(b)). Losses of 156 Da can be associated with the presence of a glucuronide group in the molecule. In the product ion scan $MS_3$ of ion 226 the most abundant ions (m/z 132, 135 and 107) are similar to the DMAC fragmentation and corresponded to the DMAC glucuronide formation (Figure 4(c)).




(A)




(B)



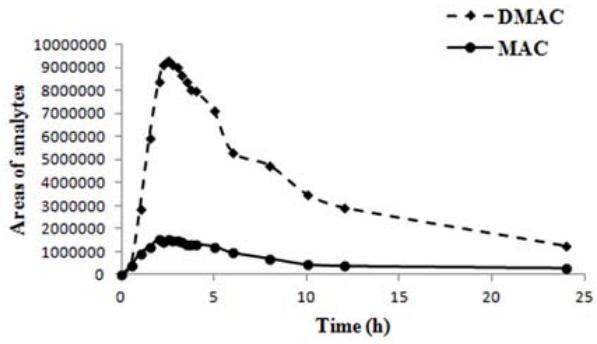

(C)

***Figure 4.*** *HR-MS, MS2 and MS3 spectra of DMAC-Glu: desmethylmebeverine acid glucuronic acid.*

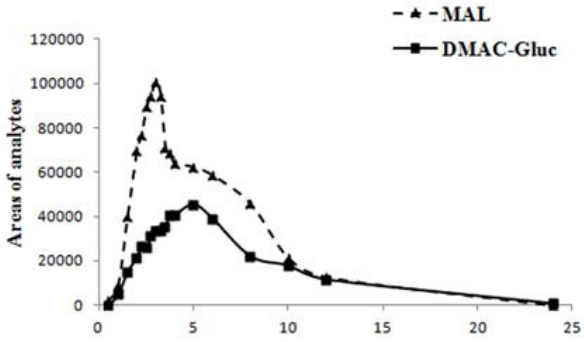
The IT-TOF mass measurements played a critical role in properly interpreting the fragmentation of mebeverine and proposing suitable structures for detected metabolites. The molecular mass of DMAC is close to MAL (Table 1), and identification must be confirmed by the accurate mass of parent ions and its fragments.

The resulted metabolism for mebeverine is depicted in Figure 6. Plasma LC-MS\MS part is investigated in this article, and GC-MS urine results are summarized from previous studies [6-9].

Once the main metabolites had been identified, the whole set of samples corresponding to the excretion studies was analyzed. A LC/MS/MS method in MRM mode was developed for this purpose. Table 2 summarizes the most critical parameters for the four metabolites. The method was found to be selective for all the selected analytes as no interferences were found in any of the six blank samples analyzed. Figure 7 shows the time course of metabolites excretion. Although the amount excreted dramatically decreased after the first 10 h, it was possible to unequivocally identify metabolites 20 h after the intake of the drug.




(A)




(B)

***Figure 7.*** *Excretion rations of mebeverine metabolites. Areas for most abundant metabolites (A) and less abundant metabolites in human plasma (B).*

According to the data obtained, the main metabolite of mebeverine is DMAC. The concentration of MAC after mebeverine administration is almost ten times less than DMAC, the content of MAL and DMAC-Glu is insignificant, and probably does not affect the pharmacological effect of mebeverine. Therefore, the concentration of DMAC is the main parameter to be monitored in studies of the bioequivalence of mebeverine.

## 4. CONCLUSION

The in-vivo metabolism of mebeverine was studied using methods HPLC-IT-TOF and synthesis of it's metabolites. Four main metabolites were found in this study: desmethylmebeverine acid (DMAC), glucuronide product of DMAC (DMAC Glu), mebeverine acid (MAC) and mebeverine alcohol (MAL). Mebeverine is mainly metabolized by the loss of the veratric acid. According to the data obtained, the main metabolite of mebeverine in blood is DMAC. The concentration of MAC after mebeverine administration is almost ten times less than DMAC, the content of MAL and DMAC-Glu is insignificant, and probably does not affect the pharmacological effect of mebeverine. Therefore, the concentration of DMAC is the main parameter to be monitored in pharmacokinetics studies.

## 5. ACKNOWLEDGMENTS

Not applicable.

## 6. AUTHORS' CONTRIBUTION

The authors' responsibilities were as follows—NM participated in the concept and design of the study, interpreted data and was responsible for writing the article; RK and PM performed the synthesis of postulated metabolites and structure conforming. SAA, Head of the Laboratory conceptualized the pharmacokinetic study, supervised laboratory, interpreted data, and has final responsibility for all parts of the manuscript. All authors read and approved the final version of the paper.

