EVALUATION OF MICRODOSING TO ASSESS PHARMACOKINETIC LINEARITY IN RATS USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

Suresh K. Balani, Nelamangala V. Nagaraja, Mark G. Qian, Arnaldo O. Costa, J. Scott Daniels, Hua Yang, Prakash R. Shimoga, Jing-Tao Wu, Liang-Shang Gan, Frank W. Lee, and Gerald T. Miwa

DMPK, Drug Safety and Disposition, Millennium Pharmaceuticals, Inc., Cambridge, Massachusetts

Received September 1, 2005; accepted November 29, 2005

ABSTRACT:
The microdosing strategy allows for early assessment of human pharmacokinetics of new chemical entities using more limited safety assessment requirements than those requisite for a conventional phase I program. The current choice for evaluating microdosing is accelerator mass spectrometry (AMS) due to its ultra-sensitivity for detecting radiotracers. However, the AMS technique is still expensive to be used routinely and requires the preparation of radiolabeled compounds. This report describes a feasibility study with conventional liquid chromatography-tandem mass spectrometry (LC-MS/MS) technology for oral microdosing assessment in rats, a commonly used preclinical species. The non-labeled drugs fluconazole and tolbutamide were studied because of their similar pharmacokinetics characteristics in rats and humans. We demonstrate that pharmacokinetics can be readily characterized by LC-MS/MS at a microdose of 1 μg/kg for these molecules in rats, and, hence, LC-MS/MS should be adequate in human microdosing studies. The studies also exhibit linearity in exposure between the microdose and ≳1000-fold higher doses in rats for these drugs, which are known to show a linear dose-exposure relationship in the clinic, further substantiating the potential utility of LC-MS/MS in defining pharmacokinetics from the microdose of drugs. These data should increase confidence in the use of LC-MS/MS in microdose pharmacokinetics studies of new chemical entities in humans. Application of this approach is also described for an investigational compound, MLNX, in which the pharmacokinetics in rats were determined to be nonlinear, suggesting that MLNX pharmacokinetics at microdoses in humans also might not reflect those at the therapeutic doses. These preclinical studies demonstrate the potential applicability of using traditional LC-MS/MS for microdose pharmacokinetic assessment in humans.


This work was presented at the 13th North American ISSX meeting in Maui, Hawaii, Oct 23–27, 2005 (Oral Presentation #133; Drug Metab Rev Vol. 35, Suppl 2).

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org. doi:10.1124/dmd.105.007195.

ABBREVIATIONS: AMS, accelerator mass spectrometry; PK, pharmacokinetic; LC-MS/MS, liquid chromatography-tandem mass spectrometry; AUC, area under the curve; EMEA, European Agency for Evaluation of Medicinal Products; LLOQ, lower limit of quantitation.
only, which biases toward the use of AMS only for the microdosing purpose, even if one can establish no safety concerns at 1/100 of the projected therapeutic dose. It is prudent to demonstrate linear pharmacokinetics between microdoses and higher, therapeutically equivalent doses in an appropriate preclinical species before microdosing could be applied to humans to predict the PK at pharmacological doses. Although AMS is an excellent tool, it requires the preparation of radioactive drug and is costly for a routine use. Two orally administered drugs and an investigational compound with pharmacokinetic parameters similar in rats and humans (Table 1) were studied at microdoses in rats to determine whether conventional LC-MS/MS was sensitive enough to quantitate drug levels after microdosing. The linearity in exposures between micro- and macrodoses was also assessed, to investigate the utility of microdosing for predicting exposures at 100- to ≥1000-fold higher doses.

Materials and Methods

Fluconazole and tolbutamide were obtained from Sigma-Aldrich (St. Louis, MO). Fluconazole was obtained from MP Biomedicals (Aurora, OH). Compound MLNX was synthesized at Millennium Pharmaceuticals, Inc. All solvents and other chemicals were of analytical or HPLC grade.

