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RESEARCH ARTICLE

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Qualitative and quantitative prediction of human *in vivo* metabolic pathways in a human hepatocyte-murine stromal cell co-culture model

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Abstract

1. This study assessed the value of a static *in vitro* human hepatocyte-murine stromal cell co-culture model to qualitatively and quantitatively predict human *in vivo* metabolic clearance pathways using ^{14}C-labeled test compounds and compared these results to an *in vitro* suspended human hepatocyte model and the *in vivo* human ^{14}C ADME studies.
2. Test compounds represented a diverse set of clearance pathways (Phase I and Phase II). Compounds were incubated for 4 h in suspended human hepatocytes and for 24 and 168 h in the human co-culture model. Multivariate analysis revealed that long-term (168 h) incubation of test compounds in the co-culture had reasonable quantitative prediction of the *in vivo* human clearance pathways as compared to the 4 h suspended hepatocytes or the 24 h co-culture incubation.
3. *In vivo* and *in vitro* disconnects were observed in cases where extra-hepatic metabolism or urinary excretion was observed *in vivo*. Differences in the relative percentages of Phase I and Phase II metabolites observed were likely due to microbial β-glucuronidase hydrolysis of conjugates and microflora mediated metabolism in the gut not present in the *in vitro* systems.

Keywords

^{14}C human, ^{14}C-labeled compounds, hepatocytes, hepatocyte-stromal cell co-culture, metabolite prediction, metabolites

History

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Introduction

Information on drug metabolism in drug discovery and development plays an important role in understanding clearance mechanisms, drug safety and the potential for drug-drug interactions. One of the major tasks of drug metabolism scientists is to synthesize *in vitro* metabolism data with preclinical *in vivo* metabolism data to ultimately predict human *in vivo* metabolism. However, due to potential species differences and limitations of *in vitro* systems, the success rate of human predication can in some cases be low; in the range of 50% (Hutzler et al., 2015; Wang et al., 2010). Quantitative information on circulating and excretory metabolites in humans has ultimately relied on *in vivo* ^{14}C-radiolabeled human ADME (absorption, distribution, metabolism and excretion) studies. These clinical studies are costly, which makes many pharmaceutical companies delay initiation until the compound has shown promise in the clinic. Such delaying practices can sometime impede clinical development due to poor assumptions involving prediction of human clearance pathways. More recently the use of a microtracer dose of radiolabel compound in the clinic early in development has been utilized to gain human *in vivo* ADME data, however, this technology remains expensive (Roffel et al., 2016).

In vitro systems which predict *in vivo* human hepatic metabolism reliably are highly desirable. Currently, *in vitro* systems routinely utilized, such as microsomal preparations and hepatocytes readily provide preliminary metabolism information across species in a simple and cost effective manner, albeit with specific limitations. For example, microsomal preparations, as subcellular fractions, do not have a complete set of drug metabolizing enzymes and lack transporters which interplay with drug metabolizing enzymes for drug clearance. Therefore, microsomal incubations cannot provide a complete picture of hepatic clearance pathways. Suspended hepatocytes possess an entire array of enzymes and transporters (Brown et al., 2007; Li et al., 1999), but the relatively short window of viability available for incubation (Rodriguez-Antona et al., 2002; Smith et al., 2012) presents inadequacies for slowly metabolized compounds or compounds that undergo multiple sequential biotransformation processes. It is desirable in drug discovery for chemists to design low clearance compounds (slowly metabolized) to achieve ideal clinical pharmacokinetic properties, dose and dosing regimens. Low clearance compounds create huge challenges for satisfactory prediction of *in vivo* metabolism by *in vitro* systems (Di & Obach, 2015). Various long-term

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hepatic cell based *in vitro* models have been developed in the last decade (Bonn et al., 2016; Chan et al., 2013; Novik et al., 2010) and have been evaluated for their ability to predict human *in vivo* metabolism (Ballard et al., 2016; Darnell et al., 2012; Hultman et al., 2016; Ohkura et al., 2014). However, most of these evaluations were qualitative and not quantitative; i.e. only reporting a yes or no for the detection of human metabolites based on mass spectrometry (MS). Furthermore, due to different MS ionization efficiencies of parent compound and metabolites relying on the MS ionization response often can result in an under- or over-estimation of metabolite quantity and cause a bias for predicting the overall metabolic pathways, therefore, quantitative information is highly desirable.

The science of engineering more physiologically relevant liver models is rapidly developing and several commercial vendors offer co-cultured animal and human hepatocytes that can be acquired and delivered to the sponsor's laboratory (Cho et al, 2014; Grubb et al, 2015; Hultman et al., 2016; Novik et al., 2010). As part of our criteria for selecting a co-culture model to test, we evaluated cost, ease of use, performance, availability of various preclinical species, and multi-donor human hepatocyte systems. Our lab evaluated two commercially available hepatocyte-stromal co-culture models with commercially available drugs that are metabolically cleared via different pathways; i.e. mediated by a diverse set of Phase I and Phase II enzymes. Our initial results showed good representation of the various pathways; however, the data was not quantitative (data not published). The objective of this work was to assess the usefulness of a static human hepatocyte-stromal cell co-culture to predict human *in vivo* metabolic pathways using ^{14}C-labeled compounds in order to provide a qualitative and quantitative correlation of *in vitro–in vivo* metabolic pathway analysis. Five proprietary radiolabeled compounds were evaluated in human hepatic-stromal cell co-cultures, in suspended human cryopreserved hepatocytes and the *in vitro* metabolites were compared to the human *in vivo* excretory metabolites from the radiolabeled ADME study. To our knowledge, this is the first report using ^{14}C-labeled compounds to investigate qualitatively and quantitatively human clearance pathways in the *in vitro* long-term human hepatocyte-stromal cell co-culture model.

Materials and methods

Compounds

Five ^{14}C-labeled compounds (Compounds **1**–**5**) were obtained from Eli Lilly and Company (Indianapolis, IN).

Incubation of hepatocyte-stromal cell co-culture

The human hepatocyte-stromal cell co-culture (*HμREL* Human Pool™) or stromal cell only (control) plates were purchased from HμREL Corporation (North Brunswick, NJ). Primary cryopreserved human hepatocytes pooled from five male and five female human subjects who died of non-liver diseases were used in the preparation of the co-culture plates. The plates were shipped with warm pads (approx. 37 °C) and ready to use upon arrival at our laboratory. For Compound **4**, a 24-well plate (400 μL/well) was used and Compounds **1**–**3** and **5** a 96-well plate (80 μL/well) used. The 24-well and 96-well plates contained approximately 188 K or 30 K hepatocytes/well, respectively.

After receipt, the plates were washed with the provided maintenance media and incubated in 5% CO_2 incubator at 37 °C. After 4 h of incubation, the maintenance media were replaced with the provided dosing media containing test compounds. The concentrations of test compounds in the incubations ranged from 2 to 10 μM depending on the specific radioactivity of each compound. Incubations continued for 24 h or 168 h. At the end of each incubation period, an equal volume of ice cold acetonitrile was added to each well, the samples were centrifuged, and the supernatants were used for metabolite radioprofiling and LC/MS analysis.

Incubation in suspended human hepatocytes

Cryopreserved human hepatocytes were purchased from Bioreclamation IVT (New York City, NY). Incubations were performed in a CO_2 incubator at 37 °C with 0.5 million cells/mL and shaking at approximately 520 rpm. The concentrations of test compounds ranged from 2 to 10 μM (and were the same as for the HμREL hepatocyte-stromal cell co-culture). After 4 h of incubation, an equal volume of ice cold acetonitrile was added and the samples were centrifuged, and the supernatants were used for metabolite radioprofiling and LC/MS analysis.

Clinical studies

The clinical studies were conducted in accordance with applicable laws, good clinical practice and the Declaration of Helsinki. The results of *in vivo* human metabolism of the five ^{14}C-compounds were obtained from respective ^{14}C ADME studies and reported in clinical study reports. The ClinicalTrials.gov identifier numbers are NCT01746004, NCT01367262, NCT02242981 and NCT01981408 for Compounds **1**, **2**, **4** and **5**, respectively. There is no clinical trial identifier number for Compound **3**. Briefly, the clinical studies were conducted at Covance CRU (Clinical Research Unit) (Madison, WI). All the studies were single-center and open-label studies in healthy subjects. Each subject received an oral dose of test compound (ranging from 6 to 150 mg for different compounds) containing 80–100 μCi/subject. Venous blood samples, urine and feces were collected according to study protocols approved by Eli Lilly and Company (Indianapolis, IN) and the appropriate review boards. Blood samples were collected in heparin vacutainers, and plasma was obtained from whole blood by centrifugation. Subjects were discharged from the CRU when 24-h urine and fecal samples from two consecutive collections each had radioactivity levels less than 1.0% of the total administered radioactivity in urine and feces combined. All samples were stored in –70 °C freezer until analysis.

