Identification of ilaprazole metabolites in human urine by HPLC-ESI-MS/MS and HPLC-NMR experiments

Gan Zhou, a Shuyun Shi, a,b Wei Zhang, a* Zhirong Tan, a Yao Chen, a Dong Guo, a Honghao Zhou, a Haitang Hu c and Jin Tan c

ABSTRACT: Ilaprazole is a new proton pump inhibitor designed for the treatment of gastric ulcers, and limited data is available on the metabolism of the drug. In this article, the structural elucidation of urinary metabolites of ilaprazole in human was described by HPLC-ESI-MS/MS and stopped-flow HPLC-NMR experiments. Urinary samples were precipitated by sodium carbonate solution, and then extracted by liquid–liquid extraction after adding ammonium acetate buffer solution. The enriched sample was separated using a C18 reversed-phase column with the mobile phase composed of acetonitrile and 0.05 mol/L ammonium acetate buffer solution in a gradient solution, and then directly coupled to ESI-MS/MS detection in an on-line mode or 1H-NMR (500 MHz) spectroscopic detection in a stopped-flow mode. As a result, four sulfide metabolites, ilaprazole sulfide (M1), 12-hydroxy-ilaprazole sulfide (M2), 11,12-dihydroxy-ilaprazole sulfide (M3) and ilaprazole sulfide A (M4), were identified by comparing their MS/MS and NMR data with those of the parent drug and available standard compounds. The main biotransformation reactions of ilaprazole were reduction and the aromatic hydroxylation of the parent drug and its relative metabolites. The result testified that HPLC-ESI-MS/MS and HPLC-NMR could be widely applied in detection and identification of novel metabolites. Copyright © 2010 John Wiley & Sons, Ltd.

Keywords: HPLC-ESI-MS/MS; HPLC-NMR; ilaprazole; metabolites; human urine

Introduction

Ilaprazole (2-[(4-methoxy-3-methyl)-2-pyridinyl]methylsulfinyl-5-(1H-pyrrol-1-yl)-1H-benzimidazole, which was called IY81149 before; Fig. 1) is a new proton pump inhibitor designed for the treatment of gastric ulcers developed by I1-Yang Pharmacy Co. (Seoul, Korea). Many early in vitro biochemical studies on animals, such as mice, rats, dogs and pigs, showed that ilaprazole significantly prevented the development of reflux esophagitis and gastric secretion in a dose-dependent manner (Kil et al., 2000; Kwon et al., 2001; Kim et al., 2001), and had little effect on the animal’s cardiovascular system, autonomic nerve system or smooth muscle function, which indicated that ilaprazole was a potent and very safe antiulcer drug (Kwon et al., 2001; Kim et al., 2001). Moreover, the clinical study showed that patients with gastroesophageal reflux disease had a statistically significantly greater and prolonged suppression of gastric pH after administration of 20 mg ilaprazole than 20 mg omeprazole (another proton pump inhibitor designed before ilaprazole) (Periclou et al., 2000).

A previous report on the metabolism of ilaprazole in rat by HPLC-ESI-MS/MS experiment has been demonstrated that ilaprazole sulfone and hydroxyilaprazole were the main metabolites in the plasma sample (Myung et al., 1999). Our group also detected ilaprazole sulfone in Chinese human plasma, and we found that the CYP3A5 genetic polymorphisms impressed the pharmacokinetics of ilaprazole (Li et al., 2008). However, comprehensive investigations of its metabolism are scarce, especially for human urinary samples. Therefore, the further metabolic study of ilaprazole in human urinary sample is necessary as drug metabolism experiment plays an important role in the development of a new drug and its better clinical application (Suchanova et al., 2008).

Unambiguous identification of metabolites can usually only be accomplished by spectroscopic characterization, especially by MS and NMR experiments. Usually this necessitates the isolation and purification of the metabolites from complex biofluids. However, the isolation of metabolites is a challenging task because of the low quantities of substance. Therefore, a sensitive and specific assay method is very important for the identification of metabolites. Nowadays, the development of hyphenated systems of a highly efficient separation technique like HPLC and specific and sensitive detection by tandem MS system is a promising approach to overcoming these difficulties (Anacardio et al., 2009; Zhang et al., 2009; Nakamura et al., 2009; Song et al., 2008). However, for the exact structural elucidation of metabolites, MS data alone are insufficient. Further data, especially NMR spectra, are necessary.
Identification of ilaprazole metabolites in human urine

are demanded. Therefore, LC-NMR has rapidly gained popularity in several fields (Seger et al., 2005; Pukalskas et al., 2005).

