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Metabolism of pantoprazole involving conjugation with glutathione in rats

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Abstract

We have investigated the metabolism of pantoprazole and have provided an explanation for the formation mechanism of its metabolites. Metabolites found in the urine of rats after oral administration of pantoprazole sodium (25 mg kg^{-1}) were analysed by liquid chromatography/ion trap mass spectrometry (LC/MS^n). The *N*-acetylcysteine derivatives of benzimidazole (M1) and pyridine (M2), four pyridine-related metabolites (M3–M6), and three benzimidazole-related metabolites (M7–M9) were found, none of which had been reported previously. Five of the metabolites (M1, M2, M3, M7, and M8) were isolated from the urine of rats after oral administration of pantoprazole sodium by semi-preparative HPLC. Structures of these metabolites were identified by a combination analysis of LC/MS^n and ^{1}H NMR spectra. Structures of the remaining four metabolites (M4, M5, M6, and M9) were tentatively assigned through LC/MS^n. The metabolites M2, M3, M4, M5 and M6 and the other metabolites (M1, M7, M8, and M9) reflected the fate of the pyridine moiety and the benzimidazole moiety, respectively. The proposed formation route of M3–M6 was via initial reduction to mercaptopyridine followed by *S*-methylation, *O*-demethylation, and *S*-oxidation to the corresponding sulfoxide or sulfone. Meanwhile, M8 and M9 were formed via initial reduction to the 5-difluoromethoxy-1*H*-benzoimidazole-2-thiol (M7) followed by hydroxylation and *S*-methylation. The metabolism of pantoprazole included an attack by glutathione on the benzimidazole-2-carbon and pyridine-7′-carbon. It is an important metabolic pathway of pantoprazole in rats.

Introduction

Pantoprazole, a substituted benzimidazole sulfoxide derivative, is an irreversible proton pump inhibitor which is used clinically in the treatment of reflux oesophagitis, Zollinger-Ellison syndrome, peptic ulcers, and other acid-related, hypersecretory gastrointestinal disorders (Fitton & Wiseman 1996; Jungnickel 2000). Similar to other proton pump inhibitors, pantoprazole, a weak base that accumulates in the parietal cells of the gastric mucosa, is activated into a cationic cyclic sulfonamide in this acidic environment, and suppresses gastric acid secretion by reacting covalently with cysteine residues in the H^+, K^+-ATPase (Lindberg et al 1986). The pharmacokinetics of pantoprazole in animals and man have been extensively studied (Doyle et al 1990; Huber et al 1990). Pantoprazole is rapidly absorbed after oral administration, with a peak plasma concentration time of approximately 2.7 h. It is subjected to low first-pass hepatic extraction, as reflected in an estimated absolute oral bioavailability of 77%, and plasma concentrations decline bi-exponentially after oral administration, with a mean plasma terminal elimination half-life of 0.9–1.9 h. A few investigations on the metabolism of pantoprazole in animals and man have been reported. Metabolism of pantoprazole is independent of the route of administration (intravenous or oral) and is extensively metabolized in the liver through the cytochrome P450 (CYP450) system. In man, phase I metabolic reactions of pantoprazole include mainly sulfoxide oxidation and reduction (catalysed mainly by CYP3A4), 4′-*O*-demethylation and aromatic hydroxylation (catalysed by CYP2C19 and CYP3A4) (Simon 1995). The hydroxylated metabolites then undergo phase II metabolism, i.e. glucuronidation or sulfation, and are excreted chiefly in urine. Pantoprazole sulfone and 4′-*O*-demethylated pantoprazole sulfone were found to be major metabolites, and pantoprazole sulfide and 4′-*O*-demethylated pantoprazole sulfide were found to be minor

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metabolites in rat plasma (Masubuchi et al 1998). The main serum metabolites in man were found to be the sulfate conjugate of 4′-*O*-demethylated pantoprazole and pantoprazole sulfone (Huber et al 1996). There has been no evidence that any of the pantoprazole metabolites have significant pharmacologic activity. After a single oral or intravenous dose of ^{14}C-labelled pantoprazole to healthy, normal metabolizer volunteers, approximately 71% of the dose was excreted in the urine with 18% excreted in the faeces through biliary excretion. There was no unchanged pantoprazole found in urine or in faeces (Peeters et al 1993). Similar to other proton pump inhibitors, pantoprazole exhibits polymorphic metabolism in man (Tanaka et al 1997). CYP2C19 displays a known genetic polymorphism due to its deficiency in some sub-populations (e.g. 3% of Caucasians and African-Americans and 17–23% of Asians).

In certain cases conjugation with glutathione is an important route of metabolism for a large number of lipophilic xenobiotics and endogenous compounds possessing an electrophilic centre. It is known that in addition to mercapturic acids, several other metabolites derived from glutathione-conjugation may also be excreted in urine, such as cysteine, 3-mercaptopyruvic acid, 3-mercaptolacetic acid, and 2-mercaptoacetic acid *S*-conjugates, methylthioether-compounds, as well as their corresponding sulfoxides (Commandeur et al 1995). There are more than 15 enzymes known to be active in the formation and disposition of glutathione conjugates (Commandeur et al 1995). There has been a steady growth of interest in the metabolism of xenobiotics involving conjugation with glutathione (Mutlib et al 2000, 2002; Yin et al 2004). Beattie & Blake (1989a, b) and Weidolf et al (1992) reported that proton pump inhibitors, SK&F95448 and omeprazole, were partly metabolized by the pathway of glutathione conjugation in rats, respectively. As far as we know, until now, there have been no reports on the glutathione conjugation of pantoprazole. As there is a structural similarity between pantoprazole, omeprazole and SK&F95448, we speculated that glutathione conjugation might be an important metabolic pathway of pantoprazole. Furthermore, clarifying the metabolism of pantoprazole via glutathione conjugation may be useful to interpret the potential drug–drug interactions and to comprehend the toxicological and pharmacological mechanism of it. Therefore, this study was designed primarily to identify the metabolites formed through the pathway of pantoprazole involving conjugation with glutathione in rats, and to provide the explanation for the formation mechanism of the metabolites. A specific and sensitive liquid chromatography/ion trap mass spectrometry (LC/MS^n) was used to examine the biotransformation and characterize the structures of its metabolites formed through the metabolic pathway in rat urine after oral administration of pantoprazole sodium.

