IDENTIFICATION OF THE METABOLITES OF ROXITHROMYCIN IN HUMANS

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(Received November 10, 1999; accepted January 19, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:
The semisynthetic antibiotic roxithromycin (RXM) exists in an (E)-configuration. Metabolites of RXM in the bile of four cholecystectomy patients with T-tube drainage and in the urine and plasma of four healthy volunteers after single oral doses of 150 mg of RXM were investigated. A total of 15 metabolites were found in bile, urine, and plasma by HPLC with ion trap mass spectrometric and electrochemical detection. These metabolites were identified as descladinose derivative of RXM (M1), erythromycin-oxime (M2), N-, O-, and N,O-di-demethylated derivatives of RXM (M3, M4, and M6), and N-mono- and N-di-demethylated derivatives of erythromycin-oxime (M5 and M7), as well as the (Z)-isomers (M8–M15) of RXM and metabolites M1 to M7, respectively. Structures of six major metabolites (M1–M4, M8, and M10) were established by chromatographic and mass spectrometric determination and comparison with synthesized standards. The stability of RXM and the six synthesized substances was investigated to exclude artifact products. These results, together with previous findings, suggest that biotransformation pathways elucidated for RXM include: 1) isomerization of RXM derivatives, from E-isomer to Z-isomer; 2) O-demethylation; 3) N-demethylation; 4) hydrolysis of the cladinose moiety; and 5) dealkylation of the oxime ether side chain. Secondary metabolism via these pathways was also evidenced. The O-demethylation and isomerization of RXM derivatives represent two novel biotransformation pathways recovered for RXM.

Roxithromycin (RXM), chemically designated as (E)-erythromycin-9-[O-[[2-methoxyethoxy]methyl]oxime], is a semisynthetic, orally administered antibacterial macrolide structurally related to erythromycin. It has in its antibacterial profile similar to that of erythromycin, with activity against Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pneumoniae, Streptococcus pyogenes, Branhamella catarrhalis, Mycoplasma pneumoniae, some anaerobes and other less common pathogens. Clinical efficacy has been confirmed in the treatment of respiratory tract infections, genitourinary tract infections, and skin and soft tissue infections (Young et al., 1989; Markham and Faulds, 1994). RXM has better bacteriologic activity than erythromycin, and the presence of the oxime ether side chain at the 9-position confers greater acid stability to the erythronolide ring, thereby preventing formation of the inactive 6,9,12-spiroketal derivative (Gase et al., 1991).

A few investigations in animals and humans have reported that after oral administration of RXM, four metabolites have been identified in urine and feces (Esumi et al., 1988; Koyama et al., 1988; McLean et al., 1988). These are erythromycin-9-oxime (ERY-oxime), a descladinose derivative (to which a sugar residue of RXM is lost), and N-mono- and N-di-demethylated derivatives of RXM. The metabolites of RXM in human bile have not been investigated. The biotransformation of RXM is poorly understood; this is due in part to difficulties that have been encountered in establishing a sensitive and specific assay for RXM and its metabolites. Because of the lack of a strong chromophore, development of a suitable analytical method for metabolic studies using HPLC with UV or fluorescence detection is not practical. Reported thin-layer chromatography methods (Esumi et al., 1988; Koyama et al., 1988; McLean et al., 1988) had low specificity and sensitivity, and HPLC with electrochemical detection (HPLC-ECD; Shirotsuka et al., 1988) could not respond to those metabolites that have low electrochemical activity. Thus, although the pharmacokinetic properties of RXM have been investigated, many questions remain as to its fate after administration. To gain additional insight into its metabolism, we examined the biotransformation of RXM and characterized the structures of its metabolites in bile, urine, and plasma after oral administration in humans. A specific and sensitive liquid chromatography-mass spectrometry at stage n (LC/MSn) assay technique, as well as HPLC-ECD, was used in this study so that even small amounts of RXM and its metabolites could be detected. To exclude artifact products, the stability of RXM and its six major metabolites in freshly collected human bile, urine, and plasma was investigated under the current experimental conditions.