Measurement of total radioactivity

Total radioactivity in urine, feces, and *in vitro* samples (supernatants from suspension hepatocyte incubations or co-cultures) were determined using liquid scintillation counting (LSC) techniques. Feces were combusted, and the

resulting $^{14}CO_2$ was trapped in a mixture of Carbo Sorb E and Perma Fluor E+ (Perkin Elmer, Waltham, MA) and analyzed. All other samples were directly analyzed by LSC.

Sample preparation for metabolite radioprofiling and identification

Fecal samples from all five compounds were extracted using organic solvents (acetonitrile and/or methanol) or solid phase extraction (SPE) prior to HPLC analysis. Supernatants generated from *in vitro* incubations after quench and urine samples were directly analysed by HPLC.

Metabolite radioprofiling by HPLC and quantitative estimation

Metabolite radioprofiles were generated by HPLC fraction collection followed by off-line radioactivity counting. The total HPLC flow rate was 1 mL/min, which was split post column so that approximately 10–20% of the total flow was introduced to the mass spectrometer interface for metabolite identification, and the remaining flow was collected into 96-well LumaPlates (Perkin Elmer, Waltham, MA) with scintillation coating at 10–15-second intervals. The LumaPlates were dried under centrifugal vacuum and counted for radioactivity on a TopCount NXT (Perkin Elmer, Waltham, MA) counter. Radioactivity (CPM) against time (min) was plotted to generate radiochromatograms. Chromatographic peaks below 10 cpm were considered background and not integrated for peak areas. The peak area for each integrated peak was expressed as % ROI (region of interest), where the total peak area for all integrated peaks constituted 100% ROI.

The % of total radioactivity for each peak was calculated by peak area of a given peak divided by total integrated peak area. The % of the dose for a metabolite in excreta was calculated by its % of total radioactivity multiplied with % of the total dose in the analyzed sample (urine or feces) and multiplied with extraction recovery if sample was extracted.

Metabolite identification by LC–MS analysis

Metabolite identification was achieved by LC–MS and LC–MS/MS either using high resolution accurate mass on a Thermo Finnigan LTQ Oritrap or using nominal mass on a Thermo Finnigan LCQ Advantage (ThermoFinnigan, Waltham, MA). When available, synthetic standards were used for definitive identification of metabolites by comparison of HPLC retention times, MS and MS/MS data.

Multivariate analysis

Multivariate analysis was conducted in JMP® 13.1.0. The percent dose in Total Urine + Feces, % ROI from 4 h hepatocytes, 24 h co-culture and 168 h co-culture for all metabolites and across all compounds were analysed using the Restricted Maximum Likelihood Method (REML).

Results

Compound selection

Five compounds were selected for this study based on the following criteria: compounds that represented both CYP and non-CYP clearance mechanisms were taken into consideration for compound selection when *in vivo* human metabolism packages and compound access were available. When available the *in vitro* clearance rates, major metabolic pathways in human and enzymes involved are listed in Table 1.

*Compound **1***

A single dose of 150 mg (100 μCi) of ^{14}C-Compound **1** was orally administrated to healthy male human subjects. Radioactivity was almost completely recovered in excreta with approximately 65% of the dose recovered in feces and 37% in urine. Compound **1** was extensively metabolized with only 13% of the dose eliminated in excreta as parent drug (Table 2). The major biotransformation pathways observed for Compound **1** are shown in Figure 1(B). A pyrrolidine ring and a 2-methyl substituted pyridine moiety were the main metabolic sites on this compound. The major metabolic pathways included (1) oxidation of the carbon on the pyrrolidine ring alpha to a nitrogen resulting in ring opening to presumed intermediate aldehyde (not detected) followed by reduction to an alcohol (1-M14) or oxidation to a carboxylic acid (1-M6) and subsequent glucuronidation of the alcohol and acid (1-M3 and 1-M1, respectively); (2) allylic oxidation on the 3-position of pyrrolidine ring (1-M8) and (3) oxidation of the 2-methyl group on the pyridine ring (1-M10) with subsequent glucuronidation (1-M4). These three pathways accounted for approximately 34%, 15% and 10% of the eliminated dose, respectively (Table 2 and Figure 1A).

Table 1. Clearance rates and metabolic pathways of the selected compounds.

Compound #	*In vitro* hepatocyte Clint (μL/min/million cells)	Major *in vivo* metabolic pathways in human	Known enzyme(s) involved
Compound **1**	2.7	Aliphatic hydroxylation, pyrrolidine ring oxidative opening with subsequent reduction or oxidation to alcohol or carboxylic acid, glucuronidation secondary to Phase I reactions	CYP3A4
Compound **2**	<1.8	Hydrolysis of amide to aniline with subsequent N-acetylation and N-glucuronidation	Hydrolase
Compound **3**	NA	Aliphatic or aromatic hydroxylation	2D6, 3A4, SULTs
Compound **4**	NA	Oxidative dealkylation with loss of ethylene from morpholine ring; N-dealkylation with loss of morpholine ring; and cleavage of urea substituent	CYP3A4
Compound **5**	11.3	N-demethylation; hydroxylation of methyl substituent of pyridinone ring	CYP3A4, UGT

NA: not available.

Table 2. Metabolites of Compound **1** observed in human *in vivo*, in human hepatocyte co-culture, and in human suspension hepatocytes.

		% of the dose in excreta (mean, $n=6$)			% ROI *in vitro*		
Peak name	Description	Urine	Feces	Total	Hepatocyte suspension (4 h)	Co-culture (24 h)	Co-culture (168 h)
1-M6	+2O	2.7	14.4	17.1	NQ	4.3	15.9
1-M1	M6 + gluc	2.1	NQ	2.1	NQ	NQ	NQ
1-M2	M6 + O	1.4	3.2	4.6	NQ	NQ	3.3
1-M14	+H2O	NQ	7.2	7.2	NQ	0.4	1
1-M3	M14 + gluc	3.0	NQ	3.0	NQ	4.6	22.4
1-M9/1-M10/1-M11	M9(–NH2 + OH) + M10 (+O) + M11 (+O)	2.2	7.7	9.9	NQ	3.7	5.7
1-M4	M10 + gluc	1.8	NQ	1.8	NQ	NQ	7.1
1-M8	+O	3.6	11.3	14.9	NQ	7.0	9.7
1-M5	+O (N-oxide)	2.8	NQ	2.8	NQ	2.2	4.6
1-M7	+O	2.0	2.1	4.1	NQ	2.9	7.0
1-M12	+2O	1.0	1.3	2.3	NQ	NQ	0.5
1-M13	+2O	1.2	1.6	2.8	NQ	NQ	0.5
1-Parent	NA	6.2	6.8	13.0	100%	72.8	10.3

NQ: not quantified (peak height <10 cpm); gluc: glucuronide; ROI: region of interest.
Only metabolites above 2% of the dose in excreta are listed in the table.

Additional primary metabolic pathways involved N-oxidation to 1-M5 and mono-oxidation to 1-M7, however, the sites of oxidation were not determined.

[^{14}C] Compound **1** (10 μM, 31 nCi/mL) was incubated in pooled human liver hepatocytes co-cultured with murine stromal cells (*HμREL* HumanPool™) for 24 and 168 h. Metabolite formation was low after 24 h, however, Compound **1** was extensively metabolized after 168 h of incubation (Figure 1A). Metabolites that resulted from the major metabolic pathways 1, 2 and 3 above contributed to approximately 43%, 10% and 12% of total radioactivity in the 168 h incubation, and remaining parent compound accounted for 10% of the total radioactivity.

The extent of metabolism of [^{14}C] Compound **1** (10 μM) in suspension human hepatocytes for 4 h was low with no quantifiable metabolites detected (Table 2, Figure 1A).

*Compound **2***

A single dose of 25 mg (80 μCi) of ^{14}C-Compound **2** was orally administrated to healthy male human subjects. Approximately, 95% of radioactivity was recovered in excreta with approx. 86% of the dose recovered in urine and 9% in feces. Compound **2** was extensively metabolized with only 12% of the dose eliminated in excreta as parent drug. The primary clearance pathway was hydrolysis of an amide linkage with subsequent N-acetylation or glucuronidation, resulting in major metabolites 2-M2 (amine), 2-M3 (N-acetyl ofamine) and 2-M1 (N-glucuronide of amine) (Figure 2B). Metabolites 2-M2, 2-M3 and 2-M1 accounted for 36%, 21% and 1% of the dose in excreta (Table 3, Figure 2A).

