Identification of novel metoclopramide metabolites in humans: \textit{in vitro} and \textit{in vivo} studies

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List of non-standard abbreviations:

MCP: metoclopramide
HLM: human liver microsome
HLC: human liver cytosol
CYP: cytochrome P450
UGT: uridine diphosphoglucuronosyltransferase
SULT: sulfotransferase
UDPGA: uridine-5’- diphosphoglucuronic acid
PAPS: 3’-phosphoadenosine 5’-phosphosulfate
DMSO: dimethyl sulfoxide
ESI: electrospray ionization
CID: collision-induced dissociation
DIMD: drug-induced movement disorder
Abstract

Metoclopramide (MCP) is frequently used to treat gastroparesis patients. Previous studies have documented MCP metabolism, but systematic structural identification of metabolites has not been performed. This study aims to better understand MCP metabolism in humans. For examination of \textit{in vivo} metabolism, a single oral 20 mg MCP dose was administered to 8 healthy male volunteers, followed by complete urine collection over 24 hours. \textit{In vitro} incubations were carried out in human liver microsomes (HLM) to characterize metabolism via cytochromes P450 (CYPs) and uridine diphosphoglucuronosyltransferases (UGTs), and in human liver cytosol (HLC) for metabolism via sulfotransferases (SULTs). Urine and sub-cellular incubations were analyzed for MCP metabolites on a Thermo Orbitrap® mass spectrometer with accurate mass measurement capability. Five MCP metabolites were detected \textit{in vivo}, and 5 additional metabolites were detected \textit{in vitro}. The 5 metabolites of MCP identified both \textit{in vitro} and \textit{in vivo} were: an N-O-glucuronide (M1), an N-sulfate (M2), a des-ethyl metabolite (M3), a hydroxylated metabolite (M4), and an oxidative de-aminated metabolite (M5). To our knowledge, metabolites M1 and M4 have not been previously reported. M2 urinary levels varied 22-fold and M3 levels varied 16-fold among 8 subjects. \textit{In vitro} studies in HLMs revealed following additional metabolites - two ether glucuronides (M6, M8) possibly on the phenyl ring post-oxidation, an N-glucuronide (M7), a carbamic acid (M9), and a nitro metabolite (M10). Metabolites M6 – M10 have not been reported previously. In conclusion, this study describes the identification of MCP metabolites \textit{in vivo} and \textit{in vitro}, in humans.
Introduction

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 suffers from gastroparetic symptoms (Abell et al., 2006). Gastroparesis can be diabetic, post-surgical, or idiopathic. Treatment options include dietary management, pharmacologic agents, and surgical procedures such as gastric electrical stimulation and feeding jejunostomy (Friedenberg and Parkman, 2007).

MCP was approved by the 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 anti-emetic. It is associated with central nervous system side effects. Clinically, neurologic effects of MCP result in a broad spectrum of drug induced movement disorders (Pasricha et al., 2006). These can range from acute dystonic reactions and Parkinsonism to chronic tardive dyskinesia.

MCP follows linear pharmacokinetics. It is administered orally, and is rapidly absorbed (Bateman, 1983). It is not highly protein bound (fraction of drug unbound in plasma = 0.6) and distributes rapidly (volume of distribution = 2.2 – 3.4 L/kg upon an IV dose, with alpha-phase half life of 5 – 21 minutes) (Bateman, 1983). Elimination of the unchanged drug (fraction of drug eliminated unchanged in urine = 0.2) as well as its metabolites is predominantly via urine, and its elimination half life is 5 – 6 hours.
MCP is extensively metabolized in humans. Metabolism has been thought to be predominantly via conjugation to sulfated and glucuronidated metabolites, although oxidative metabolites are also known (Bakke and Segura, 1976; Bateman, 1983; Desta et al., 2002; Yu et al., 2006). Although MCP has been prescribed for decades, its exact metabolic profile in humans remains to be elucidated. While older reports state that a sulfated metabolite predominates, recent in silico and in vitro studies have noted major oxidative metabolites (Bakke and Segura, 1976; Bateman, 1983; Desta et al., 2002; Yu et al., 2006). Cytochrome P450 2D6 (CYP2D6) is thought to catalyze MCP oxidation (Desta et al., 2002). Relative contribution of other CYP isozymes and identity of specific conjugating enzymes is as yet unknown.