Materials and Methods

Drugs and chemicals

Pantoprazole sodium (purity 99.5%) and omeprazole magnesium (purity 99.2%) were purchased from Dongyu Pharmaceutical Co. Ltd (Shenyang, China). Methanol and acetonitrile, purchased from Concord Technology Co. Ltd (Tianjin, China), were of HPLC grade. Other reagents were commercially available and of analytical grade.

Dosing procedure and urine collection

The experiments in this study were carried out according to the Guidelines for the Care and Use of Laboratory Animals of Shenyang Pharmaceutical University. Thirty-six male Wistar rats (230–270 g) were purchased from the Experimental Animal Center of Shenyang Pharmaceutical University (Shenyang, China). The animals were fasted overnight before dosing and for at least 2 h post-dose with water freely available. For the isolation of pantoprazole metabolites, 30 rats were given a single oral administration of 150 mg kg^{-1} pantoprazole (pantoprazole sodium was dissolved in saline containing 0.5% 1 mol L^{-1} NaOH and 5% ethanol). Over the first 12-h post-dose urine was collected and pooled at room temperature (below 25°C). For the study of the metabolic profile of pantoprazole, the remaining rats were housed in metabolic cages, which enabled the separate collection of urine. The rats were administered a single oral dose of 25 mg kg^{-1} pantoprazole, and urine samples were collected and pooled over 0 to 12 h post-dose at room temperature (below 25°C). All urine samples were collected in plastic tubes to which 1 mL aqueous Na_2CO_3 (2 mol L^{-1}) had been added to prevent the excreted metabolites from being degraded by acid (Weidolf et al 1992). All pooled urine samples were stored frozen at −20°C until analysis.

Isolation of metabolites

The semi-preparative HPLC system (Shimadzu, Kyoto, Japan) consisted of two LC-6AD solvent delivery units, a DGU-14A degasser unit, an SCL-10A VP system controller, an SPD-10A VP UV-vis detector, a CLASS-VP LC workstation, and an FRC-10A fraction collector. With the UV detector set at 290 nm, preparation was accomplished on a Shim-Pack PRC-ODS column (20 × 250 mm, Shim-Pack) preceded by a GPRC-ODS precolumn (8 × 1.5 mm). Methanol–water (40:60, v/v) was used as the mobile phase with the flow rate set at 10.0 mL min^{-1}.

Sample preparation

A 1.0-mL sample of rat urine (25 mg kg^{-1}), after oral administration of pantoprazole, was filtered through a cellulose ester micropore membrane (Φ 10 mm, 0.45-μm pore size, purchased from Jiuding High-Technique Filtrating Equipment Co. Ltd (Beijing, China)). The filtrate was applied to a preconditioned Bond Elute C18 cartridge (Teda Fuji, Tianjin, China). The cartridge was washed with 1.0 mL water, and metabolites were eluted with 1.0 mL methanol. The eluate was evaporated to dryness under a gentle stream of nitrogen at 40°C, and the residue was reconstituted by addition of 100 μL acetonitrile–water (30:70, v/v) without formic acid. A 20-μL sample was injected onto the LC/MS^n system.

LC/MS^n analyses

A Finnigan LCQ ion trap mass spectrometer (San Jose, CA) equipped with an electrospray ionization (ESI) source system and a data system (Xcalibur 1.2) was employed. The interface was adjusted to the following conditions: ion mode, positive; spray voltage, 4.25 kV; capillary temperature, 200°C; sheath gas (nitrogen), 0.75 L min^{-1}; auxiliary gas (nitrogen), 0.15 L min^{-1}. The full-scan mass spectrum to obtain the protonated molecules $[M+H]^+$ of each metabolite was collected in the mass range from m/z 150 to 700. Furthermore, MS/MS and MS^3 spectra were obtained for selected precursor ions through incidental collision with neutral gas (helium) molecules in the ion trap. The relative collision energy was set at 25–50%. The LC conditions were as follows. The column was Diamonsil C18 column (250 × 4.0 mm, 5 μm, Dikma, Beijing, China) preceded by a Hypersil BDS-C18 precolumn (10 × 4.6 mm, 5 μm). The mobile phase (optimized according to Oiveira et al (2003)) consisting of acetonitrile–water–formic acid (30:70:0.2, v/v/v) was delivered by a Shimadzu LC-10AD (Kyoto, Japan) pump, at a flow rate of 0.5 mL min^{-1}. The amount of metabolites in rat urine was determined by a LC-MS-MS method. The parameters of the selected reaction monitoring transitions for the $[M+H]^+$ to selected product ions were optimized with the following typical values for the analytes and internal standard (each at its optimum collision energy): pantoprazole m/z 384 to 200; M1 m/z 346 to 217; M2 m/z 315 to 186; M3 m/z 216 to 153; M4 m/z 200 to 184; M5 m/z 186 to 138; M6 m/z 232 to 153; M7 m/z 217 to 197; M8 m/z 233 to 213; M9 m/z 247 to 227; and the internal standard omeprazole m/z 346 to 198. Urine concentrations of pantoprazole and its metabolites (M1–M3, M7 and M8) were quantified according to standard curves. As a result of structural similarity, we presumed that the ionization efficiency of M4–M6 was similar to M3, and the ionization efficiency of M9 was similar to M8. Therefore, the amounts of M4–M6 and M9 were estimated with the aid of M3 and M8, respectively.

