Identification of Novel Metoclopramide Metabolites in Humans: In Vitro and In Vivo Studies

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ABSTRACT:

Metoclopramide (MCP) is frequently used to treat gastroparesis. Previous studies have documented MCP metabolism, but systematic structural identification of metabolites has not been performed. The aim of this study was to better understand MCP metabolism in humans. For examination of in vivo metabolism, a single oral 20-mg MCP dose was administered to eight healthy male volunteers, followed by complete urine collection over 24 h. In vitro incubations were performed in human liver microsomes (HLM) to characterize metabolism via cytochromes P450 and UDP-glucuronosyltransferases and in human liver cytosol for metabolism via sulfotransferases. Urine and subcellular incubations were analyzed for MCP metabolites on a mass spectrometer with accurate mass measurement capability. Five MCP metabolites were detected in vivo, and five additional metabolites were detected in vitro. The five metabolites of MCP identified both in vitro and in vivo were an N-O-glucuronide (M1), an N-sulfate (M2), a des-ethyl metabolite (M3), a hydroxylated metabolite (M4), and an oxidative deaminated metabolite (M5). To our knowledge, metabolites M1 and M4 have not been reported previously. M2 urinary levels varied 22-fold and M3 levels varied 16-fold among eight subjects. In vitro studies in HLM revealed the following additional metabolites: two ether glucuronides (M6 and M8), possibly on the phenyl ring after oxidation, an N-glucuronide (M7), a carbamic acid (M9), and a nitro metabolite (M10). Metabolites M6 to M10 have not been reported previously. In conclusion, this study describes the identification of MCP metabolites in vivo and in vitro in humans.

Gastroparesis is a chronic stomach motility disorder and is characterized by slow emptying of solids in the absence of mechanical obstruction. It is estimated that up to 4% of the population in the United States have gastroparetic symptoms (Abell et al., 2006). Gastroparesis can be diabetic, postsurgical, or idiopathic. Treatment options include dietary management, pharmacologic agents, and surgical procedures such as gastric electrical stimulation and feeding jejunostomy (Friedenberg and Parkman, 2007). Metoclopramide (MCP) was approved by the U.S. Food and Drug Administration (FDA) in 1979 and has been available as a generic drug for many years. To date, MCP is the only FDA-approved drug for the treatment of gastroparesis (Smith and Ferris, 2003).

MCP is a dopamine receptor antagonist. In addition to being commonly prescribed for management of gastroparesis, it is also widely used as an antiemetic. It is associated with central nervous system side effects. Clinically, the neurologic effects of MCP result in a broad spectrum of drug-induced movement disorders (Pasricha et al., 2006).

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ABBREVIATIONS: MCP, metoclopramide; FDA, U.S. Food and Drug Administration; P450, cytochrome P450; HLM, human liver microsome(s); UDPGA, UDP-diphosphoglucuronic acid; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry; UGT, UDP-glucuronosyltransferase; SULT, sulfotransferase; MS, mass spectrometry; ESI, electrospray ionization; RF, radiofrequency; FTMS, Fourier transformation mass spectrometry; CID, collision-induced dissociation; amu, atomic mass units; DIMD, drug-induced movement disorder.
Identifying the specific enzymes responsible for drug metabolism further enables prediction of drug-drug interactions and interindividual variability due to enzyme polymorphisms. These are poorly studied aspects of MCP. The present study sought to identify metabolites of MCP in humans. To this end, human urine samples were collected and analyzed after oral MCP administration. In addition, in vitro incubations were performed to identify human metabolites in hepatic subcellular fractions and to corroborate in vivo findings.

Materials and Methods

Chemicals and Reagents. Pure metoclopramide hydrochloride was purchased from Sigma-Aldrich (St. Louis, MO). An NADPH-regenerating system (a system that generates NADPH in situ using an enzymatic reaction), pooled human liver cytosol (HLC), and pooled human liver microsomes (HLM) were purchased from BD Biosciences (San Jose, CA). The cofactor for glucuronidation incubations UDP-glucuronic acid (UDPGA), the pore-forming antibiotic alamethicin, 3'-phosphoadenosine-5'-phosphosulfate, and triethylamine were purchased from Sigma-Aldrich (St. Louis, MO). An NADPH-regenerating system further enables prediction of drug-drug interactions and interindividual variability due to enzyme polymorphisms. These are poorly studied aspects of MCP. The present study sought to identify metabolites of MCP in humans. To this end, human urine samples were collected and analyzed after oral MCP administration. In addition, in vitro incubations were performed to identify human metabolites in hepatic subcellular fractions and to corroborate in vivo findings.