Characterizing the metabolic profile of a drug is critical in understanding its pharmacokinetics as well as its adverse effect profile. Identifying the specific enzymes responsible for drug metabolism further enables prediction of drug-drug interactions and inter-individual 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 vitro incubations were additionally carried out 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 co-factor for glucuronidation incubations uridine-5’- diphosphoglucuronic acid (UDPGA), the pore-forming antibiotic alamethicin, 3’-phosphoadenosine 5’-phosphosulfate (PAPS), and triethylamine were purchased from Sigma-Aldrich (St. Louis, MO). HPLC grade solvents acetonitrile, methanol, and analytical grade dimethyl sulfoxide (DMSO) were from Mallinckrodt Baker Inc. (Phillipsburg, NJ). Analytical grade buffer reagents were from Thermo Fisher Scientific Inc. (Waltham, MA). All other reagents were HPLC grade.

Human subjects

The study was approved by Temple University Institutional Review Board. Eight healthy male subjects between the ages 18 – 65 years were recruited. Subjects were non-smokers and not on any other medication. Subjects did not ingest any caffeinated or alcoholic beverages, and any product containing grapefruit juice for 5 days prior to the start of the study. Exclusion criteria included sensitivity/allergy to MCP, subjects with known renal failure, pheochromocytoma, history of Parkinson’s disease, history of clinical depression, subjects with suspected bowel obstruction, patients taking other medications, and subjects with any 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 MCP (Reglan®) with 100 ml water. This was followed by a standard meal 30 minutes later, and at regular intervals until the end of the study. Urine was collected until 24 hours post-dose. 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, 5 ml urine sample was thawed and centrifuged at 14000 rpm for 10 minutes. The supernatant was directly analyzed.

In vitro incubations

CYP incubations

CYP-mediated metabolism of MCP was characterized in HLM using methods previously described (Ung et al., 2009). Briefly, 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 μl and incubated at 37°C for 5 hours. 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 μl ice cold acetonitrile. Reactions were then centrifuged at 14000 rpm for 5 minutes and the supernatant collected for further analysis on HPLC.
UGT incubations

Glucuronidation reactions were conducted either as one-step UGT incubations, or two-step mixed CYP+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 hour, the reaction was terminated with 50 µL ice cold acetonitrile, and prepared for further analysis. For the two-step CYP+UGT incubations, a total volume of 500 µl containing 0.5 mg/ml HLM, 1mM MCP, and NADPH regenerating system in 50 mM potassium phosphate buffer (pH 7.4) was first incubated at 37°C for 3 hours prior to the addition of alamethicin (10µg/ml) and UDPGA (5mM) 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 14000 rpm for 5 minutes. The supernatant was collected for further analysis on LC-MS/MS.

SULT incubations

Sulfation reactions were conducted using human liver cytosol (HLC). Briefly, using a modified assay (Ung and Nagar, 2007), a total incubation volume of 500 µl contained 1 mg/ml HLC, 1mM MCP, and 4 mM PAPS in 10 mM potassium phosphate pH (6.5). Incubations were carried out for 3 hours 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 14000 rpm for 5 minutes 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 reversed-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 x 4.6 mm, 5 μm; Agilent Technologies), and an ultraviolet detector set at 274 nm for detection of MCP and its metabolites. The software ChemStation for LC (Version A.08.01, Agilent Technologies) performed 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/minute. The supernatants (100 ul injection) of CYP-mediated reactions were analyzed and two separate metabolite peaks were detected at 20.7 and 22.9 minutes, respectively. Fractions containing each respective metabolite were collected for structural elucidation by LC-MS/MS. Similarly, supernatants from other in vitro incubations were initially run on the HPLC-UV assay, and all putative metabolite peaks were further tested 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 previously reported method (Gunduz et al., 2010). Urine collected over a period of 24 hours was aliquoted and centrifuged at 6440 x g for 5 minutes prior to analysis. The respective supernatants were removed and transferred to 1 mL conical
glass inserts for LC-MS\textsuperscript{n} analysis. MCP spiked into blank urine was utilized as a control sample for background ion spectra. Samples were analyzed for presence of metabolites with the help of a Thermo LTQ Orbi-Trap mass spectrometer (Thermo Fisher Scientific, Waltham, MA) capable of MS\textsuperscript{n} scanning and accurate mass measurement interfaced with a 3X Ti HPLC pump and CTC PAL autosampler (Leap Technologies, Carrboro, NC). The chromatographic separation was carried out on a Waters Symmetry C18 analytical column (5um, 2.1 x 150 mm, Waters, Milford, MA) with a 35 minute-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/minute with 5% B for the initial 5 minutes. Thereafter, the percentage of mobile phase B was gradually increased to 40% B over 20 minutes and to 95% over 5 minutes. Following the elution of MCP and its metabolites, the column was returned over 1 minute to 5% B, where it was held for 3 minutes for re-equilibration, 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/minute, source voltage of 4.20 kV, source current of 100 µA, capillary voltage of 35 and tube lens voltage equal to 100 V. Multipole RF amplifier was set at 400 Vp-p, along with multipole ‘0’ offsets at -1.25 V 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 frequency of 2824 Hz, main RF frequency of 1187.5 Hz and pulsed Q dissociation collision energy factor of 10. Fourier Transformation Mass Spectrometry (FTMS) enabled accurate mass measurement was carried out at a mass resolution of 60,000. Ion Trap Mass Spectrometry (ITMS) based
data dependent scan after collision induced dissociation was carried out at a normalized collision energy of 35. Activation Q was set at 0.25 and activation time was 30 milliseconds.

