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Studies on the metabolism of 4-methyl-piperazine-1-carbodithioc acid 3-cyano-3,3-diphenylpropyl ester hydrochloride in rats by high-performance liquid chromatography/electrospray ionization tandem mass spectrometry

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Abstract

4-Methyl-piperazine-1-carbodithioc acid 3-cyano-3,3-diphenylpropyl ester hydrochloride(TM208) is a newly synthesized compound, which has shown excellent in vivo and in vitro anticancer activity and low toxicity. In this study, the metabolism of TM208 in rats was studied for the first time by high-performance liquid chromatography coupled with tandem mass spectrometry. Following a single oral administration to rats, TM208 was metabolized to eight metabolites (M1–M8). M1 is the desmethyl metabolite and the acylation of M1 with *N*-acetyl transferase results in M6 (*N*-acetyl metabolite), M5 is *N*-formyl metabolite; M4 is phenyl monohydroxylation metabolite, M2 is the sulfine metabolite of TM208, and M3 is also an odd-oxygen added products which the possible oxidation site has described in this paper; M8 is the metabolite resulting from the replacement of '–C=S' with '–C=O', M7 is a ring-opened piperazine oxidation products to a kind of acid.

Keywords: 4-Methyl-piperazine-1-carbodithioc acid 3-cyano-3,3-diphenylpropyl ester hydrochloride; Metabolism; High-performance liquid chromatography; Tandem mass spectrometry

1. Introduction

4-Methyl-piperazine-1-carbodithioc acid 3-cyano-3,3-diphenylpropyl ester hydrochloride (TM208, Fig. 1) is one of dithiocarbamic acid esters which has been prepared in this laboratory. To the best of our knowledge, dithiocarbamic acid esters exhibit a variety of valuable biological effects, including antibacterial activity [1,2], antifungal activity [3], the ability to chelate heavy metals and to function as NO scavengers [4–6]. Recently, we discovered several kinds of dithiocarbamic acid esters possessing significant anticancer activity [7,8] and TM208 is the prominent example in them which showed excellent anticancer activity in vitro and in vivo, with inhibition rates of 46–60% ($P < 0.01$–0.001), 39–52% ($P < 0.05$–0.001) and 18–59% against S_{180}, H_{22} and implanted human gastric carcinoma in nude mice, respectively [9]. Toxicological study showed that in mice after intragastric administration of TM208, the MTD (maximal tolerance dose) was more than 1000 mg/kg, and the chronic toxicity was relatively low. However, so far, no studies have been carried out to determine its metabolism and pharmacokinetics. In the present study, following a single oral 250 mg/kg dose of TM208 to rats, fecal metabolites were characterized by high-performance liquid chromatography coupled with mass spectrometry (LC–MS and LC–MS^n). LC–MS-based methods have been proven highly effective for identifying and quantifying drugs and drug metabolites in biological samples.

2. Experimental

2.1. Chemicals and reagents

TM208 (99.5%) was synthesized in our research group, and the structure was confirmed by ^{1}H NMR, ^{13}C NMR, ESI-MS,

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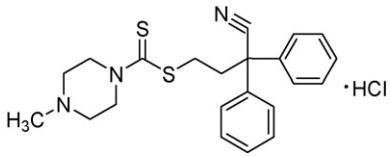


Fig. 1. Structure of TM208.

and elemental analysis. Acetonitrile (HPLC grade) was obtained from Fisher Scientific (Loughborough, UK). Ammonium acetate (99.999%) was obtained from Sigma (St. Louis, MO, USA). Distilled water, prepared from demineralized water, was used throughout the study, and other chemicals used were of analytical grade.

2.2. Liquid chromatography/mass spectrometry

The liquid chromatographic system (Agilent, USA) consisted of a gradient pump, a dynamic mixer, a PDA detector, column oven, and Agilent Chromatographic workshop. Mass spectrometric detection was carried out by an ion trap-based Agilent LC-MSD-Trap-XCT system (Agilent, USA), using an electrospray ionization source operated in the positive ionization mode.

