Determination of *in vitro* metabolic half life \( (t_{1/2}) \) and *in vitro* hepatic intrinsic clearance \( (Cl_{int}) \)
3.1 Introduction

Metabolic stability (defined as the rate of metabolism) assay is performed to screen compounds and identify the lead candidate compound. These assays are usually performed using product monitoring approach [1] or in vitro $t_{1/2}$ approach [2]. In vitro $t_{1/2}$ approach involves the quantification of substrate consumption with respect to time at a single drug concentration below $K_m$ [2, 3]. Thus, in vitro $t_{1/2}$ and in vitro $Cl_{int}$ associated with metabolic stability data helps in ranking compounds prior to development by pharmaceutical companies [2]. Moreover, in vitro $Cl_{int}$ is used for prediction of in vivo behavior of the compound.

3.2 Experimental

3.2.1 Materials

Chemicals

CDRI 99/411 (Figure 14 of chapter 1) and CDRI 99/357 (used as an internal standard, IS; Figure 1) were synthesized by the Medicinal and Process Chemistry division of CDRI. HPLC-grade acetonitrile (ACN), tris (hydroxymethyl)aminomethane (tris base), KCl, MgCl$_2$·6H$_2$O, and a reduced form of β-nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sisco Research Laboratory (Mumbai, Maharashtra, India). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was obtained from USB Corporation (Cleveland, Oh., USA). Male human liver microsomes were procured from Sigma (St Louis, MO, USA).

Instrument

HPLC-PDA (Shimadzu, Kyoto, Japan), refrigerated centrifuge (Sigma 15K, Osterode am Harz, Germany).
3.2.2. Chromatographic Conditions

The HPLC system (Shimadzu, Kyoto, Japan) consisted of a binary pump (UFLC, LC 20AD), a vacuum degasser (DGU-20A5), an autosampler with temperature-controlled peltier tray (SIL-HTC) and a photodiode array detector (SPD-M20A). The system was operated in isocratic mode in a 90:10 ratio of ultrasonically degassed ACN and triple-distilled water, respectively. The mobile phase was filtered through a 0.22μm Millipore filter (Billerica, MA, USA) prior to use. Chromatographic separations were achieved at a flow rate of 1.5 mL/min on a Spheri-5 ODS Perkin Elmer Brownlee Column (5 μm, 220 × 4.6 mm) with a run time of 15 min. The wavelength was set at 247nm and the injection volume was 50 μL [4].

3.2.3. Stock Solutions and Sample

Primary stock solution of calibration curve (CC) and quality control (QC) samples of CDRI 99/411 were prepared separately (from separate weighings). The primary stock solution of 1 mg/mL of CDRI 99/411 and IS were prepared in ACN and stored at 4°C. Working stock solutions of 2, 4, 8, 20, 40, 160 and 200 μg/mL were prepared by appropriate dilutions of primary stock solution in ACN. This set of working stock was used to prepare standards for the CC. Another set of working stock solutions of QC (8, 40
and 160 µg/mL) samples of CDRI 99/411 was prepared in ACN. On the day of analysis, working stock solutions of CC and QC were spiked into heat-denatured microsomes for preparation of CC and QC samples.

Calibration standards (CS) were prepared by spiking 5 µL of appropriate working stock solution of CC in 195 µL of diluted heat denatured microsomes (1 mg/mL). Similarly, samples for the determination of precision and accuracy were prepared by spiking 5 µL of working stock solution of QC at three final concentrations of 0.2 µg/mL (QC low), 1.0 µg/mL (QC medium) and 4.0 µg/mL (QC high) in diluted heat denatured microsomes (1 mg/mL).

### 3.2.4. Sample Preparation

The samples were prepared by simple 1:1 protein precipitation method. Ice-cold ACN spiked with 2 µg/mL of IS was used as the precipitating solvent. Protein-precipitated samples were vigorously vortex mixed for 4 min at room temperature and centrifuged at 15,500g at 4°C for 10 min.

### 3.2.5. Assay Validation

**Recovery.** The recoveries of CDRI 99/411 and IS were determined by comparing the response of the extracted analytes (from biological matrix) of replicate QC samples (n = 6) with the replicate (n = 6) response of analytes prepared at equivalent concentrations in ACN (analytical standard). Recoveries of CDRI 99/411 were determined at QC low, QC medium and QC high. The recovery of IS was determined at a single concentration of 2 µg/mL.

**Calibration curve.** The CC was acquired by plotting the ratio of sum of peak area of CDRI 99/411 to that of IS against the nominal concentration of CS. The final concentrations of CS were obtained by plotting the CC from 0.1 to 5.0 µg/mL (0.1, 0.2, 0.5, 1.0, 4.0 and 5.0 µg/mL). The results were fitted to linear regression analysis using 1/X² as the weighting factor.
Accuracy and precision. Accuracy and precision were determined at QC low, QC medium and QC high. Six replicates of each QC sample analyzed on single day were used for the determination of intra-day accuracy and precision. Six replicates of each QC sample analyzed on three different days were used for the determination of inter-day accuracy and precision. Accuracy and precision were expressed in terms of percentage bias and percentage relative standard deviation (RSD).