## 7. ETHICAL APPROVAL

Written informed consent was received from all participants before inclusion in the study. Studies were carried out according to "The code of ethics of the World Medical Association (Declaration of Helsinki)" and all experimental protocols were approved by the Human Investigation Ethical Committee at the I.M. Sechenov First Moscow State Medical University, Moscow, Russia.

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***Table 1.*** *Ion observed for mebeverine metabolites and its retention time (RT)*

| Metabolite | RT (min) | Precursor Ion (*m/z*) | Calc. (*m/z*) | Molecular Formula | Error (mDa) | Product Ion (*m/z*) | Abundance (%) | Error (mDa) | Molecular Formula |
|---|---|---|---|---|---|---|---|---|---|
| DMAC ($MS^2$) | 1.05 | 266.1774 | 266.1751 | $C_{15}H_{23}NO_3$ | 2.3 | 132.1068 | 100 | 4.9 | $C_6H_{13}NO_2$ |
| | | | | | | 135.0851 | 54 | 4.7 | $C_9H_{10}O$ |
| | | | | | | 107.0523 | 50 | 3.2 | $C_7H_6O$ |
| | | | | | | 114.0943 | 9.4 | 3 | $C_6H_{11}NO$ |
| MAC ($MS^2$) | 1.28 | 280.1920 | 280.1907 | $C_{16}H_{25}NO_3$ | 1.3 | 149.0997 | 100 | 3.6 | $C_{10}H_{12}O$ |
| | | | | | | 121.0687 | 73 | 3.9 | $C_8H_8O$ |
| MAL ($MS^2$) | 1.2 | 266.2136 | 266.2115 | $C_{16}H_{27}NO_2$ | 2.1 | 132.1066 | 1 | 4.9 | $C_6H_{13}NO_2$ |
| | | | | | | 149.0999 | 100 | 3.8 | $C_{10}H_{12}O$ |
| | | | | | | 121.0683 | 71 | 3.5 | $C_8H_8O$ |
| DMA-Glu ($MS^2$) | 0.4 | 442.2067 | 442.2072 | $C_{21}H_{31}NO_9$ | -0.5 | 266.1781 | 100 | 3 | $C_{15}H_{23}NO_3$ |
| DMA-Glu ($MS^3$) | 0.4 | 266.1781 | 266.1751 | $C_{15}H_{23}NO_3$ | 3.0 | 132.1051 | 100 | 3.2 | $C_6H_{13}NO_2$ |
| | | | | | | 135.0836 | 79 | 3.2 | $C_9H_{10}O$ |
| | | | | | | 107.0515 | 37 | 0.8 | $C_7H_6O$ |

***Table 2.*** *HPLC and MS parameters for detection of mebeverine metabolites*

| Analyte | Retention time (min) | Precursor (m/z) | Product (m/z) | Q1 Pre Bias (V) | CE (V) | Q1 Pre Bias (V) |
|---|---|---|---|---|---|---|
| MAL | 2.5 | 266.1 | 149.00 | -14.0 | -14.0 | -18.0 |
| | | 266.1 | 121.00 | -16.0 | -16.0 | -12.0 |
| MAC | 1.8 | 280.1 | 149.00 | -15.0 | -16.0 | -18.0 |
| | | 280.1 | 121.00 | -16.0 | -18.0 | -12.0 |
| DMAC | 0.8 | 266.1 | 135.1 | -14.0 | -15.0 | -26.0 |
| | | 266.1 | 132.1 | -15.0 | -18.0 | -10.0 |
| $^{2}H_{5}$-DMAC (IS) | 0.8 | 271.1 | 135.1 | -14.0 | -15.0 | -26.0 |