Chromatography was performed on a Diamonsil ODS column (C_{18}, 250 mm × 4.6 mm i.d.; 5 μm particle size, Dikma, USA) fitted with a C_{18} guard column (40 mm × 3.0 mm i.d., 5 μm particle size, Tianjin, China). The column temperature was maintained at 30 °C the maximum pressure was 200 bar and the run time was 50 min. The HPLC mobile phase flow-rate was set at 0.8 mL/min, with gradient elution starting at 40% acetonitrile and 60% 0.05 M ammonium acetate aqueous solution for 10 min, followed by a linear increase of acetonitrile composition to 80% in 40 min.

The HPLC system was connected to the mass spectrometer via an ESI source. Nitrogen was used as the sheath gas (80 psi) and auxiliary gas (3 L/min) for nebulization. The spray and capillary voltage were set at 4.25 kV and 30 V, respectively. The heated capillary temperature was set to 325 °C. Samples were injected by an autosampler and the injection volume was 20 μL. The MS^n spectra were obtained by collision-induced dissociation (CID) with helium after isolation of the precursor ions of interest.

2.3. Samples preparation

2.3.1. Faeces sample

Adult healthy male Sprague–Dawley rats weighing 280–300 g were supplied from the Animal Service of Health Science Center, Peking University. The rats were housed in individual metabolism cage and allowed to acclimate for one week under standardized temperature (25–28 °C), humidity (50–60%) and light (12 h light/12 h dark) environment with free access to standard food and tap water before being used in the study. Food was withheld the night before the study, but water was freely avalable, and the control faeces was collected. On the study day, a single oral dose (250 mg/kg) of TM208 suspension with distilled water was given to rats by gavage and faeces samples approximately 20 h after dosing were collected. All samples were stored at −20 °C until required for analysis.

For qualitative analysis of metabolites, air-dried rat control faeces and post-administration faeces samples (0.5 g, respectively) was vortex mixed with 4 mL of methanol for 5 min and then sonicated for 10 min. After centrifugation at 4000 rpm at room temperature for 10 min, the supernatant was collected and filtered through a 0.45 μm PTFE, HPLC membrane prior to LC–MS analysis.

2.3.2. Plasma sample

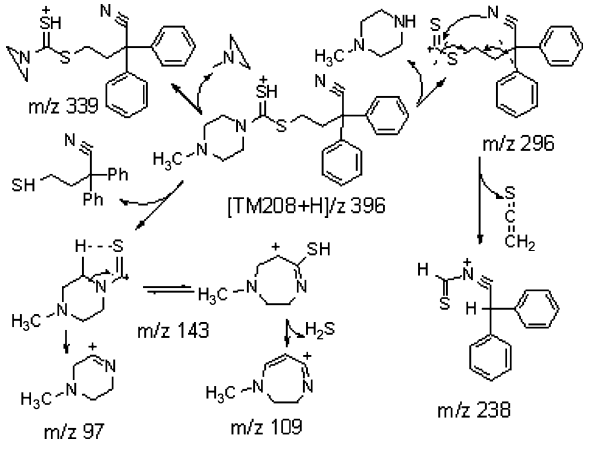
To a 200 μL aliquot of rat plasma, 400 μL distilled water were added. This mixture was extracted with 2 mL redistilled ethyl acetate by vortex mixing for 1 min. After centrifugation at 1800 × *g* for 5 min, the upper organic layer was transferred to another tube and evaporated to dryness at 37 °C under a gentle stream of nitrogen. The residue was dissolved in 200 μL of methanol and 50 μL aliquot of the solution was injected into the HPLC system for analysis.

2.3.3. Urine sample

To a 200 μL aliquot of rat urine, 400 μL distilled water were added. This mixture was extracted with 2 mL redistilled ethyl acetate by vortex mixing for 1 min. After centrifugation at 1800 × *g* for 5 min, the upper organic layer was transferred to another tube and evaporated to dryness at 37 °C under a gentle stream of nitrogen. The residue was dissolved in 200 μL of methanol and 50 μL aliquot of the solution was injected into the HPLC system for analysis.

3. Results and discussion

Shown in Figs. 2–4 are the total ion chromatogram (bottom) and the reconstructed ion chromatograms (RIC) generated by monitoring only the detected ions of TM208 and its metabolic products in rat faeces, plasma and urine, respectivly. The figure shows that eight metabolites were present in the post-administration faeces sample extracts, and all of these peaks were ascertained to be absent in control faeces samples, four metabolites in rat plasms and three metabolites in rat urine. The



Scheme 1. Fragmentation patterns of $[TM208 + H]^+$.