In the present study, LC-ESI-MS/MS and LC-NMR experiments were used to identify and elucidate the structures of four in vivo metabolites in human urine after oral administration of ilaprazole by combining MS/MS and NMR data with those of the parent drug and available standard samples.

Experimental

Chemicals and Reagents

Ilaprazole (5 mg enteric-coated tablet), together with ilaprazole sulfide (purity of 99.5%) were provided by Liyzon Pharmaceutical Group Inc. (Zhuhai, China), which signed a licence agreement and got the patent from II Yang Pharmacy Co. (Seoul, Korea). HPLC-grade acetonitrile was bought from Dikma Co. (Guangzhou, China). Deuterated acetonitrile used for NMR was bought from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). All aqueous solutions used in the experiments were prepared with pure water produced by Milli-Q water system (Millipore, Bedford, MA, USA). Other analytical grade chemicals were purchased from the Chemical Reagent Factory of Hunan Normal University (Changsha, Hunan, China).

Subjects

Six healthy Chinese volunteers participated in these studies, who were nonsmokers with no history of significant medical illness. The study was approved by the Ethics Committee Board of Xiangya School of Medicine, Central South University, Hunan, China. All volunteers provided written informed consent. Physical examination, blood chemistries screen (including a complete blood count and liver function test), urinalysis and electrocardiogram were performed before the study. Volunteers receiving proton pump inhibitors or any other drugs within the last month, consuming alcohol within 2 weeks or with hypersensitivity to drugs were not included. All volunteers were hospitalized at 10:00 p.m. the night before the study and fasted for 10 h before drug administration. All subjects received a single dose of ilaprazole (three 5 mg tablets) with 250 mL of water at 8:00 a.m. and standardized meals 1, 4 and 10 h after dosing. During the 24 h period after drug administration, no strenuous physical or mental activity was permitted. The use of alcohol, tea, coffee, cola and fruit juice was forbidden throughout the study.

Sample Collection and Pre-treatment

Urinary samples were collected at pre-dose and 0–24 h after dosing, and stored at approximately −40°C before the samples were extracted and analyzed. Urinary samples for volunteers not taking ilaprazole were screened for ilaprazole metabolites as a control.

For extraction of ilaprazole metabolites from urine, all 24 h urinary samples of six volunteers were combined and 1 mL of 1 mol/L sodium carbonate solution was added into 10 mL urinary samples. After centrifugation for 10 min at 3000 rpm, the supernatant was added with 2 mL of 0.05 mol/L ammonium acetate buffer and extracted by liquid–liquid extraction twice with 10 mL methylene chloride. The organic phase containing the metabolites was transferred to a new Eppendorf tube and evaporated to dryness under a gentle stream of N\textsubscript{2} at 40°C and the residue was dissolved in 50 μL of the initial mobile phase (0.05 mol/L ammonium acetate buffer–acetonitrile, 7:3, v/v), and 20 μL of the sample solution was injected for HPLC analysis.

For HPLC-NMR analysis, all 24 h urinary samples of six volunteers were combined and evaporated under reduced pressure (1 L, 40°C), and then 100 mL of 1 mol/L sodium carbonate solution was added. After centrifugation for 10 min at 3000 rpm, the supernatant was added with 200 mL of 0.05 mol/L ammonium acetate buffer and then partitioned with methylene chloride (500 mL) three times successively. All the methylene chloride solution were combined and concentrated to dryness (7 mg), and the residue was dissolved in 0.2 mL acetonitrile-d\textsubscript{6} for analysis.

Chromatographic and Spectroscopic Methods

The HPLC-ESI-MS/MS system comprised a ThermoFinnigan Surveyor liquid chromatography and a LCQ Deca XP instrument with ESI interface (ThermoFinnigan, San Jose, CA, USA). The data acquisition and control systems were created using Xcalibur 1.3 software (Finnigan, San Jose, CA, USA). The metabolites were separated using a reversed-phase column (Hypersil BDS C\textsubscript{18}, 200 × 4.6 mm i.d., 5 μm, Yilite, Daliang, China) and a security guard C\textsubscript{18} ODS (4.0 × 3.0 mm i.d.) from Phenomenex (Torrance, CA, USA). The mobile phase consisted of 0.05 mol/L ammonium acetate buffer solution (solvent A) and acetonitrile and acetoni­trile (solvent B) in a gradient elution mode. Gradient elution was as follows: initial 30% B maintained for 10 min, then increased to 50% B in 2 min; maintained at 50% B for 6 min. The flow rate was kept at 1.0 mL/min, while the column temperature was set at 30°C using a column oven. The chromatogram was acquired at 306 nm. The effluent was on-line transferred to an ESI-MS system with a post-column splitter. The post-column splitter delivered a rate of 5 μL/min into the electrospray needle. The operational parameters of the mass spectrometer were as follows. Positive ion mode for ESI-MS was selected. Mass detection was performed in full-scan mode for m/z in the range 100–400. Capillary and cone voltage were 2500 and 40 V, respectively; nebulizer nitrogen gas flow rate was 500 L/h; the ionization sources were worked at 120°C. The desolvation temperature was 450°C. The fragment ions were produced by collision-induced dissociation (CID) mode of the selected metabolites. Collision energy was 36 eV. Data acquisition was performed in full-scan LC-MS and tandem MS mode.

