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Fungal biotransformation of mosapride by *Cunninghamella elegans*

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ABSTRACT

The filamentous fungus, *Cunninghamella elegans* AS 3.156, was used as a microbial model of mammalian metabolism to transform mosapride, a selective 5-HT_4-receptor agonist. The fungal metabolites of mosapride were separated and detected by ultra performance liquid chromatography–tandem mass spectrometric method. After incubation for 120 h, the parent drug was metabolized to thirteen metabolites, one of which was known major mammalian metabolite, des-*p*-fluorobenzyl mosapride, while the others were all novel metabolites. Four major metabolites including three new metabolites and one known major metabolite in mammals were isolated using preparative high-performance liquid chromatography and identified by 1D- and 2D-nuclear magnetic resonance, mass and UV spectroscopic analysis. Other metabolites were characterized according to their chromatographic behavior, mass and UV spectral data. The major metabolic pathways of mosapride transformed by the fungus were *N*-oxidation, morpholinyl ring cleavage, *N*-dealkylation of p-fluorobenzyl and further *N*-formylation, while minor metabolic pathways were *N*-dealkylation, glucoside conjugation, sulfate conjugation, formylation and *N*-dealkylation of the morpholinyl ring opened metabolite, *N*-dealkylation followed by acetylation. The fungi belonging to *Cunninghamella* species could not only be used to generate mammalian metabolites of mosapride in microgram scale, but also facilitate the production of putative metabolites or novel drug derivatives. The isolates could be used as reference standards for identification and analytical tests of mammalian metabolites of mosapride.

1. Introduction

Mosapride citrate (mosapride), known as (±)4-amino-5-chloro-2-ethoxy-*N*-[[4-(4-fluorobenzyl)-2-morpholinyl]methyl]benzamide (Fig. 1), is a potent and selective gastroprokinetic agent that enhances the gastrointestinal motility by stimulating the 5-hydroxytryptamine 4 (5-HT_4) receptor [1]. Unlike metoclopramide and cisapride, mosapride has no affinity for dopamine D_2 receptor [2]. Mosapride has been reported to improve gastrointestinal symptoms in patients with gastroesophageal reflux disease and to ameliorate constipation and response fluctuations in Parkinsonian patients [3,4] without causing the extrapyramidal syndrome associated with dopamine-D_2-receptor blockage or adverse effects such as QT cardiac risks [2,5]. In addition, mosapride citrate has beneficial effects on glycemic control in patients with Type 2 diabetes mellitus by improving insulin sensitivity [6]. This compound also has a potential to be used in the field of veterinary medicine and animal science [7].

Studies of mosapride metabolism in animals and humans have shown extensive metabolism mainly by *N*-dealkylation catalyzed by CYP3A4 [8,9]. Four phase I metabolites, des-*p*-fluorobenzyl mosapride, 5′-oxo-des-*p*-fluorobenzyl mosapride as well as the corresponding 3-hydroxylation metabolites of the above two were isolated from rat urine after oral administration of mosapride [8].

There were still other metabolites detected but not identified or characterized in plasma and urine of experimental animals and in urine of humans [8,10,11]. All these reports clearly indicated that mosapride was metabolized to other metabolites except the four reported phase I metabolites. More importantly, some metabolites accounted for possibly larger proportion, such as the polar metabolite(s) detected in male rat bile [8]. However, there is no information on the identities of these unknown metabolites. The number of mosapride metabolites is very small compared with the chemically related gastrokinetic benzamides, clebopride and cisapride, which has 25 and 31 reported metabolites, respectively [12,13]. Given the widespread use of mosapride and insufficient information on its metabolism, we think it is necessary to isolate and identify new metabolites for fully understanding the metabolism, safety and efficacy of mosapride.

As is known to all, drug metabolism is considered as a series of enzymatic transformation in vivo which may produce pharmacological active or toxic metabolites. It is necessary to "detect, identify

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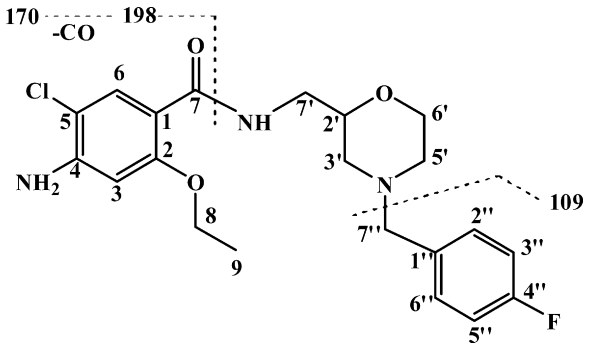

Fig. 1. Structure of mosapride with numbering system and its major ESI(+)–MS/MS fragmentation patterns.

and characterize metabolites and allow for better understanding of the role metabolites play in drug safety assessments" [14]. Hence, it is essential to obtain sufficient reference standards of metabolites. Traditionally, this has depended on the model systems such as small laboratory animal model, perfused tissues and microsomal preparations. Those methods have limitations, such as cost of experimental animals, ethical concerns, small amount of metabolites isolated which is not sufficient for structure identification and further use in pharmacological, toxicological and analytical tests. As regards the method of chemical synthesis, it can produce larger amount of metabolites based on the known structure. However, it has disadvantages such as complex steps or procedures needed, environmental pollution and high cost.

Nowadays, biotransformation is becoming a significant tool in structural modification and metabolism study of natural or synthetic organic compounds. The application of microbial transformation as a complementary in vitro model for drug metabolism has drawn considerable attention in recent years [15–18]. The main advantage of microbial system over traditional mammalian system or chemical synthesis is the ability to produce milligram quantities of putative metabolites or novel drug derivatives which are difficult to be synthesized.

The objective of this work was to examine the abilities of the filamentous fungus *Cunninghamella* (*C.*) *elegans* to metabolize mosapride, isolate and identify the major metabolites which would be used as reference standards in further study on mammalian metabolism of mosapride.