Experimental Procedures

Chemicals and Drugs. RXM tablets (150-mg tablets) were supplied by Aim Pharmaceutical Inc. (Shenyang, China). RXM and (E)-ERY-oxime reference substances were supplied by Haatai Drug Research Institute (Shenyang, China). (E)-N-demethyl RXM (RU44981) was obtained from Hoechst Marion Roussel (Romainville Cedex, France). (E)-O-demethyl RXM was synthesized at the Department of Pharmaceutical Chemistry, Shenyang Pharmaceutical...
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University (Shenyang, China). Methanol and acetonitrile were HPLC grade; water was double distilled in our laboratory. All other chemicals were analysis grade and commercially obtained.

Chemical Syntheses. (E)-descladinose derivative of RXM, (Z)-RXM, and (Z)-ERY-oxime were synthesized using the published procedures (Gase et al., 1991) as described below. Identity and purity were confirmed by examining the mass spectra and NMR spectra.

Synthesis of (E)-descladinose of RXM (M1 Reference). RXM (0.8 g) was dissolved in 150 ml of 0.012 M hydrochloric acid and the solution was allowed to stand at 45°C for 48 h. The acid solution was poured into a solution of 2.5 g of sodium bicarbonate in 25 ml of water; this was followed by extraction with four 40-ml portions of methylene chloride. From the combined extracts, methylene chloride was evaporated to obtain 0.45 g of the raw product. Crystallization from a toluene-petroleum ether (60°C–90°C) mixture (1:5) gave 0.28 g (43.2%) product.

Synthesis of (Z)-RXM (M8 Reference). A mixture of 1.89 g of (Z)-ERY-oxime, 25 ml of acetone, 1 g of sodium bicarbonate, and 0.36 ml of (methoxythio)ethylmethyl chloride was refluxed under an inert atmosphere (N₂) for 3 h. The mixture was poured into 25 ml of 0.012 M hydrochloric acid and the solution was allowed to stand at 25°C and then overnight at 0 ± 5°C. The mixture was evaporated to dryness.

H NMR spectra of reference compounds were recorded at 300 MHz on a Bruker AMX 300 NMR spectrometer (Bruker, Faellanden, Switzerland). All compounds were dissolved in CDCl₃. All chemical shifts (δ) are reported in ppm relative to tetramethylsilane as an internal standard. The NMR spectra are not discussed in detail; essential data are summarized in Table 1.

Chromatography. The HPLC system (Shimadzu Corp., Kyoto, Japan) consisted of a Shimadzu 10AD pump, a 7125 Rheodyne injector, a Shimadzu UV detector, a L-ECD-6A amperometric electrochemical detector, and a C-R6A computing integrator. Samples were analyzed on a Kromasil ODS column (particle size, 5 μm; 20 cm × 4.6 mm i.d., Hi-Tech Scientific Instrument Corp., Tianjin, China). The HPLC system (Shimadzu Corp., Kyoto, Japan) consisted of a Shimadzu 10AD pump, a 7125 Rheodyne injector, a Shimadzu UV detector, a L-ECD-6A amperometric electrochemical detector, and a C-R6A computing integrator. Samples were analyzed on a Kromasil ODS column (particle size, 5 μm; 20 cm × 4.6 mm i.d., Hi-Tech Scientific Instrument Corp., Tianjin, China).
China). The mobile phase A for ECD consisted of acetonitrile/methanol/aqueous buffer containing 3 mM EDTA and 60 mM NaH₂PO₄, and using NaOH to bring the pH to 7.0 (39:11:49, v/v). The flow rate was 1.0 ml/min and the potential of the working electrode was set to 1.20 V. The mobile phase B for MS detection consisted of acetonitrile/methanol/10 mM ammonium acetate (43:10:47, v/v). The flow rate was isocratic at 0.4 ml/min.

Subjects and Dosing Procedure. Biliary excretion studies. Four patients (two females, two males) aged 16 to 66 years and weighing 40 to 70 kg participated in this study. All were nonsmokers with normal renal function as assessed by serum creatinine levels, and none had severe hepatic disease as assessed by clinical symptoms and conventional laboratory tests. All patients had undergone cholecystectomy because of symptomatic choledocholithiasis. Bile was collected via a T-tube that was left in situ for at least 7 days after surgical intervention. No medications or ethanol consumption were allowed for 48 h before or during the study period. Each patient received a single oral dose of RXM (150-mg tablet) in the morning with 100 ml of plain water after an overnight fast. Bile samples were collected via the T-tube before drug administration (blank sample) and during 0- to 1.5-, 1.5- to 5-, and 5- to 10-h intervals after drug intake. The volume of each sample was duly recorded. All samples were kept at 2–30°C until analysis.

