INTRODUCTION

With the dramatic increase in the number of new chemical entities (NCEs) arising from combinatorial chemistry and high-throughput processes, novel bioanalytical techniques are required for the rapid determination of the metabolites of these NCEs. Knowledge of the metabolic sites of the NCEs in early drug discovery is essential for selecting compounds with favorable pharmacokinetic. In development, elucidation of biotransformation pathways of a drug candidate it is important to understand its physiological effects. Liquid chromatography (LC) coupled with atmospheric pressure ionization (API) MS has now become the most powerful tool for the rapid detection, structure elucidation, and quantification of drug-derived material within various biological fluids [1].

Metabolism is a biochemical process by which endogenous compounds and xenobiotics are converted to more hydrophilic (water soluble) entities, which enhance their elimination from the body. In general, metabolites are pharmaceutically less active and less toxic than their corresponding parent compound. However, it is not uncommon that biotransformation reactions also lead to formation of pharmacologically active metabolites, drug–drug interactions via inhibition or induction of drug metabolizing enzymes and/or formation of toxic metabolites. Therefore, determination of an NCE’s metabolic rate, biotransformation pathways in animals and humans, and pharmacological and toxicological consequences of its metabolites are very critical to pharmaceutical research and compound progression in early discovery [2-4].

Simple in vitro systems, such as subcellular liver fractions or hepatocytes, are used to determine the metabolic fates of NCEs. The information from these studies can assist medicinal chemists to synthesize rationally metabolically stable analogs by blocking the site of metabolism, which could result in the discovery of NCEs with superior pharmacology and safety. Metabolic pathways of drug candidates elucidated in laboratory animals provide guidance for the selection of animal species used for safety evaluation studies to ensure that the selected animal species are exposed to all major metabolites formed in humans. In the development phase, the metabolic profiles of an NCE are determined definitively in preclinical species and humans following its radiolabel administration. Subsequently, major circulatory metabolites in humans can be synthesized for the evaluation of their pharmacological activity. Therefore, determination of an NCE’s metabolic rate, biotransformation pathways in animals and humans, and pharmacological and toxicological consequences of its metabolites are very critical to pharmaceutical research and compound progression [5].

The Cytochrome P450 is a super family of haeme proteins that catalyze the metabolism of a large number of xenobiotics and endobiotics. The type and amount of the CYP enzymes expressed, primarily in the liver, determine the metabolic responses in that species. A majority of the CYP enzymes involved in hepatic drug metabolism has been identified and about 12 human drug-metabolizing CYP enzymes have been characterized at the molecular level. The various CYP enzymes differ in their substrate specificity and hence the metabolism of the probe substrate in various species [6]. Thus by observing the role of CYP enzymes in the metabolism of the Test compound help in identifying the safe behaviour of the compound in the body aiding it to move on to the next step in the drug discovery.

The Food and Drug Administration in 1999 indicates that investigators can use in vitro drug interaction data to conclude that a new drug does not inhibit a specific P450 activity. In practice, the in vitro evidence is usually collected from one probe reaction per enzyme, and the conclusion is extrapolated to all such substrates for that particular enzyme [7].

The work up would promote
1. Identification of the substrate metabolic pathway of the potential drug candidate at an early stage using analytical techniques.
2. Develop in vitro results that provide a reliable extrapolation to in vivo stating a clear mechanistic way of CYP role.
3. Aid in preclinical screening of the NCE’s metabolism without utilization of animals

Sample preparation and the concentrations of the drug in the presence of the specific CYP help in determining the mechanistic pathway of the metabolism and the potential safety of the Test compound.

This study characterizes the metabolic pathway of the new drug and the potential for the Test Compound to stand as new drug using Tandem Mass spectrometry. The prediction of the potential of the new drug in withstanding the perpetuated screening process while competing with other drugs relies on the evaluation of the new drug on the rate of a reaction that represents a specific P450 enzyme activity, and these studies are adding value and made mandatory. [8-11].

**EXPERIMENTAL SECTION**

**Chemicals:** Test Compound from GPRCP, Potassium Phosphate Mono Basic (PBS) from Sigma Aldrich, Potassium Hydroxide (KOH) and Magnesium Chloride (MgCl2) from Merck, NADPH Regenerating Solution (NRS), Glucose - 6- Phosphate Dehydrogenase and Ammonium formate obtained from Sigma Aldrich, Formic acid from Fluka and the Microsomes from Xenotech. Methanol (MeOH), Acetonitrile of analytical grade and supplied by J.T Bakers. Probe Substrates were obtained from Sigma.

*Note:* No specific safety considerations apply to any of these agents, although the agents shall be handled with care in a safety fume hood to avoid inhalation and contamination.