[^{14}C] Compound **2** (2 μM, 68.4 nCi/mL) was incubated in pooled human liver hepatocytes co-cultured with murine stromal cells (*HμREL* HumanPool™) for 24 and 168 h. Metabolite formation was low after 24 h, and was extensively metabolized after 168 h of incubation with only 7% of parent remaining (Table 3, Figure 2A). Major metabolites observed in the 168 h incubation were 2-M2 (31% of the total radioactivity) and 2-M3 (62% of the total radioactivity).

[^{14}C] Compound **2** (2 μM) was incubated in suspension human hepatocytes for 4 h. Metabolite 2-M2 was the only metabolite observed in the suspension hepatocyte incubation and no Phase II conjugates were observed.

*Compound **3***

A single dose of 6 mg (100 μCi) of ^{14}C-Compound **3** was orally administrated to healthy human subjects. The mean recovery of radioactivity was 99% of the dose with 68% in feces and 30% in urine 168 h postdose. Of the total recovery, almost 90% was eliminated within 72 h postdose. Aromatic hydroxylation on a benzo-benzoazepine ring and aliphatic hydroxylation on a methyl group of a dimethyl propanoic acid moiety were two metabolic pathways, which give major excretory metabolites 3-M1 (P + O on the benzene ring of the benzo-benzoazepine ring), 3-M3 (P + O on a methyl group of the dimethyl propanoic acid moiety) and 3-M4 (P + 2O on the benzo-benzoazepine ring) as shown in Figure 3(B). Metabolites 3-M1, 3-M3 and 3-M4 accounted for approx. 38%, 5% and 8% of the dose in urine and feces. Metabolite 3-M5 (P + O on the benzene ring of benzo-benzoazepine ring, different position than 3-M1) was also observed (approx. 4% of the dose in urine and feces) as shown in Table 3. Several additional trace metabolites observed *in vivo*.

[^{14}C] Compound **3** (10 μM, 28.4 nCi/mL) was incubated in pooled human liver hepatocytes co-cultured with murine stromal cells (*HμREL* HumanPool™) for 24 and 168 h. Metabolite formation was low after 24 h with 80% of parent Compound remaining, and extensively metabolized after 168 h of incubation with no parent remaining (Table 4). Metabolites 3-M1 and 3-M2 (M1 + sulfate) were the largest metabolites in the 168 h incubation, accounting for 22% and 21% of the total radioactivity, respectively. Metabolites 3-M3, 3-M4 and 3-M5 were also observed (5%, 6% and 1% of the total radioactivity, respectively).

The extent of metabolism of [^{14}C] Compound **3** (10 μM) in suspension human hepatocytes for 4 h was low with 95% of parent Compound remaining. Metabolite 3-M1 was the only

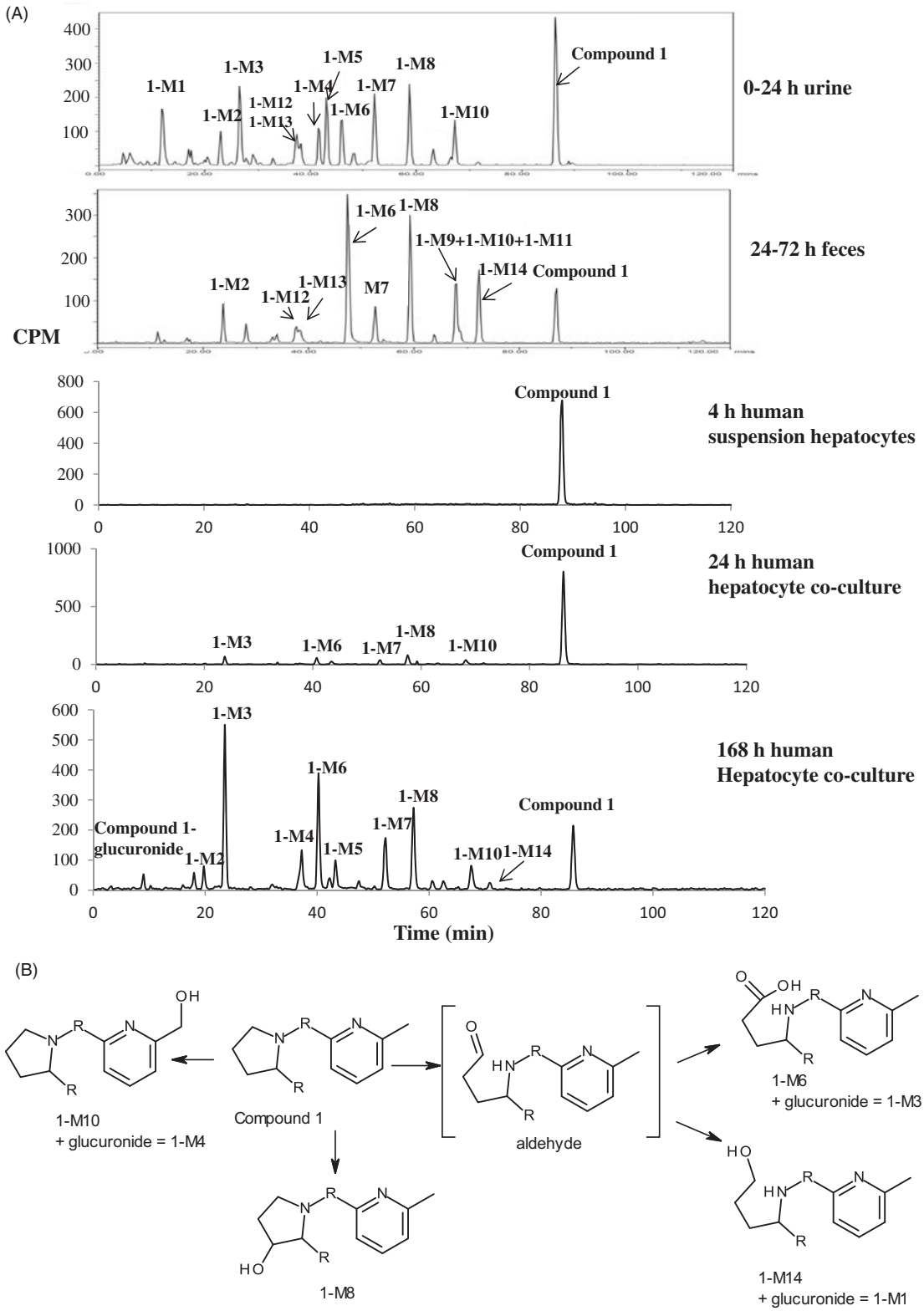


Figure 1. (A) Selected radiochromatograms for Compound **1**. (B) Metabolic pathways for Compound **1**.

metabolite observed in the suspension hepatocyte incubation (4% of the total radioactivity).

*Compound **4***

A single dose of 25 mg (100 μCi) of ^{14}C-Compound **4** was orally administrated to healthy male human subjects. The administered radioactivity was excreted slowly and approximately 62% of the dose was recovered in urine and feces though 504 h postdose (the last collection interval) with fecal excretion being the major elimination route accounting for 61% of the dose. Compound **4** was extensively metabolized with only 2% of the dose eliminated in excreta as intact parent drug. A morpholine ring and a urea moiety