FIG. 1. Putative metabolites of MCP, identified in vitro (in HLM and HLC) and in vivo (in human urine) by MSn analysis. The intermediates postulated in brackets were not detected.

Human Subjects. The study was approved by Temple University Institutional Review Board. Eight healthy male subjects between the ages 18 and 65 years were recruited. Subjects were nonsmokers and not on any other medication. Subjects did not ingest any caffeinated or alcoholic beverages or any product containing grapefruit juice for 5 days before the start of the study. Exclusion criteria included sensitivity/allergy to MCP, known renal failure, pheochromocytoma, history of Parkinson’s disease, history of clinical depression, suspected bowel obstruction, use of other medications, and history of seizures. Subjects were admitted to the General Clinical Research Center at Temple University Hospital for the duration of the study. Subjects were fasted overnight, and a baseline urine sample was collected before drug administration. Subjects were administered a single oral dose of 20 mg of MCP (Reglan) with 100 ml of water. This treatment was followed by a standard meal 30 min later and at regular intervals until the end of the study. Urine was collected until 24 h postdose. Urine samples were collected in 1000-ml capacity Nalgene glass bottles and were stored immediately upon collection at −20°C until further analysis. For LC-MS/MS analysis, a 5-ml urine sample was thawed and centrifuged at 14,000 rpm for 5 min. The supernatant was collected for further analysis on HPLC.

In Vitro Incubations. P450 incubations. P450-mediated metabolism of MCP was characterized in HLM using methods described previously (Ung et al., 2009). In brief, incubation preparations contained HLM (0.5 mg/ml) with a NADPH-regenerating system (final concentrations of components in reactions are 1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, and 3.3 mM magnesium chloride) and 50 mM potassium phosphate buffer (pH 7.4). To initiate reactions, MCP (1 mM final concentration) was added for a total incubation volume of 500 l, and the mixture was incubated at 37°C for 5 h. A high substrate concentration was intended to avoid substrate depletion. Because enzyme kinetics were not evaluated, incubations were not limited to initial rate conditions but were conducted for high metabolite yield. Reactions were terminated by addition of 100 l of ice-cold acetonitrile. Reactions were then centrifuged at 14,000 rpm for 5 min, and the supernatant was collected for further analysis on HPLC.
Glucuronidation reactions were conducted either as one-step UGT incubations or two-step mixed P450/HLM UGT incubations. For one-step glucuronidation (Iwuchukwu and Nagar, 2008), incubations in a total volume of 500 μl contained HLM (0.5 mg/ml), 1 mM MCP, 5 mM MgCl₂, 10 μg/ml alamethicin (pore-forming agent to “activate” latent UGTs), and 5 mM UDPGA in the reaction buffer, 0.1 M Tris-HCl (pH 7.4 at 37°C). After 1 h, the reaction was terminated with 50 μl of ice-cold acetonitrile and prepared for further analysis. For the two-step P450/HLM UGT incubations, a total volume of 500 μl containing 0.5 mg/ml HLM, 1 mM MCP, and an NADPH-regenerating system in 50 mM potassium phosphate buffer (pH 7.4) was first incubated at 37°C for 3 h before the addition of alamethicin (10 μg/ml) and UDPGA (5 mM) for further incubation overnight at 37°C. Because enzyme kinetics were not evaluated, incubations were not limited to initial rate conditions. The reaction was terminated the next morning by addition of 100 μl of ice-cold acetonitrile and centrifuged at 14,000 rpm for 5 min. The supernatant was collected for further analysis on LC-MS/MS.

Sulfation reactions were conducted using HLC. In brief, by use of a modified assay (Ung and Nagar, 2007), a total incubation volume of 500 μl contained 1 mg/ml HLC, 1 mM MCP, and 4 mM 3'-phosphoadenosine-5'-phosphosulfate in 10 mM potassium phosphate (pH 6.5). Incubations were performed for 3 h at 37°C and then terminated with ice-cold acetonitrile (200 μl). Because enzyme kinetics were not evaluated, incubations were not limited to initial rate conditions. The sample was centrifuged at 14,000 rpm for 5 min, and the supernatant was collected for analysis on LC-MS/MS.

**HPLC Instrumentation and Metabolite Detection.** Samples from in vitro incubations were initially analyzed by reverse-phase high-performance liquid chromatography using a slightly modified method (Desta et al., 2002). The HPLC system (Hewlett-Packard 1100 series; Agilent Technologies, Santa Clara, CA) components were a vacuum degasser, a quaternary pump, an autosampler, a Zorbax Rx-CN column (250 × 4.6 mm, 5 μm; Agilent Technologies), and an ultraviolet detector set at 274 nm for detection of MCP.