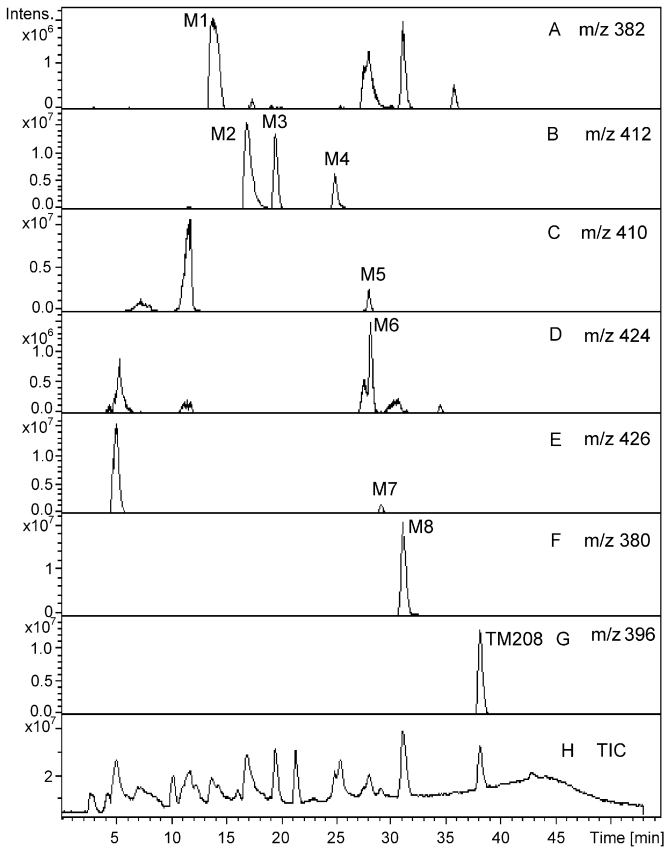


Fig. 2. Total ion chromatogram (TIC, H) and reconstructed ion chromatograms (RIC, A–G) of rat faeces. The identified metabolites are labeled M1–M8 and the parent compound is labeled TM208.

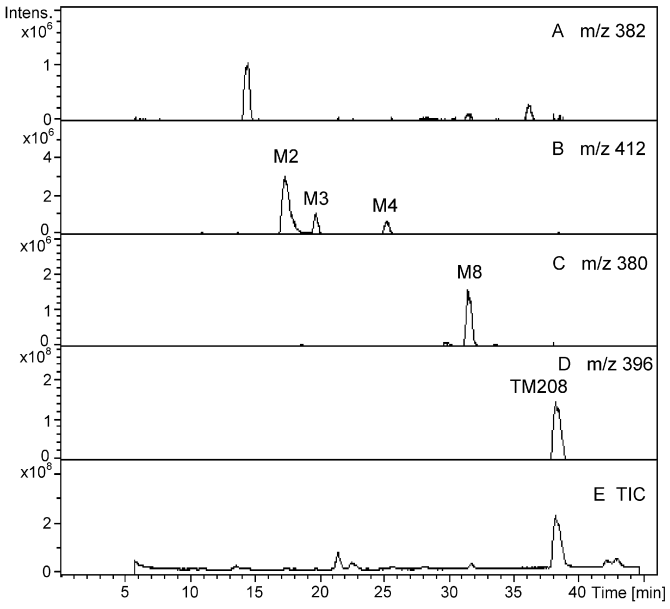


Fig. 3. Total ion chromatogram (TIC, H) and reconstructed ion chromatograms (RIC, A–G) of rat plasma. The identified metabolites are labeled M2–M4, M8 and the parent compound is labeled TM208.

protonated molecule ($[M+H]^+$) and the characteristic fragment ions of the parent compound and all identified metabolites are summarized in Table 1.

To characterize the structures of the metabolites that often contain diagnostic fragment ions related to those of the parent compound, we first studied the product ion spectrum derived from the protonated molecule of TM208 (*m/z* 396). There were four fragment ions at *m/z* 296, 238, 143 and 339 in the MS^2 spectra of $[TM208+H]^+$ ion, as shown in Table 1 (TM208). Further investigation of the ion at *m/z* 296 in the MS^3 spectrum resulted in only one ion *m/z* 238 as shown in Table 1 (TM208), which was also observed in the MS^2 spectra of $[TM208+H]^+$ ion. Its fragmentation patterns have already been elucidated by Hou et al., as shown in Scheme 1 [10,11]. These ions were subsequently used as diagnostic fragment ions for the identification of metabolites whose product ion spectra may contain one or more of the same fragments, or fragments that have been modified by hydroxylation or other metabolic reactions.