2. Experimental

2.1. Apparatus

Melting points (mp) were determined on a X-4 melting point apparatus (Beijing, China) and were uncorrected. Q-TOF/MS experiments were performed on a Bruker Daltonics micrOTOF-Q mass spectrometer (Billerica, MA, USA). The software micrOTOF control (Version 2.2) was applied for system operation and data collection. Ultra performance liquid chromatography–photodiode array detector–electrospray ionization–tandem mass spectrometric (UPLC–PDA–ESI–MS/MS) analyses were performed on a Waters Micromass® Quattro micro™ API mass spectrometer (Manchester, UK) and an ACQUITY™ UPLC system equipped with ACQUITY™ UPLC® Photodiode Array (PDA) Detector (Milford, MA, USA). Data were acquired and processed using MassLynx™ NT 4.1 software (Waters Corp., Milford, MA, USA). An Agilent preparative HPLC system (Palo Alto, CA, USA) consisting of a G1361 prep-pump, a G1365B multiple wavelength detector and a G1364B automatic fraction collector was used. 1D and 2D NMR (nuclear magnetic resonance) spectra were recorded in dimethyl-*d*6-sulfoxide (DMSO-*d*6) using TMS as internal standard on a Bruker ARX 600 NMR spectrometer (Faellanden, Switzerland).

2.2. Reagents

Mosapride citrate was supplied by Kangning Pharmaceutical Co. (Sanmen County, China). Methanol, acetonitrile and formic acid of high-performance liquid chromatography (HPLC) grade were purchased from Dikma (Richmond Hill, USA). Peptone and yeast extract were biochemical grade and obtained from Aoboxing Co. (Beijing, China). All other chemicals were of analytical grade.

2.3. Microorganisms

C. elegans AS 3.156, *C. elegans* AS 3.2028, *C. echinulata* AS 3.2004, *C. blakesleeana* AS 3.970 and *C. blakesleeana* AS 3.910, were provided by the Institute of Microbiology, Chinese Academy of Sciences (Beijing, China). Stock cultures were maintained on potato dextrose agar (Aoboxing Co.) slants at 4 °C and were transferred every 6 months to maintain activity.

2.4. Culture medium

Microbial transformation was carried out in a liquid medium. It was prepared by mixing glucose 20 g, peptone 5.0 g, yeast extract 5.0 g, K_2HPO_4 5.0 g and NaCl 5.0 g per liter of double-distilled water. The pH of the suspension was adjusted to 6.5 with 6.0 M HCl. The medium was autoclaved in Erlenmeyer flasks at 115 °C for 30 min and cooled before inoculation.

2.5. Culture and biotransformation procedures

Biotransformation of mosapride was performed according to the two-stage fermentation procedure [19]. For screening experiments, a loop of spore suspension obtained from freshly grown slant was transferred to 50 mL of sterile liquid medium in 250 mL Erlenmeyer flask. The flask was incubated at 220 rpm and 28 °C for 24 h (stage I). Then a 1.0 mL portion of stage I culture was transferred into 20 mL of liquid medium contained in 100 mL Erlenmeyer flask. The flask was incubated at 220 rpm and 28 °C for 24 h (stage II). Mosapride (4 mg) in dimethylformamide (DMF) (50 μL) was added into each flask to give a final concentration of 200 mg/L. After additional incubation for 120 h, the culture was centrifuged at 2000 × *g* for 20 min. The supernatant was transferred to tubes and kept at −20 °C until analysis. The left fungal mycelia were washed with ethyl acetate (three times, 200 mL each time) and the combined organic layers were kept at −20 °C.

Culture controls consisted of fermentation blanks in which microorganisms were grown under identical conditions but without substrate, i.e. mosapride. Substrate control experiments were performed with substrate dispersed in un-inoculated sterile media under otherwise identical conditions.

Preparative scale biotransformation of mosapride by *C. elegans* AS 3.156 was carried out in sixty 250 mL Erlenmeyer flasks. A 2.5 mL portion of stage I culture was transferred into 50 mL of medium in 250 mL Erlenmeyer flask. Solution of mosapride (10 mg) in DMF (125 μL) was added into each flask to give a concentration of 200 mg/L, the cultures were incubated for an additional 120 h. Other procedures were same with those in screening experiments.

2.6. Analysis methods

In screening experiments, solid phase extraction was used for sample pretreatment. Oasis MCX® mixed-mode cation-exchange cartridges (1 mL volume, Waters Corp., USA), which possess mixed modes of both reversed phase and cation exchange, were chosen. A

1.0 mL aliquot of each sample or control was filtered through precut membrane (0.45 μm). The filtrate was loaded onto a preconditioned Oasis MCX® cartridge. The column was washed with 1.0 mL of 1% formic acid aqueous solution, 1.0 mL of methanol and eluted with 1.0 mL of methanol containing 5% ammonia water. Then the eluate was evaporated to dryness under a gentle stream of nitrogen at 40 °C, and the residue was dissolved in 200 μL of mobile phase (acetonitrile: 0.2% formic acid aqueous, 22:78 [vol/vol]). A small aliquot of 10 μL of the solution was injected into the UPLC–PDA–MS system. The total transformation yield was defined as the ratio of the UV peak areas of all metabolites by PDA detector at 274 nm (in gradient condition 1 as described in Section 3.1) to those of the sum of metabolites and mosapride.

High-resolution mass spectra (HRMS) were recorded in the positive ESI mode with micrOTOF-mass spectrometer. High purity nitrogen was used as desolvation and nebulizer gas. Mass spectrometric parameters were set as follows: capillary voltage 4500 V, hexapole Rf 300.0 Vpp, nebulizer gas pressure 0.4 bar, dry gas flow rate 4.0 L/min and dry temperature 190 °C.

ACQUITY UPLC™ BEH C_{18} column (2.1 by 100 mm; particle size, 1.7 μm; Waters Corp., Milford, MA, USA) was employed with the column temperature set at 40 °C. The metabolites were eluted at a flow rate of 0.25 mL/min with a linear gradient. The mobile phase composed of solvent A, acetonitrile and solvent B, 0.2% formic acid aqueous. Two gradient conditions were used. For UPLC–PDA analysis performed at 274 nm, solvent A ran from 22 to 46% in 7 min, and then returned to the initial condition in 3 min for reequilibration (gradient condition 1). For UPLC–MS/MS analysis, solvent A ran from 22 to 46% in 3 min and then returned to the initial condition in 3 min for reequilibration (gradient condition 2). As for the mass spectrometric condition in positive ESI mode, parameters were as follows: capillary voltage 3.0 kV, cone voltage 30 kV, source temperature 100 °C and desolvation temperature 300 °C. Nitrogen was used as the desolvation and cone gas with a flow rate of 350 and 50 L/h, respectively. MS/MS spectra were produced by collision-induced dissociation of the selected precursor ions with argon as the collision gas, and the collision energy ranged from 18 to 50 eV for all metabolites.

