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Metabolism of Roxithromycin in the Isolated Perfused Rat Liver

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Abstract

Roxithromycin is a macrolide antibiotic with high clinical potency. *N*-Demethylation is considered to be one of the main pathways of roxithromycin metabolism in rats. We have studied the hepatic metabolism of roxithromycin in the isolated perfused rat liver.

After addition of roxithromycin (30 μM) to the perfusion medium the parent compound and one major metabolite were detected in bile by high-performance liquid chromatography. The metabolite was identified as monodesmethylated roxithromycin by mass spectrometric analysis. Onset of biliary excretion of native roxithromycin was fast, reaching a maximum ($130{\cdot}52 \pm 43{\cdot}88$ pmol g^{-1} min^{-1}) after only 10 min, whereas excretion of the metabolite was delayed (maximum $75{\cdot}83 \pm 11{\cdot}92$ pmol g^{-1} min^{-1} at 30 min). The cumulative excretion of roxithromycin and its metabolite into bile during the 60 min of application amounted to only $1{\cdot}09 \pm 0{\cdot}30$ and $0{\cdot}64 \pm 0{\cdot}22$% of the roxithromycin cleared from the perfusate during the same time. The liver content was 0·48 μmol $(g\,liver)^{-1}$, indicating high retention within the organ. No release of the metabolite into the perfusate was detected.

In conclusion, this study has demonstrated the importance of phase-I metabolism for the biliary excretion of roxithromycin in rat liver. These findings might be predictive of roxithromycin biotransformation and biliary excretion in man.

Roxithromycin, an ether-oxime derivative of the naturally occurring macrolide antibacterial drug erythromycin, has proven clinically efficient in upper and lower respiratory infection, skin and soft-tissue infections, urogenital infections and orodental infections (Markham & Faulds 1994). Studies have also been conducted to assess the potential of roxithromycin in the treatment of cutaneous manifestations of *Lyme borreliosis* (Hansen et al 1992) and of opportunistic infections in patients with HIV (Durant et al 1994), and as part of an eradication regimen for *Helicobacter pylori* in patients with peptic ulceration or gastritis (Labenz & Börsch 1994).

Although both compounds have very similar antibiotic properties in-vitro, the in-vivo antimicrobial potency of roxithromycin is greater. This difference might be because of the slower rate of metabolism of roxithromycin, which include mono- and didemethylation or cleavage of the cladinose (Birkett et al 1990). These biotransformation products have been identified in the urine and faeces of man whereas only the parent drug was detected in plasma (McLean et al 1987). In contrast with erythromycin, roxithromycin does not induce cytochrome P450 (CYP) 3A1/2 and CYP3A4, enzymes catalysing the *N*-demethylation of all macrolide antibiotics in the liver microsomes of rat and man, respectively.

Recent studies of liver microsomes of rat and man have monitored the *N*-desmethylation of roxithromycin by measuring the formation of formaldehyde; however, the amounts of mono- and didemethylation products in the liver are not known (Yamazaki et al 1996). In addition, there is no information on the extent to which hepatically-generated *N*-desmethylated metabolites undergo biliary excretion. In this study we therefore used a recirculating isolated perfused rat-liver model to assess the hepatic disposition of roxithromycin and its metabolites in bile and perfusate. It was intended

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that possible metabolites would be isolated and their structure determined by mass spectrometry.

Materials and Methods

Chemicals and reagents

Roxithromycin (erythromycin 9-{*O*-[(2-methoxyethoxy)methyl]oxime}) was a gift from Hoechst Marion Roussel (Vienna, Austria). Ammonium acetate was obtained from Sigma (Munich, Germany). Methanol and water were of high-performance liquid chromatography- (HPLC) grade; other chemicals and solvents were of analytical grade and purchased from Merck (Darmstadt, Germany).