NMR measurements

^{1}H NMR spectra measurements of pantoprazole metabolites isolated from urine were carried out at 300 MHz on an ARX 300 NMR spectrometer (Bruker, Faellanden, Switzerland). Compounds were dissolved in CD_3OD. All chemical shifts (δ_H) are reported in part per million (ppm) relative to tetramethylsilane as an internal standard.

Results

To obtain enough material for ^{1}H NMR analyses, the highest dose used in the lifespan safety evaluation (150 mg kg^{-1}) was used. However, to study the metabolic profile of pantoprazole, urine samples (25 mg kg^{-1}, a pharmacodynamic dose) were analysed by LC/MS^n. Five metabolites (M1, M2, M3, M7, and M8) of pantoprazole were isolated from urine of Wistar rats after oral administration of pantoprazole (150 mg kg^{-1}) and identified unambiguously by a combination analysis of LC/MS^n and ^{1}H NMR spectra. The ^{1}H NMR spectra of these metabolites determined in CD_3OD are shown in Figure 1, and summarized in Table 1. Moreover, structures of four other metabolites (M4, M5, M6, and M9) were tentatively assigned using LC/MS^n. The full MS^2 scan chromatogram of a pooled urine sample collected over 0 to 12 h from a rat (25 mg kg^{-1}) after oral administration of pantoprazole is shown in Figure 2. The LC retention times and main characteristic fragment ions in the MS^n spectra of pantoprazole and its metabolites are summarized in Table 2. Key parameters of method validation and recovery percentage of pantoprazole and its metabolites in rat urine are shown in Table 3. The assay has been shown to be reproducible with precision below 14.5% and accuracy between −14.2% and 12.4%, which were calculated from the blank urine sample mixed with pantoprazole and metabolites M1–M3, M7 and M8 (all at three concentrations of 0.1, 0.5, and 4.0 μg mL^{-1}). The lower limit of quantification was 100 ng mL^{-1} for the parent drug and all its metabolites.

M0 (parent drug)

Single-stage full scan mass spectrum of M0 gave the protonated molecule at m/z 384. The MS^2 spectrum of the protonated molecule only provided one characteristic fragment ion at m/z 200, which was formed by the loss of 5-(difluoromethoxy)-1*H*-benzimidazole. The MS^3 spectrum of m/z 200 yielded fragment ions at m/z 185, 182, 168, and 138 formed by the losses of 15 ($\cdot CH_3$), 18 (H_2O), 32 (CH_3OH), and 62 Da (CH_2=S=O) from m/z 200, respectively. The fragment ion at m/z 110 was generated from the further loss of 28 Da (CH_2CH_2) from m/z 138. Protonated molecule, MS^2, MS^3 fragment ions of M0 were consistent with those of pantoprazole. Thus it was identified as pantoprazole, further confirmed by comparison of its retention time (t_R = 12.8 min) on LC/MS^n with that of pantoprazole.

M1 and M2 (*N*-acetylcysteine metabolites)

Two metabolites of *N*-acetylcysteine derivatives of benzimidazole (M1) and pyridine (M2) were isolated from rat urine after pantoprazole administration (150 mg kg^{-1}, p.o.), and characterized by ^{1}H NMR (Figure 1A, B). The retention times of M1 and M2 by LC/MS^n analysis were 14.5 and 4.7 min, respectively.

M1

The single-stage full scan mass spectrum indicated a molecular weight of 345 ($[M+H]^+$ m/z 346). The MS^2 spectrum of m/z 346 generated two fragment ions at m/z 217 and 130 that were generated by the losses of 129 [CH_2=C(COOH)NHCOCH$_3$] and 216 Da (5-difluoromethoxy-1*H*-benzoimidazole-2-thiol), respectively. The MS^3 mass spectrum at m/z 217 of M1 was common to the MS^2 mass spectrum at m/z 217 of M7. In accordance with the mass spectral fragment pattern and ^{1}H NMR data, its structure was safely assigned as 2-acetylamino-3-(5-difluoromethoxy-1*H*- benzoimidazol-2-ylsulfanyl)-propionic acid.