3.2.6 Estimation of in vitro (t$_{1/2}$) and in vitro hepatic Cl$_{int}$

CDRI 99/411 and testosterone were incubated in a shaking water bath at concentrations of 2 and 4 μM, respectively, in an incubation mixture of 0.1 M Tris buffer, 5 mM MgCl$_2$·6H$_2$O and 0.4 mg/mL of microsomes (rat and human and only human for testosterone) at 37 ± 1°C. The reaction was commenced with NADPH (2mM) after a preincubation of 4 min. Table 1 summarizes the incubation milieu. Summary of assay conditions used for the determination of metabolic stability CDRI 99/411 and testosterone is shown in Table 2.

<table>
<thead>
<tr>
<th>Incubation Composition</th>
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</thead>
<tbody>
<tr>
<td>Tris buffer</td>
<td>0.1M, pH 7.4</td>
</tr>
<tr>
<td>MgCl$_2$·6H$_2$O</td>
<td>5 mM</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.4 mg/mL</td>
</tr>
<tr>
<td>NADPH</td>
<td>2 mM</td>
</tr>
<tr>
<td>Preincubation time</td>
<td>4 min</td>
</tr>
<tr>
<td>Temperature</td>
<td>37 ± 1°C</td>
</tr>
</tbody>
</table>

Table 1. Incubation milieu
Table 2. Summary of assay conditions used for the determination of metabolic stability CDRI 99/411 and testosterone.

<table>
<thead>
<tr>
<th>Analytical conditions for CDRI 99/411</th>
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<tbody>
<tr>
<td>Analyte</td>
</tr>
<tr>
<td>Internal Standard</td>
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<tr>
<td>Column</td>
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<tr>
<td>Flow rate</td>
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<tr>
<td>Injection volume</td>
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<tr>
<td>Mobile phase</td>
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<tr>
<td>Isocratic</td>
</tr>
<tr>
<td>Lambda Max</td>
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<table>
<thead>
<tr>
<th>Analytical conditions for Testosterone (Positive Control)</th>
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<tbody>
<tr>
<td>Analyte</td>
</tr>
<tr>
<td>Internal Standard</td>
</tr>
<tr>
<td>Column</td>
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<tr>
<td>Flow rate</td>
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<tr>
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<tbody>
<tr>
<td>CDRI 99/411</td>
</tr>
<tr>
<td>Testosterone</td>
</tr>
<tr>
<td>Microsomes</td>
</tr>
<tr>
<td>Incubation time</td>
</tr>
<tr>
<td>Sample preparation</td>
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<tr>
<td>Incubation sets</td>
</tr>
</tbody>
</table>
Calculation

a) *In vitro* metabolic $t_{1/2}$ was calculated using GraphPad Prism 4.0 software. Data was fit to One Phase Exponential Decay

\[ Y = \text{Span} \cdot \exp(-K \cdot X) + \text{Plateau} \]

Starts at $\text{Span} + \text{Plateau}$ and decays to $\text{Plateau}$ with a rate constant $K$.

The half life is $\frac{0.69}{K}$. \hspace{1cm} \text{Equation 1}

b) *In vitro* hepatic $Cl_{int}$ was calculated from the equation

\[ Cl_{int} = \frac{0.693}{in \text{ vitro } t_{1/2}} \cdot \frac{\mu L \text{ incubation}}{mg \text{ protein}} \hspace{1cm} \text{Equation 2} \]

Where, $Cl_{int}$ is *in vitro* hepatic intrinsic clearance, *in vitro* $t_{1/2}$ is *in vitro* metabolic half life of drug, $\mu L \text{ incubation}$ is the volume of incubation and $mg \text{ protein}$ is the amount of microsomal protein in incubation.

3.3 Results and Discussion

3.3.1 Chromatographic Conditions

Chromatographic conditions were optimized by the selection of appropriate column and mobile phase composition. It was observed that 90% ACN and 10% of triple distilled water was suitable (peak shape and resolution) for the elution of CDRI 99/411 and IS (Figure 2).
Figure 2. HPLC-PDA chromatograms of extracted blank and extracted QC high. The chromatographic conditions are written in the text.

3.3.2 Assay Validation

Recovery. Recovery of the CDRI 99/411 was expressed as percentage recovered at QC low, QC medium and QC high (Table 3). Recovery of IS was expressed as percentage recovered at 2 μg/mL. The average recovery of IS was 95% ± 0.64 (mean ± SD).

Calibration curve, accuracy and precision. The CC was constructed using six calibration standard (0.1–5 μg/mL). The CS curve was reproducible at the concentration range 0.1–5.0 μg/mL. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) vs concentration and using the linear regression equation $y = mx + c$ with $1/X^2$ as the weighting factor. The coefficient of correlation was found to be ≥0.99 for the range 0.1–5.0 μg/mL.