**FIG. 2.** LC-MS chromatograms of MCP and MCP metabolites. Left, metabolites formed in vivo in human urine. Right, metabolites additionally formed in vitro in HLM and HLC. Peaks from three unique types of in vitro incubations (single- or two-step HLM and HLC) are overlaid. The figure depicts true retention times of each metabolite formed in vitro. Chromatograms depict a 16-min segment of a 34-min run; no parent-related peaks were observed between 16 and 34 min.
and its metabolites. The software ChemStation for LC (version A.08.01; Agilent Technologies) was used to perform integration of peaks. At 25°C, metabolites were eluted isocratically with a mobile phase consisting of 20% acetonitrile and 80% buffer (100 mM sodium acetate plus 0.05% v/v triethylamine, pH adjusted to 7.0 with acetic acid), at a flow rate of 1.0 ml/min. The supernatants (100-μl injection) of P450-mediated reactions were analyzed and two separate metabolite peaks were detected at 20.7 and 22.9 min, respectively. Fractions containing each respective metabolite were collected for structural elucidation by LC-MS/MS. Likewise, supernatants from other in vitro incubations were initially run on the HPLC-UV assay, and all putative structural elucidation by LC-MS/MS. Fractions containing each respective metabolite were collected for two separate metabolite peaks were detected at 20.7 and 22.9 min, respectively. Fractions containing each respective metabolite were collected for structural elucidation by LC-MS/MS.

LC-MS<sup>n</sup> Method for Metabolite Identification. A sensitive LC-MS<sup>n</sup> method was developed for identification of MCP metabolites, by modification of a method reported previously (Gunduz et al., 2010). Urine collected over a period of 24 h was aliquoted and centrifuged at 6440g for 5 min before analysis. The respective supernatants were removed and transferred to 1-ml conical glass inserts for LC-MS<sup>n</sup> analysis. MCP spiked into blank urine was used as a control sample for background ion spectra. Samples were analyzed for the presence of metabolites with the help of a Thermo LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) capable of MS<sup>n</sup> scanning and accurate mass measurement interfaced with a <span>3×/Ti</span> HPLC pump and CTC PAL autosampler (Leap Technologies, Carrboro, NC). The chromatographic separation was carried out on a Symmetry C18 analytical column (5 μm, 2.1 × 150 mm; Waters, Milford, MA) with a 35-min gradient elution method. Mobile phase A consisted of 10 mM ammonium formate in MS grade water with 0.1% formic acid. Mobile phase B consisted of MS-grade acetonitrile with 0.1% formic acid. The sample aliquots were eluted with a flow rate of 0.25 ml/min with 5% B for the initial 5 min. Thereafter, the percentage of mobile phase B was gradually increased to 40% B over 20 min and to 95% over 5 min. After the elution of MCP and its metabolites, the column was returned over 1 min to 5% B, where it was held for 3 min for reequilibration, before the next injection. ESI in positive mode was carried out at a capillary temperature of 375°C, a sheath gas flow rate of 35 ml/min, source voltage of 4.20 kV, source current of 100 μA, capillary voltage of 35, and tube lens voltage equal to 100 V. A multipole RF amplifier was set at 400 Vp-p, along with multipole 0 offsets at −1.25 and −4.75 V, lens 0 voltage at −6.50 V, lens 1 voltage at −13 V, gate lens offset at −62 V, multipole 1 offset at −7 V, and front lens at −5 V. The instrument calibration was set at a multiple RF of 2824 Hz, main RF of 1187.5 Hz, and pulsed Q dissociation collision energy factor of 10. Fourier transformation mass spectrometry (FTMS)-enabled accurate mass measurement was performed at a mass resolution of 60,000. An ion trap mass spectrometry-based data-dependent scan after collision-induced dissociation was performed at a normalized collision energy of 35. Activation Q was set at 0.25 and activation time was 30 ms.

Comparison of MCP Urinary Metabolites across Subjects. Urinary MCP and metabolite absolute peak areas were compared across all subjects (n = 8) for intersubject variability in the formation of each metabolite. It was not possible to compare relative formation of the different metabolites because synthetic metabolite standards were not available. The MS signals obtained for each chemical moiety could not be compared across chemicals because of a lack of equimolar responses across the chemical matter. Therefore, for each metabolite, the fold variability was calculated by normalizing the highest absolute peak area to the lowest absolute peak area for that metabolite among eight subjects.