2.7. Isolation, purification and identification of major metabolites

A preparative scale biotrasformation allowed for the isolation of the major metabolites of mosapride in sufficient quantities for structure elucidation. After biotransformation, the culture media and mycelia were separated by centrifugation at 2000 × *g* for 20 min. The mycelia were washed with ethyl acetate and the supernatant was adjusted to pH 9 with 2 M NaOH and was extracted with twice the volume of ethyl acetate for two times. The organic layer was dried over sodium sulfate and evaporated to dryness under reduced pressure. The brown oily crude extract (308 mg) dissolved in methanol was injected onto the Agilent preparative HPLC system. Separation was achieved using Zorbax C_{18} column (10.7 mm × 250 mm; particle size, 7 μm; Agilent, CA, USA). The mobile phase consisted of methanol: 10 mM NH_4Ac water (45:55 [vol/vol]) for 45 min at a flow rate of 20 mL/min. The wavelength was set at 274 nm. The methanol solution of the extract was injected repeatedly and major peaks with the same retention time were pooled. After the organic layer was removed by evaporation under reduced pressure, the aqueous residues were lyophilized to afford (isolated yields in parenthesis) unchanged mosapride 96 mg (31.2%) and four major metabolites, M4 56 mg (18.2%), M5 18 mg (5.8%), M6 16 mg (5.2%) and M7 10 mg (3.3%). Among 96 mg of the recovered mosapride, 12 mg (3.9%) was from the supernatant and 84 mg (27.2%) was from the mycelia by extraction with ethyl acetate.

For structural elucidation, the purified metabolites M4–M7 and mosapride were dissolved in DMSO-*d*6 for 1D, 2D NMR spectral analyses, including heteronuclear multiple quantum coherence spectroscopy (HMQC, one-bone carbon–proton correlation), heteronuclear multiple-bond correlation spectroscopy (HMBC, long-range carbon–proton correlation) and H–H correlation spectroscopy (H–H COSY). HRMS were recorded in order to determine the elemental composition thereby confirming the structures.

3. Results and discussion

3.1. Preliminary screening for biotransformation

Five strains of *Cunninghamella* were screened for their abilities to transform mosapride by UPLC–PDA–MS analyses. In the chromatograms of culture controls, there was no interference from endogenous or secondary metabolites from *C. elegans* for the analyses of mosapride and its metabolites. The substrate controls showed there was no chemical decomposition or any transformation of mosapride under medium and fermentation conditions. The results of UPLC–PDA analyses indicated that mosapride was highly metabolized in the supernatant after centrifugation, and the percentages of transformation were *C. elegans* AS 3.156 (90.35%), *C. elegans* AS 3.2028 (59.29%), *C. echinulata* AS 3.2004 (63.87%), *C. blakesleean*a AS 3.970 (64.71%) and *C. blakesleeana* AS 3.910 (80.70%), respectively. A typical UPLC–PDA chromatogram (gradient condition 1) of mosapride and its metabolites after transformation by *C. elegans* AS 3.156 is shown in Fig. 2. For its highest transformation percentage, *C. elegans* AS 3.156 was finally selected for preparative scale biotransformation. TIC and selected ion reaction (SIR) chromatograms (gradient condition 2) of mosapride and its 13 metabolites (metabolite order consistent with that marked in SIR chromatograms) in the incubation sample are shown in Fig. 3.

3.2. Identification and characterization of mosapride metabolites

The structures of four major metabolites (M4–M7) were identified by 1D, 2D NMR (Table 1), HRMS, UPLC–ESI–MS/MS and UV spectroscopic analyses (Table 2, retention times were given according to the UPLC–ESI–MS/MS analyses performed in gradient condition 2). The structures of the other metabolites in small amounts were proposed by HRMS, UPLC–ESI–MS/MS and UV spectra (Table 2).

The compound eluted at 2.75 min showed UV absorption at 217, 273 and 308 nm. HRMS showed a quasi-molecular ion $[M+H]^+$ at *m*/*z* 422.1636 (calc. 422.1641), indicating the molecular formula of $C_{21}H_{25}ClF\ N_3O_3$. In full-scan HRMS/MS spectra, it had typical fragment ion at *m*/*z* 198.0313 (calc. for $C_9H_9ClNO_2$, 198.0316, error 1.7 ppm) by cleavage at C7–N, which further lost a moiety of CO to form the ion at *m*/*z* 170.0359 (calc. for C_8H_9ClNO, 170.0367, error 4.8 ppm). The fragment ion at *m*/*z* 109.0441 (calc. for C_7H_6F, 109.0448, error 6.5 ppm) represented the *p*-fluorobenzyl portion formed by *N*-dealkylation (Fig. 1). The fragment ions at *m*/*z* 198 and 170 were characteristic of the benzamide part, while the one at *m*/*z* 109 represented the *p*-fluorobenzyl part. All the chromatographic, mass and UV spectroscopic properties were identical to those of authentic mosapride.

Metabolites M1, M2, M3 and M4 with retention times of 2.21, 2.34, 2.56 and 2.81 min, respectively, had similar UV absorption spectra with λ_{max} values of 273 and 308 nm. Their HRMS spectra showed $[M+H]^+$ ions at *m*/*z* 438.1581, 438.1592, 438.1586 and 438.1592, (calc. 438.1590), suggesting identical molecular formula of $C_{21}H_{25}ClF\ N_3O_4$. Their MS/MS spectra showed different fragment ions, indicating they were isomers. Their quasi-molecular ions were 16 Da higher than that of the parent drug, hence, they were proposed to be *N*-oxidation or mono-hydroxylation metabolites of mosapride.