Urinary and plasma studies. Four healthy volunteers aged 21 to 26 years and weighing 45 to 75 kg (two males and two females) participated in this study. Subjects were judged to be in good health based on a medical history, physical examination, and laboratory profiles that were performed within 2 weeks before the study. Subjects fasted, except for water, for 12 h before drug administration. Each subject was given an oral 150-mg dose of RXM tablet. Urine samples were collected at 0 to 3, 3 to 12, and 12 to 24 h post drug administration. All urine samples were frozen immediately after collection. Five-milliliter blood samples were collected by venipuncture into evacuated heparinized blood collection tubes at 0, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 11, 15, and 24 h after dosing. Plasma samples were prepared by centrifugation of the blood at 2000 g for 10 min. The organic layer was then vortexed vigorously for 3 min and afterward centrifuged for 10 min at 2000 g. The organic layer was transferred into a conical test tube and evaporated to dryness under a stream of nitrogen at 25°C. The residue was dissolved in 100 µl of the HPLC mobile phase, and an aliquot of 20 µl was injected onto the chromatograph. Other portions of plasma, bile, or urine samples (0.5 ml) were added in a glass tube, to which was added 100 µl of 0.1 M sodium carbonate followed by 3 ml of fresh distilled diethyl ether. Samples were then vortexed vigorously for 3 min and afterward centrifuged for 10 min at 2000 g. The organic layer was transferred into a conical test tube and evaporated to dryness under a stream of nitrogen at 25°C. The residue was dissolved in 100 µl of the HPLC mobile phase, and an aliquot of 20 µl was injected onto the chromatographic system.

Extraction of Metabolites from Biological Samples. A 0.5-ml portion of bile or urine collected from each subject was diluted with 0.5 ml of distilled water and filtered through precut membranes (0.45 µm). The filter was applied to a preconditioned 1.5-ml Sep-Pak C₁₈ cartridge (J. T. Baker, Phillipsburg, NJ). The column was washed with water, and the metabolites were eluted with methanol. A small aliquot of the methanol solution (20 µl) was injected onto the chromatograph. Other portions of plasma, bile, or urine samples (0.5 ml) were added in a glass tube, to which was added 100 µl of 0.1 M sodium carbonate followed by 3 ml of fresh distilled diethyl ether. Samples were then vortexed vigorously for 3 min and afterward centrifuged for 10 min at 2000 g. The organic layer was transferred into a conical test tube and evaporated to dryness under a stream of nitrogen at 25°C. The residue was dissolved in 100 µl of the HPLC mobile phase, and an aliquot of 20 µl was injected onto the chromatographic system.

Analysis of Stability of RXM and its Major Six Metabolites by LC/MS. To assess whether any artifact product of RXM had been produced during the intervals of sample collection and extraction procedure before LC/MS analysis, RXM, M1, M2, M3, M4, M8, and M10 were added to freshly collected bile, urine, and plasma, respectively, in concentrations of 10 and 1 µg/ml, followed by standing at room temperature (below 25°C) for 4 or 10 h, or by three freeze-thaw cycles. Each mixture was then extracted and was subjected to LC/MS analysis as described above.

Results

Metabolite Profiles in Bile, Urine, and Plasma Samples. The collected bile, urine, and plasma samples were extracted and analyzed as described in Experimental Procedures. In addition to unchanged RXM, a total of 15 metabolites were found in bile, 10 metabolites in urine, and four metabolites in plasma (Fig. 1). No additional peaks corresponding to the more polar metabolites such as the glucuronide-conjugated metabolites were observed in the solid-phase extraction elute using the previously described method (Gu et al., 1999).

To examine the stability of RXM and the metabolites in biological matrices as well as to evaluate the sensitivity of the method, the chromatographic peak height and area of the six reference substances and RXM at the same concentration were compared. It was observed...
that the peak heights were similar, whereas the peak areas were quite different. Concentrations of each metabolite were therefore estimated with reference to RXM on the basis of peak height ratios of their [M + H]⁺ selected ion monitoring chromatograms. The lower limit of detection (S/N = 3) of the LC/MS method for each reference substance was about 10 ng/ml. The reproducibility of the method was observed with a day-to-day variation of less than 8%.