**Results**

A total of 10 metabolites of parent MCP were detected in the in vitro and in vivo studies together (Fig. 1). The types of metabolites, their retention times, and key MS and MS<sup>n</sup> fragments are listed in Table 1. Human urine samples exhibited a total of five MCP metabolites (labeled M1 through M5) (Fig. 2, left). The structures of these five metabolites were characterized as described in detail below. In addition, all five metabolites (M1–M5) formed in vivo were confirmed with in vitro incubations for specific metabolic pathways. Thus, for example, in vitro SULT incubations in human liver cytosol resulted in the formation of only a sulfated metabolite, which exhibited exactly the same MS/MS spectrum as the sulfated metabolite observed in human urine. The in vitro incubations resulted in five

![Fig. 3. MS<sup>2</sup> spectrum of MCP obtained in positive ion mode ESI. The parent (MCP) is seen at m/z 300 at approximately 5% signal intensity compared with the base peak m/z 227. Other diagnostic fragments include ions obtained from N,N-diethylamino-ethylamine and 4-amino-5-chloro-2-methoxy-benzoyl moieties.](image-url)
FIG. 4. Mass spectra for metabolites M1 to M5 formed in vivo. Ions indicated by * denote unchanged fragmentation compared with the parent. A, M1 was observed to be formed after heteroatom oxidation followed by glucuronidation. The CID product ion spectrum of M1 afforded MCP (+16 + 176), suggesting an oxidative addition followed by conjugation with glucuronic acid. A peak at m/z 316 was also observed as a single peak in a neutral loss scan of 176. B, CID of m/z 380 (M2), with ESI revealed a base peak ion of m/z 300, corresponding to the protonated molecular ion of MCP. A peak at m/z 300 was also observed as a single peak in a neutral loss scan of 80 amu, a diagnostic loss of the sulfonic acid group, suggesting that M2 was a MCP sulfate.
C, MS² spectrum of m/z 272 (M3) in positive mode at a collision energy of 35 afforded m/z 227 as the base peak ion, resulting from the loss of ethyl amine moiety. D, M4 was an oxidative metabolite of MCP, possibly on the 4-amino-5-chloro-2-methoxy-benzoyl moiety. MS/MS of m/z 316 (M4) compared with that of the parent (at m/z 300) suggested a base peak ion shift from m/z 227 to m/z 243, accounting for oxidation.
additional metabolites not observed in vivo (M6–M10) (Fig. 2, right), for a total of 10 MCP metabolites. The structural rationalization of each metabolite is explained in detail below.

**Structural Rationalization of MCP.** With ESI in positive ion mode at a collision energy of 35, MCP showed a protonated molecular ion \([M+H]^+\) of \(m/z\) 300. The LC retention time was approximately 10.4 min (Fig. 2). The CID product ion spectrum of \(m/z\) 300 (Fig. 3) afforded a base peak ion of \(m/z\) 227 resulting from a neutral loss of a diethyl amine moiety. Other fragment ions in this spectrum were \(m/z\) 272, 184, 143, 117, 115, 100, and 89. The fragment ion \(m/z\) 272 resulted from the cleavage of the C–N bond of one of the ethyl groups attached to the aliphatic amino group. Subsequent loss of the aromatic ring from \(m/z\) 300 resulted in ions of \(m/z\) values 143 and 115. Fragment ion \(m/z\) 184 was formed from \(m/z\) 300 by loss of the N,N-diethylamino-ethylamine moiety (Fig. 3). Fragment ions \(m/z\) 89, 100, and 117 were derived from the aliphatic side chain of the parent molecular ion \(m/z\) 300 and corresponded to the loss of the 4-amino-5-chloro-2-methoxy-benzoyl moiety (Fig. 3). The fragment ions were confirmed by accurate mass measurement.