PK Studies. Male jugular vein-cannulated Sprague-Dawley rats (n = 3–9; Hilltop Labs, Scottsdale, PA) were fasted overnight before oral dosing of the compounds. The compounds were formulated with 0.5% hydroxypropylmethylcellulose + 0.2% Tween 80 solution doses for oral administration. Fluconazole was administered at 5, 0.05, 0.005, and 0.001 mg/kg; tolbutamide at 1, 0.1, 0.01, 0.002, and 0.001 mg/kg; and MLNX at 10, 1, 0.1, and 0.01 mg/kg.

To minimize variability, a stock solution of each compound was prepared in 0.1% formic acid and each was supplemented with 0.1% formic acid. A YMC ODS-AQ column (55, 200A, 2.1 × 50 mm) (Waters, Milford, MA) was used for the separation at a flow rate of 0.4 ml/min under ambient temperature. An MDS SCIEX API–4000 mass spectrometer (MDS SCIEX, Toronto, ON, Canada) was used for analysis. Each compound was tuned on the mass spectrometer to establish a quantification method under the multiple reaction monitoring mode. For high dose (≥1 mg/kg) studies, the calibration standard curve ranged from 1 to 1000 nM. A 0.1 to 10 nM range was used for low MLNX dose studies. The accuracy of measurement was within ±20% of the nominal concentrations.

PK and Statistical Analysis. Noncompartmental PK parameters (AUC, CL, Vd, t1/2) were calculated using WinNonLin V4.1 (Pharsight, Mountain View, CA). Dose linearity tests on AUCinf, Cmax, and t1/2 were carried out by the regression of log-transformed data (power regression model) (Gough et al., 1995; Smith et al., 2000). Doses and PK parameters were log-transformed, and correlation coefficient (R2), slope, and 95% confidence intervals were calculated using the Data Analysis tool in Microsoft Excel (Microsoft, Redmond, WA). Inferences were made based on the theoretical slope of 1, and confidence limits of 0.8 and 1.25.

Results

Sensitive and specific bioanalytical assay methods were developed using LC-MS/MS. Using 100 μl of plasma sample and the API–4000 mass spectrometer, a concentration range of 1 to 1000 nM was readily established for all the compounds. The quantification of MLNX and fluconazole was extended to a lower quantification limit of 0.1 nM. However, because of the carryover, two separate concentration ranges were established (0.1–10 and 1–1000 nM) for analyzing samples from high and low doses of MLNX in rats.

Fluconazole. The plasma concentration-time profiles of fluconazole in rats after single oral doses are shown in Fig. 1, and the PK parameters are summarized in Table 2. The exposure increased proportionally in the dose range of 0.001 to 5 mg/kg.

Tolbutamide. The plasma concentration-time profiles of tolbutamide in rats after single oral doses are shown in Fig. 2, and the PK parameters are summarized in Table 2. The exposure increased proportionally in the dosing range of 0.001 to 1 mg/kg.

MLNX. The plasma concentration-time profiles of MLNX in rats after single oral doses are shown in Fig. 3, and the PK parameters are

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose Regimen</th>
<th>Bioavailability</th>
<th>CLp</th>
<th>Vdss</th>
<th>t1/2</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>400, q.d.</td>
<td>&gt;90</td>
<td>0.016</td>
<td>0.6</td>
<td>32</td>
<td>Debruyne and Ryckelynck (1993)</td>
</tr>
<tr>
<td>Tolbutamide</td>
<td>500, b.i.d.</td>
<td>93</td>
<td>0.014</td>
<td>0.1</td>
<td>6</td>
<td>Balant (1981)</td>
</tr>
<tr>
<td>MLNX</td>
<td>500, b.i.d.</td>
<td>&gt;90</td>
<td>0.42</td>
<td>–1</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

* Predicted.
The model compounds are summarized in Table 2. Plots of log concentration-time profiles of tolbutamide in rats (n = 3) after single oral doses of 0.001 (●), 0.002 (○), 0.01 (▲), 0.1 (▼), and 1 (▲) mg/kg. summarized in Table 2. The exposure was found to increase linearly over the dose range of 0.01 to 1 mg/kg, but nonlinearly between 1 and 10 mg/kg.

