High-Resolution Mass Spectrometry for Rapid Metabolite Characterization and Reaction Phenotyping: A Case Study with Repaglinide

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Introduction

Assessing the involvement of specific cytochrome P450 (P450) enzymes in biotransformation of a drug is an important step in evaluating overall disposition and victim potential for clinically-relevant drug-drug interactions.[1] Current methods for biotransformation pathway identification (reaction phenotyping) rely on prior knowledge of metabolites, biotransformation routes, and availability of metabolite reference standards or radioisotopically-labeled drug. Synthesis of metabolite standards and radiolabeled compounds is time-consuming and expensive and the material is typically not available in early preclinical testing. Reaction phenotyping can be achieved by monitoring loss of the parent drug, but that approach suffers from a lack of specificity. Consequently, it is preferable to monitor the formation of one or more metabolites to obtain a comprehensive biotransformation map for the drug of interest. In general, this requires radiolabeled drug or metabolite reference standard material, potentially adding several months to the drug development timeline before biotransformation pathway assignments can be made.

High-resolution mass spectrometry (HRMS) is a powerful tool for a priori metabolite profiling and characterization because complex data sets comprising information on all of the components in a sample, within a specified mass range, are acquired.[2] HRMS data can be employed for relative quantitation of all detected components and have potential for derivation of earlier stage reaction phenotyping information without the need for metabolite reference standards. In the present study, the prandial glucose regulator repaglinide was incubated with various in vitro reaction phenotyping test systems, and the samples were analyzed by HRMS metabolite profiling approaches to characterize all of the repaglinide metabolites formed. Relative amounts of each individual metabolite detected in different incubation test systems were compared to establish relationships between specific P450 enzymes and repaglinide metabolites. In the absence of metabolite reference standards, the abundance values were not compared across different metabolites since ionization efficiency for each metabolite is unknown. The approach was assessed for its suitability for rapid determination of specific biotransformation pathways for a drug in development without the additional time and cost associated with generating radiolabeled drug or metabolite reference standards.

Materials and Methods

Repaglinide was purchased from Toronto Research Chemicals (Ontario, Canada). Human liver microsomes (HLM; n=1 or n=200) were prepared and characterized in-house. Recombinant cytochrome P450 enzymes co-expressed with reductase were purchased from Cypex (Dundee, Scotland).

Repaglinide (1 or 10 µM) was incubated for up to 30 min (37°C; pH 7.4) with NADPH-fortified HLM (0.1 or 0.5 mg protein/mL; pooled or from individual donors) or recombinant enzymes, namely CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, or 2J2 (10 pmol/mL). For the inhibition experiments, pooled HLM were pre-incubated with mibefradil (1 µM) or gemfibrozil glucuronide (100 µM), metabolism-dependent inhibitors of CYP3A4 and CYP2C8, respectively, for 30 min before incubation with repaglinide. Following protein precipitation with acetonitrile, all samples were analyzed for metabolite profiling with reverse-phase gradient ultra-performance LC and HRMS on a Waters Synapt G2 HDMS quadrupole time of flight mass spectrometer equipped with a Waters Acquity LC (Milford, MA). An unrelated internal standard (1'-hydroxymidazolam) was employed for relative quantitation.

Metabolites were separated on a Waters Acquity BEH column (1.7 µm, 2.1 x 100 mm) at 50°C with 0.1% formic acid in water or acetonitrile (0.4 mL/min) using the following gradient: 10% organic held for 1 min, increased to 35% at 7 min, 42% at 12 min, 73% at 20 min, and 90% at 25 min. The mass spectrometer was operated in positive, resolution elevated energy mass spectrometry (MS^E) or MSMS mode with electrospray ionization. All samples were analyzed with full scan MS^E. The low energy scan data were employed for quantitation. Generic ionization parameters were used throughout. Fexofenadine was employed for real-time mass calibration. Data processing employed MetaboLynx XS and QuanLynx subroutines of Waters MassLynx version 4.1 (Milford, MA). Metabolite structural elucidation was performed manually.

Results

Eleven repaglinide metabolites were characterized by full scan accurate MS^E in the HLM incubations as summarized in **Table 1**. Five were well-established, previously described metabolites,[3,4] namely M0-OH and M4 (hydroxylation metabolites), M1 (the N-dealkylated primary amine metabolite), M5 (*O*-desethyl repaglinide) and M2 (the ring-opened dicarboxylic acid and major human metabolite formed in vivo.[5] Six other metabolites (C1 through C6) were observed. Four (C1, C4, C5 and C6) were formed by dehydrogenation. C2 was formed from M5 by hydroxylation. C3 was consistent with the alcohol intermediate involved in formation of M2.