**Procedure For In Vitro Sample Preparation for Metabolite Identification**

A typical incubation (final volume 0.2 ml) consisted of 0.1 mg of microsomal protein in 100 mM potassium phosphate buffer (pH 7.4).

Stock solution of Test compound was prepared in methanol (final concentration of methanol was less than 0.3% v/v) (approximately 10µM/incubation) drug. The drug, buffer, and microsomes were mixed and pre incubated at 37°C for 4 min.

Then the incubations were initiated by the addition of the NADPH, and conducted at 37°C for 60 min. For control incubations, NADPH was omitted. Reactions were terminated upon addition of 200 ml of Acetonitrile, after which samples were vortexed and centrifuged for 15 minutes at 14,000 rpm. The subsequent supernatants were evaporated and reconstituted using 50µL of 90: 10 water and Acetonitrile with 0.1% Formic acid and 10µL solution was injected onto LC Q TOF for analysis.

**UPLC:** Analysis of the Test compound was achieved using the Waters Acquity UPLC system. The Waters Acquity system consisted of a binary UPLC Pump, column oven, a sample manager, and a dual UV detector.

Separation was carried out on an analytical column of X Bridge C18 (250 × 4.6 mm, 3 µ 5 mm particle size) in a column oven maintained at 25°C.

The mobile phase used consisted of solvent A (10 mM Ammonium formate in water) and solvent B (0.1% Formic acid in Acetonitrile).

Initial mobile phase conditions (100% solvent A) at a rate of 0.2 ml/min were held for 5 min, followed by a step gradient to 30% solvent B in 45 min, followed by a second step gradient to 95% solvent B in 10 min, the final conditions were held for 5 min, then returned to the original starting conditions.

**Mass Spectrometry:** High-resolution mass spectrometric measurement was performed using Quadruple Time of Flight mass spectrometer with dual orthogonal Z Spray ESI Source. The Q TOF was operated under V-Mode and calibrated with polyethylene glycol, 50 pg/µL Leucine Enkephalin was used as lock spray at a flow rate of 3 µL/min, Electron Spray Ionization under positive ion mode with collision energy ramp of 20 to 40 eV under MS1 scan were used all along the retention window of 0 – 60 mins. The capillary and tube lens voltages were 32 and 60 V respectively. Nitrogen was used as a drying gas at a sheath pressure of 78 psi with auxiliary flow.

**Drug Metabolite Identification for Test Compound**

The Test compound is an NCE / KSL 03 synthesized with an amino linkage in between main scaffold and a Glycol linkage found to be showing therapeutic effect and is considered for metabolic studies. Figures 1& 2.
Figure 1: Test Compound
1-(2-(2-(3, 4-dichlorophenyl)-N-methylacetamido)-2-phenylethyl)-3-((2-(2-(2-
methoxyethoxy)ethoxy)ethyl)amino)pyrroloidin-1-ium
Chemical Formula: C_{28}H_{40}Cl_{2}N_{3}O_{4}^+ Exact Mass: 552.24

Figure 2: MS and MS/MS spectra of Test Compound
The incubated sample were run on analytical tool LC-Q-TOF Mass spectrometry. The MS spectrum acquired after 60 minutes of incubation along the retention times for the resolved chromatographic peaks depicted the formation of various metabolites, Figures 3 & 4.

Figure 3: Chromatograph of Blank Sample of Rat Liver Microsomes (RLM)
The hypothetical proposed metabolic changes which are likely are the
1. oxidative cleavages and carboxylate metabolites formation on one side and
2. Aromatic Hydroxylation along with the oxidative cleavages and carboxylate metabolites
   formation on other side.
These two types of oxidative metabolites observed were confirmed by taking the MS/MS and Accurate
mass analysis data.

RESULTS AND DISCUSSION

The MS spectrum acquired after 60 minutes of incubation along the retention times for the resolved
chromatographic peaks depicted the formation of various metabolites; the metabolite numbering was
given based on their retention times starting from the retention time of Test compound, Table 1.

**Oxidation Cleavage by the CYP Enzymes**

The MS spectrum acquired after 60 minutes of incubation, is observed all along the retention times for
the resolved chromatographic peaks. The peaks depicted the formation of various metabolites, and the
approach for drug metabolite identification with HR-MS, is accomplished via post-acquisition data mining
where the chromatograph obtained were checked of their full-scan HR-MS and MS/MS data sets over the
entire retention window. Once metabolite ions were found, multistage product ion scans (MSn) were
carried out to obtain more detailed fragmentation pathways for structure elucidation. HR-MS instrument
is utilized for the determination of MS/MS and empirical formulae of metabolites. The mass-based
identification was observed as the main annotation technique for, all the resolved chromatographic
peaks.