**Structural Rationalization of M1.** ESI of M1 in positive ion mode at a collision energy of 35, MCP showed a protonated molecular ion \([M+H]^+\) of \(m/z\) 300. The LC retention time was approximately 10.4 min (Fig. 2). The CID product ion spectrum of \(m/z\) 300 (Fig. 3) afforded a base peak ion of \(m/z\) 227 resulting from a neutral loss of a diethyl amine moiety. Other fragment ions in this spectrum were \(m/z\) 272, 184, 143, 117, 115, 100, and 89. The fragment ion \(m/z\) 272 resulted from the cleavage of the C–N bond of one of the ethyl groups attached to the aliphatic amino group. Subsequent loss of the aromatic ring from \(m/z\) 300 resulted in ions of \(m/z\) values 143 and 115. Fragment ion \(m/z\) 184 was formed from \(m/z\) 300 by loss of the N,N-diethylamino-ethylamine moiety (Fig. 3). Fragment ions \(m/z\) 89, 100, and 117 were derived from the aliphatic side chain of the parent molecular ion \(m/z\) 300 and corresponded to the loss of the 4-amino-5-chloro-2-methoxy-benzoyl moiety (Fig. 3). The fragment ions were confirmed by accurate mass measurement.

**Structural Rationalization of M2.** A peak corresponding to \(m/z\) 380 was observed at 5.7 min (Fig. 2, left). The CID of \(m/z\) 380 (M2), with ESI at a collision energy of 35, revealed a base peak ion of \(m/z\) 300, corresponding to the protonated molecular ion of MCP. In addition, \(m/z\) 227, a fragment ion that corresponded to MCP without the ethyl amine moiety, was also observed. A peak at \(m/z\) 300 was also observed as a single peak in a neutral loss scan of 80 amu, a diagnostic loss of the sulfonic acid group. FTMS-based accurate mass measurement
ment confirmed M2 to be a MCP sulfate (Fig. 4B). Comparison with in vitro SULT incubations in HLC corroborated these findings.

**Structural Rationalization of M3.** An N-des-ethy1 metabolite of MCP was observed at 6.2 min (Fig. 2, left), both in human urine samples and in in vitro P450 incubations. The MS\(^2\) spectrum of \(m/z\) 272 in positive mode at a collision energy of 35 afforded \(m/z\) 227 as the base peak ion, resulting from the loss of diethyl amine moiety (Fig. 4C). Fragment ions \(m/z\) 272, 184, and 115 seemed to be unchanged, pointing toward the absence of an ethyl group on M3. Compared with MCP, other fragment ions such as \(m/z\) 143, 100, and 117 containing the \(N,N\)-diethyl amino group were not detected. Therefore, M3 was determined to be an oxidative deethylation product of MCP. These observations were further confirmed by accurate mass-based calculation of the empirical formula and mass defect measurement.

**Structural Rationalization of M4.** MS\(^2\) scans revealed a single peak corresponding to \(m/z\) 316 at 6.5 min (Fig. 2, left). MS/MS of \(m/z\) 316 compared with the parent (at \(m/z\) 300) suggested that the base peak ion had shifted from \(m/z\) 227 to \(m/z\) 243, accounting for oxidation (Fig. 4D). A fragment ion \(m/z\) 184 also revealed addition of 16 amu and was observed as \(m/z\) 200. Other fragment ions \(m/z\) 100 and 117 were unchanged. This result suggested that M4 was an oxidative metabolite of MCP, possibly on the 4-amino-5-chloro-2-methoxy-benzoyl moiety, as confirmed by FTMS. M4 was also formed with in vitro P450 incubations. Neither was a peak corresponding to MCP, \(m/z\) 300 observed at 6.5 min, nor did the MS\(^2\) spectrum reveal fragments corresponding to the unchanged 4-amino-5-chloro-2-methoxy-benzoyl moiety of MCP. These data collectively point to M4 being an oxidation on a carbon atom and not a heteroatom, i.e., the aromatic amino group.

**Structural Rationalization of M5.** M5 was identified to be an oxidative metabolite of MCP, resulting from an oxidative deamination of the diethyl amine moiety followed by subsequent oxidation. In addition, M5 was observed in P450 incubations in vitro. ESI in positive mode at collision energy of 35 resulted in an \(m/z\) of 259 at 12.0 min (Fig. 2, left). MS/MS of \(m/z\) 259 resulted in the MS\(^2\) spectrum as shown in Fig. 4E. The base peak ion changed from \(m/z\) 227 to \(m/z\) 184, which corresponds to the 4-amino-5-chloro-2-methoxy-benzoyl moiety. Fragment ion \(m/z\) 184 was confirmed by accurate mass measurement to be the same \(m/z\) 184 as that from MS/MS of MCP. Fragment ion \(m/z\) 115 also appeared to be an unchanged ion compared with the parent. No fragment ions corresponding to the \(N,N\)-diethyl amine moiety were observed. This finding, along with FTMS-based accurate mass determination, verified M5 as 4-amino-5-chloro-N-carboxymethyl-2-methoxy-benzamide.

