Short Communication

Generation of Major Human Excretory and Circulating Drug Metabolites Using a Hepatocyte Relay Method

ABSTRACT

The prediction of human drug metabolites using in vitro experiments containing human-derived reagents is an important approach in modern drug research; however, this can be challenging for drugs that are slowly metabolized. In this report, we describe the use of a recently developed human hepatocyte relay method for the purpose of predicting human drug metabolite profiles. Five compounds for which in vivo human metabolism data were available were selected for the investigation of this method, and the results were compared with data gathered in hepatocyte suspensions as well as previous data from a micropatterned hepatocyte coculture method. The hepatocyte relay method demonstrated an improved performance (generation of 75% of human in vivo metabolites) for those drugs for which previous methods showed a relatively low rate of success (50% of human in vivo metabolites). Metabolites included those arising from both oxidative and conjugative reactions and metabolites that required sequential reactions. Two 4-hour relays were shown to adequately generate metabolites, and no further benefit was derived from more relays. Overall, it can be concluded that the hepatocyte relay assay method can be successfully used in the generation of relevant human metabolites, even for challenging drugs.

Introduction

Early identification of major human metabolic pathways and biotransformation liabilities for new chemical matter is a critical part of drug research (Lin and Lu, 1997; Baillie, 2008). During early to mid-stage drug discovery and structure-activity-relationship investigations, subcellular fractions (liver microsomes and S-9 fractions) and hepatocyte suspensions are commonly used to better understand and predict human in vivo clearance mechanisms (Plant, 2004; Pelkonen and Raunio, 2005; Kerns and Di, 2008). The ability of these in vitro tools to accurately predict the major human metabolite profile in circulation (>10% total drug-related material) or in excreta (>10% of dose) varies from system to system and begins to falter more significantly when the metabolism is multistep/multiphase (Anderson et al., 2009; Dalvie et al., 2009; Wang et al., 2010). Occasionally, a compound displays differential metabolic profiles in vivo versus in vitro, and avoiding this situation is an important goal for all drug research programs. Currently, hepatocyte suspensions and micropatterned coculture (MPCC) systems provide the most complete in vitro drug metabolic profile. Hepatocyte suspensions, however, still fail to capture more complex secondary metabolites under a standard incubation time of 4 hours (Skett, 1994; Gebhardt et al., 2003). Although the application of a micropatterned hepatocyte coculture system greatly improved the predictive capabilities, secondary metabolites were still underpredicted (Wang et al., 2010).

Recently, we have been investigating the use of a novel hepatocyte suspension relay system to provide extended drug residence time in viable hepatocytes providing reliable in vitro clearance data for compounds with slow hepatic turnover (Di et al., 2012). Although the relay method was primarily used to investigate substrate depletion of low-clearance compounds, it was hypothesized that it could also be used to produce metabolic profiles of highly metabolized drugs comparable to the hepatocyte MPCC system. To this end, we sought to investigate a set of structurally diverse compounds subject to a range of biotransformation reactions in a modified version of the hepatocyte relay (Fig. 1). Capromorelin, CP-122721 (2-methoxy-5-trifluoromethoxy-benzyl)-(2S,3S)-2-phenyl-piperidin-3-yl-amine, and ziprasidone were chosen specifically based on their extensive metabolism (each having 4 major human in vivo metabolites) and predominant secondary metabolite profiles that were under-represented in other in vitro systems including the MPCC. For this set of compounds, hepatocyte suspensions identified 25% (3/12) of the major human in vivo metabolites, whereas the MPCC produced 50% (6/12) (Wang et al., 2010). The metabolite profiles generated from the relay method are compared with the MPCC 7-day incubation and ultimately compared with the radiolabeled human absorption, distribution, metabolism, and excretion data focusing on the major human excretory and circulating metabolites (Dalvie et al., 2009).