The full-scan HR-MS and MS/MS data sets acquired and processed were with an intensity-dependent
method. This process help in finding expected metabolites by following predicted molecular masses
based on the similarity of metabolites to that of the parent drug. To state, more clearly - the fragmentation
of the parent would give m/z 320 which can be cross checked in the MS/MS of the metabolites formed
during the biotransformation process.

Fragment ion which is likely to be observed during fragmentation of parent is m/z 320

**Figure 4: Chromatograph of KSL 03 upon 60 min incubation in RLM**

**Figure 5.0: MS/MS Fragment of Parent**
So metabolites formed from parent shall have $m/z$ 320 as fragment at most of the times, as they are formed from parent, this is taken as an important annotation technique for identification of metabolites formed all along the Chromatogram.

Hence the use of MS/MS of the Test compound and the metabolites is important in detecting the metabolites, while the elemental compositions and their respective accurate masses with a mass difference of less than 35 mDa within a PPM error of less than 20 would give, a high assurance of the data generated thus aiding in confirmation of the structures proposed.

The MS/MS Spectra of Oxidative Metabolites were shown from Figure 5.1 to 5.7 respectively.

Figure 5.1: MS/MS spectra of Parent $m/z$ 552.24

Figure 5.2: MS/MS spectra of $m/z$ 538.22 M1

Figure 5.3: MS/MS spectra of $m/z$ 552.20 M2
Note: The carboxylate metabolite of the parent with m/z 552 in Figure 5.3 observed can be differentiated from parent by its retention time and the elemental composition.

Figure 5.4: MS/MS spectra of m/z 494.20 M3

Figure 5.5: MS/MS spectra of m/z 508.18 M4

Figure 5.6: MS/MS spectra of m/z 450.17 M5
The Test compound is having two aromatic rings which make it prone to aromatic hydroxylation by the microsomal enzymes. Hence the fragmentation of the parent with aromatic hydroxylation can be observed after microsomal incubation.

The Test compound upon aromatic Hydroxylation would be with m/z 568, so when the metabolite, m/z 568 undergoes fragmentation it would give m/z 326 ion as fragment by the MS. Implies all the metabolites formed after aromatic hydroxylation from metabolite m/z 568 shall have m/z 326 as the fragment.

Fragment ion to be observed for Aromatic Hydroxylated Test compound is m/z 326.
Figure 6.2: MS/MS spectra of m/z 554.22  M8

Figure 6.3: MS/MS spectra of m/z 568.23  M9

Note: Similar metabolites with m/z 568 were observed in Figure 6.3 and Figure 6.1. The aromatic hydroxylated metabolite is differentiated from its carboxylate metabolite by its retention time and the elemental composition.

Figure 6.4: MS/MS spectra of m/z 510.11  M10
Figure 6.5: MS/MS spectra of m/z 524.19  M11

Figure 6.6: MS/MS spectra of m/z 466.17  M12

Figure 6.7: MS/MS spectra of m/z 478.17  M13
Elemental Composition of the Metabolites Confirmed for Oxidation

The use of MS/MS data for the test compound and the metabolites is important in detecting the metabolites, while the elemental compositions and their respective accurate masses with a mass difference of less than 35 mDa within a PPM error of less than 20 would give a high assurance of the data generated thus aiding in confirmation of the structures proposed.

The co-injection of analyte and the lock mass compound directly into ion source give authenticated mass measurements all through the MS and MS/MS scans which is an instrument driven check for the reliability of results.

The Elemental Composition of all the metabolites observed were shown from Figure 7.1 to 7.15 respectively.

![Figure 6.8: MS/MS spectra of m/z 422.14 M14](image)

![Figure 7.1: Elemental Composition of Parent](image)

![Figure 7.2: Elemental Composition of M1](image)
Figure 7.3: Elemental Composition of M2

Figure 7.4: Elemental Composition of M3

Figure 7.5: Elemental Composition of M4
Figure 7.6: Elemental Composition of M5

Figure 7.7: Elemental Composition of M6

Figure 7.8: Elemental Composition of M7
Figure 7.9: Elemental Composition of M8

Figure 7.10: Elemental Composition of M9

Figure 7.11: Elemental Composition of M10
Figure 7.12: Elemental Composition of M11

Figure 7.13: Elemental Composition of M12

Figure 7.14: Elemental Composition of M13
The Accurate mass analysis of Test Compound and its metabolites are listed under Table 1.