Results from regression analyses of the $C_{\text{max}}$, AUC_{int}, and $t_{1/2}$ data of the model compounds are summarized in Table 3. Plots of log-transformed $C_{\text{max}}$ and AUC_{int} data and the regression lines are shown in Fig. 4.

**Discussion**

Microdosing allows determination of pharmacokinetic parameters in humans at subpharmacological doses, which may then be extrapolated to those at therapeutically relevant doses. Currently, AMS is the only technology that offers the ultrasensitivity required for the quantitation of drugs using radiotracers. The level of sensitivity is reported to be 100- to 1000-fold lower than that which can be achieved using traditional mass spectrometry (Garner, 2000). However, AMS analysis is expensive and requires radiolabel drug, and, therefore, limits the broader application of microdosing studies in drug development. We have assessed the use of the traditional LC-MS/MS for microdosing in rats for two drugs, fluconazole and tolbutamide, which are known to show similar pharmacokinetic properties in humans and rats (Balant, 1981; Sawada et al., 1985; Debruyne and Ryckelynck, 1993; Yamao et al., 1994; Yang et al., 1996). The microdoses for rats were approximated on a mg/kg body weight basis (Fan et al., 1995) from the clinical doses of the two drugs. Thus, an oral microdose of 1 µg/kg for tolbutamide and fluconazole was studied in rats. For fluconazole and tolbutamide, the lower limit of quantitation was 0.1 and 1 nM, respectively. Because of the low plasma clearance, low volume of distribution, and high oral bioavailability recorded in the literature for these compounds, the plasma concentrations in rats declined slowly and were easily quantifiable in 24-h samples (Figs. 1 and 2). Thus, an LC-MS/MS sensitivity of 0.1 to 1 nM was adequate to support microdosing studies for these nonlabeled compounds in rats. By analogy, microdosing should also be assessable by LC-MS/MS in humans.

An aspect of microdosing is the evaluation of linearity of pharmacokinetics between the microdoses and the therapeutically equivalent doses of new chemical entities, leading to predictability of pharmacokinetic parameters at high doses from the microdose data. Before this aspect is applied in the clinical setting, it is prudent to demonstrate linearity in pharmacokinetic parameters in a relevant preclinical model that shows pharmacokinetic properties and metabolism broadly similar to those projected in humans. Preclinical species that can be used for allometric scaling (Boxenbaum, 1984) to project human pharmacokinetic parameters may be used as a preclinical model for microdosing. More than one preclinical species could also be used to strengthen the projection of linearity in humans, thereby increasing confidence in the utility of microdosing studies in humans. In the current study, demonstration of linear increases in exposure upon oral administrations between microdoses and higher doses in rats for tolbutamide and fluconazole, which are reported to show linear increases in exposure after oral doses in humans (Physicians’ Desk reference, www.pdr.net; Balant, 1981), would corroborate this system of microdosing for projecting pharmacokinetic parameters at high doses. These drugs were ideally suited for showing linear pharmacokinetics after administration of microdoses since both drugs are reported to show low CLP, low Vd, and high oral bioavailability and similar metabolism in rats and humans (Balant, 1981; Sawada et al., 1985; Debruyne and Ryckelynck, 1993; Yamao et al., 1994; Ashforth et al., 1995; Yang et al., 1996). Unlike high clearance compounds, these drugs were not expected to exhibit saturation of first-pass effect and nonlinear kinetics. However, the microdosing assessment using LC-MS/MS is expected to be applicable to compounds with varied pharmacokinetic properties. Tolbutamide was tested in rats with higher oral doses of 0.002, 0.01, 0.1, and 1 mg/kg, and fluconazole was tested at higher doses of 0.005, 0.05, and 5 mg/kg. Doses higher...
than 1 mg/kg were not tested for tolbutamide because comparison of exposure with a published report (Yamao et al., 1994) showed that in rats, this compound, at a dose of 13 mg/kg, gave a linear increase in AUC, within a 2-fold margin of error. The exposures at various doses of these molecules in rats are included in Table 2. The terminal disposition phases at different doses were parallel to each other. The exposures as measured by \(C_{\text{max}}\) and AUC\(_{\text{0-inf}}\) were linear over the \(\approx 1000\)-fold dose ranges studied for these compounds, allowing for 2-fold variability. This was also evident by the power regression analysis of the plots of exposure versus dose (Fig. 4; Table 3). These results establish the use of LC-MS/MS for microdosing assessments.