Identification of RXM and Metabolites. The structures of metabolites were elucidated by a combination analysis of their electrospray ionization-MS and MS/MS spectra, chromatographic behavior, in response to the HPLC-ECD, as well as chromatographic and mass spectral comparison to several synthesized reference substances. The main characteristic fragment ions in the MS/MS spectra of RXM and its metabolites as well as of the reference substances were summarized in Tables 2 and 3.

Parent drug and M8. Single-stage full scan mass spectra of RXM and M8 gave abundant protonated molecular ions [M + H]⁺ at m/z 837. The retention times of RXM and M8 by HPLC were 31.5 and 17.0 min, respectively. RXM was present in all samples, whereas M8 was only present in bile and urine samples. The MS/MS spectra of the [M + H]⁺ species provided a number of characteristic fragment ions at m/z 679, 558, 540, and 522 (Fig. 2). RXM and M8 displayed the same protonated molecular ions as well as MS/MS fragment ions, and both substances could be detected by HPLC-ECD, suggesting that they were stereoisomers with tertiary amino groups in their structure. They were thus identified as RXM and its Z-stereoisomer, respectively, confirmed by comparison of their retention times on HPLC and mass spectra with synthesized reference substances.

Metabolites M1 and M9. M1 was detected in bile, urine, and plasma samples, whereas M9 was found only in bile and urine. The retention times of M1 and M9 by HPLC were 13.4 and 10.5 min, respectively. Both showed protonated molecular ions [M + H]⁺ at m/z 679 as well as identical MS/MS fragment ions, indicating that they were stereoisomers. Their protonated molecular ions were 158 Da lower (characteristic loss of the alkylether side chain) than that of the parent drug, indicating that they were O-dealkylated metabolites. The MS/MS spectra of both M1 and M9 (parent ion at m/z 749) gave fragment ions at m/z 591 and 434. The former ion was 158 Da lower (loss of cladinose) than that of the precursor ion, whereas the latter ion was subsequently at loss of 157 Da (loss of desosamine) from the former ion. M2 and M10 could also be detected by HPLC-ECD. Based on these data, they were identified as (E)- and (Z)-ERY-oxime, respectively. The identifications were also confirmed by a comparison of the retention times on HPLC and mass spectra with synthesized reference substances.

Metabolites M2 and M10. M2 was present in bile, urine, and plasma samples, whereas M10 was found only in bile and urine. The retention times of M2 and M10 by HPLC were 14.5 and 11.1 min, respectively. Both showed protonated molecular ions [M + H]⁺ at m/z 749 as well as identical MS/MS fragment ions, indicating that they were stereoisomers. Their protonated molecular ions were 88 Da lower (characteristic loss of the alkylether side chain) than that of the parent drug, indicating that they were O-dealkylated metabolites. The MS/MS spectra of both M2 and M10 (parent ion at m/z 749) gave fragment ions at m/z 591 and 434. The former ion was 158 Da lower (loss of cladinose) than that of the precursor ion, whereas the latter ion was subsequently at loss of 157 Da (loss of desosamine) from the former ion. M2 and M10 could also be detected by HPLC-ECD. Based on these data, they were identified as (E)- and (Z)-ERY-oxime, respectively. The identifications were also confirmed by a comparison of the retention times on HPLC and mass spectra with synthesized reference substances.

Metabolites M3, M11, M4, and M12. The retention times of M3, M11, M4, and M12 by HPLC were 19.8, 13.7, 14.1, and 10.2 min, respectively. M3 and M4 were present in bile, urine, and plasma samples, whereas M11 and M12 were found only in bile. They all exhibited the protonated molecular ion [M+H]⁺ at m/z 823, yet each had different MS/MS fragment ions, indicating that they were stereoisomers. The protonated molecular ion was 14 Da lower than that of the parent drug, indicating the loss of a methyl group from RXM. The MS/MS spectra of both M3 and M11 (parent ion at m/z 823) gave ions at m/z 665, 544, 526, and 508, whereas for M4 and M12 the MS/MS spectra gave ions at m/z 665, 558, 540, and 522. The presence of the fragment ion at m/z 665, with loss of 158 Da from the parent ion, suggested also that the cladinose in the molecule was unaltered. The diagnostic ions at m/z 544, 526, and 508 for both M3 and M11 were all 14 Da lower than those fragment ions in the MS/MS spectra of RXM and M8, respectively. The presence of fragment ions at m/z 544, 121 Da lower than the precursor ion, indicated that the oxime alkylether side chain was unaltered. The basis of these data and a comparison of the retention times on HPLC and mass spectra with reference standard, M3 was identified as the N-demethylated metabolite of (E)-RXM, and M11 was tentatively assigned as (Z)-stereoisomer of M3. In the MS/MS spectra of both M4 and M12, the presence