**Structural Rationalization of M6 and M8.** In in vitro incubations, M6 and M8 were formed only in two-step P450/UGT incubations but not in one-step UGT incubations. ESI of M6 and M8 in positive mode at a collision energy of 35 resulted in protonated molecular ions of \(m/z\) value 492 each, higher than MCP by 192 amu (Fig. 5, A and C). M6 was detected at a retention time of approximately 1.8 min, whereas M8 eluted at 2.7 min (Fig. 2, right). The CID product ion spectra of M6 and M8 were identical and afforded MCP (+ 16 + 176), suggesting an oxidative addition followed by conjugation with glucuronic acid. A peak at \(m/z\) 316 was also observed as a single peak in a neutral loss of 176 scan. Unlike for M1, a peak at \(m/z\) 300 was not observed as a single peak in a neutral loss of 192 (176 + 16) scan. MS\(^1\) of \(m/z\) 316, corresponding to addition of 16 amu to MCP, revealed fragments ions with \(m/z\) values 200 and 243, 16 amu higher than 184 and 227, respectively. In addition, fragment ions \(m/z\) 376 and 419, equivalent to addition of 192 amu (16 + 176 amu) to184 and 227, respectively, were also observed in the composite spectrum generated from MS/MS of \(m/z\) 492. This is a characteristic fragmentation pattern for \(O\)-glucuronides. Taken together, these data along with accurate mass-based evidence suggests oxidation followed by subsequent glucuronidation on the 4-amino-5-chloro-2-methoxy-benzoyl moiety of MCP.

**Structural Rationalization of M7.** M7 was formed in one-step in vitro UGT incubations. With ESI of M7 in positive ion mode ionization at a collision energy of 35, a protonated molecular ion of \(m/z\) 476 was observed. Fragment ion \(m/z\) 476 was higher than MCP by 176 amu (Fig. 5B). This was detected at retention time of approximately 2.1 min (Fig. 2, right). The product ion spectrum of M7 afforded fragment ions \(m/z\) 360 and 403, corresponding to addition of 176 amu to fragment ions \(m/z\) 184 and 227. A peak at \(m/z\) 300 was also observed as a single peak in a neutral loss of 176 scan. These data point to M7 being a direct glucuronide of MCP, possibly at the aromatic amino group of the 4-amino-5-chloro-2-methoxy-benzoyl moiety of MCP. Empirical formulae for the product ions have been verified by FTMS-based accurate mass measurement.

**Structural Rationalization of M9.** M9 was formed in in vitro P450 incubations. MS\(^2\) scans revealed a single peak corresponding to \(m/z\) 344 at 10.0 min (Fig. 2, right). MS/MS of \(m/z\) 344 compared with the parent (at \(m/z\) 300) suggested that the base peak ion had shifted from \(m/z\) 227 to \(m/z\) 271, accounting for addition of carbon dioxide (Fig. 5D). Fragment ion \(m/z\) 184 also revealed addition of 44 amu and was observed as \(m/z\) 228. Other fragment ions, \(m/z\) 100, 117, and 143, were unchanged. This suggested that M9 was a carbamate of MCP, possibly on the 4-amino-5-chloro-2-methoxy-benzoyl moiety. Accurate mass measurement confirmed the formation of MCP carbamoyl metabolite. A peak corresponding to MCP, \(m/z\) 300 was observed at 10.0 min, and the MS\(^2\) spectrum did not reveal fragments corresponding to the unchanged 4-amino-5-chloro-2-methoxy-benzoyl moiety of MCP, confirming that M9 was not formed in the MS source.

**Structural Rationalization of M10.** M10 was formed in in vitro P450 incubations. A stable nitro metabolite of MCP was observed at 12.0 min (Fig. 2, right). The MS\(^2\) spectrum of \(m/z\) 330 in positive mode at a collision energy of 35 afforded \(m/z\) 257 as the base peak ion, resulting from addition of 30 amu to fragment ion \(m/z\) 227 (Fig. 5E). Likewise, fragment ion \(m/z\) 214, equivalent to addition of 30 amu, was observed in the MS\(^2\) spectrum of \(m/z\) 330. This pointed to M10 being a stable nitro metabolite of MCP. FTMS-based accurate mass detection revealed that the measured accurate mass was \(m/z\) 330.1220. The theoretical accurate mass for a nitro metabolite of MCP was calculated to be 330.1215. Based on these \(m/z\) values, the mass accuracy value (6ppm) for M10 was found to be 6 ppm. Therefore, M10 was determined to be a stable nitro metabolite of MCP.