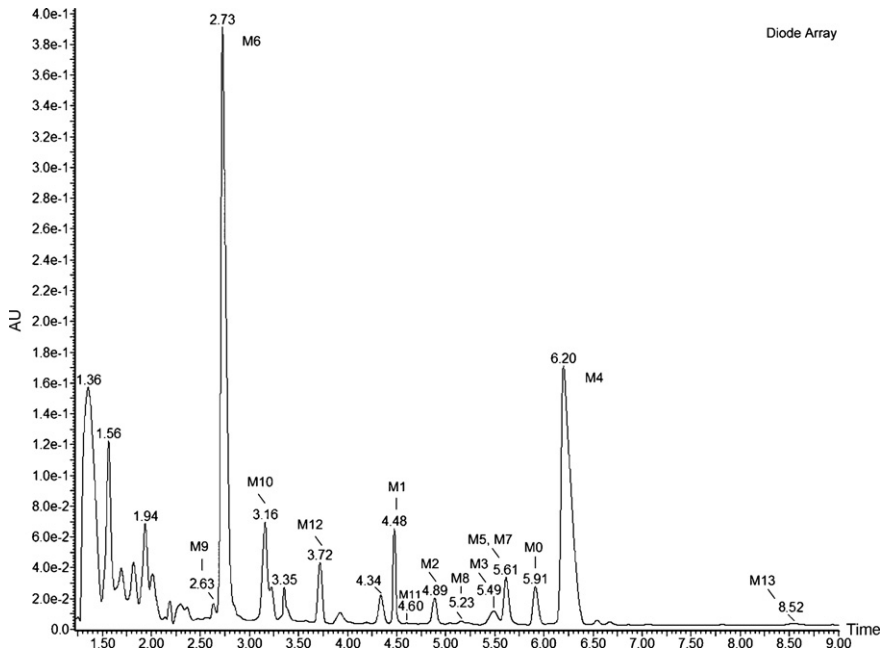

Fig. 2. UPLC–PDA profile of the metabolic products (in gradient condition 1) of mosapride after incubation with *C. elegans* AS 3.156 for 120 h.

The MS/MS of M1 showed fragment ions at *m*/*z* 314, 198, 170 and 125, suggesting the addition of O at the 4-fluorobenzyl moiety (125 = 109 + 16), but the exact substituted position could not be determined from the data of current study. M1 was supposed to be 4-fluorobenzyl-hydroxylated mosapride.

The MS/MS of M2 showed fragment ions at *m*/*z* 214, 186 and 109, which indicated the hydroxylation occurred at the benzamide moiety (214 = 198 + 16, 186 = 170 + 16). The fragment ion at *m*/*z* 109 represented the unaltered 4-fluorobenzyl moiety. The substituted position could be in either C-3 or C-6, but the exact structure could not be identified from these data. M2 was assumed to be 3-hydroxylated mosapride or 6-hydroxylated mosapride.

The MS/MS of M3 showed fragment ions at *m*/*z* 420, 269, 224, 206, 198, 170 and 109. From the presence of ions at *m*/*z* 198, 170 and 109, the addition of O was deduced to be neither in the benzamide moiety nor in the 4-fluorobenzyl moiety. The diagnostic ion at *m*/*z* 420 with about 20% relative abundance suggested water loss from the hydroxylated morpholinyl, for the aliphatic hydroxylation favored loss of water in ESI–MS/MS spectra as reported by Ramanathan et al. [20]. The fragment ion at *m*/*z* 420 further cleaved at C7′–N bond to produce the base fragment ion at *m*/*z* 206 and it represented the morpholinyl and 4-fluorobenzyl moiety. The fragment ion at *m*/*z* 224, which was 18 Da more than the one at *m*/*z* 206, represented the morpholiny moiety formed by cleavage at the C7′–N bond without loss of water. Thus, M3 was characterized as morpholinyl-hydroxylated mosapride.

M4 was isolated as a white powder, mp: 141–143 °C. The MS/MS spectra showed fragment ions at *m*/*z* 420, 329, 280, 215, 198, 170, 159 and 109. The presence of fragment ions at *m*/*z* 198, 170 and 109 suggested the addition of O was neither in the benzamide moiety nor in the 4-fluorobenzyl moiety. The ion at *m*/*z* 420 formed by a loss of water was present in quite a small abundance percentage of 2%, which indicated *N*-oxidation instead of hydroxylation at the morpholinyl [20]. Identification of the structure of M4 was based on the 1D and 2D NMR (HMQC, HMBC, COSY and DEPT 135) data. Compared with the ^{1}H and ^{13}C NMR data of mosapride [21], the proton and carbon resonances of 3′ and 5′, 7″, 2″ and 6″ positions had downfield shifts to different degrees, while those of 6′, and 1″ had upfield shifts. The proton of 2′ had a downfield shift from 3.5 to 4.2 ppm, and the corresponding carbon had an upfield shift from 73.9 to 69.6 ppm. All these changes may be attributed to the effect of *N*-oxidation of morpholinyl, which made the N more electrophilic. Compared with the parent drug, no significant shift change of other protons and carbons was observed. There were long-rang correlations between the signal of H-6′ at 3.4 ppm and the signal of C-2′ at 69.61 ppm, as well as the correlations between the signal of H-5′ at 4.10 ppm and the signal of C-3′ at 64.66 ppm in HMBC experiment. The signals of H-5′ and H-6′ were correlated in the COSY spectrum. The DEPT135 spectrum showed only the signal of C-2′ was upward, while the signals of C-3′, 5′ and 6′ was downward. This clearly indicated no substitution at any carbons of the morpholinyl. Confirmed by HRMS, HMBC and UV spectra data, metabolite M4 was identified as mosapride-4′-*N*-oxide.

Metabolite M5 was isolated as a white powder, mp: 147–149 °C. Its HRMS spectra showed a quasi-molecular ion $[M+H]^{+}$ at *m*/*z* 396.1488, which corresponded to a molecular formula of $C_{19}H_{23}ClFN_3O_3$ (calc. 396.1485). Its molecular weight was 26 Da lower than that of mosapride. The MS/MS spectra of M5 showed fragment ions at *m*/*z* 378, 271, 225, 215, 198, 181 and 170. The presence of fragment ions at *m*/*z* 198 and 170 suggested the unaltered benzoyl moiety, while the one at *m*/*z* 378 from a loss of water was indicative of aliphatic hydroxylation. The ^{1}H and ^{13}C NMR spectra of M5 lacked the signals of position 5′ and 6′ observed for the parent mosapride, while the characteristic proton signals for NH and OH appeared at 1.8–2.0 ppm and 3.8–4.8 ppm with broad peak shapes in ^{1}H NMR spectra, respectively. Thus, M5 was assumed to be the ring opening metabolite by cleavage at both C6′–O and C5′–N bonds of morpholinyl, that is, a loss of $CH_2=CH_2$ (395 = 421-28 + 2H). The following data confirmed the assumption. Due to the inductive effects of OH and NH groups, carbon resonances of 2′, 3′ and 7″ positions had upfield shifts to some degree, while those of 7′ and 1″ had downfield shifts, and the corresponding proton resonances changed too. The proton signal of H-3′ at 2.50 ppm was significantly overlapped with the signal of DMSO, and those of H-2′ and H-7′ were overlapped. These signals were further assigned by HMQC. In addition, the diagnostic ion at *m*/*z* 271 was the benzamide side formed by cleaving at C3′–NH bond, 225 the benzene side by cleaving at C1–C=O bond, and