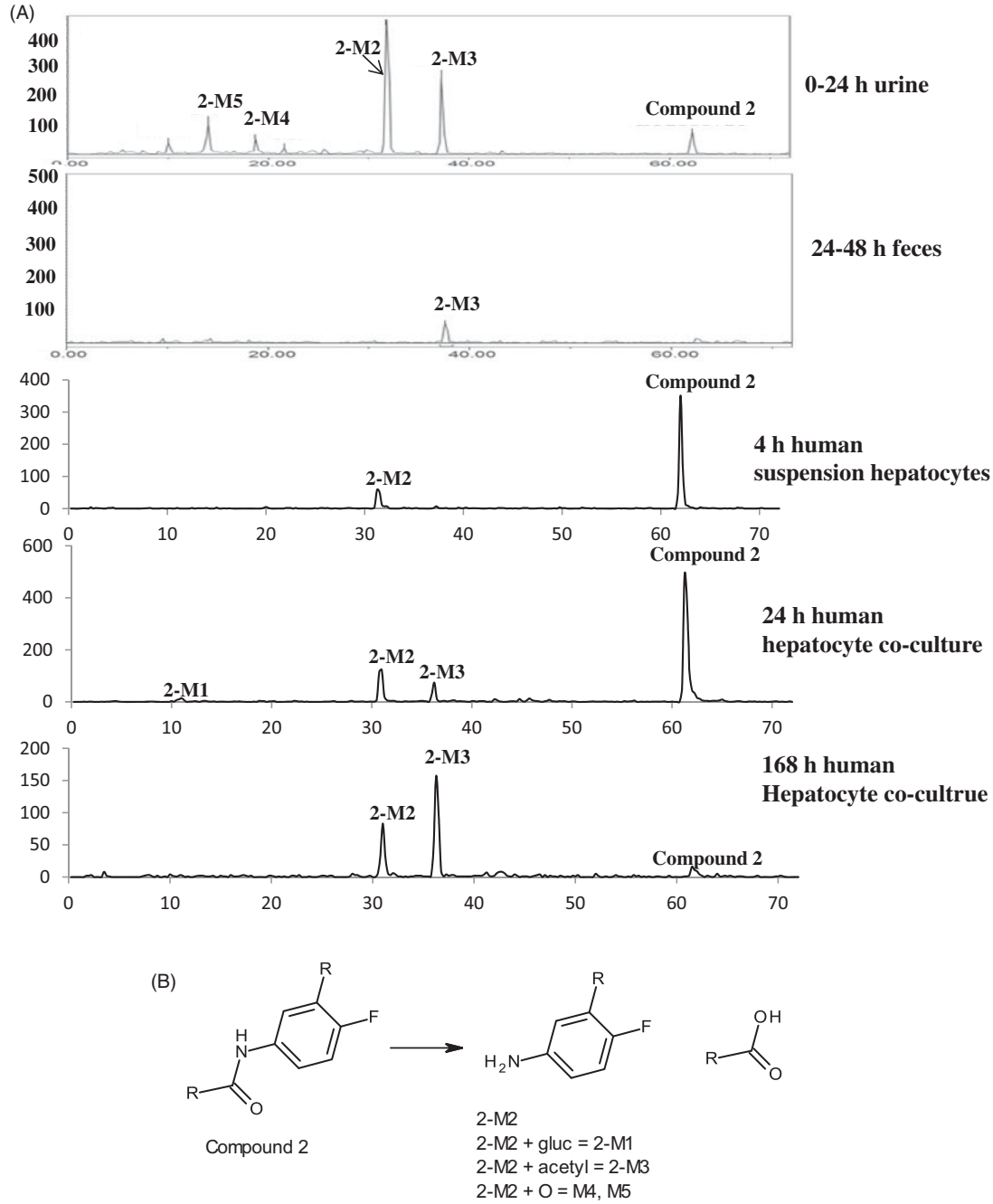


Figure 2. (A) Selected radiochromatograms for Compound **2**. (B) Metabolic pathways for Compound **2**.

Table 3. Metabolites of Compound **2** observed in human *in vivo*, in human hepatocyte co-culture, and in human suspension hepatocytes.

		% of the dose in excreta (mean, $n = 6$)			% ROI *in vitro*		
Peak name	Description	Urine	Feces	Total	Hepatocyte suspension (4 h)	Co-culture (24 h)	Co-culture (168 h)
2-M1	M2 + gluc	1.3[a]	NQ	1.3	NQ	2.3	NQ
2-M2	amide hydrolysis to amine	36.4	NQ	36.4	18.5	16.9	30.6
2-M3	M2 + acetyl	17.2	4.1	21.3	NQ	7.1	62.2
2-M4	M2 + O	3.8	NQ	3.8	NQ	NQ	NQ
2-M5	M2 + O	8.8	NQ	8.8	NQ	NQ	NQ
2-Parent	NA	12.3	NQ	12.3	81.5	70	7.2

NQ: not quantified (peak height <10 cpm); ROI: region of interest.
Only metabolites above 2% of the dose in excreta are listed in the table.
[a]M1 was only detected in one subject urine at 7.5% of the dose and M1 in other subjects was treated as 0, when mean value was calculated.

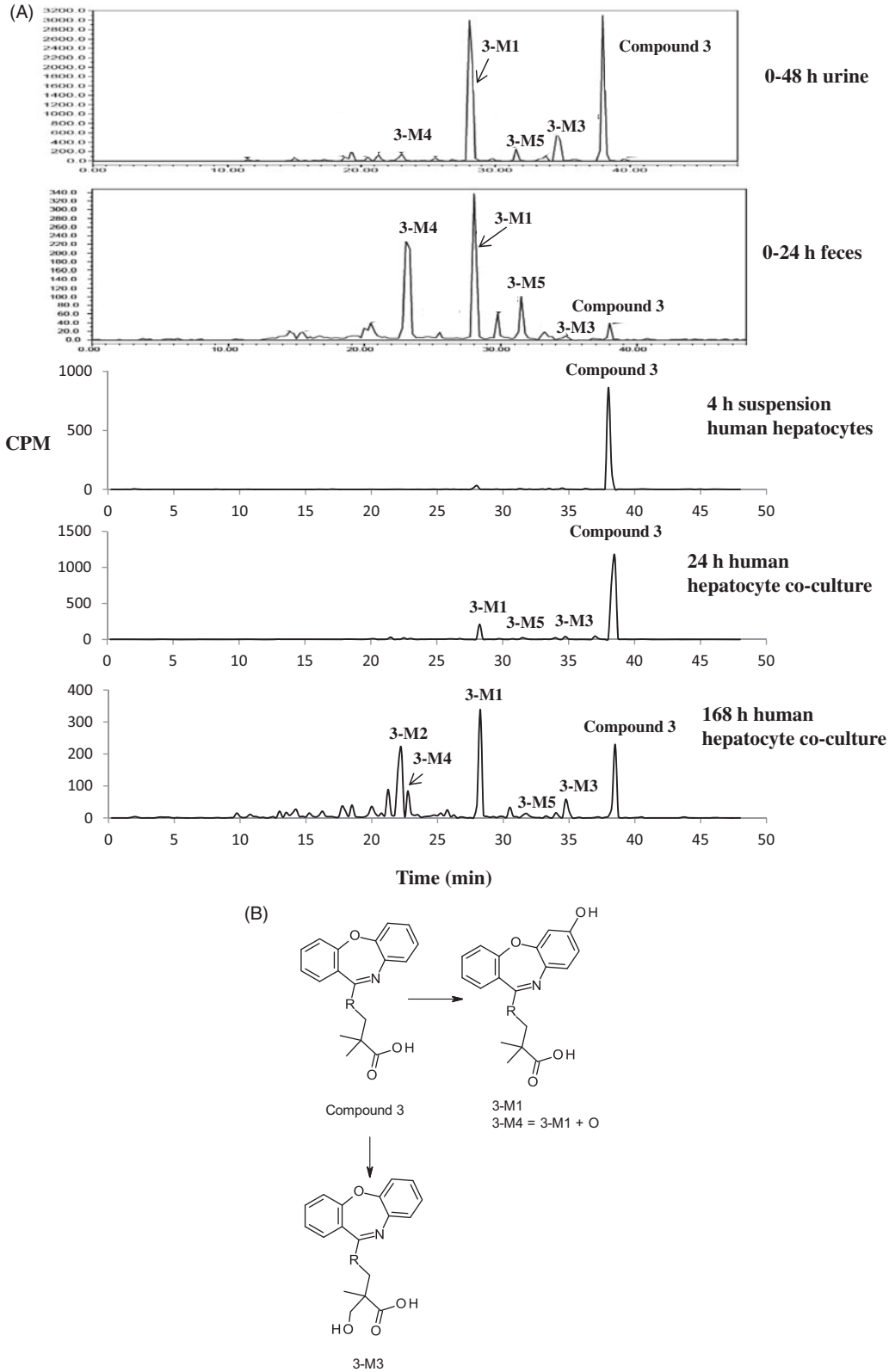


Figure 3. (A) Selected radiochromatograms for Compound **3**. (B) Metabolic pathways for Compound **3**.

were the primary sites of metabolism for this molecule (Figure 4B). N,O-dealkylation from the morpholine ring formed a primary alcohol (4-M1) and urea hydrolysis that formed a primary amine (4-M7) constituted two major metabolites in feces, accounting for 13% and 12% of the dose, respectively. Oxidative deamination with loss of the morpholine ring formed a second alcohol metabolite (4-M5), accounting for 6% of the dose. All other metabolites in feces accounted for less than 5% of the dose (Table 5, Figure 4A).

[^{14}C] Compound **4** (5 µM, 87 nCi/mL) was incubated in pooled human liver hepatocytes co-cultured with murine stromal cells (*HµREL* HumanPool^{TM}) for 24 and 168 h.

Table 4. Metabolites of Compound **3** observed in human *in vivo*, in human hepatocyte co-culture, and in human suspension hepatocytes.