**Intersubject Variability in MCP Metabolism.** A total of eight individuals were evaluated for urinary MCP and metabolites M1 to M5 levels in a full urine (24-h) sample. As depicted in Fig. 6, there was high intersubject variability among the subjects for each chemical moiety. The most marked variability was found in levels of metabolite M2 (21-fold) and metabolite M3 (16-fold).

**Discussion**

MCP is the only FDA-approved drug for patients with gastroparesis. MCP is associated with neurologic side effects because of its action on central dopaminergic receptors. These neurologic effects consist of a broad spectrum of syndromes known as drug-induced movement disorders (DIMDs) (Pasricha et al., 2006). DIMDs can consist of a broad spectrum of syndromes known as drug-induced movement disorders (DIMDs) (Pasricha et al., 2006). DIMDs can be accounted for 8% of all movement disorders in a study recruiting 125 patients prescribed neuroleptic drugs (Miller and Jankovic, 1990). In a larger study involving 434 patients with tardive dyskinesia, MCP
FIG. 5. Mass spectra for metabolites M6 to M10 formed in vitro. A, ESI of M6 resulted in protonated molecular ions of m/z value 492, higher than MCP by 192 amu. Fragment ions m/z 227 and 300 showed addition of 16 amu, corresponding to oxidation. Neutral loss of 176 afforded m/z 316, corresponding to MCP (m/z 300) + 16, similar to M8. B, with ESI of M7 a protonated molecular ion of m/z value 476 was observed. Fragment ion m/z 476 was higher than MCP by 176 amu. A peak at m/z 300 was also observed as a single peak in a neutral loss of 176 scan.
C, ESI of M8 in positive ion mode resulted in a protonated molecular ion of m/z value 492, higher than MCP by 192 amu. Similar to M6, FTMS-based accurate mass measurement confirmed m/z 492 to be a glucuronide. D, MS/MS of m/z 344 (M9) compared with the parent (at m/z 300) suggested that the base peak ion had shifted from m/z 227 to m/z 271, accounting for the addition of carbon dioxide. Fragment ion m/z 184 also revealed addition of 44 amu and was observed as m/z 228. Other fragment ions m/z 100, 117, and 143 were unchanged, and are illustrated by *. This suggested that M9 was a carbamate of MCP, possibly on the 4-amino-5-chloro-2-methoxy-benzoyl moiety.
E, MS² spectrum of m/z 330 (M10) afforded m/z 257 as the base peak ion, resulting from addition of 30 amu to fragment ion m/z 227. Accurate mass data suggested addition of 2 oxygen atoms and loss of 2 hydrogen atoms, accounting for 30 amu and pointing toward M10 being a stable nitro metabolite of MCP. F, the measured accurate mass for the putative nitro metabolite M10 was 330.1220. The empirical formulae for the theoretical and measured masses are shown in the figure. The theoretical accurate mass for a nitro metabolite of MCP was calculated to be 300.1215. Based on these m/z values, the mass accuracy value (1.5 ppm) for M10 was found to be −1.5 ppm.
was found to be one of the most common medications associated with tardive dyskinesia (Kenney et al., 2008). Therefore, it is critical to understand the disposition of MCP and to determine its metabolites and their activity/toxicity in humans. Even if all MCP metabolites are inactive, enzyme induction or inhibition with coadministered drugs might affect the disposition of MCP and therefore its efficacy/side effect profile. Furthermore, variability in MCP efficacy and toxicity might be due to genetic variability in its metabolizing enzymes, and knowledge of MCP metabolism is necessary to elucidate these effects.

The pharmacokinetics of MCP have been characterized in humans and other mammalian species (Bakke and Segura, 1976; Bateman, 1983; McDermed et al., 1985; Lamparczyk et al., 2001; Kim et al., 2002; Vlase et al., 2006; Zaki et al., 2006). Significant interspecies differences have been reported in MCP metabolism (Bakke and Segura, 1976; Cowan et al., 1976; Bateman, 1983). Approximately 80% of MCP is reportedly metabolized in humans (Bateman, 1983). However, to our knowledge, its metabolism in humans has not been described in detail. Conjugated metabolites (an N-sulfate and a glucuronide) and an oxidative deamination product (labeled M5 in the present study) have been reported in humans (Bateman, 1983). Conjugated metabolites were previously discerned indirectly by subjecting samples to deconjugation reactions, and the exact structure of conjugates was not described previously (Bateman et al., 1980). A recent study reported a deethylated MCP metabolite (labeled M3 in the present study) in vitro (Desta et al., 2002), but this metabolite was not reported in vivo. Finally, an arylhydroxyamino metabolite was reported in vitro (Yu et al., 2006) and in human urine (Maurich et al., 1995) but was not detected in the present study. However, this metabolite might be a precursor for the N-O-glucuronide (M1), which we report for the first time. M1 is formed by sequential metabolism via P450 followed by UGT. Thus, of the five in vivo metabolites (Table 1) we report here (M1–M5), metabolites M2 and M5 have been reported previously in vivo. Metabolites M1 and M4 are reported here for the first time.