Based on the tandem mass spectra collected for each unknown peak separated by HPLC and detected by the mass spectrometer, structural information was obtained and eight metabolites were tentatively or assuredly identified, the protonated forms of which gave rise to fragment ions that were related to the diagnostic fragment ions of the parent compound, TM208. Some typical product ion spectra are presented in Table 1 (M1–M8) for illustration.

In Table 1 (M1), the metabolite identified as M1 shows its $[M+H]^+$ ion at *m/z* 382, 14 u lower than that of TM208. A comparison of Table 1 (M1) and (TM208) reveals that the fragment ion at *m/z* 129 for M1 is also 14 u lower than the fragment ion at *m/z* 143 for TM208, whereas the other two fragment ions are identical (*m/z* 296, 238) in their MS^2 spectra, and in their MS^3

Table 1
$[M+H]^+$ and the characteristic fragment ions (*m/z*) of TM208 and its eight identified metabolites

Compound	Label	$[M+H]^+$	Diagnostic product ions		
			MS^2 (% relative abundance)	MS^3	
M1	*N*-Desmethyl (TM208)	382	296(100), 238(38), 129(41)	296 → 238	
M2	TM208 (sulfine)	412	312(100), 336(56), 254(58), 280(47)	312 → 279, 245	
M3	TM208 (S or C)	412	312(100), 336(8), 254(3), 280(3)	312 → 279, 245	
M4	Phenyl Monohydroxylation (TM208)	412	312(96), 254(100), 143(98)	312 → 254	254 → 237
M5	*N*-Formyl (TM208)	410	296(38), 238(37), 157(100)	296 → 238	157 → 129
M6	*N*-Acetyl (TM208)	424	296(100), 238(62), 171(91)	29 → 238	171 → 129
M7	Ring-opened piperazine oxidation (TM208)	426	323(100), 254(77), 173(87)	323 → 193	173 → 113
M8	Carboxyl group (TM208)	380	280(100), 127(4)	280 → 246	
Parent (TM208)	TM208	396	296(97), 238(83), 143(100)	296 → 238	