		% of the dose in excreta (mean, $n=6$)			% ROI *in vitro*		
Peak name	Description	Urine	Feces	Total	Hepatocyte suspension (4 h)	Co-culture (24 h)	Co-culture (168 h)
3-M1	+O	28.2	9.84	38.04	4.3	9.0	21.7
3-M2	M1 + sulfate	NQ	NQ	NA	NQ	0.9	20.6
3-M4	+2O	1.30	7.17	8.47	NQ	0.6	5.5
3-M3	+O	5.26	0.10	5.35	NQ	1.6	4.5
3-M5	+O	1.72	1.94	3.66	NQ	1.5	1.3
3-Parent	NA	22.91	0.39	23.30	94.7	80.0	NQ

NQ: not quantified (peak height <10 cpm); NA: not applicable; ROI: region of interest.
Only metabolites above 2% of the dose in excreta are listed in the table.

Table 5. Metabolites of Compound **4** observed in human *in vivo*, in human hepatocyte co-culture, and in human suspension hepatocytes.

		% of the dose (mean, $n=6$)	% of radioactivity		
Peak name	Description	Feces	Hepatocyte suspension (4 h)	Co-culture (24 h)	Co-culture (168 h)
4-M1	N-desethyl	12.7	3.8	6.9	34.2
4-M2/4-M3	M2 (M1 + CO) + M3 (+O-2H, aldehyde)	5.4	NQ	NQ	NQ
4-M4	N-dealkyl + aldehyde	2.9	NQ	NQ	NQ
4-M5	N-dealkyl + alcohol	6.4	NQ	5.5	19.0
	M5 + gluc	NQ	NQ	NQ	6.3
4-M6	N-dealkyl + aldehyde	1.3	6.0	2.5	1.0
4-M7	urea cleavage	12.4	NQ	NQ	NQ
4-Parent	NA	2.1	77.7	78.3	1.8

NQ: not quantified (peak height <10 cpm); ROI: region of interest.
Only metabolites above 2% of the dose in excreta are listed in the table.
Total dose in urine was approx. 1% of the dose, therefore urine was not profiled.

Metabolite formation was low after 24 h with 78% of parent compound remaining, and was extensively metabolized after 168 h of incubation with only approx. 2% of parent remaining (Table 5, Figure 4A). The two most prominent metabolites observed in the 168 h incubation were 4-M1 and 4-M5 (34% and 19% of the total radioactivity, respectively). 4-M6 (N-dealkylation at the morpholine ring to an aldehyde) was also observed.

The extent of metabolism of [^{14}C] Compound **4** (5 μM) in suspension human hepatocytes for 4 h was low with 78% of parent compound remaining. The largest metabolites were 4-M1 and 4-M6 which accounted for 3.8% and 6.0% of the total radioactivity, respectively (Table 5, Figure 4A).

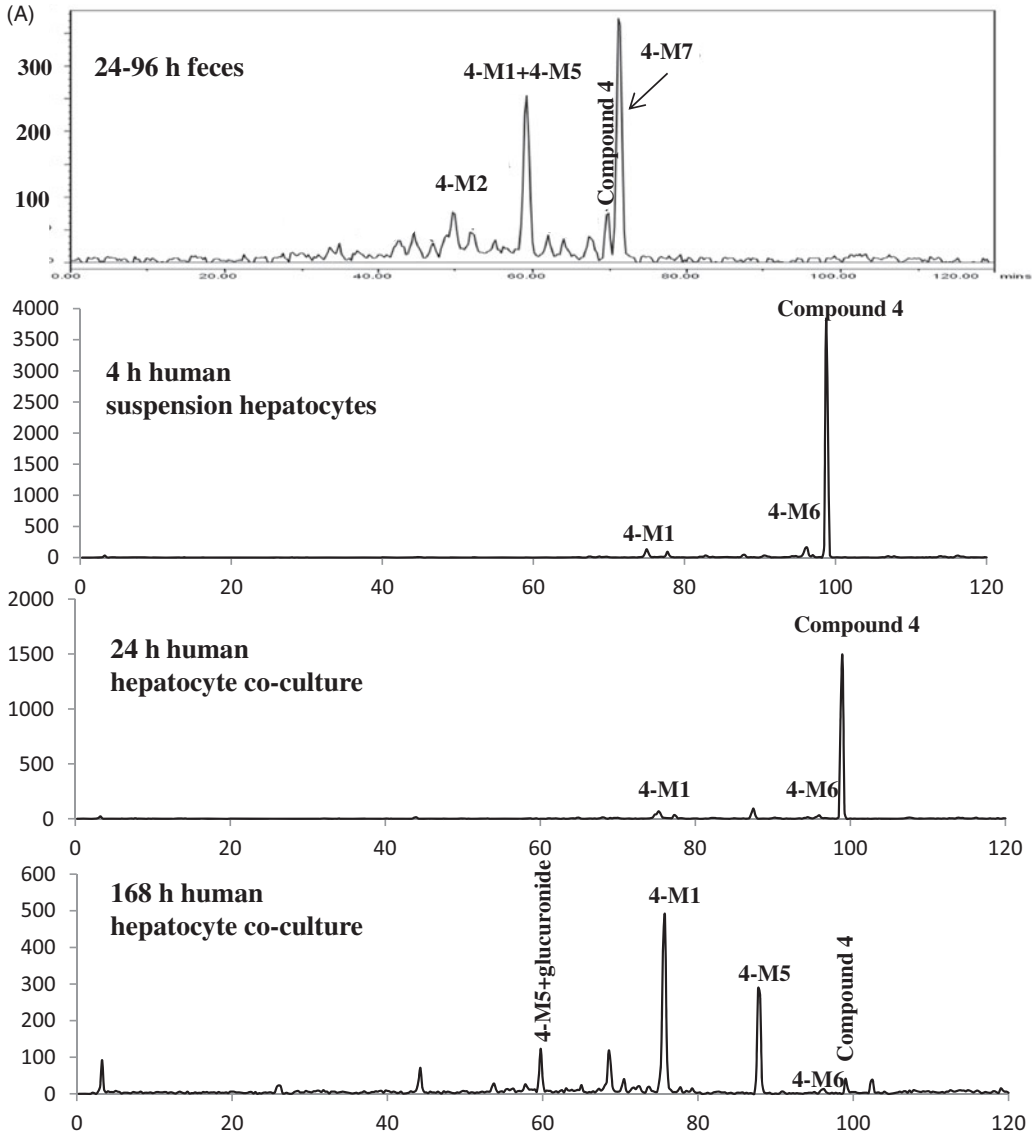
*Compound **5***

A single dose of 120 mg (100 μCi) of ^{14}C-Compound **5** was orally administrated to healthy female human subjects. The mean recovery of radioactivity after 196 h postdose was approx. 99% of the dose with 96% in feces and 3% in urine. N-demethylation at a methylindazole moiety and hydroxylation at the methyl group of a 6-methyl-2-oxo-pyridine moiety were two primary metabolic pathways, which gave two major fecal metabolites 5-M1 and 5-M2, respectively (Figure 5B). Further hydroxylation at the methyl group of the 6-methyl-2-oxo-pyridine moiety of 5-M1 formed another significant fecal metabolite 5-M4. Metabolites 5-M1, 5-M2 and 5-M4 accounted for approx. 8%, 43% and 14% of the dose in feces. Several Phase II metabolites (glucuronide conjugates) were observed in urine as minor metabolites (<1% of the dose, individually, Table 6).

[^{14}C] Compound **5** (2 μM, 72 nCi/mL) was incubated in pooled human liver hepatocytes co-cultured with murine stromal cells (*HμREL* HumanPool™) for 24 and 168 h. Compound **5** was extensively metabolized after both 24 h and 168 h of incubations with approx. 5% of parent remaining after 24 h and no parent remaining after 168 h (Table 6, Figure 5A). Glucuronide conjugates direct to parent or following Phase I oxidation were major metabolites observed in HμREL human hepatocytes. Parent + glucuronide and glucuronides of 5-M1 (5-M5), 5-M2 (5-M6 + 5-M9) and M4 (5-M8) accounted for 26%, 15%, 9 + 11% and 13% of the total radioactivity in the 24 h incubation. Parent + glucuronide, 5-M5, 5-M8 and 5-M9 accounted for approx. 31%, 21%, 20 and 29% of the total radioactivity in 168 h incubation. 5-M1 and 5-M2 were detected in the 24 h incubation as minor metabolites.

[^{14}C] Compound **5** (2 μM) was incubated in suspension human hepatocytes for 4 h. The extent of metabolism was moderate with 40% of parent Compound remaining. Three metabolites (5-M1, 5-M2 and parent + glucuronide) were observed, accounting for 11%, 4% and 22% of the total radioactivity, respectively.