Materials and Methods

Test compounds were obtained from Pfizer Global Material Management (Groton, CT). A mixed-gender, 10-donor pooled cryopreserved human hepatocyte, Lot# RTH, was purchased from Celsis IVT (Baltimore, MD) and was used in all of the following studies. All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Metabolite Identification Relay Method Using Human Hepatocytes. Williams’ E medium (custom formula number 91-5233EC; Invitrogen, Grand Island, NY) supplemented with 26 mM sodium bicarbonate and 50 mM HEPES was warmed to 37°C and bubbled with 95% O2/5% CO2 30 minutes prior to each use. Twenty-four-well hepatocyte plates containing 0.5 × 10^6 cells/ml

ABBREVIATIONS: CLint, intrinsic clearance; CP-122721, (2-methoxy-5-trifluoromethoxy-benzyl)-(2S,3S)-2-phenyl-piperidin-3-yl-amine; MPCC, micropatterned coculture; MS, mass spectrometry; UHPLC, ultra-high-performance liquid chromatography.
in Williams’ E medium were exposed to compounds at a final concentration of 10 μM (dimethylsulfoxide, final concentration 0.025%; methanol, final concentration 0.125%), in a final volume of 0.5 ml. The plates were placed on an orbital shaker at 120 rpm and incubated at 37°C with 95% O2/5% CO2, 75% relative humidity for 4 hours. At time 0 hour, 500 μl of hepatocyte suspension was mixed and transferred to a new 24-well plate, centrifuged (Eppendorf, Hauppauge, NY) at 1500 g for 10 minutes at 4°C, and 450 μl of the supernatant was removed and snap frozen. At time 4 hours, separate hepatocyte suspensions were centrifuged at 1500 x g for 10 minutes at 4°C, and supernatants (300 μl) were transferred to a new plate and stored at −80°C overnight for the next relay. For the second relay experiment, the supernatant plates were warmed to 37°C for 30 minutes, and fresh hepatocytes were thawed and added to the supernatant samples to give a final cell density of 0.5 x 10^6 cells/ml and a total of 0.5 ml. The plates were incubated at 37°C for 4 hours, sampled, and processed as described above (8 hours; 2-relay time point). Five relays were performed to give a total incubation time of 20 hours. Thawed samples were mixed with 4 volumes of acetonitrile and centrifuged at 1700 x g for 5 minutes, and the supernatant was decanted into 15-ml glass tubes and concentrated to dryness in a vacuum centrifuge (Genevac Inc, Valley Cottage, NY) system under reduced pressure. The residues were reconstituted in 5% acetonitrile/H2O (0.4 ml), centrifuged (1700 x g) for 5 minutes, and the supernatants were analyzed.

**UHPLC–Tandem Mass Spectrometry Analysis.** Reconstituted samples were analyzed by ultra-high-performance liquid chromatography (UHPLC)–UV–mass spectrometry (MS) operated in positive ion mode using an Orbitrap Elite mass spectrometer (Thermo Scientific, Somerset, NJ). For UHPLC-UV-MS analysis, the capillary temperature was set at 265°C, the source potential was 3500 V, and the source heater was set at 400°C. The mass spectrometer was operated in a data-dependent scanning mode to MS^3 with dynamic exclusion enabled (repeat count: 2; repeat duration: 5.0 seconds; list size: 500; exclusion duration: 7.5 seconds). The normalized collision energy for the data-dependent scans was 35%. Other potentials were adjusted to obtain optimal ionization and fragmentation of the parent compound. UV absorption spectra were obtained by an in-line Accela photodiode array detector (Thermo Scientific). A Kinetex C18 100 Å column (Phenomenex, Torrance, CA) was used (2.1 x 150 mm, 1.7 μm) with a flow rate of 0.4 ml/min at room temperature (25°C). Mobile phase A was composed of 0.1% formic acid, and mobile phase B was composed of acetonitrile. The gradient system used was as follows: initially, 5% B held for 0.8 minute followed by a linear gradient to 60% B from 0.8 to 8.25 minutes, a second linear gradient to 95% B at 8.5 to 8.75 minutes, a 0.25-minute wash at 95% B, a third linear gradient to 5% B at 9 to 9.2 minutes, and finally, a 0.8-minute re-equilibration period at 5% B. Injections of 10 μl were made by a CTC Prep and Load (PAL) autosampler (CTC Analytics, Zwingen, Switzerland).