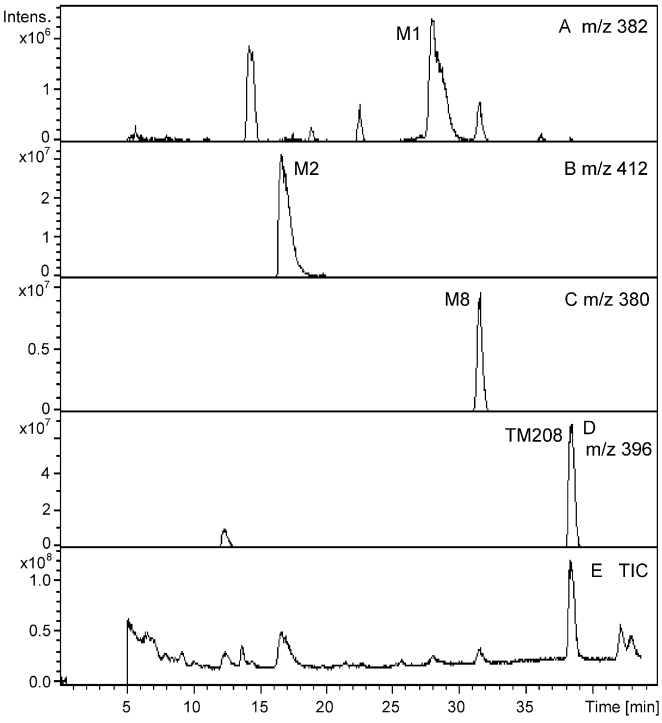
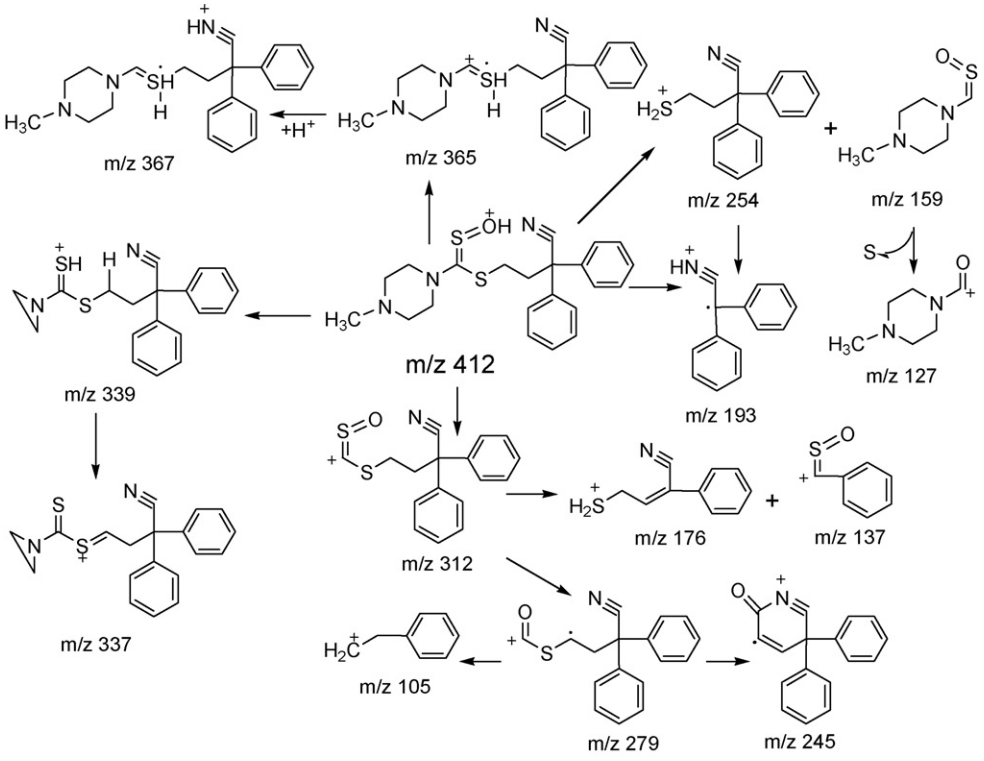


Fig. 4. Total ion chromatogram (TIC, H) and reconstructed ion chromatograms (RIC, A–G) of rat urine. The identified metabolites are labeled M1, M2, M8 and the parent compound is labeled TM208.

spectra, the ion at *m/z* 296 identically showed only one ion *m/z* 238. Hence, *N*-demethylation of the piperazine ring of TM208 appears to be the only structural modification observed in M1. In the same way, the protonated M5 at *m/z* 410 and M6 at *m/z* 424 are 14 and 28 u higher than $[TM208 + H]^+$ at *m/z* 396. In their MS2 spectra, the fragment ion at *m/z* 157 for M5 and *m/z* 171 for M6 are also 14 and 28 u higher than the ion at *m/z* 143 for TM208, and the other two fragment ions are identical (*m/z* 296, 238), furthermore, the MS^3 spectra of M5 and M6 are identical at *m/z* 129 indicating an intact piperazinyl thiocarbonyl cation (129 u). Therefore, M5 is tentatively identified as the metabolite resulting from the oxidation of TM208 to an *N*-formyl, and M6 is proposed to be *N*-acetyl metabolite accounted for acylation of M1 (desmethyl metabolite) with *N*-acetyl transferase.

Three metabolites (M2, M3 and M4) seem to be the odd-oxygen added products since their $[M + H]^+$ ions are all at *m/z* 412, 16 u higher than $[TM208 + H]^+$. M4 appears to be consistent with a structure resulting from monohydroxylation of TM208. Comparing the product ion spectrum of M4 (Table 1) with that of TM208, the fragment ion *m/z* 143 is identical, indicating the intact alkylpiperazinyl thiocarbonyl cation on the metabolite. Further, the fragment ions at *m/z* 312 and 254 for M4 correspond to those at *m/z* 296 and 238 in the MS^2 spectra of TM208, and in M4 the MS^3 of ion *m/z* 312 resulted in one product ion at *m/z* 254 (Table 1 (M4)). So M4 is a phenolic metabolite, the site of monohydroxylation is most likely on the *p*-phenyl location, which is frequent reaction in vivo-metabolism of some drugs. A comparison of the MS^n ($n = 2, 3$) spectra of M2–M4 reveals that the structures of M2 and M3 are much different from the structure of M4 and that is to say that M2 and M3 are impossibly the aromatic monohydroxylation metabolites of TM208. In the MS^2 spectra of M2 and M3, the fragment ion at *m/z* 312 is observed to be the base peak, which is 100 u lower than their $[M + H]^+$ ion at *m/z* 412, suggesting that the oxidation site is not on the methylpiperazinyl moiety (100 u). Since some



Scheme 2. Proposed fragmentation patterns of $[M2 + H]^+$ at *m/z* 412.