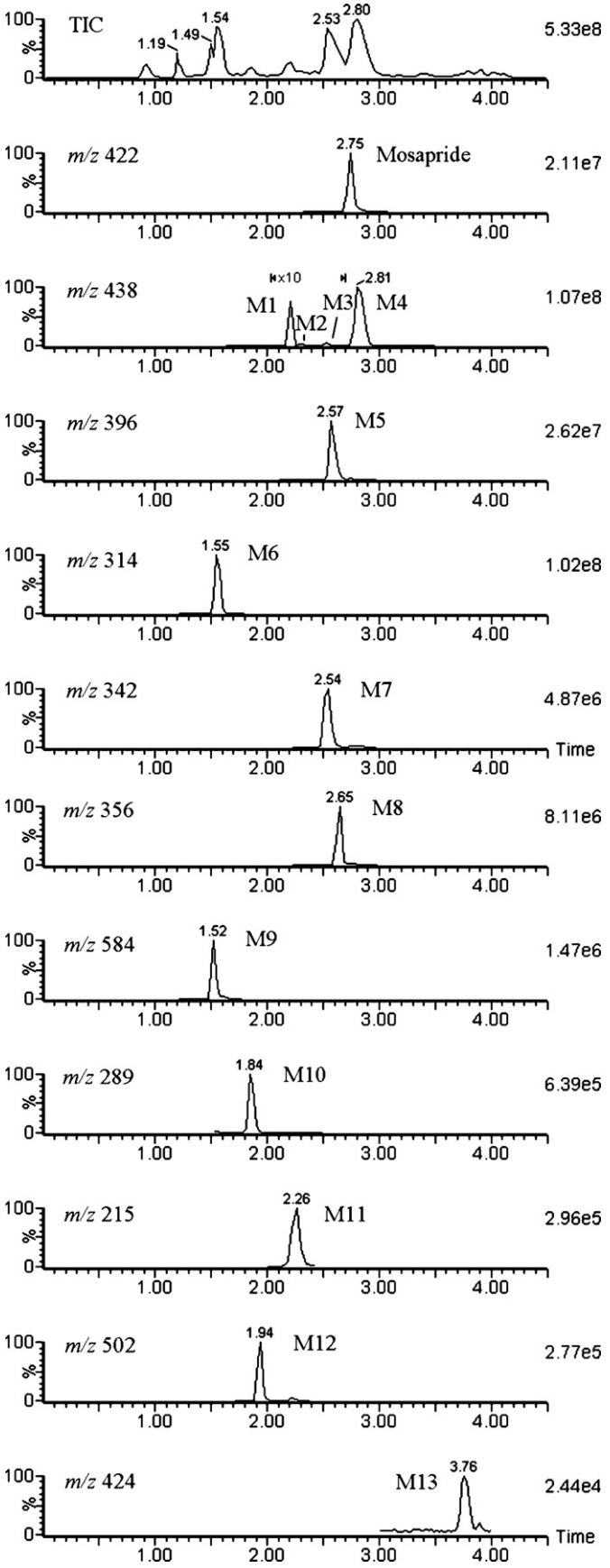

Fig. 3. TIC and SIR chromatograms of mosapride and its metabolites (in gradient condition 2) in the sample after incubation of mosapride with *C. elegans* AS 3.156 for 120 h.

181 the 4-fluorobenzyl side by cleavage of C7–NH along with a loss of water. With the consistent information afforded by HMBC spectra, M5 was identified as 4-amino-5-chloro-2-ethoxy-*N*-[3-(4-fluorobenzylamino)-2-hydroxypropyl] benzamide.

Metabolite M6 was isolated as a white powder, mp: 94–96 °C. It had a molecular formula of $C_{14}H_{20}ClN_3O_3$ as determined from the quasi-molecular ion $[M+H]^+$ at *m*/*z* 314.1266 (calc. 314.1266) in its HRMS spectra. The molecular weight was 108 Da less than that of mosapride. The presence of fragment ions at *m*/*z* 198 and 170 in MS/MS spectra suggested the unaltered benzamide moiety, while the loss of 108 Da corresponded to *N*-dealkylation of 4-fluorobenzyl moiety. In the ^{1}H NMR spectrum, the signals for the *p*-fluorobenzyl moiety were absent and a singlet with broad sharp peak shape was present at 6.79 ppm, which was consistent with the proton connected to nitrogen atom of morpholinyl. Therefore, M6 was unambiguously identified as des-*p*-fluorobenzyl mosapride, that is, 4-amino-5-chloro-2-ethoxy-*N*-(2-morpholinylmethyl)benzamide, which has been reported to be the major mammalian metabolite [8].

Metabolite M7, isolated as a white powder, mp: 99–101 °C, displayed a quasi-molecular ion $[M+H]^+$ at *m*/*z* 342.1212 in HRMS spectrum, corresponding to a molecular formula of $C_{15}H_{20}ClN_3O_4$ (calc. 342.1215). The MS/MS spectra showed fragment ions at *m*/*z* 314, 198 and 170, indicative of formylation of M6. Compared with ^{1}H- and ^{13}C-NMR spectra of mosapride, the H and C signals for the *p*-fluorobenzyl moiety were absent for M6 and a singlet representing the proton of 7″-formyl was present at 8.02 ppm. The proton resonances of 3′, 5′ and 6′ positions had obvious upfield shifts ranged from about 8 to 19 ppm, while the carbon resonances of 3′ and 5′ positions had downfield shifts, all of these changes might be due to the addition of formyl at N-4′. The overlapped proton signals of H-8 and 3′ at 4.06 ppm were assigned by HMQC data. In HMBC experiment, there were long-rang correlations between the signal of the H-7′ and those of C-7, 2′ and 3′, as well as the correlations between the H-5′ signal and the C-6′ and 7″ signals. Confirmed by HMQC and HMBC spectra, M7 was identified as *N*-formyl-des-*p*-fluorobenzyl-mosapride, that is, 4-amino-5-chloro-2-ethoxy-*N*-[(4-formyl-2-morpholinyl) methyl] benzamide.