Liver perfusion

Livers, 9·8–11·3 g, of male Wistar rats, 210–265 g, raised at the Institut für Versuchstierzucht und -haltung, University of Vienna, Himberg, Austria, were perfused in a recirculating system as described previously (Thalhammer et al 1994). The total volume of the perfusion reservoir was 1000 mL. Perfusion was conducted using Krebs Henseleit bicarbonate–CO_2 buffer (118 mM NaCl, 4·5 mM KCl, 2·75 mM $CaCl_2$, 1·19 mM KH_2PO_4, 1·18 mM $MgSO_4$ and 25 mM $NaHCO_3$, equilibrated with 95% O_2/5% CO_2) at a constant flow rate of 3·1 mL (g liver)$^{-1}$ min^{-1} in a recirculating mode in which hepatic venous outflow was returned to the perfusate reservoir. The temperature of the perfusion cabinet and perfusion medium was thermostatically controlled at 37°C. Single drops of bile falling from the bile duct cannula were weighed (approx. 8 mg) and collected. Bile flow was determined from the time-interval between drops and from liver weight and is given in mg (g liver)$^{-1}$ min^{-1}. After 30 min perfusion with control medium, perfusion was continued with 30 μM roxithromycin medium for 60 min. Control experiments were performed by application of the same solution to the perfusion model without a liver present. Neither significant binding of roxithromycin to the perfusion system nor degradation of roxithromycin in the perfusion medium was detected.

Roxithromycin in bile and perfusate

Bile samples (30–50 μL) collected over a period of 5 min were immediately frozen on dry ice, and stored at −20°C. Before analysis samples were centrifuged (5000 *g* for 5 min). From the clear supernatant 5 μL were diluted with 95 μL distilled water. Perfusate (approx. 1·5 mL) collected every 5 min was stored at −20°C. Before analysis a sample (997 μL) was mixed with phosphoric acid (1 M; 3 μL) and centrifuged as described above. Diluted bile samples (50 μL) and perfusate supernatant (100 μL) was injected on to the HPLC column by use of conical glass inserts in amber glass autosampler vials capped with open screw-caps with PTFE seals (Chromacol, London, UK).

Chromatographic conditions

HPLC was performed with a Kontron 325 pump, a Kontron HPLC UV 430 detector set at 214 nm (Kontron Instruments, Neufarn, Germany), a model BFO-04 column thermostat set at 35°C, (Industrial Electronics, Langenzersdorf, Austria) and a LaChrom L-7200 autosampler (Merck, Hitachi, Kyoto, Japan), using the software Nina (Nuclear Interface, Münster, Germany), for integration of peak areas. Roxithromycin was separated from interfering plasma compounds on a Hypersil BDS-C18 column (5 μm; 250 mm × 3·6 mm i.d.; Astmoor, UK); the mobile phase was 5·5 : 4·5 (v/v) methanol–10 mM ammonium acetate buffer (adjusted to pH 5·0 with acetic acid and filtered through a 0·45-μm filter (HVLPO4700, Millipore, Vienna, Austria)). Isocratic elution was performed at 1·0 mL min^{-1}.

Calibration was accomplished by use of an external standard. Because a standard of the metabolite was not available, quantification of roxithromycin was based on the assumption that the unknown metabolite had a molar extinction coefficient similar to that of roxithromycin. Linear calibration curves were constructed from the ratios of the peak areas of roxithromycin and its metabolite to that of the external standard roxithromycin by adding standard solutions of roxithromycin to drug-free rat bile and perfusate to give a concentration range of 0·2 to 30 μg mL^{-1}. Liver weights and bile flows or perfusion rate were included in all calculations.

Isolation and purification of the bile metabolite

Four bile samples were re-combined (400 μL) and diluted with water (1 : 2; v/v). Samples of this solution (100 μL) were injected on to a Spherisorb ODS column (10 μm, 200 mm × 8 mm i.d.; SRD, Vienna, Austria) and the metabolite separated by HPLC using the equipment described above (flow rate 2·5 mL min^{-1}). The peaks corresponding to the metabolites from each chromatographic run were collected, pooled and lyophilized. The dry residue was dissolved in 50 : 50 (v/v) methanol–water (1 mL) and the solution was analysed by mass spectrometry.