As shown in **Figure 1** (for M1 and M4), metabolite formation data (by peak area ratio) were derived for the HLM incubation time-course to establish initial rate conditions for subsequent experiments. Formation over time was linear up to 20 min for the five established metabolites at 0.1 mg/mL protein and 10 µM repaglinide. Deviations from linearity were observed in the 0.5 mg/mL protein samples. Some of the metabolites (M1, M2 and M5) were not detected or were present at low abundance in several of the 1 µM incubations. Subsequent experiments were performed with repaglinide (1 and 10 µM) incubated for up to 20 min with 0.1 mg/mL protein or 10 pmol/mL recombinant cytochrome P450.

Recombinant P450 enzymes which formed each of the five established metabolites are summarized in **Table 2**. Abundance data were normalized to the maximum observed amount of the specific metabolite of interest. With the exception of C2, all of the repaglinide metabolites were also detected in the recombinant enzyme incubations. Metabolite M1 was detected at similar levels in incubations with recombinant CYP3A4 and recombinant CYP2C8, while M4 was predominantly observed in the recombinant CYP2C8 incubations (**Figure 2**). CYP2C8 formed M0-OH, M1 and M4. CYP3A4 formed M1, M2, M5 and a small amount of M0-OH. Recombinant enzymes are not always representative of more complete test systems so additional experiments were performed.

Representative correlation plots for metabolite formation with CYP2C8 and CYP3A4/5 activities are shown as **Figure 3**. Following incubation with HLM from the individual typed donor panel, relative abundance data were derived and the peak area ratios associated with detection of each individual metabolite were plotted against the measured activity of specific P450 enzymes in HLM from each donor. M0-OH and M4 formation showed poor correlation with CYP3A4 activity and good correlation with CYP2C8 activity, while M1 formation correlated well with CYP3A4 activity and poorly with CYP2C8 activity. M2 and M5 formation correlated best with CYP3A4/5 activity.

P450 enzymes that showed correlation with formation of each individual established metabolite are summarized in **Table 2**. The recombinant enzyme and correlation data were in good agreement for M0-OH (CYP2C8), M2 (CYP3A4), M4 (CYP2C8) and M5 (CYP3A4), but were conflicting for M1. Therefore, an inhibition experiment employing irreversible inactivation of CYP3A4 by mibefradil and CYP2C8 by gemfibrozil glucuronide, respectively, prior to incubation of repaglinide with HLM was performed.

The inhibition results are summarized in **Figure 4** and **Table 2**. By comparing the extent of formation of the five metabolites in HLM incubated in the presence of the inhibitors to the extent observed in the absence of inhibitor, involvement of CYP2C8 in the formation of M0-OH and M4 and of CYP3A4 in formation of M1 and M5 was established. The inhibition data were inconclusive for M2.

Table 2 summarizes the results derived with the HRMS approach (**Figure 5**) compared with literature results derived by traditional methods. Consistent with the literature, the approach resulted in the identification of CYP2C8 as the primary mediator of M0-OH and M4 formation and CYP3A4 as primarily involved in formation of M1 and M5. Although only CYP2C8 and 3A4 were implicated in formation of M2 in HLM, the data were ambiguous as to the extent of involvement of either enzyme. This is likely to be due to the established fact that cytosolic aldehyde dehydrogenase can play a role in M2 formation.[4]

Tables and Figures

Table 1. Accurate mass metabolite profile for repaglinide (1 or 10 µM) incubated with NADPH-fortified human liver microsomes (0.1 or 0.5 mg/mL) for up to 30 min