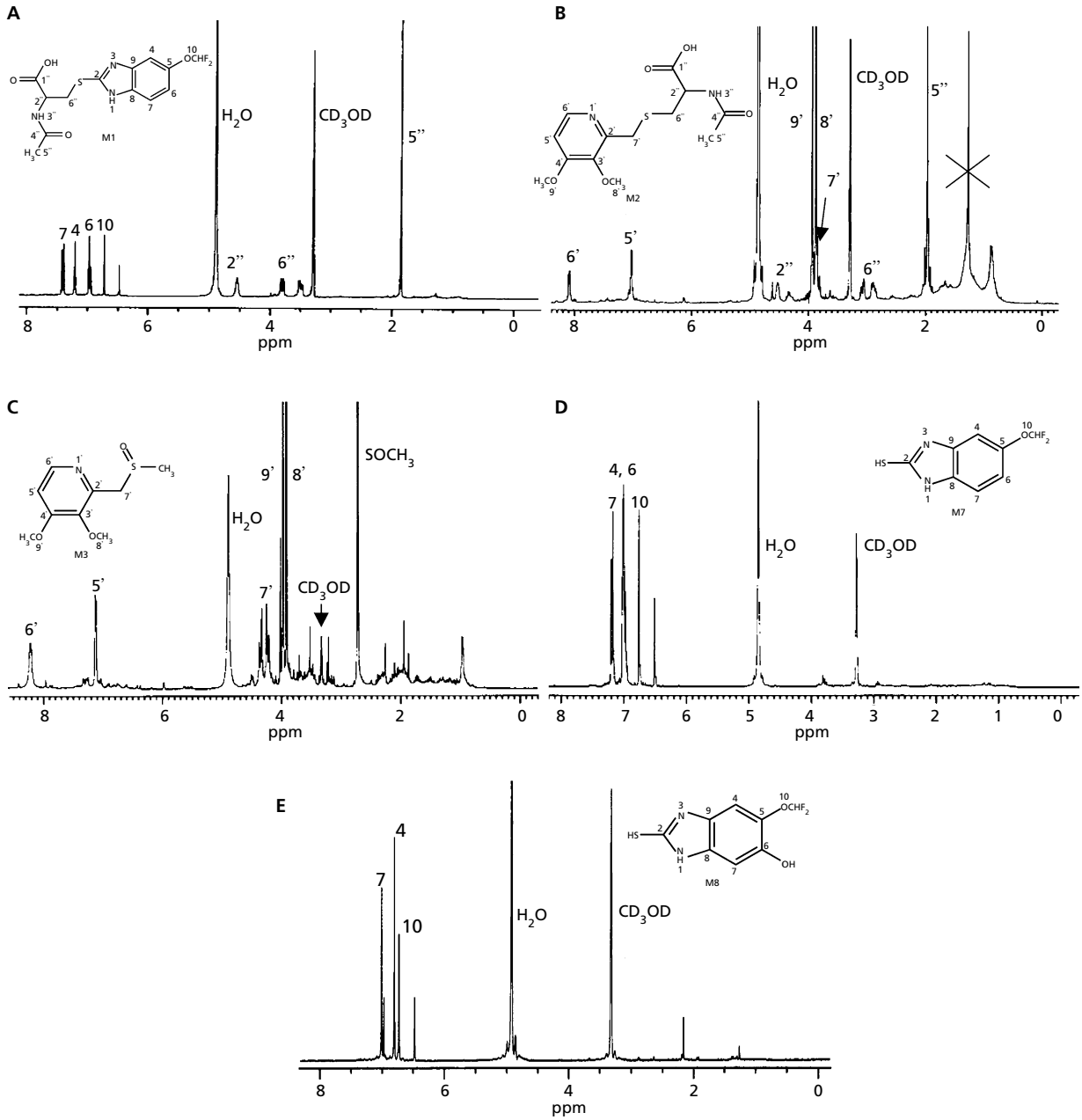

Figure 1 The ^{1}H NMR spectra of M1(A), M2 (B), M3 (C), M7 (D), and M8 (E) isolated from the urine of rats determined in CD_3OD.

M2

The single-stage full scan mass spectrum indicated a molecular weight of 314 ($[M+H]^+$ m/z 315), whereas the only significant fragment ion produced was the protonated pyridine (m/z 186). The MS^3 full scan mass spectra of the ion (m/z 186) were at m/z 170, 152, 140, 122. The fragment ions at m/z 170, 152 and 140 were formed by the losses of 16 (CH_4), 34 (SH_2) and 46 Da (CH_2=S), respectively. The fragment ion at m/z 122 was generated from the further loss of H_2O from ion at m/z 140. Based on the mass spectral and ^{1}H NMR data, its structure was identified as 2-acetylamino-3-(3,4-dimethoxy-pyridin-2-ylmethylsulfanyl)-propionic acid.

M3–M6 (pyridine-related metabolites)

The retention times of M3 ($[M+H]^+$ m/z 216), M4 ($[M+H]^+$ m/z 200), M5 ($[M+H]^+$ m/z 186), and M6 ($[M+H]^+$ m/z 232) by LC/MS^n analysis were 5.2, 8.4, 8.4, and 8.3 min, respectively.

M3

Fragmentation of the protonated molecule (m/z 216) of M3 produced ions at m/z 153, 138, and 107. The radical fragment ion at m/z 153 was formed through fission of the methylene-sulfur bond, and other two fragment ions at m/z 138 and 107 were formed by the sequential losses of

Table 1 Proton NMR data of pantoprazole and its five metabolites isolated from urine of rats[a]

^{1}H	Pantoprazole[b]	M1	M2	M3	M7	M8
4	7.28 (1H, *d*, $J = 2.1$ Hz)	7.24 (1H, *d*, $J = 1.7$ Hz)			7.00 (1H, *d*, $J = 2.1$ Hz)	6.78 (1H, *s*)
6	6.75 (1H, *dd*, $J = 8.6$, 2.1 Hz)	7.00 (1H, *dd*, $J = 8.7$, 1.7 Hz)			6.98 (1H, *dd*, $J = 8.5$, 2.1 Hz)	
7	7.47 (1H, *d*, $J = 8.6$ Hz)	7.44 (1H, *d*, $J = 8.7$ Hz)			7.18 (1H, *d*, $J = 8.5$ Hz)	7.00 (1H, *s*)
10	7.06 (1H, *t*, $J = 75.5$ Hz)	6.76 (1H, *t*, $J = 74.6$ Hz)			6.75 (1H, *t*, $J = 74.2$ Hz)	6.70
5′	7.09 (1H, *d*, $J = 5.6$ Hz)		7.03 (1H, *d*, $J = 5.5$ Hz)	7.07 (1H, *d*, $J = 5.5$ Hz)		(1H, t, J = 75.2 Hz)
6′	8.24 (1H, *d*, $J = 5.6$ Hz)		8.10 (1H, *d*, $J = 5.5$ Hz)	8.17 (1H, *overlapped*)		
7′	4.50 (2H, *dd*, $J = 56.0$, 5.6 Hz)		3.86 (2H, *overlapped*)	4.26 (2H, *dd*, $J = 37.6$, 12.4 Hz)		
8′	3.77 (3H, *s*)		3.89 (3H, *s*)	3.89 (3H, *s*)		
9′	3.77 (3H, *s*)		3.95 (3H, *s*)	3.94 (3H, *s*)		
2″		4.56 (1H, *brs*)	4.53 (1H, *brs*)			
5″		1.86 (3H, *s*)	1.98 (3H, *s*)			
6″-H		3.51 (1H, *dd*, $J = 13.1$, 6.6 Hz)	2.89 (1H, *dd*, $J = 13.1$, 6.6 Hz)			
6″-H		3.82 (1H, *dd*, $J = 13.1$, 3.8 Hz)	3.08 (1H, *dd*, $J = 13.1$, 3.8 Hz)			
SCH_3				2.70 (3H, *s*)		

[a] Values are chemicals shifts (ppm); [b] ^{1}H NMR spectrum measurement was carried out at 500 MHz (dissolved in DMSO-d_6).