Mass spectrometry

MS-MS measurements of roxithromycin and the isolated metabolite (approx. 4 μg absolute) were obtained with a Finnigan MAT (Bremen Germany) 95S double-focusing instrument of reverse geometry (BE) fitted with a second-generation atmospheric pressure ionization (API) source for electrospray ionization (ESI). In continuous-infusion mode a flow rate of 50 μL min^{-1} was used. The ESI conditions were: sheath gas pressure, 8 bar N_2; accelerating voltage, +4·75 kV; capillary temperature, 250°C; capillary exit voltage, 27 V; tube-lens voltage, 39 V; skimmer voltage, −1·1 V; rf-octapole voltage, −6·0 V for maximum intensity of $[M+H]^+$ adduct ions; resolution, approximately 1100 (10% valley definition); scan, linear profile mode (1 s/100 u); averages of 10–20 scans, positive mode. Product-ion scans were performed in the first field-free region (FFR 1; daughter ions at B/E = constant) using helium as a collision gas at a collision gas pressure of 8×10^{-5} mbar corresponding to a precursor ion reduction of 80%.

Results

Analysis of roxithromycin and its metabolite in bile and perfusate

Addition of roxithromycin induced brief stimulation of bile flow during the first 6 min from 0·94 ± 0·10 mg (g liver)$^{-1}$ min^{-1} to a peak value of 1·14 ± 0·20 mg (g liver)$^{-1}$ min^{-1}. Then bile flow returned to control values within 15 min. Metabolite formation was assessed every 5 min (for a time period of 60 min). One biotransformation product (retention time, t_R, 35·72 min) and native roxithromycin (t_R 27·93 min) were separated by HPLC (Figure 1).

Figure 2 shows that the biliary excretion of this metabolite reached a maximum, 75·83 ± 11·93 pmol g^{-1} min^{-1} after 30 min whereas that of roxithromycin reached a plateau (130·52 ± 43·88 pmol g^{-1} min^{-1}) after only 10 min. After reaching its maximum the excretion of roxithromycin and its biotransformation product further decreased to 65·47 ± 9·33 and 47·59 ± 4·90 pmol g^{-1} min^{-1}, respectively. The total amount of roxithromycin and its metabolite in bile during the 60 min of perfusion were equivalent to means of 1·09 ± 0·30% and 0·64 ± 0·22% of the dose cleared from the perfusate. During recirculating perfusion of the livers with roxithromycin, the perfusate concentration of roxithromycin declined; 18·83% (5·65 μmol) of the substrate was cleared from the perfusate within 60 min. In contrast with the bile, no roxithromycin metabolite could be detected in the perfusate.