Accuracy and precision data for intra-day and inter-day are presented in Table 3. The assay values on both the occasions (intra-day and inter-day) were found to be within the accepted variable limits.
Table 3. Intra-day and inter-day accuracy and precision and percentage recovery of CDRI 99/411 in rat liver microsomes (n = 3 days with six replicates per day)

<table>
<thead>
<tr>
<th>QC (ng/mL)</th>
<th>Accuracy (% bias)</th>
<th>Precision (% RSD)</th>
<th>% Recovery mean ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Intra-day</td>
<td>Inter-day</td>
<td>Intra-day</td>
</tr>
<tr>
<td>LQC (200)</td>
<td>-4.11</td>
<td>4.39</td>
<td>5.57</td>
</tr>
<tr>
<td>MQC (1000)</td>
<td>5.42</td>
<td>5.03</td>
<td>2.27</td>
</tr>
<tr>
<td>HQC (4000)</td>
<td>-2.74</td>
<td>-2.32</td>
<td>2.78</td>
</tr>
</tbody>
</table>

% bias is [(Final concentration-Nominal concentration) / Nominal concentration] * 100
% RSD is (Standard deviation / Mean)
S.D. is Standard deviation

3.3.3 Estimation of in vitro \( t_{1/2} \) and in vitro hepatic \( C_{l\text{int}} \) with rat and human liver microsomes

Inter-species profiling of metabolic stability of a drug candidate is performed to assess its potential to form undesirable metabolites (potentially toxic or pharmacologically inactive metabolites due to phase I metabolism) or accumulate in the body due to lacking or negligible metabolic degradation.

Metabolic stability of CDRI 99/411 was determined in liver microsomes of rat and human at a concentration of 2 \( \mu \text{M} \). CDRI 99/411 metabolized in the presence of NADPH in liver microsomes of both species (rat and human). This was inferred from experimental observations of NADPH supplemented and deficient incubation sets \( (p < 0.05, \text{ Student } t \text{ test}) \). The data shown in Figure 3a and Figure 3b was fit to mono-exponential decay model (with 1/Y weighting factor) as described in equation (1) and the coefficients of correlation \( (r) \) were better than \( \geq 0.98 \).

In vitro \( t_{1/2} \) and in vitro \( C_{l\text{int}} \) (intrinsic clearance is a measure of enzyme kinetics towards substrate independent of physiological determinants like flow of blood and binding of protein) were determined from equations 1 and 2, respectively. The observed in vitro \( t_{1/2} \) (13 and 19 min in rat and human, respectively) indicates moderate
degradation of CDRI 99/411 in liver microsomes of rat and human. Similarly, the observed \textit{in vitro} \( Cl_{int} \) as shown in Figure 4 (130.7 ± 25.0 and 89.3 ± 17.4 \( \mu \text{L/min/mg} \) of microsomes in rat and human, respectively with \( p > 0.05 \) between rats and human as determined by Student \( t \) test) indicates moderate hepatic \textit{in vitro} \( Cl_{int} \) of CDRI 99/411 [5-7]. The experimental determinations suggest insignificant difference in the metabolic stability profile of CDRI 99/411 in rats and humans. Hence, rat can be selected as an animal model for preliminary evaluation of pharmacokinetics and metabolism (\textit{in vitro} and \textit{in vivo}) of CDRI 99/411.

Testosterone was taken as a positive control. Depletion data of testosterone was fit to mono-exponential decay equation (equation 1) with \( 1/Y \) weighting factor. At \( r \geq 0.97 \) the \textit{in vitro} \( t_{1/2} \) of testosterone was 31 min in male human liver microsomes. Hence, the calculated value of \textit{in vitro} \( Cl_{int} \) (equation 2) of testosterone was 54.8 ± 4.2 \( \mu \text{L/min/mg} \) which was in the reported range 60 ± 18 \( \mu \text{L/min/mg} \) in human liver microsomes [6].
Figure 3. Depletion profile of CDRI 99/411 in (a) rat and (b) human liver microsomes (mean ± S.E. of triplicate experiments).

(a)

\[ Y = \text{Span} \times e^{-K \times X} + \text{Plateau} \]
Starts at \( \text{Span} + \text{Plateau} \) and decays to \( \text{Plateau} \) with a rate constant \( K \)

\[ t_{1/2} = \frac{0.69}{K} \]

Best-fit values

\[ K = 0.05231 \]
\[ \text{HalfLife} = 13.25 \]

(b)

\[ Y = \text{Span} \times e^{-K \times X} + \text{Plateau} \]
Starts at \( \text{Span} + \text{Plateau} \) and decays to \( \text{Plateau} \) with a rate constant \( K \)

\[ t_{1/2} = \frac{0.69}{K} \]

Best-fit values

\[ K = 0.03577 \]
\[ \text{HalfLife} = 19.38 \]
Figure 4. *In vitro* hepatic intrinsic clearance in rat and human liver microsomes (mean ± S.E. *p* > 0.05 between rat and human).

3.4 Conclusion

*In vitro* metabolic investigations of CDRI 99/411 were conducted by using a validated HPLC-PDA method. It was observed that CDRI 99/411 exhibited moderate metabolic stability with male rat and human liver microsomes.
References