78 (CH_3SOCH_3) and 31 Da ($\cdot OCH_3$) from *m/z* 216, respectively. As shown in Figure 1C, the ^{1}H NMR spectrum of M3 consisted of three "singlet" signals at 2.70 (*s*, 3H), 3.89 (*s*, 3H), and 3.94 (*s*, 3H), two "doublet" signals at 7.07 (*d*, 1H, $J = 5.5$ *Hz*) and 8.17 (1H, *overlapped*), and the prochiral methylene protons attached to the sulfoxide group which gave an AB system appearing almost as a quartet located at 4.26 ppm ($J = 12.4$, 37.6 *Hz*). Based on MS and ^{1}H NMR data, M3 was identified as 2-methanesulfinyl-methyl-3,4-dimethoxy-pyridine.

M4

The molecular ion of M4 was at *m/z* 200, 16 Da lower than that of M3. The MS^2 spectrum of *m/z* 200 yielded fragment ions at *m/z* 184, 170, 152, and 140 formed by the losses of 16 (CH_4), 30 (CH_2O), 48 ($HSCH_3$), 60 Da (CH_2SCH_2) from *m/z* 200, respectively. The fragment ion at *m/z* 122 was generated from the further loss of 62 Da (CH_3SCH_3) from *m/z* 184. The losses of 48 ($HSCH_3$), 60 (CH_2SCH_2), and 62 Da (CH_3SCH_3) suggested that M4 was a sulfide derivative. Based on the MS data, M4 was tentatively assigned as 3,4-dimethoxy-2-methylsulfanylmethyl-pyridine.

M5

The molecular ion of M5 was at *m/z* 186, 14 Da lower than that of M4, suggesting M5 was the monodemethylated derivative of M4. Same as the formation of the MS^2 spectrum at *m/z* 200 of M4, the MS^2 spectrum of *m/z* 186 yielded fragment ions at *m/z* 170, 156, 138, and 126 formed by the losses of 16 (CH_4), 30 (CH_2O), 48 ($HSCH_3$) and 60 Da (CH_2SCH_2) from *m/z* 186, respectively. The fragment ion at *m/z* 110 was formed by the further loss of 16 Da (CH_4) from *m/z* 126 or 60 Da (CH_2SCH_2) from *m/z* 170. The losses of 48 ($HSCH_3$) and 60 (CH_2SCH_2) suggested that $-CH_2SCH_3$ of M4 was not changed and M5 was *O*-demethyl-2-methylsulfanyl-methyl-pyridine. However, it was impossible on the basis of MS data alone to determine whether the site of *O*-demethylation was in position 3′ or 4′. In our study, we isolated and identified other metabolites of pantoprazole, one of which was 2-(5-difluoromethoxy-1*H*-benzoimidazol-2-ylsulfanylmethyl)-3-methoxy-pyridin-4-ol (data not reported). Based on the fact that the site of *O*-demethylation was in position 4′, M5 was tentatively assigned as 3-methoxy-2-methylsulfanylmethyl-pyridin-4-ol.

M6

The molecular ion of M6 was at *m/z* 232, 16 Da higher than that of M3. Fragmentation of the molecular ion (*m/z* 232) of M6 produced ions at *m/z* 214, 184, 168, 153, 138, 122, and 107. Similar to M3, the fragment ions at *m/z* 153, 138, 107 formed by the losses of 79 ($\cdot SO_2CH_3$), 94 ($CH_3SO_2CH_3$), and 125 Da ($CH_3SO_2CH_3$ and $\cdot OCH_3$) from *m/z* 232 suggested that the structure of 3,4-dimethoxy-pyridine was not changed and a sulfone derivative was formed. The loss of 64 Da corresponded to the loss of SO_2 (Wang et al 2003), therefore the fragment ion