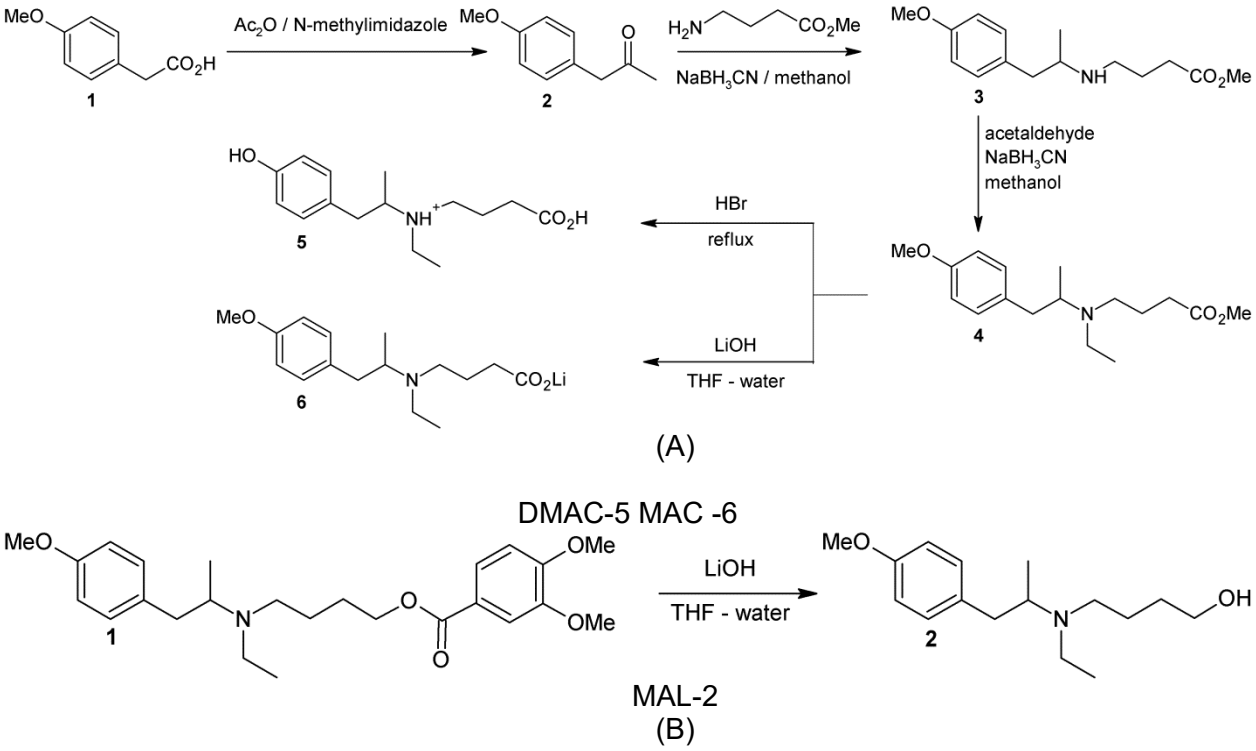


***Figure 1.*** *The scheme of synthesis of mebeverine metabolites.*

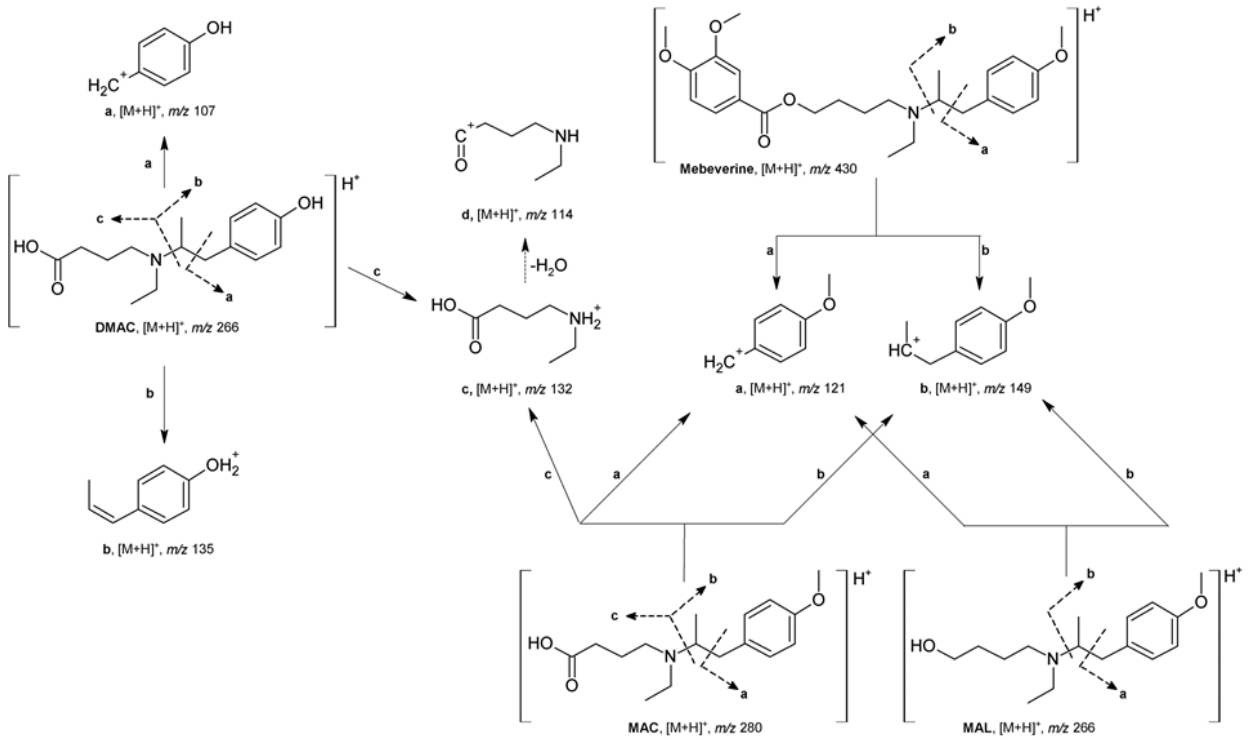


***Figure 5.