### Table 2

Summary of key LC/MS data of the biliary metabolites after single oral dose of 150 mg RXM to a cholecystectomy patient with T-tube drainage

<table>
<thead>
<tr>
<th>Compound</th>
<th>Proposed Configuration</th>
<th>MS [M + H]⁺</th>
<th>MS/MS (Fragment Ions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RXM</td>
<td>(E)</td>
<td>837</td>
<td>679 (100), 558 (9), 540 (4), 522 (6)</td>
</tr>
<tr>
<td>M8</td>
<td>(Z)</td>
<td>837</td>
<td>679 (100), 558 (9), 540 (4), 522 (5)</td>
</tr>
<tr>
<td>M3</td>
<td>(E)</td>
<td>823</td>
<td>665 (100), 544 (10), 526 (2), 508 (8)</td>
</tr>
<tr>
<td>M11</td>
<td>(Z)</td>
<td>823</td>
<td>665 (100), 544 (7), 526 (2), 508 (4)</td>
</tr>
<tr>
<td>M4</td>
<td>(E)</td>
<td>823</td>
<td>665 (100), 558 (13), 540 (5), 522 (4)</td>
</tr>
<tr>
<td>M12</td>
<td>(Z)</td>
<td>823</td>
<td>665 (100), 558 (9), 540 (4), 522 (5)</td>
</tr>
<tr>
<td>M6</td>
<td>(E)</td>
<td>809</td>
<td>651 (100), 544 (10), 526 (3), 508 (12)</td>
</tr>
<tr>
<td>M14</td>
<td>(Z)</td>
<td>809</td>
<td>651 (100), 544 (6), 526 (2), 508 (9)</td>
</tr>
<tr>
<td>M2</td>
<td>(E)</td>
<td>749</td>
<td>591 (100), 558 (34), 540 (9), 522 (7), 434 (5)</td>
</tr>
<tr>
<td>M10</td>
<td>(Z)</td>
<td>749</td>
<td>591 (100), 558 (8), 540 (2), 522 (2), 434 (5)</td>
</tr>
<tr>
<td>M5</td>
<td>(E)</td>
<td>735</td>
<td>577 (100), 544 (14), 526 (6), 508 (10), 434 (12)</td>
</tr>
<tr>
<td>M13</td>
<td>(Z)</td>
<td>735</td>
<td>577 (100), 544 (5), 526 (2), 508 (4), 434 (13)</td>
</tr>
<tr>
<td>M7</td>
<td>(E)</td>
<td>712</td>
<td>563 (100), 530 (16), 512 (8), 494 (25), 434 (7)</td>
</tr>
<tr>
<td>M15</td>
<td>(Z)</td>
<td>721</td>
<td>563 (100), 530 (4), 512 (4), 494 (8), 434 (8)</td>
</tr>
<tr>
<td>M1</td>
<td>(E)</td>
<td>679</td>
<td>558 (73), 540 (38), 522 (100)</td>
</tr>
<tr>
<td>M9</td>
<td>(Z)</td>
<td>679</td>
<td>558 (63), 540 (32), 522 (100)</td>
</tr>
</tbody>
</table>

* Chromatographic and spectroscopic conditions, see Experimental Procedures.
of fragment ions at $m/z$ 558, 107 Da lower than the precursor ion, indicated the loss of a methyl group occurring on the oxime alkylether side chain. Based on these data and a comparison of the retention times on HPLC and mass spectra with synthesized standard, M4 was identified as the O-demethylated metabolite of (E)-RXM, and M12 was tentatively assigned as (Z)-stereoisomer of M4.