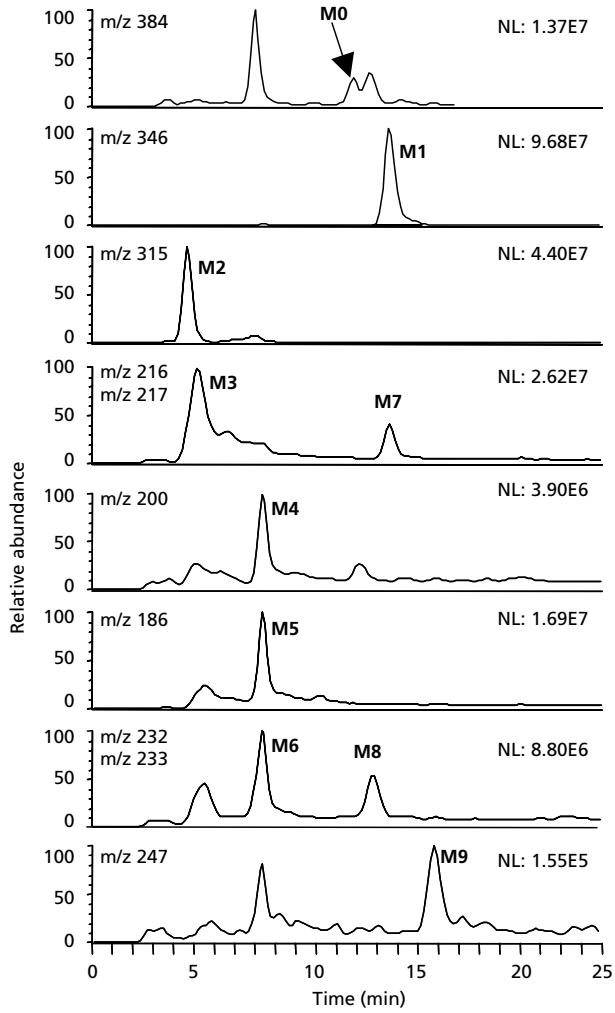

Figure 2 The full MS^2 scan chromatogram of a pooled urine sample collected over 0–12 h from a rat (25 mg kg^{-1}) after oral administration of pantoprazole sodium.

at *m/z* 168 formed by the loss of 64 Da (SO_2) from *m/z* 232 further confirmed that M6 was a sulfone derivative. Based on the MS data, M6 was tentatively assigned as 2-methanesulfonyl-methyl-3,4-dimethoxy-pyridine.

M7–M9 (benzimidazole-related metabolites)

The retention times of M7 ($[M+H]^+$ *m/z* 217), M8 ($[M+H]^+$ *m/z* 233), and M9 ($[M+H]^+$ *m/z* 247) by LC/MS^n were 14.6, 13.8, and 16.8 min, respectively. M7 and M8 were isolated from rat urine after administration of pantoprazole (150 mg kg^{-1}, p.o.), and characterized by ^{1}H NMR (Figure 1D, E).

M7

Fragmentation of the protonated molecule (*m/z* 217) of M7 by CID produced ions at *m/z* 197, 184, 177, 167, 159, 150, and 149. The fragment ions at *m/z* 184, 150, and 149 were formed by the losses of 33 (HS), 67 (·$OCHF_2$), and 68 Da ($HOCHF_2$), respectively. The sequential losses of 20 (HF), 20 (HF), and H_2O from the ion at *m/z* 217 formed the ions at *m/z* 197, 177, and 159, respectively. The fragment ion at *m/z* 167 was generated from the further loss of 30 Da (CH_2O) from *m/z* 197. Based on the MS and ^{1}H NMR data, M7 was identified as 5-difluoromethoxy-1*H*-benzoimidazole-2-thiol.

M8

The protonated molecule was 16 Da higher than that of M7, suggesting M8 was the monohydroxylated derivative of M7. The MS^2 mass spectrum of M8 at *m/z* 233 yielded one ion at *m/z* 213 formed by the loss of 20 Da (HF). The MS^3 mass spectrum of ions at *m/z* 213 yielded fragment ions at *m/z* 193, 180, and 165 formed by the losses of 20 Da (HF), 33 (SH), and 48 Da (CHFO) from *m/z* 213, respectively. The fragment ion at *m/z* 149 was generated from the further loss of CO_2 from *m/z* 193. The loss of 33 (SH) indicated that the -*SH* was unchanged. According to the ^{1}H NMR, the site of hydroxylation occurred at the

Table 2 Summary of key LC/MS^n data of pantoprazole and its metabolites

Compound	t_R (min)	MS[M + H]$^+$	MS^2 [*m/z* (relative abundance, %)]	MS^3 [*m/z* (relative abundance, %)]
M0	12.8	384	200 (100)*	185 (20), 182 (75), 168 (15), 138 (100), 110 (6)
M1	14.5	346	217 (100)*, 130 (6)	The same as MS at *m/z* 217 of M7
M2	4.7	315	297 (10), 273 (10), 186 (100)*, 152 (5)	170 (30), 152 (100), 140 (16), 122 (6)
M3	5.2	216	153 (100), 138 (10), 107 (2)	
M4	8.4	200	184 (100), 170 (6), 152 (14), 140 (6), 122 (2)	
M5	8.4	186	170 (30), 156 (40), 138 (100), 126 (4), 100 (6)	
M6	8.3	232	214 (10), 184 (10), 168 (14), 153 (100), 138 (30) 122 (4), 107 (2)	
M7	14.6	217	197 (100), 184 (50), 177 (10), 167 (95), 159 (35), 150 (25), 149 (60)	
M8	13.8	233	213 (100)*	193 (22), 180 (60), 165 (100), 149 (20), 138 (10)
M9	16.8	247	227 (100)*	212 (100), 207 (10), 180 (20), 207 (10), 180 (20), 179 (20), 163 (10)

[a]Chromatographic and spectroscopic conditions, see Materials and Methods. * Represents the MS^3 fragment ion produced from the ion.

Table 3 Key parameters of method validation and recovery percentage of pantoprazole and its metabolites in rat urine[a]

Compound	Added concn ($\mu g\,mL^{-1}$)						LLOQ ($\mu g\,mL^{-1}$)	Dose percentage (%)
	0.1	0.5	4.0	0.1	0.5	4.0		
	Precision (RSD, %)			Accuracy (RE, %)				
M0	12.1	8.7	10.2	−13.4	12.4	−3.7	0.1	0.9
M1	9.3	3.7	13.7	5.7	−5.8	6.6	0.1	9.8
M2	14.5	13.0	3.4	4.3	−7.3	7.2	0.1	4.2
M3	10.3	5.6	7.2	−14.2	9.1	−7.1	0.1	2.2
M4	–	–	–	–	–	–	–	2.7
M5	–	–	–	–	–	–	–	3.4
M6	–	–	–	–	–	–	–	2.3
M7	13.9	9.8	5.4	−13.7	6.7	−4.7	0.1	2.9
M8	11.0	4.9	8.1	12.1	7.8	−8.1	0.1	1.9
M9	–	–	–	–	–	–	–	0.8

[a]For chromatographic and MS conditions see Materials and Methods. LLOQ, lower limit of quantification.