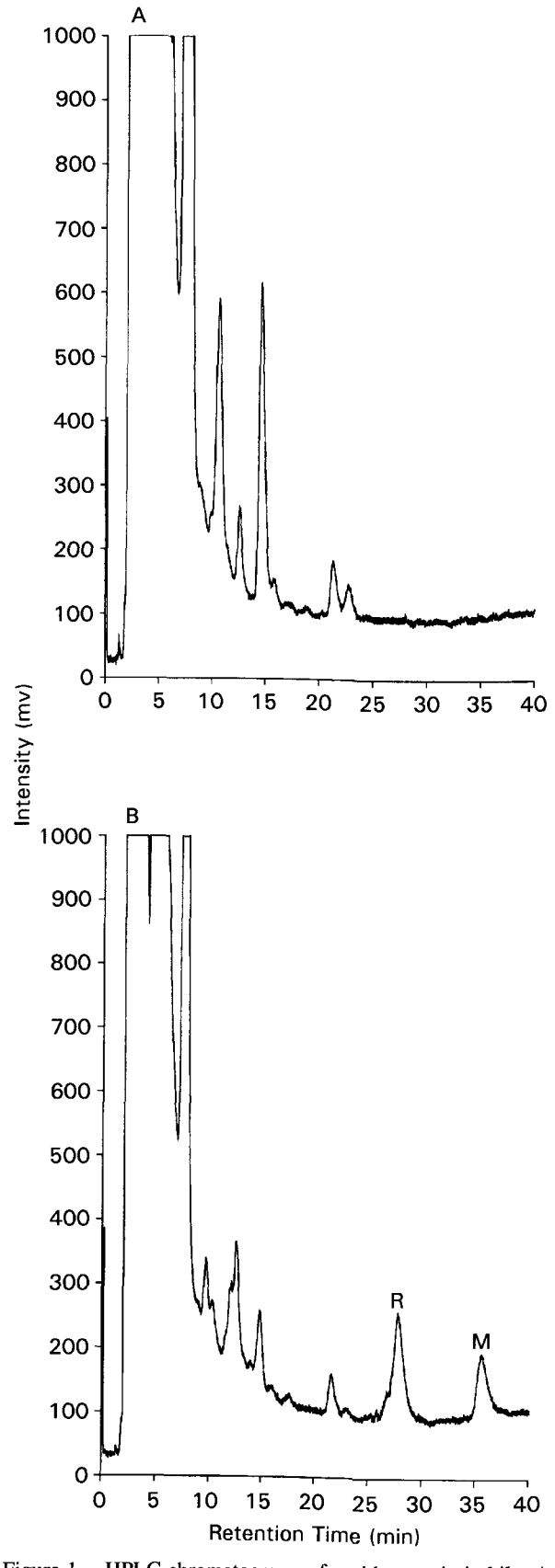

Figure 1. HPLC chromatograms of roxithromycin in bile: A, blank bile sample; B, bile sample collected 30 min after addition of roxithromycin to the perfusate. M, metabolite; R, roxithromycin.

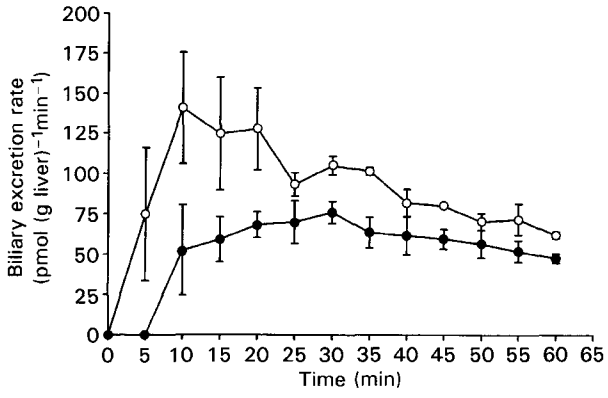

Figure 2. Time-course of the biliary excretion of roxithromycin (○) and roxithromycin metabolite (●) in the isolated perfused rat liver.

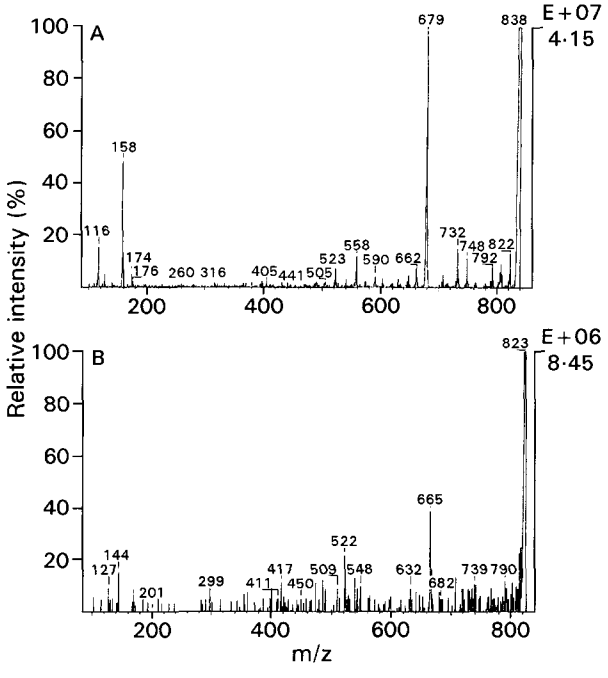

Figure 3. Mass spectra of roxithromycin and of its metabolite purified by reversed-phase HPLC. Roxithromycin and the biotransformation product were dissolved in methanol–water (1 : 1) and analysed in positive-ion mode with a double-focusing mass spectrometer. A, MS-MS mass spectrum of roxithromycin; B, MS-MS mass spectrum of the protonated metabolite.