The published plasma \(C_{\text{max}}\) for fluconazole after a 400-mg oral dose to human subjects is 62 to 100 \(\mu\)M (Grant and Clissold, 1990). Assuming pharmacokinetic linearity to microdose, the plasma \(C_{\text{max}}\) after a 100-\(\mu\)g dose (current maximum microdose limit; EMEA and Food and Drug Administration guidelines) would be 15 to 25 nM. Given its monoexponential decay and a long elimination half-life of \(\approx 30\) h, it could be adequately monitored by LC-MS/MS with an LC-MS/MS sensitivity of 1 nM. Thus, with an LC-MS/MS sensitivity of 1 nM, tolbutamide could also be adequately monitored by LC-MS/MS for characterization of its pharmacokinetics at therapeutic doses.

We have applied the microdosing approach on an investigational compound, MLNX, which in rats showed CL\(_{\text{p}}\) of 1.0 l/h/kg, \(V_d\) of 1.2 l/kg, and \(F\) of \(>95\%\), properties broadly similar to those projected by allometric scaling for humans (Table 1), and the in vitro metabolism of MLNX also was similar in the two species (internal communication). The clinical oral dose of MLNX is predicted to be 10 mg/kg b.i.d. Accordingly, the doses of MLNX studies in rats were 0.01, 1, and 10 mg/kg. MLNX is ionized well on LC-MS/MS and gave a strong signal, leading to an LLOQ of 0.1 nM. The two methods used for plasma collection at the lowest dose of 0.01 mg/kg, sample pooling, and individual animal sampling per time point with blood transfusion from donor rats generally gave similar results (Table 2). Thus, either method for obtaining larger volumes of plasma could be adopted for sample collections and could possibly enhance the LLOQ.

At low doses, MLNX showed quantifiable concentrations only up to 8 h. Because the half-life of the compound was short, extrapolation of AUC to infinity added only a fractional area (\(\leq 10\%\)) to AUC\(_{0–8\text{h}}\). Therefore, an AUC\(_{0–\text{inf}}\) comparison across dose groups was considered appropriate for this compound. The increase in exposure in rats was linear in the dose range of 0.01 to 1 mg/kg (Fig. 4); however, nonlinearity in the exposure was observed between 1 and 10 mg/kg. Because of this nonlinearity at the high dose, it was not considered necessary to assess doses lower than 10 \(\mu\)g/kg. These results showed that MLNX was assessable for microdosing using LC-MS/MS, and also suggested that microdosing to determine the pharmacokinetics at therapeutic doses might not work for this investigational compound in humans.

Overall, oral microdose pharmacokinetics of tolbutamide, fluconazole, and MLNX were successfully characterized in rats using the LC-MS/MS approach. The results establish the use of LC-MS/MS for microdosing assessments.
conventional LC-MS/MS technique. Linearity of exposure between microdoses and ≥1000-fold higher doses in rats was demonstrated for tolbutamide and fluconazole, which are known to show linear pharmacokinetics in humans, providing a validation of the system. Application of this preclinical approach to the investigational compound MLNX showed that the exposures at therapeutic doses may not be predictable from the microdoses in humans. Thus, with the current trends toward higher-sensitivity LC-MS/MS systems, the potential of conducting microdosing studies in the clinic without radiolabeled compounds is becoming feasible.

Acknowledgments. We thank Kym Cardoza, Emily Guan, and Matt Gallacher of Comparative Medicine for expert handling of the in-life portion of rat studies, and Susan Colson of Drug Safety and Disposition for expert proofreading.

References

Address correspondence to: Dr. S. K. Balani, DMPK, Drug Safety & Disposition, Millennium Pharmaceuticals, Inc., 45 Sidney Street, Cambridge, MA 02199. E-mail: suresh.balani@mpi.com