Identity	Retention Time (min)	Experimental *m/z*	Mass shift from repaglinide	Proposed elemental composition	Theoretical *m/z*	Mass error (ppm)	Proposed biotransformation
M0-OH [a]	5.48	469.2693	+15.9940	$C_{27}H_{36}N_2O_5$	469.2702	-1.9	Hydroxylation
C1	5.94	451.2590	-2.0156	$C_{27}H_{34}N_2O_4$	451.2597	-0.2	Dehydrogenation
C2	6.83	441.2369	-12.0384	$C_{26}H_{32}N_2O_5$	441.2389	4.7	O-Deethylation + hydroxylation
M4 [a]	7.50	469.2700	+15.9947	$C_{27}H_{36}N_2O_5$	469.2702	-0.4	Hydroxylation
M1 [a]	7.95	385.2112	-68.0641	$C_{22}H_{28}N_2O_4$	385.2127	-3.9	N,N-Didealkylation
M5 [a]	8.26	425.2436	-28.0317	$C_{25}H_{32}N_2O_4$	425.2440	-4.5	O-Deethylation
Repaglinide	8.98	453.2738	-0.0015	$C_{27}H_{36}N_2O_4$	453.2753	-3.3	Not applicable
C3	10.09	471.2848	+18.0095	$C_{27}H_{38}N_2O_5$	471.2859	-0.9	Hydroxylation + reduction
M2 [a]	10.30	485.2642	+31.9889	$C_{27}H_{38}N_2O_6$	485.2652	-5.1	N-Dealkylation + oxidation to the carboxylic acid
C4	16.09	451.2583	-2.0170	$C_{27}H_{34}N_2O_4$	451.2597	-3.1	Dehydrogenation
C5	17.23	451.2591	-2.0162	$C_{27}H_{34}N_2O_4$	451.2597	-1.3	Dehydrogenation
C6	17.52	451.2599	-2.0154	$C_{27}H_{34}N_2O_4$	451.2597	0.4	Dehydrogenation

[a] Corresponds to a metabolite previously established in the literature

Table 2. Comparison of cytochrome P450 enzymes identified with full scan accurate mass spectrometry and relative quantitation as contributing to repaglinide biotransformation with enzyme involvement established with traditional methods

	M0-OH	M1	M2	M4	M5
Recombinant P450 enzyme experiment	CYP2C8, Minor contribution from CYP3A4	CYP2C8, CYP3A4	CYP3A4	CYP2C8	CYP3A4
Correlation experiment with human liver microsome panel	CYP2C8	CYP3A4	CYP3A4	CYP2C8	CYP3A4
Chemical inhibition in human liver microsomes	CYP2C8	CYP3A4	CYP2C8, CYP3A4	CYP2C8	CYP3A4
Overall conclusion	CYP2C8	CYP3A4	CYP2C8, CYP3A4	CYP2C8	CYP3A4
Literature	CYP2C8 [3] Minor contribution from CYP3A4 [3]	CYP3A4 [3,4] Minor contribution from CYP2C8 [3]	CYP2C8 [3,4], CYP3A4 [3,4] Aldehyde dehydrogenase [4]	CYP2C8 [3,4] Minor contribution from CYP3A4 [3]	CYP3A4 [3]

[3] Bidstrup et al (2003)
[4] Säll et al (2012)

Figure 1. Representative plots showing relative abundance changes with time for M1 formed from repaglinide incubated at (a) 1 µM and (b) 10 µM and M4 formed from repaglinide incubated at (c) 1 µM and (d) 10 µM with NADPH-fortified HLM (0.1 and 0.5 mg/mL) for up to 30 min

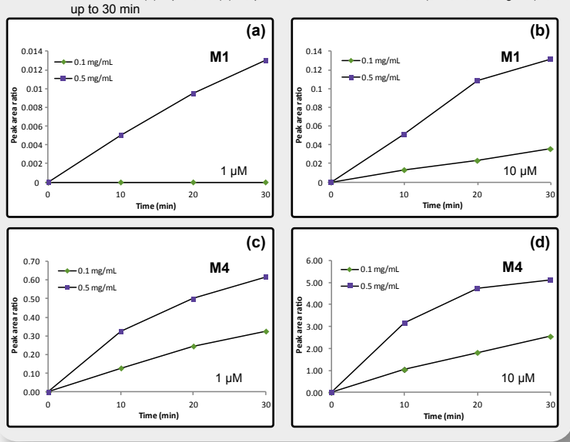


Figure 2. Extracted normalized ion chromatograms for (a) M1 (*m/z* 385.2127 ± 20 mDa) and (b) M4 (*m/z* 469.2702 ± 20 mDa) in samples of repaglinide (10 µM) incubated with NADPH-fortified recombinant cytochrome P450 enzymes (10 pmol/mL) for 20 min