![Figure 6](image_url)
Of the five additional metabolites (Table 1) we observed in vitro (M6–M10), metabolites M6, M8, M9, and M10 have never been reported. Metabolite M7 was reported in vivo, but it is possible that an N-O-glucuronide (M1) may have been mistaken for an reported. Metabolite M7 was reported in vivo, but it is possible that an (M6–M10), metabolites M6, M8, M9, and M10 have never been metabolite or may even result in subsequent formation (Gunduz et al., 2010). A stable nitro metabolite (M10) formed by six-electron oxidation of the MCP aniline group was also detected in vitro. Both metabolites M9 and M10 were chemically stable enough to be identified by MS in vitro but were not detected in vivo. Therefore, pharmacologic or toxicologic inferences about M9 and M10 cannot be made at this time. Mechanistic studies on the formation of the nitro metabolite and trapping of possible reactive intermediate(s) are currently being conducted in our laboratory. We observed metabolites M6 to M10 only in vitro but not in human urine. It is possible that these metabolites are formed in vitro because of to the relatively high drug concentration that liver preparations are directly exposed to, a scenario that might not occur in vivo. It is also possible that these metabolites are formed to a very small extent in vivo and were therefore not detected. Finally, only human urine samples were collected in the present study, and it is possible that some metabolites are excreted via nonrenal pathways and therefore were not detected in human urine. This first study with noninvasive sample collection will form the basis of future work in which plasma levels of circulating metabolites will be discerned.

The goal of the present study was identification of all human urinary MCP metabolites. Therefore, it was a small study recruiting only eight healthy male subjects. Because MCP metabolites were not available as synthetic standards, it was not possible to determine the extent of metabolite formation or to compare metabolites and determine quantitatively major metabolites. The average elimination half-life of MCP is approximately 5 h (Bateman, 1983; Lamparczyk et al., 2001; Vlase et al., 2006); therefore, full urine collection for 5 half-lives (24 h) was conducted. In the urine samples, MCP levels were found to be 3.6-fold variable in the eight subjects. There was high variability in all the metabolites (M1–M5) (Fig. 6), the M2 (21-fold) and M3 (16-fold) being the most variable. Metabolite M2 was reported to be a major metabolite of MCP (Bateman, 1983) and is a sulfated conjugate. Genetic variability in human SULTs is well understood (Nowell and Falany, 2006), and identification of specific SULT isozymes that catalyze MCP sulfation in future studies will shed light on the role of SULT pharmacogenetics in variable MCP metabolism. Metabolite M3 was reported to be formed via human cytochrome P450 CYP2D6 in vitro (Desta et al., 2002). CYP2D6 is a well known, highly polymorphic enzyme, and this genetic variation might contribute to variable MCP metabolism as well. Based on our in vitro assays, all of the reported MCP metabolites are formed via P450, UGT, and SULT enzymes. It is possible that M5 might also be formed via mitochondrial monoamine oxidase (MAO) enzymes. MAOs, present in HLMs as contaminants, can catalyze the oxidative deamination of MCP with subsequent oxidation to form the corresponding acid (M5). MAO involvement in MCP metabolism would be clinically important in predicting drug-drug interactions of MCP with MAO inhibitors. Whether MAOs are involved in MCP metabolism and the specific isozymes from each enzyme superfamily (P450, UGT, and SULT) responsible for MCP metabolism are currently being characterized in our laboratory. Pharmacogenetic variability in these isozymes and their association with MCP metabolism (systemic MCP and metabolite levels) will also be evaluated in a larger study.

In summary, the metabolism of MCP was determined in humans and in vitro with MS identification and characterization. A total of 10 metabolites were identified, of which 5 metabolites (M1–M5) were formed in vivo. Of these, M1 and M4 are novel and have not been reported previously. Among the metabolites also identified in vitro (M6–M10), M6, M8, M9, and M10 have not been reported previously. These studies have allowed deeper insight into the metabolism of MCP in humans than reported previously.

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References
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