The HPLC-NMR system comprised a Prostar-230 liquid chromatography and a Varian Inova-500 NMR spectrometer (Varian Corporation, Palo Alto, CA, USA). The stationary phase and the elution gradient were the same as those in the HPLC-ESI-MS/MS system; however, the mobile phase consisted of 0.05 mol/L ammonium acetate buffer in D\textsubscript{2}O (solvent A) and acetonitrile-d\textsubscript{6} (solvent B). The chromatographic conditions were used in a stopped-flow mode. 1H NMR spectra were measured with a spectral width of 12 000 Hz, and 32 K data points were acquired. An acquisition time of 1.5 s and a relaxation delay of 1.8 s were used. Chemical shifts were expressed in ppm relative to internal acetonitrile.

Results and Discussion

To obtain HPLC chromatograms with good resolution of adjacent peaks within a reasonably short analysis time, different mobile phases, such as acetonitrile–water, methanol–water, acetonitrile–buffer solution and methanol–buffer solution in various proportions, were tested. It was found that the presence of 0.05 mol/L ammonium acetate buffer solution in mobile phase leads to a significant improvement on the peak shape and resolution of the metabolites. Therefore, a gradient solvent system composed of 0.05 mol/L ammonium acetate buffer solution–acetonitrile was chosen as the mobile phase. DAD detection was employed at wavelength range of 200–400 nm to investigate the UV spectra of the target metabolites. It was found that 306 nm showed good
sensitivity for the target peaks. A column temperature of 30°C was beneficial to the separation time and resolution (Fig. 2).

Because of the large throughput needed for HPLC-NMR, a loadability experiment was used. Loadability for separation is the maximum sample capacity for the target analytes of interest. Therefore, the scaleup process was performed by injecting increasingly larger amounts of material on column until the resolution became so poor that purity was compromised.

UV spectrum and the full-scan mass spectrum of human urinary samples after administration of ilaprazole were compared with those of blank urinary samples to screen and identify the possible metabolites. Carefully mining the data collected in the HPLC-PDA-ESI-MS/MS experiments resulted in the discovery of four metabolites (M1–4) in methylene chloride extract; however, the parent drug was not found in the urinary samples (Fig. 2). Then the possible metabolites were analyzed by HPLC-ESI-MS/MS and HPLC-NMR. Combining with the MS/MS data and 'H NMR data, the structures of the four metabolites could be unambiguously elucidated.

At first, the possible structures of metabolites for ilaprazole were speculated according to the rule of drug metabolism and the structure of parent drug (Zhang and Li, 1999; Myung et al., 1999). As metabolites often contain product ions related to those of parent compounds, the parent compound’s fragmentation behavior was first studied by HPLC-ESI-MS/MS to characterize these structures. As indicated in Fig. 3, the protonated parent molecule shows m/z 367. The dominant fragment ion at m/z 184 in the MS/MS spectrum is postulated to result from fragmentation between the sulfur atom and benzimidazole. Then the CID pathway of ilaprazole could be proposed.

M1 was observed at a retention time of 15.26 min with a protonated ion [M + H]+ at m/z 351 (Fig. 4A). A loss of 16 Da (m/z 367 → 351) by comparison with the parent compound, ilaprazole, indicated the loss of an oxygen group. The MS/MS spectra of m/z 351 displayed the ions at m/z 318, 184 and 168. The product ion at m/z 318 [M + H – 33]+ suggested the presence of a sulfide bond in the metabolite, and this fragmentation process was typical for a large number of substituted benzimidazoles with a –CH2–S– linkage to the pyridine ring (Hoffmann, 1986; Pearce and Lushnikova, 2006; Zhu et al., 2006). Moreover, the product ion at m/z 184, the same as the parent compound, suggested that the modification did not happen in the benzimidazole, pyrrole and pyridine rings, while the product ion at m/z 168 (16 Da less than that of ilaprazole) highly suggested that the sulfoxide group in ilaprazole was reduced to the corresponding sulfide group. Based on the data above, M1 was deduced as ilaprazole sulfide (2-[(4-methoxy-3-methyl)-2-pyridinyl]methylthio-5-(1H-pyrrol-1-yl)-1H-benzimidazole), which was confirmed by the comparison of retention time between M1 and ilaprazole sulfide standard.