Comparison of MCP urinary metabolites across subjects

Urinary MCP as well as metabolite absolute peak areas were compared across all subjects (n=8) for inter-subject variability in each metabolite’s formation. 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 due to 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 8 subjects.
Results

A total of 10 metabolites of parent MCP were detected in the *in vitro* and *in vivo* studies together (Figure 1). The types of metabolites, their retention times, key MS and MS\(^n\) fragments are listed in Table 1. Human urine samples exhibited a total of 5 MCP metabolites (labeled M1 through M5; Figure 2A). The structures of these 5 metabolites were characterized as described in detail below. Additionally, all 5 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 5 additional metabolites not observed *in vivo* (M6 – M10; Figure 2B), for a total of 10 MCP metabolites. The structural rationalization of each metabolite is explained in detail below.

*Structural rationalization of MCP*

Upon ESI in positive ion mode at collision energy of 35, MCP showed a protonated molecular ion [M+H] of m/z 300. The LC retention time was approximately 10.4 minutes (Figure 2). The CID product ion spectrum of m/z 300 (Figure 3) afforded a base peak ion of m/z 227 resulting from a neutral loss of 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 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 $N$, $N$-diethylamino-ethylamine moiety (Figure 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 (Figure 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 resulted in a protonated molecular ion of m/z value 492, higher than MCP by 192 amu (Figure 4A). This was detected at retention time of approximately 2.9 minutes (Figure 2A). The CID product ion spectrum of M1 afforded MCP (+ 16 + 176) and m/z 419 (i.e m/z 227 + 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. A peak at m/z 300 was also observed as a single peak in a neutral loss of 192 (176 + 16) scan. MS$^3$ of m/z 316 revealed exactly the same fragmentation pattern as that of m/z 300, i.e ions m/z 143, 184 and 227 as the base peak ion (Figure 3). The same fragment ions were also detected in the MS/MS spectrum of m/z 492, as shown in Figure 4A. Additionally, ions m/z 243 and 316 were also observed in the composite spectrum generated from MS/MS of m/z 492. This characteristic fragmentation pattern of loss of 16 amu leads to a fragmentation pattern similar to that of the parent during MS$^n$ and is a diagnostic pattern of heteroatom oxidation. This metabolite formation was confirmed with *in vitro* incubations in HLM. No glucuronide was formed upon one-step UGT incubation. A glucuronidated metabolite was formed only upon two-step CYP/UGT incubation, indicating that sequential oxidation followed by glucuronidation had taken place.
Collectively, this data points toward an N-O-glucuronide of MCP, verified by accurate mass.

**Structural rationalization of M2**

A peak corresponding to m/z 380 was observed at 5.7 minutes (Figure 2A). CID of m/z 380 (M2), upon 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 MCP sans 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 confirmed M2 to be a MCP sulfate (Figure 4B). Comparison with *in vitro* SULT incubations in HLC corroborated these findings.

**Structural rationalization of M3**

An N-des-ethyl metabolite of MCP was observed at 6.2 minutes (Figure 2A), both in human urine samples and in *in vitro* CYP incubations. MS$^2$ spectrum of m/z 272 in positive mode at collision energy of 35, afforded m/z 227 as the base peak ion, resulting from the loss of diethyl amine moiety (Figure 4C). Fragment ions m/z 272, 184 and m/z 115 appeared to be unchanged, pointing toward the absence of ethyl group on M3. As compared to 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 de-ethylation product of MCP. These observations were further confirmed by accurate mass based calculation of empirical formula and mass defect measurement.
Structural rationalization of M4