Multivariate analysis. The plots in Figure 6 show all of the combinations of correlating the % metabolites (% dose or % ROI) either excreted *in vivo* or observed *in vitro* for the different matrices across all compounds. Results from the analysis showed that when combining the data from all five



Note: Different HPLC condition was used for fecal samples than other matrices,

Figure 4. (A) Selected radiochromatograms for Compound **4**. (B) Metabolic pathways for Compound **4**.

compounds the 168 h co-culture results (% ROI for each metabolite) correlated to the Total Urine and Feces percent dose excreted with an *R* value of 0.5338. Correlations of the 4 h hepatocyte or 24 h co-culture to Total Urine and Feces were essentially 0. No other statistical analysis was conducted.

Discussion

Predicting human *in vivo* clearance pathways in early drug discovery and development remains challenging. Metabolism in suspension hepatocytes has been routinely used for predicting preclinical and clinical metabolism of drug candidates since this model is simple and cost effective. However, suspension hepatocytes have a small window of viability and therefore this model often does not provide adequate prediction of *in vivo* metabolic pathways, especially for slowly metabolized compounds. Several *in vitro* hepatic systems with competency of long-term incubations have been developed in the last decade and evaluated in our lab. Among these new systems, we have found that the commercially available hepatocyte-stromal cell co-culture offers flexibility (multiple preclinical species and pooled human cells), convenience and is relatively cost effective if utilized in a judicious manner.

Claims that hepatocytes in the co-culture remain viable and liver-specific functions for several weeks have been substantiated though out the literature (Cho et al., 2014; Hultman et al., 2016).

The purpose of this study was to evaluate qualitative and quantitative prediction of human *in vivo* metabolism in human hepatocyte co-cultures using ^{14}C-labeled compounds. Human *in vivo* metabolism data of these compounds were already generated in clinical ^{14}C ADME studies. As shown in Table 1, these compounds represented a diverse array of metabolic clearance pathways. Major biotransformation enzymes, including CYPs, UGT and hydrolase(s), were involved in the metabolism of these compounds. A limitation of the compound set studied is that no compounds metabolized by

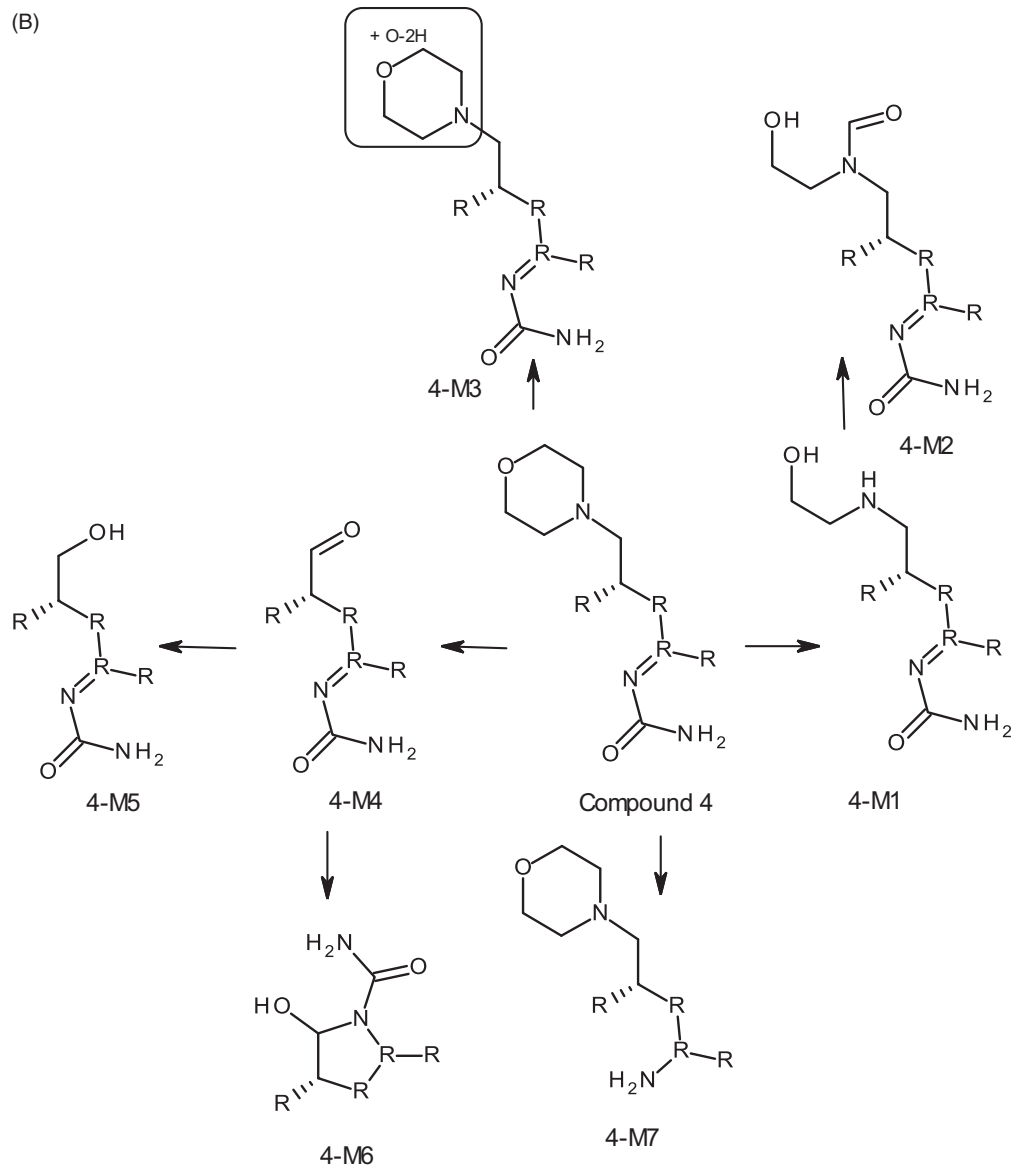


Figure 4. Continued.

aldehyde oxidase (AO) were studied. AO has become major enzyme in biotransformation of drug candidates due to chemists improving microsomal metabolic stability. However, we did not have a clinical data set available where AO was a major metabolic pathway.

Compound **1** (Figure 1B) contains a 2-methyl substituted pyridine ring, and a pyrrolidine ring as part of the chemical backbone. The human ^{14}C ADME study showed that the primary clearance routes of Compound **1** were mostly oxidative with only 13% of the dose recovered unchanged in excreta. The major metabolic clearance pathways for Compound **1** were represented by: (1) oxidative ring opening of the pyrrolidine ring to form an alcohol (1-M14) or a carboxylic acid (1-M6) with secondary Phase II glucuronidation (1-M3 and 1-M1) (together 1-M1, 1-M3, 1-M6 and 1-M14, 2) allylic oxidation on the 3-position of pyrrolidine ring (1-M8) and (3) oxidation of the substituted 2-methyl group on the pyridine ring (1-M10) with subsequent glucuronidation (1-M4) The absorption of Compound **1** was considered high in the human ADME study (13% dose excreted unchanged). Despite that the major clearance routes for Compound **1** were metabolic, incubation of Compound **1** in the suspended human cryopreserved hepatocyte model showed no quantifiable metabolites after a 4 h incubation. This result corresponded with the calculated *in vitro* intrinsic clearance in human hepatocytes of 2.7 μL/min/million cells, which is considered slow and near the limit of assay detection (1.8 μL/min/million cells). This apparent *in vitro* to *in vivo* disconnect led us to investigate the utility of the commercially available hepatocyte co-culture model. After incubation of Compound **1** for 24 h in the co-culture, several minor metabolites were observed in the radiochromatogram, however, the extent of metabolism and metabolic pathways observed in the 168 h co-culture incubation better represented the *in vivo* human metabolism (Table 2 and Figure 1A). The three major metabolic pathways in the 168 h co-culture incubation corresponded to the same three major metabolic pathways *in vivo*. However, upon close inspection into the