The retention time of M2 was 8.47 min, and M2 showed a protonated ion [M + H]+ at m/z 367 identical to that of ilaprazole (Fig. 4B). However, M2 was more hydrophilic than the parent drug during the HPLC analysis. The appearance of the product ion m/z 334 [M + H – 33]+ in the MS/MS spectra indicated a sulfide rather than a sulfoxide in M2 (Hoffmann, 1986; Pearce and Lushnikova, 2006; Zhu et al., 2006), which suggested that a hydroxylation reaction had occurred in other part of M2. The MS/MS spectrum of M2 displayed two characteristic product ions at m/z 168 and 200. The ion at m/z 168, the same as that of M1, suggested that the substituted pyridine moiety was not metabolically modified. The ion at m/z 200, 16 Da higher than that of M1, supported the notion that the hydroxylation occurred in the benzimidazole ring or pyrrole ring. However, the
position of the hydroxyl group could not be deduced only from the HPLC-ESI-MS/MS experiment. In the $^1$H NMR spectra, M2 showed signals for three aromatic hydrogens in an ABX system with resonances at $\delta_H 7.61$ (1H, s, H-4), $\delta_H 7.41$ (1H, d, $J_\text{H} = 8$ Hz, H-7) and $\delta_H 7.30$ (1H, d, $J_\text{H} = 8$ Hz, H-6), which suggested that the hydroxylation did not occur in the benzimidazole ring. However, in the pyrrole ring, only three aromatic hydrogen signals displayed at $\delta_H 6.13$ (1H, d, $J_\text{H} = 2$ Hz, H-13) and $\delta_H 7.29$ (2H, br s, H-11 and H-14); therefore, it can be concluded that H-12 was hydroxylated. M2 was then deduced as 12-hydroxy-ilaprazole sulfide $\{2-[(4-methoxy-3-methyl)-2-pyridinyl]methylthio-5-(3-hydroxy-1H-pyrrol-1-yl)-1H-benzimidazole\}$.

The mass spectra of M3 that was detected at a retention time of 4.04 min gave an ion $[\text{M} + \text{H}]^+$ at m/z 383 (Fig. 4C). The protonated molecule at m/z 383 $\{\text{M} + \text{H} - 33\}$ in the MS/MS spectrum also indicated the presence of a sulfide bond (Hoffmann, 1986; Pearce and Lushnikova, 2006; Zhu et al., 2006). The protonated molecular at m/z 383 was 32 Da higher that of M1, suggesting the presence of hydroxyl groups. The characteristic product ion of M3 in MS/MS experiment existed at m/z 168, which was the same as that of M1, indicating that there was no change at pyridine ring. The other product ion at m/z 216 (32 Da higher than that of M1, Fig. 4A) indicated the presence of two hydroxyl groups in the benzimidazole or pyrrole ring. According to $^1$H NMR (Table 1), two hydroxyl groups were located at C-11 and C-12 of pyrrole ring because of the existence of only two aromatic hydrogens at $\delta_H 6.27$ (1H, d, $J_\text{H} = 2$ Hz, H-13) and $\delta_H 7.56$ (1H, d, $J_\text{H} = 8$ Hz, H-14). Therefore, M3 was elucidated as 11,12-dihydroxy-ilaprazole sulfide $\{2-[(4-methoxy-3-methyl)-2-pyridinyl]methylthio-5-(3,4-dihydroxy-1H-pyrrol-1-yl)-1H-benzimidazole\}$.