MS² scans revealed a single peak corresponding to m/z 316 at 6.5 minutes (Figure 2A). MS/MS of m/z 316 as compared to 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 (Figure 4D). 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 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 upon in vitro CYP incubations. Neither was a peak corresponding to MCP, m/z 300 observed at 6.5 minutes, nor did MS² spectrum reveal fragments corresponding to the unchanged 4-amino-5-chloro-2-methoxy-benzoyl moiety of MCP. This data collectively points toward 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. M5 was additionally observed in CYP incubations in vitro. ESI in positive mode at collision energy of 35, resulted in an m/z of 259 at 12.0 minutes (Figure 2A). MS/MS of m/z 259 resulted in the MS² spectrum as shown in Figure 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 an
unchanged ion, as compared to the parent. No fragment ions corresponding to the N, N-diethyl amine moiety were observed. This 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 CYP/UGT incubations but not in one-step UGT incubations. ESI of M6 and M8 in positive ion mode at a collision energy of 35 resulted in protonated molecular ions of m/z value 492 each, higher than MCP by 192 amu (Figures 5A and 5C). M6 was detected at a retention time of approximately 1.8 minutes, whereas M8 eluted at 2.7 minutes (Figure 2B). 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 M1, a peak at m/z 300 was not observed as a single peak in a neutral loss of 192 (176 + 16) scan. MS3 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. Additionally, fragment ions m/z 376 and 419, equivalent to addition of 192 amu (16 + 176 amu) to 184 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. Collectively, this 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 \textit{in vitro} UGT incubations. Upon ESI of M7 in positive ion mode ionization at collision energy of 35, a protonated molecular ion of m/z value 476 was observed. Fragment ion m/z 476 was higher than MCP by 176 amu (Figure 5B). This was detected at retention time of approximately 2.1 minutes (Figure 2B). 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. This data points toward 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 \textit{in vitro} CYP incubations. MS$^2$ scans revealed a single peak corresponding to m/z 344 at 10.0 minutes (Figure 2B). MS/MS of m/z 344 as compared to 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 (Figure 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, 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 observed not at 10.0 minutes 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* CYP incubations. A stable nitro metabolite of MCP was observed at 12.0 minutes (Figure 2B). MS² spectrum of m/z 330 in positive mode at 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 (Figure 5E). Similarly fragment ion m/z 214, equivalent to addition of 30 amu was observed in the MS² spectrum of m/z 330. This pointed toward M10 being a stable nitro metabolite of MCP. FTMS based accurate mass detection revealed that the measured accurate was mass m/z 300.1220. 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 (δppm) for M10 was found to be -1.5 ppm (Figure 5F). Therefore, M10 was determined to be a stable nitro metabolite of MCP.

**Inter-subject variability in MCP metabolism**

A total of 8 individuals were evaluated for urinary MCP and metabolites M1-M5 levels in a full urine (24-h) sample. As depicted in Figure 6, there was high inter-subject 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 due to 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 be acute or chronic, and vary in their severity. MCP-induced DIMDs accounted for 8% of all movement disorders in a study recruiting 125 patients prescribed neuroleptic drugs (Miller and Jankovic, 1990). In a bigger study involving 434 tardive dyskinesia patients, MCP was found to be one of the most common medications associated with tardive dyskinesia (Kenney et al., 2008). It therefore becomes 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 co-administered drugs might affect the disposition of MCP and therefore its efficacy / side effect profile. Further, 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 inter-species differences have been reported in MCP metabolism (Bakke and Segura, 1976; Cowan et al., 1976; Bateman, 1983). About 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 (Bateman et al., 1980). A recent study reported a deethylated MCP
metabolite (labeled M3 in the present study) \textit{in vitro} (Desta et al., 2002), but this
metabolite was not reported \textit{in vivo}. Finally, an arylhydroxyamino metabolite was
reported \textit{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 CYP followed by UGT. Thus, for the 5 \textit{in vivo} metabolites (Table 1) we
report here (M1 – M5), metabolites M2 and M5, have been previously reported \textit{in vivo}.
Metabolites M1 and M4 are reported here for the first time.