*** *ESI Fragmentation of mebeverine, DMAC: desmethylmebeverine acid, MAC: mebeverine acid and MAL: mebeverine alcohol.*

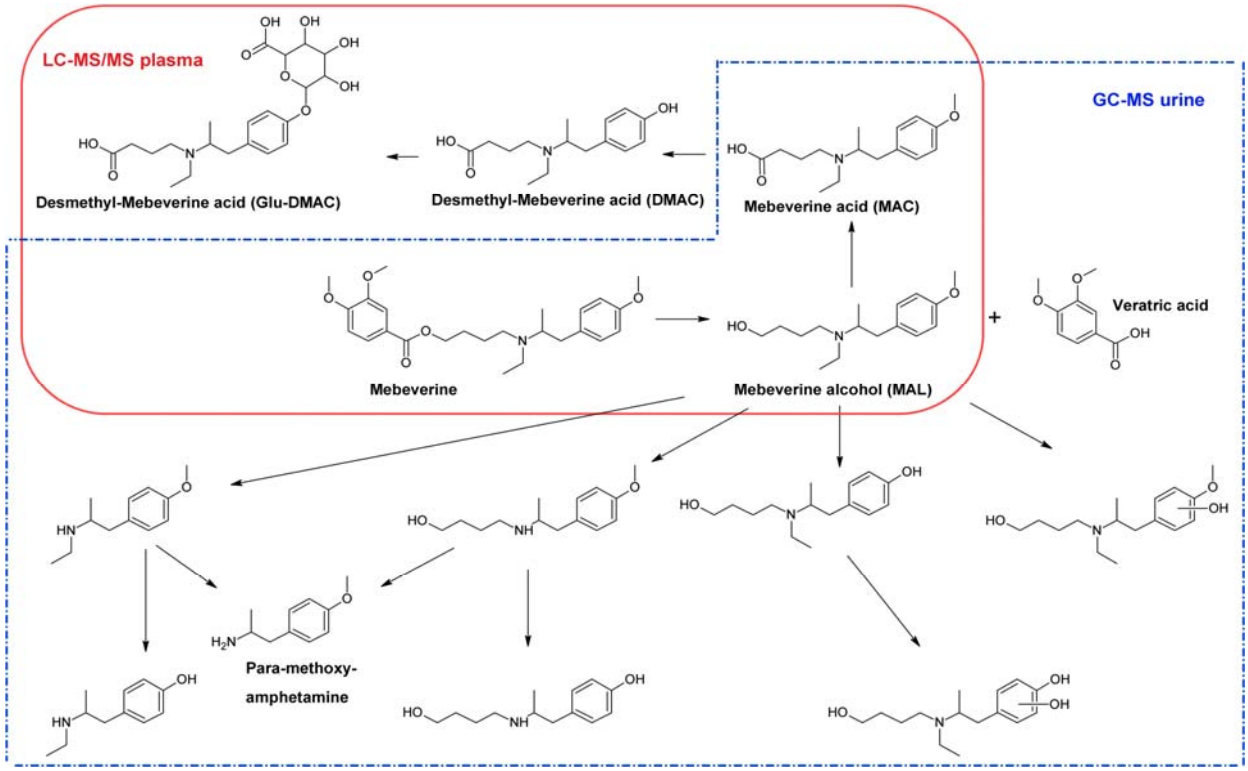


***Figure 6.*** *The main metabolic pathway of mebeverine.*