Identification of the roxithromycin metabolite

After purification of the metabolite approximately 4 μg of pure product (as judged from analytical HPLC results) was recovered. When subjected to positive-ion mass spectrometry (roxithromycin was used as control), MS-MS data (Figure 3B) showed the protonated molecular ion $[M+H]^+$ of the metabolite at m/z = 823 amu. This 14 mass unit difference from roxithromycin (m/z = 823 amu) and further abundant ions at m/z 665 (M – monodesmethyldesosamine), m/z 509 (M – cladinose and M – monodesmethyldesosamine) and m/z 144 (monodesmethyldesosamine) indicate that a CH_2 group has been lost from the parent compound, in agreement with monodesmethylroxithromycin (Figure 3A).

Discussion

The aim of this study was to evaluate hepatic disposition and metabolism of the antibacterial drug roxithromycin. We have shown that this highly lipophilic compound is moderately accumulated from the perfusate and is excreted into bile in its native form together with one metabolite. The metabolite could not be detected in the perfusate. The formation of monodemethylroxithromycin was indicated by mass spectrometry (Figures 3A, B and 4), in accordance with previous reports that describe *N*-demethylation as the major metabolic pathway of roxithromycin and for other macrolide antibiotics, for example erythromycin and troleandomycin (Larrey et al 1983; Delaforge et al 1988; Yamazaki et al 1996).

Although drug metabolites are usually more polar than the parent compound, the retention time of monodesmethylroxithromycin was greater than that of roxithromycin. At pH 5·0 silanols on the column wall act as weak acids and might interact more strongly with the more basic secondary amine residue of the metabolite (Rosing et al 1997).

N-Dealkylation of secondary and tertiary amines is a common reaction catalysed by cytochrome P450 isoenzymes in liver microsomes (Coutts et al

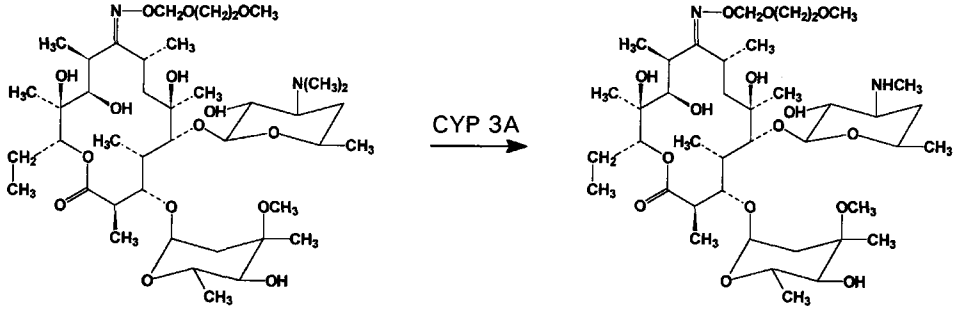

Figure 4. Proposed main metabolic pathway of roxithromycin in the isolated perfused rat liver.

1994). Several lines of evidence indicate that cytochrome P450 3A1/3A2 in rat and CYP3A4 in man are the principal liver microsomal enzymes for this biotransformation reaction: incubation of roxithromycin with either microsomes prepared from cells transfected with CYP3A4 cDNA from man or with microsomes from human livers containing high levels of CYP3A4 resulted in increased *N*-demethylation compared with metabolism in control samples with a moderate CYP3A4 content (Periti et al 1992; Yamazaki et al 1996).

McLean et al (1987) identified didesmethylated and descladinosed roxithromycin in urine samples from man; we could not detect measurable quantities of these two biotransformation products in rat livers during 60-min perfusion, findings in accordance with those of Rodrigues et al (1997) who detected no didemethylation and descladinose biotransformation products of the structurally related macrolide antibiotic clarithromycin after incubation of the compound with liver microsomes from man. Therefore, in the liver biotransformation seems to be restricted to monodemethylation. Although cladinose ring cleavage occurs non-enzymatically, it seems not to occur in the liver.

In conclusion, this study indicates the importance of phase-I metabolism in the biliary excretion of roxithromycin in rat liver and might be predictive of roxithromycin biotransformation and biliary excretion in man. This finding might also be of clinical relevance because CYP3A catalyses *N*-desmethylation. Therefore, formation of monodesmethylroxithromycin might be increased by various therapeutic CYP3A inducers, for example phenytoin and rifampicin (Guengerich 1995), although roxithromycin itself does not induce CYP3A.

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