Unique Biotransformation from Pyrimidine to Pyrazole Derivatives in Rats – Its Isolation/Identification by LC/MS and NMR

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Abstract
The test article (TA) under investigation contains a pyrimidine ring to which a carbon-containing R group is attached at the 5-position. [14C]-labeling for the TA was made on either C2 or C3 of the pyrimidine ring (see below for numbering). Both intact and bile duct cannulated rats were administered a single oral dose of 15 mg/kg (100 μCi/kg), and plasma, urine, feces, and bile were collected for metabolite profiling and identification. Metabolite profiles were determined by radio-HPLC and metabolite identification was performed by LC/MS and NMR. Major metabolites were also subjected to H/D exchange to assist in identification. In this study, oxidative metabolites were identified with a pyrimidine N-oxide as the most abundant (21% of dose) in bile. Two minor fecal metabolites (1% and 3% of dose), M1 and M2 (N-oxide of M1), and one bile metabolite (M3 – a glucuronide of M2) had mass spectra indicating that they may have been derived from the loss of a carbon in the pyrimidine moiety. M1 and M2 were isolated from ca. 100 g of rat feces by extraction, followed by SPE clean-up and preparative HPLC fractionation. NMR analysis of purified isolates confirmed that M1 had a pyrazole moiety resulting from the loss of a carbon from the pyrimidine moiety. Although it is not a commonly known pathway, the biotransformation of pyrimidine to pyrazole has been previously observed in Virciviroc, which contains a dimethyl pyrimidine moiety (Ghosal et al., 2007). M2, the aglycone of M3, may have been reduced to form M1 in GI by intestinal flora. M2 was also observed in rat hepatocyte incubations. A mechanism for the formation of pyrazole from pyrimidine is proposed. N-oxidation of pyrimidine might activate the adjacent C2 to nucleophilic attack by CYP Fe-bound peroxide, which leads to oxidative ring opening, followed by loss of one carbon and ring closure to form the observed pyrazole.

Introduction
The objective of the study was to investigate the in vivo biotransformation profiles of [14C]TA in rats.

Materials and Methods

Test System: (1) Bile and feces from male Sprague-Dawley rats
Dose: 15 mg/kg (100 μCi/kg)
Samples Used: 0-24 hr and Feces; 0-48 hr feces
Sample Processing: The bile sample was centrifuged at 10,000 g, 4 °C for 10 min. The feces sample was extracted with acetonitrile (3x, v/v), vortexed, shaken, and centrifuged. The supernatant was analyzed by HPLC/RAM or LC/MS and also used for preparative HPLC to isolate M1 and M2.
Isolation/Purification: The fecal extract (from 95 g sample) was subjected to a series of isolation/cleanup procedures using SPE as well as preparative HPLC. The flowchart of isolation and purification is depicted in Figure 1. The final purified M1 (~10 μg) and M2 (~5 μg) were subjected to NMR analysis.
Radiochromatograms: Fractions were collected into DeepWell Lumaplate™-96 plates and the collected fractions were dried and counted using a Packard TopCount™ NXT™ solid scintillation counter. Reconstructed HPLC chromatograms were prepared using the determined radioactivity in each fraction using HPLC Method 1.
HPLC System: Waters Model 2695
Column 1: ACE 3 AR-C18, 3 μm, 4.6 x 150 mm
Mobile Phase: A: 0.4% formic acid in water adjusted pH 3.2 with NH4OH; B: CH3OH/C2H5OH/C2H5CN; for HPLC Method 1 & 2
Column 2: Phenomenex Synergi Hydro PR, 4 μm, 250 x 4.6 mm
Mobile Phase: D: 10 mM Ammonium acetate in water; C: CH3CN; for HPLC Method 3

Results and Discussion

Figure 3. 1H-NMR Spectra of TA (A) and M1 (B) Isolate in Acetonitrile-d3 (500 MHz NMR)

Figure 4. 1H-NMR Spectra of M1 (B) and M2 (A) in Acetonitrile-d3 (600 MHz NMR)

The structure was further confirmed by 2D NMR (1H-1H DQF-COSY spectrum, 1H-13C multiplicity edited HSQC spectrum, 1H-12C HMBC using 600 MHz NMR; data not shown). The biotransformation of pyrimidine to pyrazole has been previously observed in Virciviroc.

Figure 5. Proposed Formation Mechanism of M1 and M2

Conclusion

Novel pyrazole metabolites, derived from a pyrimidine-containing drug, were identified in rat in vivo, structures confirmed and the formation mechanism was proposed (Figure 5). Although a definitive answer cannot be made, by making the N-oxide of the pyrimidine first, the ring becomes electrophilic and therefore susceptible to attack by the iron peroxide. But the unactivated heterocycle may not be so susceptible to the attack by nucleophiles. In addition, M2 (N-oxide) was the only detectable in bile as a glucuronide. The relationship between the three metabolites, M1, M2, and M3, is proposed in Figure 6.

References