Metabolite M8, with a retention time of 2.65 min, had a quasi-molecular ion $[M+H]^+$ at *m*/*z* 356.1363 in HRMS spectra, corresponding to a molecular formula of $C_{16}H_{22}ClN_3O_4$ (calc. 342.1372). Its molecular weight was 42 Da higher than that of M6 and there were fragment ions at *m*/*z* 314, 198 and 170. M8 was tentatively assumed as *N*-acetyl-des-*p*-fluorobenzyl-mosapride, that is, *N*-[(4-acetyl-2-morpholinyl)methyl]-4-amino-5-chloro-2-ethoxy-benzamide.

Metabolite M9, eluted at 1.52 min, had UV absorption λ_{max} values of 233, 275 and 305 nm. HRMS analysis showed a quasi-molecular ion $[M+H]^+$ at *m*/*z* 584.2156, indicating the molecular formula of $C_{27}H_{35}ClFN_3O_8$ (calc. 584.2169). The quasi-molecular ion $[M+H]^+$ was a mass shift of 162 Da compared with the parent mosapride. It had fragment ions at *m*/*z* 422, 360, 198 and 170. The fragment ion at *m*/*z* 422 resulted from a neutral loss of 162 Da and the one at *m*/*z* 360 (360 = 198 + 162) was the glucoside conjugated benzamide moiety formed by cleavage at the C7–N bond. All these data were consistent with glucoside conjugate of mosapride at 4-NH_2. Thus, M9 was tentatively characterized as 4-glucoside-mosapride.

Metabolite M10, with a retention time of 1.84 min, had a molecular formula of $C_{12}H_{17}ClN_2O_4$ (calc. 289.0950) as determined from the quasi-molecular ion $[M+H]^+$ at *m*/*z* 289.0949 in its HRMS spectra. There were fragment ions at *m*/*z* 271, 243, 198 and 170 in MS/MS spectra. The ions at *m*/*z* 198 and 170 suggested the unchanged benzoyl part, while those at *m*/*z* 271, 243 and 199 suggested loss of water from the aliphatic hydroxyl and further loss of CO

Table 1
^{1}H and ^{13}C NMR parameters of mosapride and four metabolites purified from the preparative-scale biotransformation of mosapride by *C. elegans* AS 3.156.

Position	Mosapride	M4		M5		M6	M7	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_H	δ_C	δ_H
1	–	110.0	–	110.4	–	–	109.9	–
2	–	156.8	–	156.8	–	–	156.7	–
3	6.44 (s, 1H)	98.31	6.45 (s, 1H)	98.42	6.46 (s, 1H)	6.47 (s, 1H)	98.34	6.46 (s, 1H)
4	–	148.7	–	148.5	–	–	148.7	–
5	–	109.1	–	109.1	–	–	109.1	
6	7.69 (s, 1H)	131.6	7.68 (s, 1H)	131.7	7.71 (s, 1H)	7.72 (s, 1H)	131.6	7.69 (s, 1H)
7	–	163.9	–	163.7	–	–	163.8	–
8	4.03 (q, 2H, 6.9)	64.52	4.05 (q, 2H, 6.9)	64.99	4.03 (q, 2H, 6.9)	4.05 (q, 2H, 6.8)	64.54	4.06 (q, 2H, 7.2)
9	1.36 (t, 3H, 6.9)	14.44	1.36 (t, 3H, 6.9)	14.53	1.36 (t, 3H, 6.9)	1.39 (t, 3H, 6.8)	14.50	1.40 (t, 3H, 7.2)
2′	3.43–3.50 (m, 1H)	69.61	4.16–4.24 (m, 1H)	68.49	3.67 (1H)	4.00 (m, 1H)	73.75	3.43 (m, 1H)
3′	2.70 (d, 1H, 10.7)	64.66	2.96 (d, 1H, 11.2),	52.62	2.50 (2H)	3.13 (m, 1H)	42.22	4.06 (1H), 2.55 (dd, 1H, 10.8, 10.2)
	1.94 (dd, 1H, 10.7, 10.6)		3.22 (dd, 1H, 11.3, 10.4)			2.70 (m, 1H, 12.1)		
5′	2.59 (d, 1H, 10.9), 2.13 (ddd, 1H, 11. 0, 11.1, 3.6)	60.79	3.80 (d, 1H, 12.5), 4.10 (t, 1H, 11.9, 12.0)	–	–	2.87 (m, 1H), 2.74-2.80 (m, 1H)	44.67	3.56 (br d, 1H, 12.6), 3.15 (ddd, 1H, 11. 4, 11.4, 5.4)
6′	3.80 (d, 1H, 10.8), 3.50–3.55 (m, 1H)	61.77	2.83 (d, 1H, 11.1), 3.42–3.47 (m, 1H)	–	–	3.76 (d, 1H, 10.8), 3.46–3.51 (m, 1H)	47.38	3.67 (br d, 1H, 12.0), 2.94 (dd, 1H, 10.2, 10.3)
7′	3.36–3.44 (m, 1H), 3.24 (m, 1H)	40.80	3.38–3.44 (m, 1H), 3.22 (m, 1H)	43.41	3.38–3.46 (m, 1H), 3.20 (m, 1H)	3.38–3.43 (m, 1H), 3.32–3.36 (m, 1H)	40.94	3.51 (m, 1H), 3.33 (m, 1H)
1″	–	126.0	–	137.0	–	–	–	–
2″6″	7.32 (dd, 2H, 5.9, 2.4)	135.1 (d, 33.6)	7.59 (dd, 2H)	129.8 (d, 36.0)	7.33 (dd, 2H, 5.9, 2.4)	–	–	–
3″5″	7.13 (dd, 2H, 8.9, 8. 8)	114.9 (d, 85.2)	7.20 (dd, 2H, 8.5, 8.4)	114.8 (d, 84.0)	7.08 (dd, 2H, 8.8, 8.8)	–	–	–
4″	–	162.0	–	161.1 (d, 960)	–	–	–	–
7″	3.33–3.42 (m, 2H)	72.31	4.47 (m, 2H)	52.21	3.67 (2H)	–	161.2	8.01 (s, 1H)
NH_2	5.94 (s, 2H)	–	5.94 (s, 2H)	–	5.90 (s, 2H)	5.93 (s, 2H)	–	5.95 (s, 2H)
NH	8.04 (t, 1H, 5.0, 5.2)	–	8.04 (t, 1H, 5.4, 5.3)	–	8.08 (t, 1H, 5.0, 5.3)	8.06 (t, 1H, 4.7)	–	8.04 (t, 1H)
2′-OH	–	–	–	–	3.8–4.8 (br s, 1H)	–	–	–
4′-NH	–	–	–	–	1.6–2.3 (br s, 1H)	6.2–7.4 (br s, 1H)	–	–

Table 2
UV, ESI–Q–TOF/MS and UPLC–ESI–MS/MS data of mosapride and its metabolites (in gradient condition 2) produced by incubation with *C. elegans* 3.156.