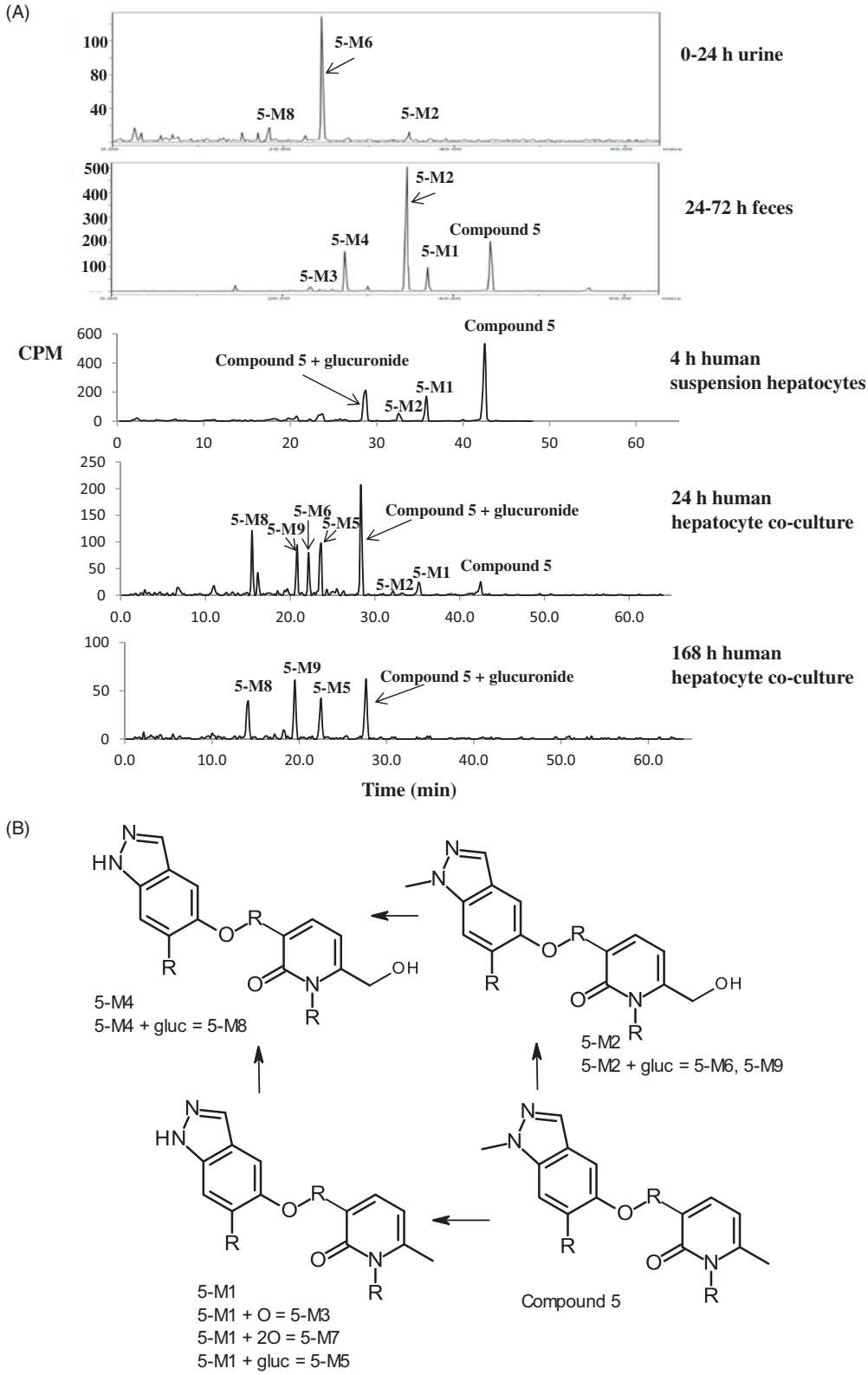


Figure 5. (A) Selected radiochromatograms for Compound **5**. (B) Metabolic pathways for Compound **5**.

distribution of individual metabolites (Phase I versus Phase II metabolites) there were indeed some differences between *in vivo* and the co-culture system. Metabolite 1-M3 (a glucuronide) was minor *in vivo* (3% of the dose), but a major *in vitro* (22% of the total radioactivity). In contrast, the aglycone 1-M14 was a large metabolite in feces (7% of the dose), but only 1% in *in vitro*. It is therefore presumed that 1-M3 *in vivo* undergoes hydrolysis to form 1-M14 in gut by microbial β-glucuronidase, but since the co-culture system lacks of microbial β-glucuronidase and the glucuronide conjugate remains intact.

Table 6. Metabolites of Compound **5** observed in human *in vivo*, in human hepatocyte co-culture, and in human suspension hepatocytes.

		% of the dose in excreta (mean, $n=6$)			% ROI		
Peak name	Description	Urine	Feces	Total	Hepatocyte suspension (4 h)	Co-culture (24 h)	Co-culture (168 h)
5-M1	N-desmethyl	NQ	8.0	8.0	11.4	4.2	NQ
5-M2	P + O	0.1	42.7	42.8	4.3	1	NQ
5-M3	N-desmethyl + O	NQ	2.2	2.2	NQ	NQ	NQ
5-M4	N-desmethyl + O	NQ	13.7	13.7	NQ	NQ	NQ
5-M5	M1 + Gluc	NQ	NQ	NA	NQ	15	20.8
5-M6	M2 + gluc	0.9	NQ	0.9	NQ	9.3	NQ
5-M7	M1 + 2O	NQ	2.2	2.2	NQ	NQ	NQ
5-M8	M4 + gluc	0.1	NQ	0.1	NQ	13.2	19.5
5-M9	M2 + gluc	NQ	NQ	NA	NQ	11	28.5
NA	Parent + gluc	NQ	NQ	NA	21.6	26.2	31.2
5-Parent	NA	NQ	13.8	13.8	40.4	4.7	NQ

NQ: not quantified (peak height < 10 cpm); NA: not applicable – metabolite not observed *in vivo*; ROI: region of interest.
Only metabolites above 2% of the dose in excreta are listed in the table.
Total dose excreted in urine up to 192 h post dose was 2.8%. No urinary metabolite was >1% of the dose.

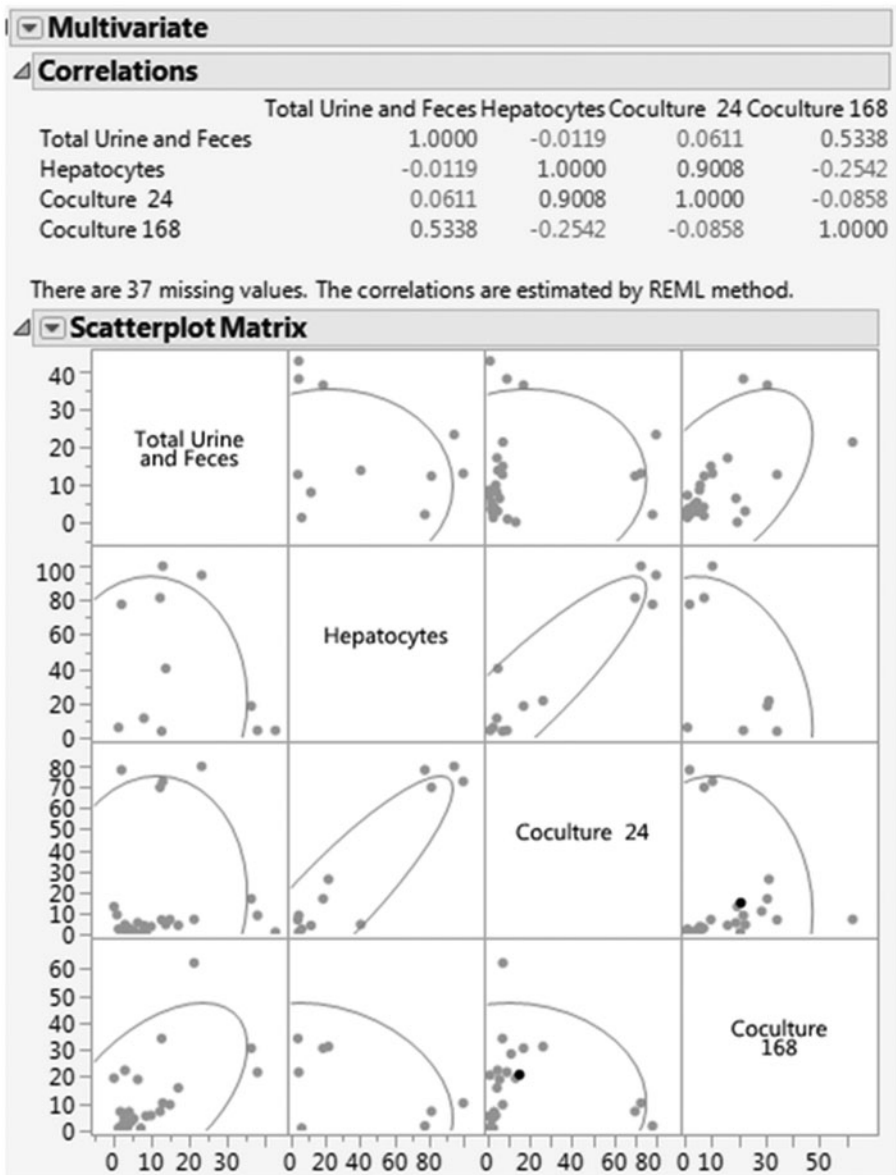


Figure 6. Multivariate analysis results; *X* and *Y* axes represent the % of each individual metabolite found in the various matrices plotted in all combinations.