Metabolites M5 and M13. The retention times of M5 and M13 by HPLC were 10.7 and 9.13 min, respectively. Both were found in bile and urine samples. They showed the same protonated molecular ions $[M + H]^+$ at $m/z$ 735 as well as identical MS/MS fragment ions, indicating that they were stereoisomers. Their protonated molecular ions were 102 Da lower than that of the parent drug, indicating a cleavage of the alkylether side chain (88 Da), followed by the loss of a methyl group. The MS/MS spectra of $m/z$ 735 gave ions at $m/z$ 577, 544, 526, 508, and 434. The diagnostic ions at $m/z$ 434 indicated that the 14-member lactone ring nucleus was unaltered. The loss of the cladinose moiety produced the base peak at $m/z$ 577, suggesting the demethylation was carried out on the desosamine.

**TABLE 3**

Summary of key LC/MS$^*$ data of roxithromycin and the reference compounds spiked in blank human bile$^a$

<table>
<thead>
<tr>
<th>Compound</th>
<th>$t_R$ (min)</th>
<th>$[M + H]^+$ $m/z$ (MS)</th>
<th>MS/MS (Fragment Ions)</th>
<th>$m/z$ (relative abundance, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E)-RXM</td>
<td>31.5</td>
<td>837</td>
<td>679 (100), 558 (9),</td>
<td>540 (4), 522 (6)</td>
</tr>
<tr>
<td>(Z)-RXM</td>
<td>17.0</td>
<td>837</td>
<td>679 (100), 558 (9),</td>
<td>540 (4), 522 (5)</td>
</tr>
<tr>
<td>(E)-N-demethyl-RXM</td>
<td>19.8</td>
<td>823</td>
<td>665 (100), 544 (10),</td>
<td>526 (2), 508 (8)</td>
</tr>
<tr>
<td>(E)-O-demethyl-RXM</td>
<td>14.1</td>
<td>749</td>
<td>591 (100), 558 (35),</td>
<td>540 (8), 522 (6), 434 (5)</td>
</tr>
<tr>
<td>(E)-ERY-oxime</td>
<td>14.5</td>
<td>749</td>
<td>591 (100), 558 (8),</td>
<td>540 (2), 522 (2), 434 (4)</td>
</tr>
<tr>
<td>(E)-descladinose-RXM</td>
<td>13.4</td>
<td>749</td>
<td>591 (100), 558 (8),</td>
<td>540 (37), 522 (100)</td>
</tr>
</tbody>
</table>

$^a$ Chromatographic and spectroscopic conditions, see Experimental Procedures.

**FIG. 2.** MS/MS spectra of $[M+H]^+$ ions ($m/z$ 837) of RXM (inset, MS) in human bile.
and with reference to the metabolite profile of clarithromycin (Ferrero et al., 1990), M5 and M13 were tentatively assigned as N-demethylated derivatives of (E)- and (Z)-ERY-oxime, respectively.

**Metabolites M6 and M14.** The retention times of M6 and M14 by HPLC were 10.2 and 9.08 min, respectively. M6 was found in bile and urine, whereas M14 was present only in bile. They displayed the same protonated molecular ions [M + H]^+ at m/z 809 as well as identical MS/MS fragment ions, indicating that they were stereoisomers. The protonated molecular ions were 28 Da lower than that of the parent drug RXM, indicating the loss of two methyl groups. The MS/MS spectra gave a prominent ion at m/z 651, with loss of 158 Da (loss of cladinose) from the precursor ion, indicating the presence of cladinose moiety in the molecules of M6 and M14. The ions at m/z 544, 526, and 508 in the MS/MS spectra of both M6 and M14 (parent ion at m/z 809) were all 14 Da lower than those fragment ions in the MS/MS spectra of M4 and M12, respectively. The presence of the fragment ion at m/z 544, 107 Da lower than the precursor ion, indicated the loss of a methyl group occurring on the oxime alkylether side chain, whereas the presence of the fragment ion at m/z 508, 143 Da lower than the precursor ion, indicated the loss of a methyl group occurring on the desosamine. On the basis of these data, M6 and M14 were tentatively identified as N,O-di-demethylated derivatives of (E)- and (Z)-RXM, respectively.