6-position of 5-difluoromethoxy-1*H*-benzoimidazole-2-thiol. Based on the MS and ^{1}H NMR data, M8 was tentatively identified as 6-difluoromethoxy-2-mercapto-3*H*-benzoimidazol-5-ol.

M9

The protonated molecule was 14 Da higher than that of M8, suggesting M9 was a methylated derivative of M8, which was confirmed by the ion at *m*/*z* 212 formed by the sequential losses of 20 (HF) and 15 Da (·CH_3) from the ion at *m*/*z* 247 of M9. The formation of the MS^3 ions at *m*/*z* 227 of M9 (*m*/*z* 207, 179, and 163) was in line with that of the MS^3 ion at *m*/*z* 213 of M8 (*m*/*z* 193, 165, and 149), which was the loss of 20 (HF), 48 (CHFO) and 64 Da (HF and CO_2), respectively, suggesting that the possible site of methylation was −*SH*. Furthermore, it was consistent with the loss of 47 Da (CH_3S) from the ion at *m*/*z* 227, which formed the ion at *m*/*z* 180. Based on the MS data, M9 was tentatively assigned as 6-difluoromethoxy-2-methylsulfanyl-3*H*-benzoimidazol-5-ol.

Metabolic profile in rats

In rat urine, in addition to the observation that several metabolites (*O*-demethylated and hydroxylated metabolites) formed via the pathways involving *O*-demethylation and aromatic hydroxylation of pantoprazole, a number of metabolites formed via a novel route, involving conjugation with glutathione. Pantoprazole formed several metabolites through fission of the molecule into pyridine- and bezimidazole-related metabolites. After administration of pantoprazole (25 mg kg^{-1}, p.o.) to rats, LC/MS^n analysis of the urine revealed two *N*-acetylcysteine derivatives, four pyridine-related metabolites, and three benzimidazole-related metabolites (Figure 2). Based on these structures, the plausible scheme for their formation pathway is shown in Figure 3. By referring to Table 3, pantoprazole was extensively metabolized in rats, with unchanged drug in only a trace amount (0.9%). The recovery percentages of its metabolites were M1 9.8%, M2 4.2%, M3 2.2%, M4 2.7, M5 3.4%, M6 2.3%, M7 2.9%, M8 1.9%, and M9 0.8%. Analyse of faeces and bile samples by LC/MS^n observed M1, M2, M6, and M8 in trace amounts, with no pantoprazole or other metabolites.

Discussion

In the pathway of glutathione conjugation, *N*-acetylcysteine derivatives are usually the end urinary products (Commandeur et al 1995). As shown in Figure 3, pantoprazole metabolism in rats involved conjugation with glutathione. Beattie & Blake (1989a, b) have reported metabolism of an analogous compound of pantoprazole (SK&F95448). Metabolites of the compound (*N*-acetylcysteine derivative of benzimidazole, mercaptobenzimidazole, *S*-methylmercapto-benzimidazole, and the corresponding sulfoxide and sulfone) were produced in suspensions of hepatocytes from several species. Weidolf et al (1992) reported that the first proton pump inhibitor (omeprazole) was partly (approximately 10% of the dose given) metabolized through the pathway involving glutathione and identified the metabolites (one *N*-acetylcysteine derivative of benzimidazole and three metabolites reflecting the fate of the pyridine moiety) excreted in the urine of rats after oral administration of a mixture of [^{3}H]- and [^{14}C]omeprazole. The identified metabolites of the two compounds were the result of the conjugation of glutathione to the benzimidazole-2-carbon, resulting in a cascade of biotransformation steps of the formed GS-benzimidazole derivative. However, in this study, apart from finding the *N*-acetylcysteine derivative of benzimidazole formed by the conjugation of glutathione to the benzimidazole-2-carbon, another *N*-acetylcysteine derivative of pyridine was found in rat urine after oral administration of pantoprazole, which was possibly formed by the conjugation of glutathione to the pyridine-7′-carbon.

There are three pathways reported for the metabolic processing of glutathione conjugates of xenobiotics (Mutlib et al 2000, 2002; Yin et al 2004). The common and