**Fig. 1. Pfizer compounds used in this analysis.**

**Results and Discussion**

Previous studies in our laboratories and others have compared the metabolite production capabilities of in vitro systems at recapitulating metabolism and excretion studies. Five diverse compounds were chosen for investigation from the Pfizer compound library: capromorelin, CP-122721, ziprasidone, celecoxib, and linezolid (Fig. 1). Three compounds (capromorelin, CP-122721, and ziprasidone) presented a significant challenge as they each produce four major metabolites (among many others) which were poorly produced from in vitro systems (Table 1). Capromorelin is prone to numerous multistep oxidative metabolism events with four metabolites present in the excreta and circulating drug-related material present in the radiolabeled human absorption, distribution, metabolism, and excretion studies. Five diverse compounds were chosen for investigation from the Pfizer compound library: capromorelin, CP-122721, ziprasidone, celecoxib, and linezolid (Fig. 1). Three compounds (capromorelin, CP-122721, and ziprasidone) presented a significant challenge as they each produce four major metabolites (among many others) which were poorly produced from in vitro systems (Table 1). Capromorelin is prone to numerous multistep oxidative metabolism events with four metabolites present in the excreta and circulating drug-related material present at >10%. Phenyl piperidone compound CP-122721 was chosen for its extensive secondary phase 2 glucuronide metabolites and an interesting salicylic acid derivative present at >50% of drug-related material in plasma derived from multiple sequential metabolic reactions. The sulfur linkage in ziprasidone is extensively metabolized to the sulfone and the sulfone, while the molecule also oxidatively cleaves into two halves. Celecoxib was chosen as a metabolism control as major metabolites are readily detectable in standard hepatocyte incubations. Linezolid was chosen based on its low clearance as metabolites are detected in 7-day hepatocyte coculture experiments but not in single-day (standard 4-hour) hepatocyte incubations.

Our initial experimental design involved minor modifications to the relay method (Di et al., 2012): substrate added at 10 μM, 500 μl volume with 300 μl of supernatant transfers (60%), using 0.5 x 10^6 cells/ml...
and only investigating four time points (0, 8, 12, and 20 hours). At each time point, the hepatocytes were centrifuged at 1500 × g to pellet the cells, and the supernatant was then transferred for the next relay step. Metabolites for both control compounds, celecoxib and linezolid, were readily detectable at the 8-hour (2 relays) time point in the supernatant (Table 1). Compound CP-122721 formed three of the four major metabolites, including the salicylic acid metabolite. The two metabolized halves of ziprasidone were detected at the 8-hour time point, whereas the smaller benzisothiazole-3-yl-piperazine sulfoxide and sulfone were not detected at any of the time points collected. Capromorelin formed all four major circulating metabolites by the 8-hour (2 relays) time point. A representative UHPLC-UV chromatogram for capromorelin (Fig. 2) shows the four major circulating metabolites of capromorelin (labeled A, B, C, and D) as well as a large UV peak (“X”), which was shown to be the glucose conjugate of N-desmethyl-O-desbenzyl-capromorelin. Small amounts of glucose-conjugated capromorelin were also detected (data not shown); however, neither of these glucose-conjugated metabolites was relevant in vivo.

Overall, our initial experiment successfully captured 81% of the important metabolites for the set of compounds investigated compared with 63% for the micropatterned coculture. Removing the positive controls from this set, the coculture captures 6/12 (50%) of the metabolites from capromorelin, CP-122721, and ziprasidone, whereas the relay produced 9/12 (75%). No additional metabolites were detected beyond the 8-hour time point (i.e., 2 relays), suggesting that the relay method for metabolite identification does not need to be run through multiple cycles like that described for intrinsic clearance (CLint measurements) (Di et al., 2012, 2013). Also, the relay method yielded a greater number of relevant human metabolites as compared with a previous evaluation of the MPCC system. This latter observation could be attributed to the 10-donor pool of hepatocytes used in the relay compared with the single-donor hepatocyte batch used in the coculture system as well as the higher number of hepatocytes used in the relay incubations (Wang et al., 2010).

In an effort to improve metabolite generation and detection, we subsequently modified the experiment. The incubation volume was doubled to provide a larger collection volume, the cell density was tripled, and the substrate transfer volume was increased from 60% transfer per day to 80% transfer per day to maximize the amount of substrate carried over to the following day. In addition, the cell fractions were included for metabolite identification. Despite the modifications, no detectable improvement was realized, with no additional metabolites being identified at any time points investigated. We hypothesized that the major influencing factor was the potential for parent and metabolites to sequester in the cells such that each transfer resulted in loss of drug-related material. Detection of a higher concentration of parent and metabolites in the cells for celecoxib, CP-122721, and ziprasidone compared with the supernatant supports this hypothesis (data not shown).