Metabolite	Retention time (min)	UV λ_{max} (nm)	MS $[M+H]^+$	Measured $[M+H]^+$	Calculated $[M+H]^+$	Error (ppm)	Formula	*m/z* (relative abundance, %) of MS^2 fragments
Mosapride	2.75	217, 273, 308	422	422.1636	422.1641	1.2	$C_{21}H_{25}ClFN_3O_3$	198 (100), 170 (5)
M1	2.21	219, 273, 308	438	438.1581	438.1590	2.1	$C_{21}H_{25}ClFN_3O_4$	314 (4), 198 (100), 170 (4), 125 (2)
M2	2.34	218, 273, 308	438	438.1592	438.1590	−0.4	$C_{21}H_{25}ClFN_3O_4$	214 (100), 186 (51), 109 (2)
M3	2.56	235, 274, 308	438	438.1586	438.1590	1.0	$C_{21}H_{25}ClFN_3O_4$	420 (20), 269 (6), 224 (4), 206 (100) 198 (39), 170 (5), 109 (2)
M4	2.81	218, 273, 308	438	438.1592	438.1590	−0.4	$C_{21}H_{25}ClFN_3O_4$	420 (2), 329 (2), 280 (43), 215 (12), 198 (100), 170 (4), 159 (39),109 (74)
M5	2.57	217, 274, 308	396	396.1488	396.1485	−1.7	$C_{19}H_{23}ClFN_3O_3$	378 (9), 271 (10), 225 (2), 215 (4), 198 (100), 181 (5), 170 (2)
M6	1.55	217, 273, 308	314	314.1266	314.1266	0.0	$C_{14}H_{20}ClN_3O_3$	198 (100), 170 (4)
M7	2.65	216, 273, 308	342	342.1212	342.1215	0.9	$C_{15}H_{20}ClN_3O_4$	314 (2), 198 (100), 170 (6)
M8	2.54	217, 273, 308	356	356.1363	356.1372	2.4	$C_{16}H_{22}ClN_3O_4$	314 (4), 198 (100), 170 (8)
M9	1.52	233, 275, 305	584	584.2156	584.2169	2.3	$C_{27}H_{35}ClFN_3O_8$	422 (3), 360 (100), 198 (47), 170 (2)
M10	1.84	216, 272, 307	289	289.0949	289.0950	0.2	$C_{12}H_{17}ClN_2O_4$	271 (6), 243 (7),198 (100), 170 (12)
M11	2.26	216, 230 (sh), 273, 308	215	215.0574	215.0582	3.6	$C_9H_{11}ClN_2O_2$	198 (84), 170 (100)
M12	1.94	216, 226 (sh), 267, 303	502	502.1198	502.1209	2.3	$C_{21}H_{25}ClFN_3O_6S$	422 (6), 278 (5), 198 (100), 170 (2), 109 (3)
M13	3.76	216, 230 (sh), 272, 308	424	424.1438	424.1434	−1.0	$C_{20}H_{23}ClFN_3O_4$	406 (42), 396 (3), 378 (5), 253 (6), 215 (8), 209 (12), 198 (100), 170 (7), 109 (2)

(243 = 271-28). Thus, M10 was proposed to be 4-amino-5-chloro-*N*-(2,3-dihydroxypropyl)-2-ethoxybenzamide. It might be produced by *N*-dealkylation of M5 to form aldehyde which further underwent reduction.

Metabolite M11, eluted at 2.26 min, showed a quasi-molecular ion $[M+H]^+$ at *m/z* 215.0574, corresponding to a molecular formula of $C_9H_{11}ClN_2O_2$ (calc. 215.0582). It had fragment ions at *m/z* 198 and 170, in consistence with the benzamide moiety by cleavage at the C7′–N bond. M11 was assumed to be 4-amino-5-chloro-2-ethoxybenzamide.

Metabolite M12 was eluted at 1.94 min and its HRMS spectra displayed a quasi-molecular ion $[M+H]^+$ at *m/z* 502.1198, suggesting a molecular formula of $C_{21}H_{25}ClFN_3O_6S$ (calc. 502.1209). The $[M+H]^+$ ion was a mass shift of 80 Da compared with the parent mosapride, which was characteristic of sulfate conjugate. There were fragment ions at *m/z* 422, 278, 225, 198, 170 and 109. The one at *m/z* 278 (278 = 198 + 80) was the sulfate conjugated benzamide moiety. Based on the above data, M12 was proposed to be mosapride-4-sulfate, that is, 2-chloro-5-ethoxy-4-[[4-(4-fluorobenzyl)-2-morpholinyl]methylcarbamoyl]phenylsulfamic acid.

A very small trace level of M13 eluted at 3.76 min showed a quasi-molecular ion $[M+H]^+$ at *m/z* 424.1438 in HRMS spectra, corresponding to a molecular formula of $C_{20}H_{23}ClFN_3O_4$ (calc. 424.1434). The molecular weight was 28 Da higher than that of M5. There were fragment ions at *m/z* 406, 396, 378, 253, 215, 209, 198, 170 and 109 in MS/MS spectra. The fragment ion at *m/z* 406 was produced by a loss of water and it further cleaved at C3′–N from the benzamide moiety to form the one at *m/z* 253. The fragment ions at *m/z* 396 and 378 arose from loss of CO, and further loss of water, respectively. The fragment ions at *m/z* 215 and 209 represented the benzamide and 4-fluorobenzyl moiety by cleavage at the C7′–N bond. Hence, M13 was characterized as 4-amino-5-chloro-2-ethoxy-*N*-[3-[*N*-(4-fluorobenzyl)formamido]-2-hydroxypropyl] benzamide, the formylated derivative of M5, that is, formylated-morpholinyl-ring-opened- mosapride.