**Metabolites M7 and M15.** The retention times of M7 and M15 by HPLC were 9.96 and 8.80 min, respectively. They were present only in bile. Both exhibited the same protonated molecular ions [M + H]^+ at m/z 721 as well as identical MS/MS fragment ions, indicating that they were stereoisomers. The protonated molecular ions were 116 Da lower than that of RXM, indicating sequential loss of the alkylether side chain (88 Da) and two methyl groups. The product ion mass spectra of m/z 721 gave fragments at m/z 563, 530, 512, 494, and 434. The diagnostic ion at m/z 434 indicated that the 14-member lactone ring was unaltered. The intense peak at m/z 563, with loss of 158 Da (loss of cladinose moiety), indicated that the loss of two methyl groups had occurred on the desosamine. On the basis of these data, M7 and M15 were tentatively assigned as N-di-demethylated derivatives of (E)- and (Z)-ERY-oxime, respectively.

In bile samples, maximal concentrations of the metabolites were reached at the 5- to 10-h interval after dosing, whereas they were not detectable at the 0- to 1.5-h interval. The difference of Z/E ratios of each pair of stereoisomers between the 1.5- to 5-h and 5- to 10-h intervals was not significant, except for one patient, in whose bile samples the Z/E ratios of each pair of stereoisomers at the 1.5- to 5-h interval were significantly greater than those at the 5- to 10-h interval. In urine samples, the major metabolite excreted was M1 with a concentration less than 30% that of RXM. The concentrations of RXM and M1 reached a maximum within 3 to 12 h after the RXM dose. Of each pair of stereoisomers in the urine, (E)-isomers were predominant throughout the sampling times. The metabolites found in plasma were M1, M2, M3, and M4. They were present in very low concentrations and could not be detected by LC/MS until 2 h after the RXM dose. M4 was the major metabolite found in plasma samples.

**Studies of Stability of RXM and Its Six Major Metabolites.** LC/MS was used to examine the stability of RXM and its six major metabolites (M1, M2, M3, M4, M8, and M10) in human bile, urine, and plasma. After three freeze-thaw cycles as well as standing for 4 or 10 h at room temperature, no degradation or isomerized product of each substance above in the spiked biological fluids was observed regardless of concentration. It was demonstrated that (Z)-ERY-oxime was liable to convert to its (E)-isomer in extraction solvent when heated above 35°C, whereas it was stable under the current experimental conditions (<25°C); in contrast, RXM, M1, M2, M3, M4, and M8 were very stable. This indicates that when the alkylether side chain is lost, the (Z)-forms of RXM metabolites become unstable and are easy to convert to the (E)-isomer, whereas the latter are rather stable.

**Discussion**

A total of 15 metabolites was found present in bile of patients, and 10 metabolites in urine and 4 metabolites in plasma of healthy volunteers. In the case of RXM and its metabolites, the [M + H]^+ decomposition was observed at ca. 30% of the maximum supplementary frequency field voltage (5 V, peak-to-peak). Under these conditions, the most intense peak was detected, corresponding to [MH – cladinose]^+ fragment, whereas [M + H]^+ had virtually disappeared. These decomposition pathways were structurally diagnostic for this class of compounds. Using the combination of HPLC and MS^n spectra, as well as information provided by ECD, we have identified RXM metabolites of various types. The identities of six major metabolites were also confirmed by chromatographic as well as mass spectral comparisons with synthesized standards.

It is very interesting to notice the stereochemical aspects of RXM and its metabolites. The (E)-isomer of RXM was reported to be the more potent of the two geometric forms in antibacterial tests (Gase et al., 1991). Therefore, RXM in clinical use exclusively contains this isomer. For the two pairs of (E)- and (Z)-isomers of RXM and ERY-oxime and (E)-descladinose-RXM, (E)-N- or (E)-O-demethylated-RXM, we observed that the (Z)-isomers always had shorter retention times under the reversed phase chromatographic conditions than their counterpart, i.e., (Z)-isomers are more polar than (E)-isomers. We speculate, therefore, that the other mono- and di-desmethyl derivatives would have the same elution order. The geometric isomerization of RXM and its metabolites with (E)-configuration to their (Z)-isomers would result in greater polarity of these molecules and easier excretion from the body.