With protonated ions $[\text{M} + \text{H}]^+$ at m/z 343, M4 was observed at a retention time of 6.39 min. The typical ions at m/z 310 ($[\text{M} + \text{H} - 33]$) and 168 showed the existence of a sulfide bond and an unchanged pyridine ring (Fig. 4D). The $^1$H NMR spectrum (Table 1) revealed signals for three aromatic hydrogens at $\delta_H 7.69$ (1H, s, H-4), $\delta_H 7.45$ (1H, d, $J_\text{H} = 8$ Hz, H-7) and $\delta_H 7.18$ (1H, d, $J_\text{H} = 8$ Hz, H-6) in the benzimidazole ring and two aromatic hydrogens at $\delta_H 8.16$ (1H, d, $J_\text{H} = 6$ Hz, H-20) and $\delta_H 6.91$ (1H, d, $J_\text{H} = 6$ Hz, H-19) for the pyridine ring; however, the aromatic hydrogens in pyrrole ring disappeared. Moreover, one $-\text{NH--CH}_2--\text{CH}_2--\text{CH}_3$ group signal ($\delta_H 1.17$ (3H, t, $J_\text{H} = 7.2$ Hz, H-13), $\delta_H 2.38$ (2H, m, H-12 and H-13), 3.65 (2H, m, H-11)) displayed in the higher field of $^1$H NMR spectrum, which suggested that the ring-opening reaction happened for the pyrrole ring, and the result was consistent with the characteristic fragment ions (m/z 176) in the MS/MS spectrum. Therefore, M4 was deduced as ilaprazole sulfide A $\{2-[(4-methoxy-3-methyl)-2-pyridinyl]methylthio-5-propylamine-1H-benzimidazole\}$.
The proposed metabolic pathways of ilaprazole in human are shown in Fig. 5. From the result, we can see that the sulfoxide group in ilaprazole was reduced in all metabolites to the corresponding sulfide, and the next biotransformation happened in hydroxylation or reduction of the molecule’s pyrrole ring.

**Conclusions**

The metabolites in human urine after oral administration of ilaprazole were elucidated by on-line HPLC-MS/MS and stopped-flow HPLC-$^1$H NMR, which is a highly desirable and most efficient technique to elucidate new interesting compounds from complex mixtures. Four metabolites identified in this study were all ilaprazole sulfide derivatives and the parent drug was not detected in the urinary sample. The important metabolic data will be useful resources for further investigation of ilaprazole.

**Acknowledgements**

This work was supported by the National Scientific Foundation of China (No. 30801421), Huge Project to Boost Chinese Drug

---

**Table 1.** $^1$H NMR spectral data for ilaprazole (DMSO-d$_6$) and metabolites (M2, M3 and M4) (MeCN-2H$_2$O) at 500 MHz

<table>
<thead>
<tr>
<th>Comp. proton</th>
<th>Ilaprazole, δ J (Hz)</th>
<th>M2, δ J (Hz)</th>
<th>M3, δ J (Hz)</th>
<th>M4, δ J (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>8.24, d, 5.5</td>
<td>8.06, d, 5</td>
<td>8.18, d, 5</td>
<td>8.16, d, 6</td>
</tr>
<tr>
<td>4</td>
<td>7.72, br s</td>
<td>7.61, s</td>
<td>7.55, s</td>
<td>7.69, s</td>
</tr>
<tr>
<td>7</td>
<td>7.70, d, 8</td>
<td>7.41, d, 8</td>
<td>7.26, d, 8</td>
<td>7.45, d, 8</td>
</tr>
<tr>
<td>6</td>
<td>7.53, d, 8</td>
<td>7.30, d, 8</td>
<td>7.21, d, 8</td>
<td>7.18, d, 8</td>
</tr>
<tr>
<td>11</td>
<td>7.36, t, 2.5</td>
<td>7.29, br s</td>
<td></td>
<td>3.65, m</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td></td>
<td>7.56, d, 2</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>6.96, d, 5.5</td>
<td>6.81, d, 5</td>
<td>6.93, d, 5</td>
<td>6.91, d, 6</td>
</tr>
<tr>
<td>12</td>
<td>6.28, t, 2.5</td>
<td></td>
<td></td>
<td>2.38, m</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>6.13, d, 2</td>
<td>6.27, d, 2</td>
<td>1.17, t, 7.2</td>
</tr>
<tr>
<td>15</td>
<td>4.80, d, 13.5</td>
<td>4.42, s</td>
<td>4.46, s</td>
<td>4.43, s</td>
</tr>
<tr>
<td>15′</td>
<td>4.75, d, 13.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>3.86, s</td>
<td>3.73, s</td>
<td>3.85, s</td>
<td>3.84, s</td>
</tr>
<tr>
<td>22</td>
<td>2.15, s</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a Signal of H-22 was imbedded in the D$_2$O peak.*

---

**Figure 5.** Proposed major metabolic pathway of ilaprazole.
Identification of ilaprazole metabolites in human urine

References