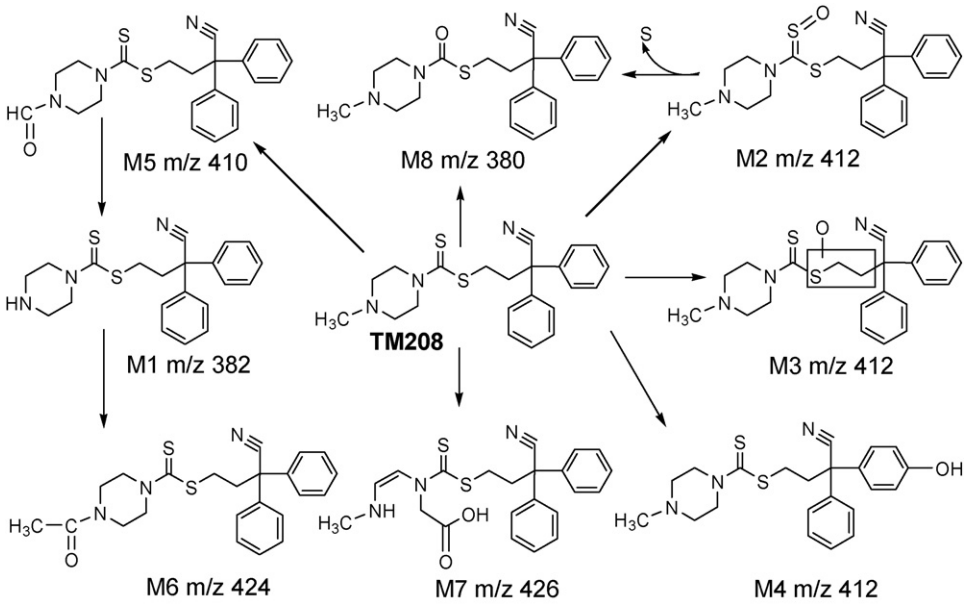


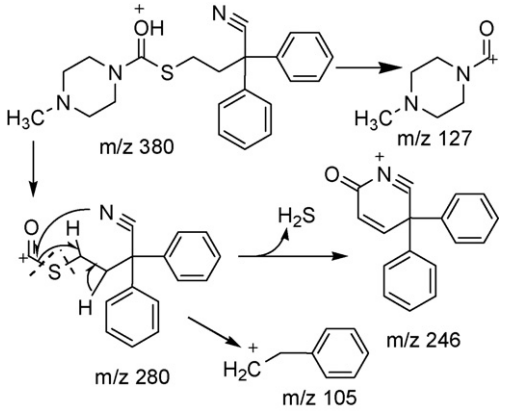
Fig. 5. Proposed metabolic pathways of TM208 in rat faeces, M1–M8 in rat bile; M1, M2 and M8 in rat urine; M2–M4, M8 in rat plasma.

literatures have reported that dithiocarbamates can be metabolized to sulfines by biotransformation [12–14]. We synthesized the sulfine of TM208 in laboratory, and its structure was confirmed using ^{1}H NMR, ^{13}C NMR and ESI-MS^{n}. Compared its chromatographic behavior and ESI mass spectrum with M2 and M3, consequently M2 is assuredly identified as the sulfine metabolite of TM208, its structure and proposed fragmentation patterns are described in Scheme 2. However, M3 is proposed to be the other S-oxidation metabolite or C-oxidation metabolite for the moment and the possible structure is described in Fig. 5.