For the 5 additional metabolites (Table 1) we observed \textit{in vitro} (M6 – M10), metabolites
M6, M8, M9 and M10 have never been reported. Metabolite M7 was reported \textit{in vivo} but
it is possible that an N-O-glucuronide (M1) may have been mistaken for an N-
glucuronide (M7) due to the use of deconjugation to identify glucuronides, and the lack
of sophisticated MS capability in earlier studies. We observed M7 only \textit{in vitro} and not in
human urine. It is interesting to note that a stable carbamic acid metabolite of MCP was
identified \textit{in vitro} in HLM incubations (M9). It is possible that carrying out MCP
incubations in a NaHCO$_3$ buffer may lead to increased formation of this metabolite, or
may even result in subsequent N-carbamoyl glucuronide 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 the metabolites – M9 and M10 were chemically stable enough to be identified by MS in vitro, but were not detected in vivo. Therefore, pharmacological or toxicological inferences about M9 and M10 cannot be made at this time. Mechanistic studies on formation of the nitro metabolite and trapping of possible reactive intermediate(s) are currently being conducted in our laboratory.

We observed metabolites M6 – M10 only in vitro but not in human urine. It is possible that these metabolites are formed in vitro due 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 non-renal pathways and therefore were not detected in human urine. This first study with noninvasive sample collection will form the basis of future work where 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 8 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 about 5 hours (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 8 subjects. There was high variability in all the metabolites (M1 – M5; Figure 6), with 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 sulfotransferases (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 the reported MCP metabolites are formed via CYP, UGT, and SULT enzymes. It is possible that M5 might additionally 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 for 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 (CYP, UGT, and SULT) responsible for MCP metabolism is 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$^a$ 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 additionally identified *in vitro* (M6 – M10), M6, M8, M9, and M10 have not been previously reported. These studies have allowed deeper insight into the metabolism of MCP in humans than previously reported.
Acknowledgments

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References


Legends for Figures

Figure 1. Putative metabolites of metoclopramide (MCP), identified *in vitro* (in HLM and HLC) and *in vivo* (in human urine) by MS\(^n\) analysis. The intermediates postulated in brackets were not detected.

Figure 2. LC-MS chromatograms of MCP and MCP metabolites. A) Metabolites formed *in vivo* in human urine, and B) Metabolites additionally formed *in vitro* in HLM and HLC. Peaks from unique three 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-minute segment of a 34-minute run; no parent-related peaks were observed between 16 – 34 minutes.

Figure 3. MS\(^2\) spectrum of MCP obtained in positive ion mode ESI. The parent (MCP) is seen at m/z 300 at approximately 5% signal intensity compared to the base peak m/z 227. Other diagnostic fragments include ions obtained from N,N-diethylamino-ethylamine and 4-amino-5-chloro-2-methoxy-benzooyl moieties.

Figure 4. Mass spectra for metabolites M1 – M5 formed *in vivo*. Ions indicated by ‘*’ denote unchanged fragmentation as compared to the parent. A. M1 was observed to be formed after heteroatom oxidation followed by glucuronidation. 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), upon 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 M2 to be a MCP sulfate. C. MS² spectrum of m/z 272 (M3) in positive mode at 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) as compared to the parent (at m/z 300) suggested a base peak ion shift from m/z 227 to m/z 243, accounting for oxidation. E. MS/MS of m/z 259 (M5) resulted in a shift of base peak ion 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.

Figure 5. Mass spectra for metabolites M6 – 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. Upon 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) as compared to 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.
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, 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 (δppm) for M10 was found to be -1.5 ppm.

Figure 6. Inter-subject variability in urinary metabolites of MCP. A) Absolute areas of MCP, M1, and M4; B) Absolute areas of M2, M3, and M5; C) Fold-variation in MCP and M1-M5 urinary levels. Data are from 8 individuals. A full urine sample (24 hours) was collected from each subject, and employed for analyses of metabolites after a single oral 20 mg dose of MCP.
### Table 1. An overview of MCP metabolites identified in human urine and subcellular fractions from human liver. N/A denotes not applicable.

<table>
<thead>
<tr>
<th>Metabolite(s)</th>
<th>Type of metabolic pathway</th>
<th>Matrix (type of in vitro incubation)</th>
<th>Key Fragments MS and MS&lt;sup&gt;n&lt;/sup&gt; (m/z)</th>
<th>Retention Time (min)</th>
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<tr>
<td>M1</td>
<td>Oxidation, followed by glucuronidation</td>
<td>HLM (CYP+UGT), human urine</td>
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<td>M7</td>
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<td>M10</td>
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Figure 1.
Figure 2.
Figure 3.
Figure 4A.
Figure 4B.
Figure 4C.
Figure 4D.
Figure 4E.
Figure 5A
Figure 5B
Figure 5C
Figure 5D
Figure 5E
Figure 5F

Observed m/z value: 330.1220
C14H21CIN3O4
Retention time: 12.0 min
Delta ppm: -1.5

Theoretical m/z value: 330.1215
C14H21CIN3O4
Figure 6

A

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