Compound **2** contained an aryl linked amide backbone as part of the chemical series. It was anticipated that hydrolase(s) were involved in metabolism of Compound **2**. In the human, ^{14}C ADME study amide hydrolysis was the primary metabolic pathway for Compound **2** (Figure 2B) Compound **2** was radiolabelled at a single site (the aniline moiety) therefore tracking carboxylic acid related metabolites by radioactivity was not possible.

In vivo in human amide hydrolysis of Compound **2** formed metabolite 2-M2 and subsequent N-acetylation and N-glucuronidation of 2-M2 formed metabolites 2-M3 and 2-M1, respectively. The extent of metabolism *in vivo* was significant as only 12% of parent was recovered unchanged in excreta. Absorption of Compound **2** was considered high in the human ADME study (12% dose excreted unchanged). Incubation of Compound **2** for 4 h in suspended hepatocytes did not predict *in vivo* metabolism well. Metabolite 2-M2 was the only metabolite detected in the 4 h incubation with no additional or secondary metabolites detected. This result corresponded with the *in vitro* intrinsic clearance in human hepatocytes which was reported as below the limit of detection or <1.8 µL/min/million cells. When Compound **2** was incubated in the long-term hepatocyte co-culture model, the results showed good congruence to the *in vivo* data. After 168 h incubation, metabolites 2-M2 and 2-M3 which were the most abundant excretory metabolites in human were also observed as the most abundant metabolites *in vitro*. These data confirmed that hepatic hydrolysis and N-acetylation were active in the co-culture. Interestingly, metabolite 2-M1 was detected *in vivo* in urine of only one subject (7.5% of the dose) and was very minor in the *in vitro* co-culture incubation (2% of the total radioactivity in 24 h incubation and not quantifiable in 168 h incubation). An *in vitro* stability assay of 2-M1 in human urine indicated that the metabolite was stable at pH 8–9, and the stability decreased when pH decreased (data not shown). Additionally, metabolite 2-M1 was not detected in urine of any other subjects or in 168 h *in vitro* incubation which could be due to pH related instability of the metabolite (a suspected N-glucuronide).

Compound **3** was cleared metabolically and by renal excretion (with 23% of the dose eliminated in urine as unchanged parent compound) in human. In co-culture, Compound **3** was extensively metabolized with no parent remaining after 168 h of incubation (Table 4, Figure 3A). Understandably due to the substantial renal elimination of Compound **3** *in vivo*, the static hepatic co-culture model over predicted the contribution of metabolism to the overall clearance. Aromatic and aliphatic hydroxylations were the major metabolic pathways *in vivo* to give metabolites 3-M1, 3-M3 and 3-M4, accounting for 38%, 5% and 8% of the dose in excreta, respectively. In co-culture 168 h incubation, 3-M1, 3-M3 and 3-M4 accounted for 22%, 5% and 6% of the total radioactivity. Metabolite 3-M2 (M1 + sulfate) was not detected *in vivo*, but represented 21% of the total radioactivity in the 168 h incubation. Presumably, 3-M2 could undergo hydrolysis of the sulfate in the gut to form 3-M1. Together 3-M1 and 3-M2 in the 168 h co-culture incubation accounted for 43% of the total radioactivity (close to the contribution of 3-M1 *in vivo*, 38% of the dose). A few minor metabolites in the 168 h co-culture incubation were not identified (Figure 3A). The 4 h suspended human hepatocyte incubation did not sufficiently predict human *in vivo* metabolism. Metabolite 3-M1 was the only metabolite observed in the suspension hepatocyte incubation (4% of the total radioactivity) with 95% of parent Compound **3** remaining after 4 h incubation.

Compound **4** contained a morpholine ring and urea in the chemical structure (Figure 4B). In the human ^{14}C ADME study after 504 h postdose (the last collection interval), the total radioactive recovery was only 62% of the dose with a majority excreted in feces (61% of the dose), and only 2.1% of the dose recovered as unchanged parent. The major *in vivo* metabolites were an N, O-desethyl metabolite (4-M1), 4-M5 (oxidative N-dealkylation of the morpholine group) and a hydrolysis of the urea to a primary amine (4-M7). Several minor metabolites were also observed. Incubation of Compound **4** for 4 h in suspended cryopreserved hepatocytes poorly predicted *in vivo* metabolism. In fact, two of the major *in vivo* metabolites 4-M5 and 4-M7 were not detected in the suspension hepatocyte incubation (Table 5, Figure 4A). In contrast, the 168 h incubation in co-culture predicted two of the three major *in vivo* metabolites, e.g. 4-M1 and 4-M5. However, the urea hydrolysis product (4-M7) was not detected in the co-cultures or suspended hepatocytes, suggesting that the hydrolytic pathway was extra-hepatic, perhaps via intestinal microflora metabolism.

The chemical structure of Compound **5** contains a methylindazole moiety and a 6-methyl-2-oxo-pyridine group (Figure 5B). The *in vitro* intrinsic clearance in hepatocytes was calculated to be 11.3 µL/min/million cells which is considered moderate. In the human, ^{14}C ADME study metabolite profiling of Compound **5** indicated that Compound **5** was extensively metabolized with 13% of the dose in feces as unchanged parent and no unchanged parent detected in urine. Oxidative Phase I metabolites were major *in vivo* metabolites (5-M2 and 5-M4, Table 6). Several Phase II glucuronides secondary to Phase I metabolism were observed, but minor (5-M6, 5-M8). No direct glucuronide of parent was detected *in vivo*. Contrasting to the *in vivo* results, Phase II glucuronide conjugates (direct to parent or secondary to Phase I oxidation) were major metabolites observed in suspended hepatocytes and hepatocyte co-culture. Interestingly, incubation of Compound **5** in suspended human hepatocytes for 4 h showed the formation of three major metabolites, 5-M1, 5-M2 and a direct glucuronide of unchanged parent compound. The direct glucuronide of parent was not observed *in vivo*. Overall, in co-culture several Phase I metabolites were detected in the 24 h incubation as minor metabolites, but not in the 168 h incubation. The discrepancy of Phase I and Phase II metabolite distribution between *in vivo* and *in vitro* is likely due to hydrolysis of the glucuronides of parent and metabolites by β-glucuronidase to aglycones by microflora in the gut. The *in vivo* formation of a direct parent glucuronide was supported by plasma and blood concentration–time curves of parent Compound **5** and total radioactivity. After an oral dose, both parent and total radioactivity concentration–time curves showed the

secondary peak around 10 h, indicating enterohepatic circulation which resulted from re-absorption of parent Compound **5** and aglycones in intestine after hydrolysis of respective glucuronide conjugates (data not shown).

The human hepatocyte-stromal cell *in vitro* co-culture system predicted *in vivo* human metabolism successfully and was superior to suspended cryopreserved hepatocytes for the compounds evaluated. Multivariate analysis (REML method) showed that the *in vivo* results correlated best to the 168 h co-culture incubation (Figure 6) with a coefficient of 0.53. As chemists synthesize drug molecules that are metabolically stable yet cleared by metabolism, long-term hepatic models will be valuable in predicting human metabolic clearance pathways. In some cases, the distribution of Phase I and Phase II (i.e. glucuronides) was dissimilar between *in vivo* and *in vitro* and this was likely due to β-glucuronidase hydrolysis of Phase II conjugates by gut microflora and the lack of β-glucuronidase in the suspension hepatocytes and hepatocyte co-culture. This nature of the hepatocyte-stromal cell co-culture may offer benefit to uncover UGTs and/or SULTs involvement *in vivo* metabolism. The *in vitro* metabolic pathway analysis using co-culture also may provide insight for possible gut microflora mediated metabolism. For example, if a major *in vivo* fecal metabolite is absent *in vitro*, this may point to possible gut microflora mediated biotransformation. A shortcoming of the co-culture method may be in determining the correct incubation time. We chose the 24 h and 168 h timepoints to hopefully capture compounds that are metabolized both quickly and slowly. Work is ongoing to determine methods for selecting incubation time points more accurately. Additionally, one must consider non-hepatic mechanisms of clearance which may include renal or biliary clearance of unchanged drug, extra hepatic CYP450 and non-CYP450 metabolism, and extra hepatic hydrolytic enzymes. It is therefore recommended that based on the data from this work, radiolabeled compound metabolism work using hepatocyte-stromal co-cultures across species (such as human, rat, dog and monkey) in parallel with radiolabelled preclinical *in vivo* metabolism studies would be useful. If a good *in vitro–in vivo* correlation is observed for preclinical species, the human *in vitro* metabolism data generated in human co-culture may provide an improved prediction for human *in vivo* metabolism prior to the ^{14}C human ADME study.

Declaration of interest

This work was supported by Eli Lilly and Company. Cassidy and Yi are employees and shareholders of Eli Lilly and Co.

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