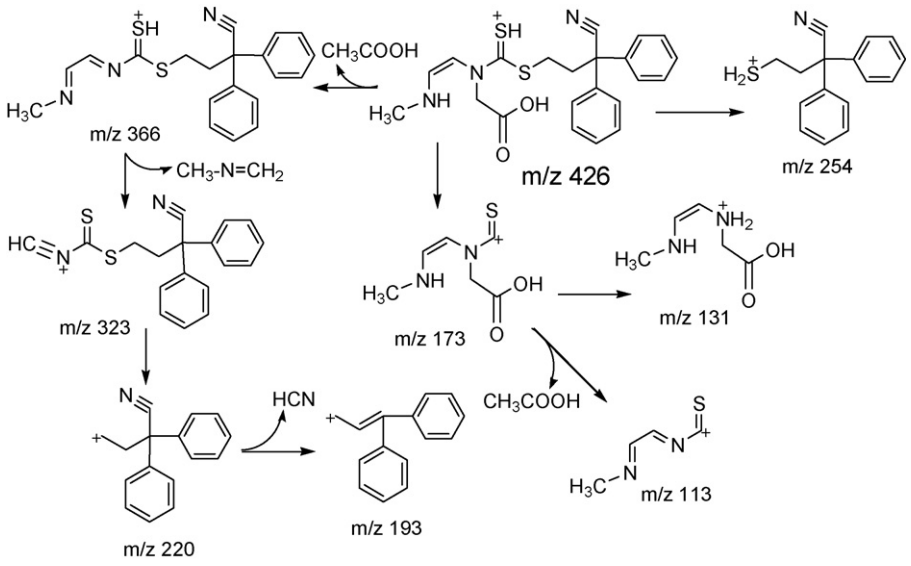
At *m/z* 380, $[M8+H]^+$ is 16 u lower than $[TM208+H]^+$, the fragment ion at *m/z* 280 as the base peak is 100 u lower than the ion at *m/z* 380, indicating the intact methylpiperazinyl moiety on the metabolite. Through analysing the structure of TM208, it is most possible that the replacement of the thiocarbonyl function (–C=S) with a carboxyl group (–C=O) leads to M8 (Fig. 5). In fact, the metabolite M2 is probably the precursor metabolite of M8 according to the loss of sulfur atom. The proposed fragmentation patterns of M8 are shown as in Scheme 3.

Protonated M7 is detected at *m/z* 426, which is 30 u higher than the parent compound, a ring-opened piperazine structure as shown in Table 1 (M7) may be proposed for M7. This structure is consistent with the observed product ion at *m/z* 366 as result of the loss acetic acid from the parent ion $[M7+H]^+$ at *m/z* 426 in MS^2 spectrum (Table 1, M7), and the product ion at *m/z* 113 resulting from the loss acetic acid from the product ion at *m/z* 173 in MS^3 spectrum (Table 1, M7). This oxidative pathway is frequently observed with either piperidine or piperazine containing compounds [15]. Furthermore, the molecular weight of M7 reveals the presence of the double bond, and the product ion at *m/z* 323 and its MS^3 spectrum (Table 1, M7) indicate the most possible position of the double. The proposed fragmentation patterns are described in Scheme 4.

In summary, TM208 is biotransformed into at least eight metabolites in rat faeces, and the proposed metabolic pathways are shown in Fig. 5. It obvious that oxydation is the main metabolic pathway and eight metabolites (M1–M8) were tentatively or assuredly identified: M1 is the desmethyl metabolite and the acylation of M1 with *N*-acetyl transferase results in M6 (*N*-acetyl metabolite), M5 is *N*-formyl metabolite; M4 is phenyl monohydroxylation metabolite, M2 is the sulfine metabolite of TM208, and M3 is also an odd-oxygen added products which the possible oxidation site has described in this paper; M8 is the metabolite resulting from the replacement of '–C=S' with '–C=O', M7 is a ring-opened piperazine oxidation products to a kind of acid. This study represents the first report on the metabolism of TM208 in rat faeces, urine and plasma, and the structures of metabolites are so complex that it would be extremely difficult to unambiguously identify metabolites solely using mass spectrometry; NMR data would be ideal for such compounds, but it may be difficult to generate sufficient amounts of metabolites. For this reason, M1, M2 and M4 have been



Scheme 3. Proposed fragmentation patterns of $[M8+H]^+$ at *m/z* 380.



Scheme 4. Proposed fragmentation patterns of $[M7+H]^+$ at *m/z* 426.

synthesized by our research group and the structures of them are assuredly identified, others have been tentatively identified based on the structural information from the tandem mass spectrum of them.

Acknowledgements

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