The identification of the O-demethylation of RXM (M4 and M12) represent another new biotransformation pathway. They are also demethylated and result in the formation of two isomers of N,O-di-demethyl RXM. In contrast with the previous reports (Koyama et al., 1988; McLean et al., 1988) that described N-demethylation as the major metabolic pathway of RXM in Caucasians and Japanese, we have demonstrated that O-demethyl RXM is one of the main metabolites in Chinese subjects, whereas N-demethylation of RXM was present only in trace amounts. It appears that the identification of the demethyl metabolites in humans with the thin-layer chromatography method may have been incorrect. However, consistent with the reports (Esumi et al., 1988, Jarukamjorn et al., 1998) that described N-demethyl RXM as the major metabolite in rats, we have demonstrated that it is indeed the main metabolite in rats according to experiments we have done. It is obvious that some species difference occurs between humans and rats. This apparent metabolic difference needs additional investigation.

McLean et al. (1988) identified N-didemethylated RXM in urine samples from humans; in this study, measurable quantities of this biotransformation product were not detected in human bile or urine, consistent with the results of Jarukamjorn et al. (1998), who detected no N-didemethylated RXM after incubation of RXM with liver microsomes from rats. In consideration of the fact that the N-demethylated RXM was present in such a small amount, it was not surprising that there was no detectable N-didemethyl RXM metabolite.

In this study, LC/MS^n analysis of RXM and its metabolites in bile provided unequivocal evidence that isomerization of the C=N double bond had occurred. No major time-dependent changes in the metabolite profiles in human bile were observed. Results from a typical bile
sample are shown in Fig. 1. The extent of the isomerization was considerably different for the derivatives. The concentrations of RXM and its metabolites with (E)-configuration were often higher than those of their (Z)-isomers. The isomerization seems to be induced after cleavage of the alkylether side chain (RXM to M2), i.e., the Z/E ratios of the ERY-oxime and its N-demethylated isomers may exceed unity (ratios of M2/M10, M5/M13, and M7/M15), indicating a highly stereoselective biotransformation pathway. But for the descladinose-RXM derivative M1, the isomerization was very limited, suggesting that M1 may be resistant to the isomerization process. To investigate whether this type of metabolic isomerization is unidirectional, (Z)-RXM was administered i.v. to male Wistar rats at a 3-mg/kg dose. Bile, urine, and plasma samples were collected for LC/MS analysis. Preliminary excretion data suggested that this isomerization may be bidirectional in rats. Additional investigation is in process.

This study demonstrates that RXM is metabolized in humans by oxidation, hydrolysis, and/or isomerization. After a single oral dose of RXM, oxidative dealkylation of the alkylether side chain, hydrolytic cleavage of the sugar cladinose, and O-demethylation at the oxime side chain are the major metabolic pathways whereas N-demethylation at the desosamine is a relatively minor route. There is stereoselectivity regarding the isomerization of the oxime, leading to the formation of Z-oxime isomers. In addition, identification of the secondary metabolites M5, M6, M7, and their Z-isomers in bile and urine indicates that the primary metabolites of RXM may act as substrates and undergo additional N-demethylation and isomerization. Based on the structures of these metabolites, a plausible scheme for the biotransformation pathways of RXM in humans is shown in Fig. 3.

Although bile and urine levels of the N-demethylated metabolites of RXM could not be determined by HPLC-ECD due to their low electrochemical activity, there was good response with LC/MS detection for these substances. In the present study with limited samples, an analysis of bile, urine, and plasma showed that considerably higher levels of metabolites were present in the bile than in urine, whereas only a trace amount could be detected in plasma.

In summary, this study has demonstrated that RXM was extensively metabolized by a variety of routes to form a large number of metabolites in humans after oral administration of a therapeutic dose. The proposed structural identities of six metabolites were supported by chromatographic and mass spectral comparison with synthesized standards, and nine additional metabolites were tentatively identified according to their LC and MS data. The LC/MS method proved to be very helpful for differentiation of the structures of isomeric oximes. These results contribute to our understanding to the metabolism of RXM in human subjects.

Acknowledgments. We thank Dr. Michael Neugebauer (University of Bonn, Germany) and Dr. Tiemin Sun, Peng Wang, and Yongyu Zhang (Shenyang Pharmaceutical University) for providing the synthesized reference substances.

References