**Conclusions**

In all but one case, the hepatocyte relay successfully generated more of the major human excretory and circulating metabolites (Table 1) for this set of compounds compared with the MPCC system. Ziprasidone was the exception as it was better covered in the MPCC system compared with the relay method. This outcome was attributed to the very poor solubility of ziprasidone causing increased loss of parent compound during relays. The hepatocyte relay produced three of the four major human circulating and excreted metabolites of CP-122721, whereas four of the four major capromorelin metabolites were formed in the relay method compared with two in the coculture system. The relay method also produced complete metabolite profiles at the 8-hour

---

**TABLE 1**

Generation of major human metabolites in the hepatocyte relay method compared to suspended and MPCC hepatocytes

<table>
<thead>
<tr>
<th>Compound Name</th>
<th>Major In Vivo Human Metabolites</th>
<th>Excreta</th>
<th>Circulating Radioactivity</th>
<th>Biotransformation Phase</th>
<th>1/2° metabolite</th>
<th>Metabolites Detected in Matrix</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>%</td>
<td>%</td>
<td></td>
<td></td>
<td>Suspended Hepatocytes</td>
</tr>
<tr>
<td>Capromorelin</td>
<td>Nitrocapromorelin's (A)</td>
<td>11</td>
<td>14</td>
<td>1</td>
<td>2°</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>O-desbenzyl hydroxycapromorelin</td>
<td>12</td>
<td>1</td>
<td>2°</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>N-desmethyl-O-desbenzyl</td>
<td>12</td>
<td>1</td>
<td>2°</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>nitrocapromorelin' (C)</td>
<td>15</td>
<td>1</td>
<td>2°</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>N-desmethyl-O-desbenzyl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>hydroxycapromorelin (D)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CP-122721</td>
<td>Desmethyl O-glucuronide</td>
<td>27</td>
<td>14</td>
<td>2</td>
<td>2°</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>N-dealkylated, reduced O-glucuronide</td>
<td>11</td>
<td>2</td>
<td>2°</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Desmethylhydroxy O-glucuronide</td>
<td>25</td>
<td>2</td>
<td>2°</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>5-trifluoromethoxysalicylic acid</td>
<td>56</td>
<td>1</td>
<td>2°</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Ziprasidone</td>
<td>Sulfoxide</td>
<td>18</td>
<td>69</td>
<td>1</td>
<td>1°</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>S-methylhydroziprasidone</td>
<td>18</td>
<td>69</td>
<td>1</td>
<td>2°</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>N-dealkylated sulfoxide</td>
<td>11</td>
<td>21</td>
<td>1</td>
<td>2°</td>
<td>Yes</td>
</tr>
<tr>
<td>Celecoxib</td>
<td>Carboxylic acid</td>
<td>73</td>
<td>20</td>
<td>1</td>
<td>2°</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>Carboxylic acid glucuronide</td>
<td>45</td>
<td>15</td>
<td>2</td>
<td>2°</td>
<td>Yes</td>
</tr>
<tr>
<td>Linezolid</td>
<td>O-dealkylation/ring opening, carboxylic acid</td>
<td>10</td>
<td>1</td>
<td>2°</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>N-dealkylation/ring opening, carboxylic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

*Percentage of total drug-related material radioactivity in circulation.

Dalvie et al., 2009.

Micropatterned coculture hepatocytes (7-day incubation).

Wang et al., 2010.

Metabolites originally reported as carboxylic acids but later determined to be nitroalkanes (Pfizer internal communication).

MS negative mode detection only.
(2 relays) time point for every compound tested. Overall, the hepatocyte relay captured 75% of the human-relevant metabolites from the difficult subset of capromorelin, CP-122721, and ziprasidone compared with 50% for the micropatterned coculture system.

The hepatocyte relay and the coculture system produce more than just the major human circulating and excreted metabolites. The relay system also appears to be better suited for compounds that are expected to undergo multiple sequential metabolism events. Prediction of the exact human major circulating metabolites is still not possible from these systems; however, they provide a much richer view of the metabolic profile, providing more opportunity to capture the major metabolic routes earlier in the drug discovery and development process.

Acknowledgments

The authors thank Dr. Li Di for input in the support of these studies.

Pharmacokinetics, Dynamics and Metabolism, Pfizer, Inc., Groton, Connecticut

T. Eric Ballard

Christine C. Orozco

R. Scott Obach

Authorship Contributions

Participated in research design: Ballard, Orozco, Obach.

Conducted experiments: Ballard, Orozco.

Performed data analysis: Ballard, Obach.

Wrote or contributed to the writing of the manuscript: Ballard, Orozco, Obach.

References


Address correspondence to: T. Eric Ballard, Pharmacokinetics, Dynamics and Metabolism, Pfizer, Inc., Groton, CT 06340. E-mail: Thomas.E.Ballard@Pfizer.com