Herein, we studied the metabolism of mosapride by microorganisms, *C. elegans*, for the first time. Thirteen metabolites including seven phase I metabolites and six phase II metabolites were detected after biotransformation of mosapride by *C. elegans* AS 3.156. In addition to M6, the known major mammalian metabolite, others were all novel metabolites produced by microbial transformation. After preparative scale biotransformation, most of the unchanged drug was recovered from the mycelia (84 mg) other than the supernatant (12 mg) by extraction with ethyl acetate, indicating adsorption and solubilization of this hydrophobic molecule into lipophilic cell membranes, possibly with limited permeation to the cytosol and microsomal compartments, as described in the literatures [22,23]. Although the isolated yield of metabolites was not high, our results still indicated that microbial transformation could produce reference standards of mammalian metabolites and/or new drug derivatives. The proposed biotransformation pathway of mosapride is shown in Fig. 4. The results of our study further confirmed that *C. elegans* is capable of metabolizing compounds, especially by *N*-oxidation and *N*-dealkylation [16,24–26].

Matsumoto et al. [8] reported that two of the four mammalian metabolites, 3-hydroxyl des-*p*-fluorobenzyl and 3-hydroxyl 5′-oxo-des-*p*-fluorobenzyl mosapride, were of great polarity and isolated from the aqueous phase of rat urine. In our study, however, except the major metabolite M6, none of other reported mammalian metabolites were observed in the screening experiments. This might be explained by two points. One was the metabolism difference between the tested microorganisms and mammalians. The other was that we only studied the organic layer of the fermentation culture not the aqueous phase which might contain

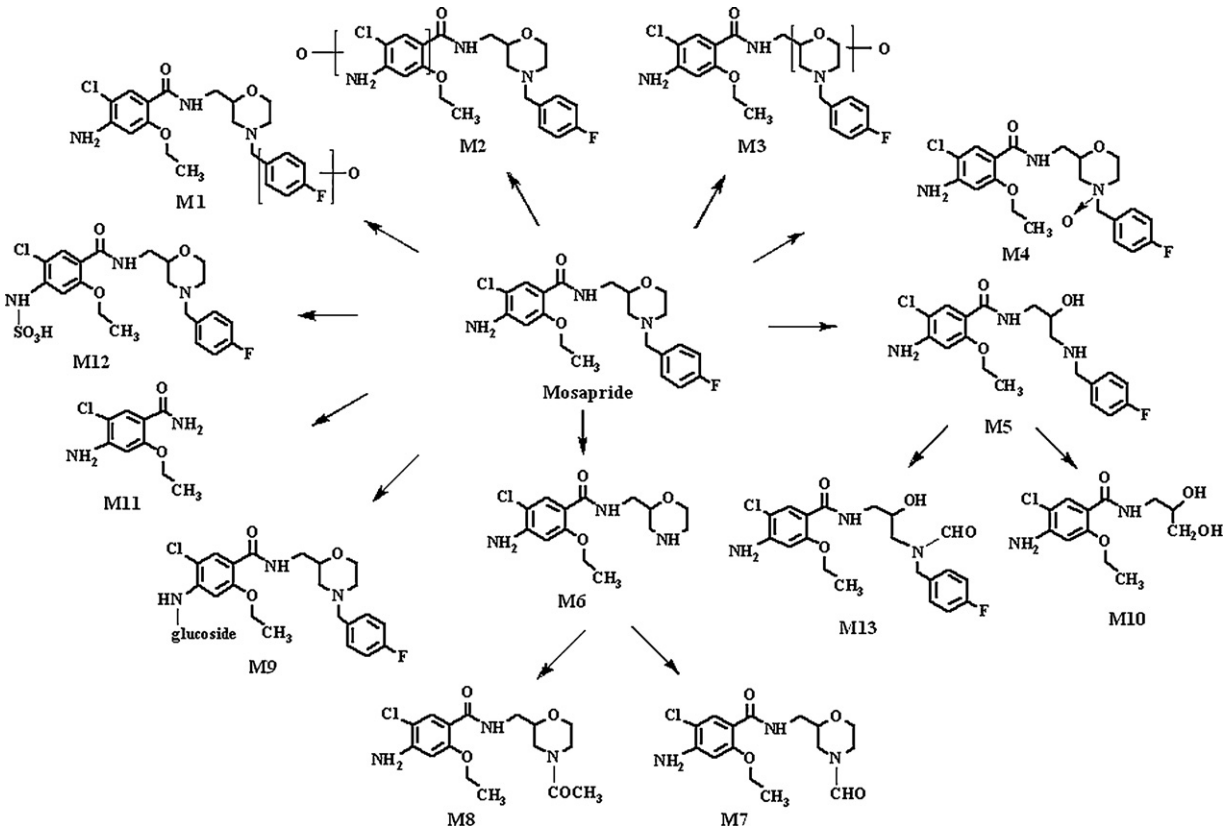

FIG. 4. Proposed metabolic profile of mosapride by *C. elegans* AS 3.156.

more polar metabolites. There were still other unknown metabolites found in the metabolism of mosapride in rats, dogs, monkeys and humans, but their identities were not mentioned in previous studies [8,10,11]. In order to know whether the metabolites found in microbial transformation of mosapride exist in the mammalian metabolism, further study is needed to give more detailed and concrete metabolic profiles of mosapride in mammalians.

4. Conclusions

In this paper, mosapride was transformed to twelve novel and one known mammalian metabolites by *C. elegans* AS 3.156. The main metabolic routes were as follows: *N*-oxylation, morpholinyl ring cleavage, *N*-dealkylation by cleavage at the C7″–N bond and *N*-dealkylation followed by formylation. Phase II metabolites such as sulfate, glucoside conjugates, formylation and acetylation metabolites were also found in our study. Three identified metabolites, M4, M5 and M7, were firstly reported and could be used as reference standards in further study on mammalian metabolism of mosapride. All the results in our study clearly indicated that transformation of mosapride by *C. elegans* AS 3.156 provided a facile way of producing known metabolites and/or novel derivatives of mosapride in sufficient amounts and purities that could be used as analytical reference standards.

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