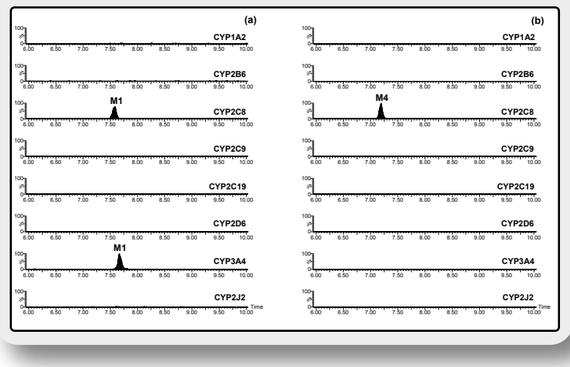


Figure 3. Variation in formation of M0-OH (a,b), M1 (c,d), M2 (e,f), M4 (g,h) and M5 (i,j) with measured CYP2C8 and CYP3A4 activity. Data correspond to repaglinide (10 µM) incubated with NADPH-fortified human liver microsomes (0.1 mg/mL) for 20 min

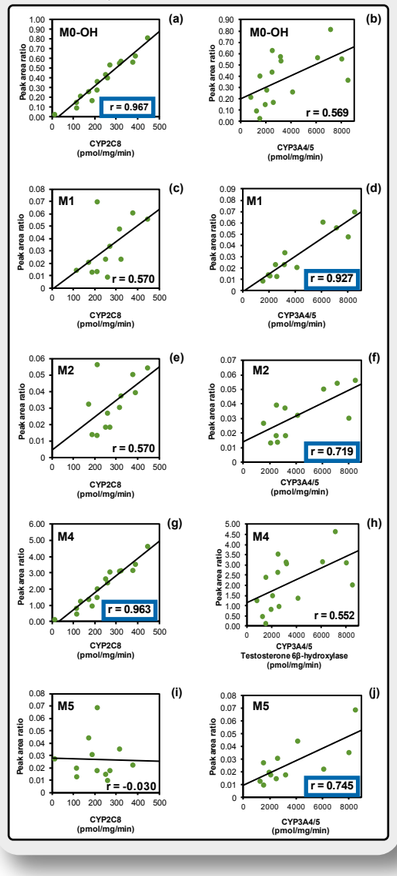


Figure 4. Effect of preincubation (30 min) of NADPH-fortified human liver microsomes (0.1 mg/mL) with the CYP3A4 inhibitor mibefradil (1 µM) and the CYP2C8 inhibitor gemfibrozil glucuronide (100 µM) on formation of five established metabolites formed from repaglinide (10 µM) incubated for 30 min

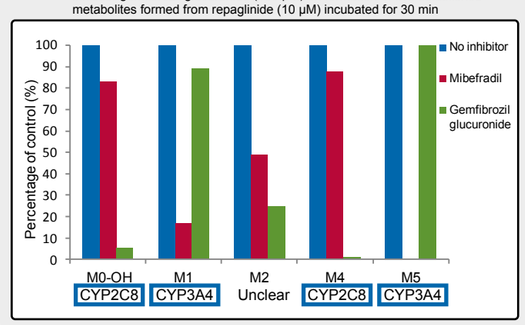
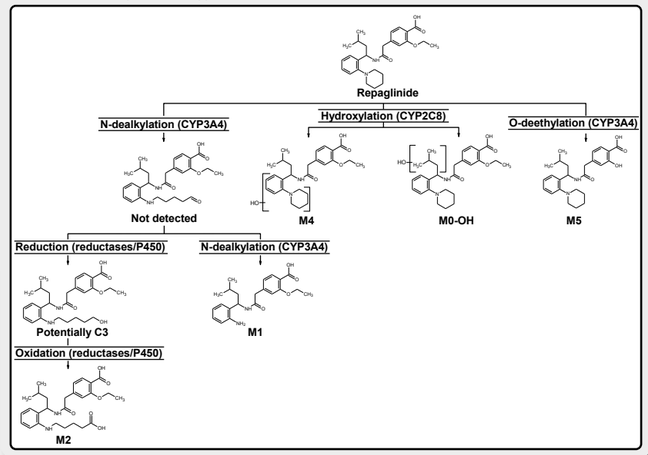


Figure 5. Biotransformation scheme for repaglinide established with the high-resolution mass spectrometry relative quantitation approach



Conclusion

- The HRMS approach was appropriate for rapid, simultaneous metabolite profiling and reaction phenotyping without the need for metabolite standards for the test compound repaglinide.
- The approach resulted in biotransformation pathway assignment for the test compound repaglinide that was in good agreement with published literature.
- In early-stage drug discovery it is not always straightforward to determine which in vitro metabolites may be relevant in an in vivo situation. Therefore the availability of the additional data for subsequent data mining is an attractive proposition. An important benefit to this strategy is that corresponding data were generated for the non-established metabolites C1 through C6 (not shown).

Acknowledgements

The authors would like to acknowledge Phyllis Yerino for assistance with sample preparation.

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