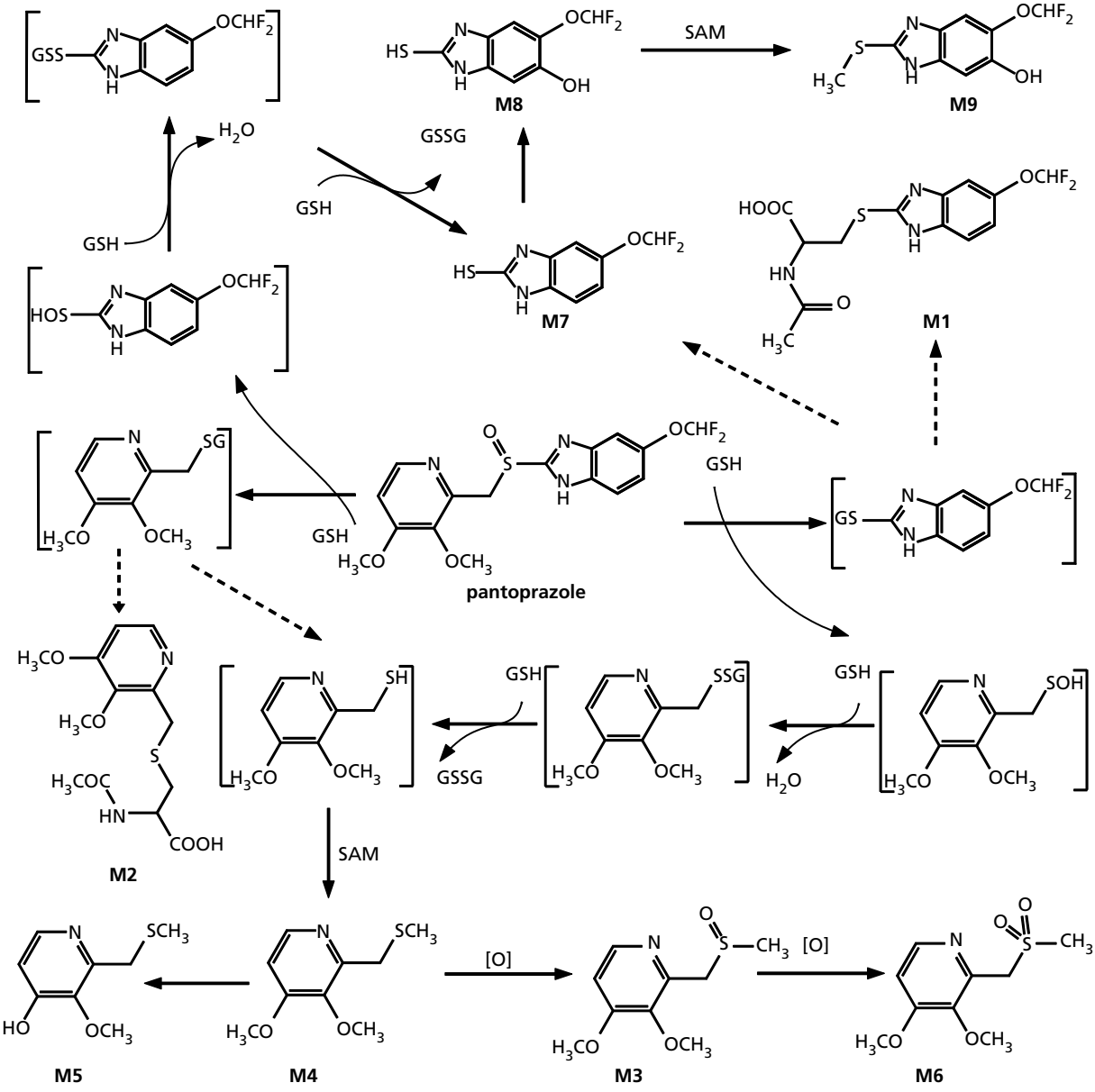

Figure 3 The proposed formation route for the identified metabolites of pantoprazole involving the conjugation with glutathione in rats (SAM, methyl transfer via *S*-adenosylmethionine and methyltransferase; [O], monooxygenase; GSH, glutathione; GST, γ-glutamyltranspeptidase; GSSG, oxidized glutathione).

important one is the so called "mercapturic acid pathway" involving sequential cleavage of the glutamic acid and glycine residues of the glutathione moiety via the enzymes γ-glutamyl transpeptidase (to form cysteinylglycine-*S*-conjugates) and aminopeptidase-M (to form cysteine-*S*-conjugates), respectively (Commandeur et al 1995). The resulting cysteine *S*-conjugates are then *N*-acetylated at the cysteine amino group via the cysteine *S*-conjugate *N*-acetyltransferase to form *N*-acetylcysteine or mercapturic acid metabolites (Commandeur et al 1995). In this study, it was possible that the formation of two *N*-acetylation derivatives (M1 and M2) and two mercapturic acid compounds (mercaptopyridine and M7) followed the common mercapturic acid pathway. Moreover, as presented in Figure 3, the mercapturic acid compounds (mercaptopyridine and M7) were formed through another route. The basis for this was that following glutathione conjugation, the groups leaving were the sulfenic acid derivatives of pyridine and benzimidazole, which rapidly reacted, consecutively, with two molecules of glutathione, to form those compounds, respectively. M3–M6 were then formed by *S*-methylation, *O*-demethylation, and *S*-oxidation from the intermediate of mercaptopyridine. M8 and M9 were formed by hydroxylation and *S*-methylation from mercaptobenzimidazole (M7). The reaction of the sulfenic acid and glutathione might proceed nonenzymatically, but further metabolism most likely

involved glutathione *S*-transferase and *S*-methyl-transferase (Weidolf et al 1992), as well as cytochrome P450 and flavin-dependent monooxygenases (Jakoby & Habig 1980; Weisiger & Jakoby 1980). The reaction of glutathione with pantoprazole may be described as a displacement of the pyridine side-chain or benzimidazole side-chain by glutathione, analogous to the methylthio displacement by glutathione from the methylthiotriazine derivative terbutryn (Huwe et al 1991). In-vitro studies of the displacement of the methylsulfinyl groups have identified methyl mercaptan and the mixed disulfide CH_3-SSG (Davis & Jenkins 1980), which agrees with the biotransformation route of the displaced pyridylmethylsulfinyl moiety or benzimidazole sulfinyl moiety of pantoprazole upon reaction with glutathione, as proposed previously.

Conclusions

Analysis of rat urine after oral administration of pantoprazole revealed nine metabolites, all previously unreported. Five of these metabolites were derived from glutathione conjugation. These five were isolated and their structures identified by LC/MS^n and ^{1}H NMR. The structures of the remaining four metabolites were tentatively assigned by LC/MS^n. The pathway of glutathione conjugation of pantoprazole was found in rats and an explanation given for the formation mechanism of the metabolites. The metabolism of pantoprazole included an attack by glutathione on the benzimidazole-2-carbon and pyridine-7′-carbon, which was mainly catalysed enzymatically by glutathione-*S*-transferase. Through several chemical and enzymatic steps, the metabolic pathway formed at least two *N*-acetylcysteine metabolites, four pyridine-related and three benzimidazole-related metabolites.

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