Table 1: The Accurate mass analysis of Test Compound and its metabolites detected along with their elemental composition

<table>
<thead>
<tr>
<th>Name</th>
<th>Observed Mass (m/z)</th>
<th>CalcMass (m/z)</th>
<th>mDa</th>
<th>PPM</th>
<th>Molecular Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>552.2170</td>
<td>552.2472</td>
<td>-30.2</td>
<td>-54.7</td>
<td>C28H40Cl2N3O4</td>
</tr>
<tr>
<td>M1</td>
<td>538.2162</td>
<td>538.2198</td>
<td>-3.6</td>
<td>-6.7</td>
<td>C27H38Cl2N3O4</td>
</tr>
<tr>
<td>M2</td>
<td>552.2094</td>
<td>552.2032</td>
<td>6.2</td>
<td>11.2</td>
<td>C27H36Cl2N3O5</td>
</tr>
<tr>
<td>M4</td>
<td>508.1933</td>
<td>508.1626</td>
<td>30.7</td>
<td>60.4</td>
<td>C25H32Cl2N3O4</td>
</tr>
<tr>
<td>M5</td>
<td>450.1739</td>
<td>450.1715</td>
<td>2.4</td>
<td>5.3</td>
<td>C23H30Cl2N3O2</td>
</tr>
<tr>
<td>M6</td>
<td>406.1441</td>
<td>406.1453</td>
<td>-1.2</td>
<td>-3.0</td>
<td>C21H26Cl2N3O</td>
</tr>
<tr>
<td>M7</td>
<td>568.2393</td>
<td>568.2406</td>
<td>-1.3</td>
<td>-2.3</td>
<td>C28H40Cl2N3O5</td>
</tr>
<tr>
<td>M8</td>
<td>554.2202</td>
<td>554.2189</td>
<td>1.3</td>
<td>2.3</td>
<td>C27H38Cl2N3O5</td>
</tr>
<tr>
<td>M9</td>
<td>568.2332</td>
<td>568.1981</td>
<td>35.1</td>
<td>61.8</td>
<td>C27H36Cl2N3O6</td>
</tr>
<tr>
<td>M10</td>
<td>510.1942</td>
<td>510.1926</td>
<td>1.6</td>
<td>3.1</td>
<td>C25H34Cl2N3O4</td>
</tr>
<tr>
<td>M11</td>
<td>524.1930</td>
<td>524.1914</td>
<td>1.6</td>
<td>3.5</td>
<td>C25H32Cl2N3O5</td>
</tr>
<tr>
<td>M12</td>
<td>466.1694</td>
<td>466.1664</td>
<td>3.0</td>
<td>6.4</td>
<td>C23H30Cl2N3O3</td>
</tr>
<tr>
<td>M13</td>
<td>478.1694</td>
<td>478.1664</td>
<td>4.0</td>
<td>7.5</td>
<td>C23H26Cl2N3O4</td>
</tr>
<tr>
<td>M14</td>
<td>422.1376</td>
<td>422.1402</td>
<td>-2.6</td>
<td>-6.2</td>
<td>C21H26Cl2N3O2</td>
</tr>
</tbody>
</table>
Metabolite Profiles of KSL 03 in Human, Rat, Mouse, Monkey Rat, and Dog Microsomes

Met ID at early stage of drug discovery was considered as a good practice for drug development and regulatory filing. The in-vitro metabolite profiling guide to select in-vivo animal model to conduct PK and TK studies. Today extensive metabolic studies of NCEs on animals lead to high cost and significant challenges in drug development when performed in vivo. Hence in vitro MET-ID studies are encouraged to successfully guide for the lead optimization by studying the metabolic profiles in different species at early stages.

The Test compound was incubated in hepatic microsomes of various species like Human (HLM), Rat (RLM), Mouse (mLM), Dog (DLM) and Monkey liver microsomes (MoLM) for 60 minutes and the metabolic pathway was observed.

The percentage of metabolites found after 60minute incubation, along with their retention times were given in Table 2.

Table 2: The percentage of metabolites found after 60minute incubation, along with their retention times

<table>
<thead>
<tr>
<th>Name</th>
<th>m/z</th>
<th>Metabolic Pathway</th>
<th>RT</th>
<th>HLM</th>
<th>RLM</th>
<th>mLM</th>
<th>MoLM</th>
<th>DLM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>552.2170</td>
<td></td>
<td>51.843</td>
<td>5.80</td>
<td>5.46</td>
<td>25.69</td>
<td>7.62</td>
<td>14.22</td>
</tr>
<tr>
<td>M1</td>
<td>538.2162</td>
<td></td>
<td>49.541</td>
<td>42.21</td>
<td>1.87</td>
<td>22.09</td>
<td>34.29</td>
<td>24.64</td>
</tr>
<tr>
<td>M2</td>
<td>552.2094</td>
<td></td>
<td>45.948</td>
<td>3.59</td>
<td>3.18</td>
<td>2.43</td>
<td>6.64</td>
<td>5.11</td>
</tr>
<tr>
<td>M3</td>
<td>494.1857</td>
<td>Side chain cleavage and carboxylation</td>
<td>41.244</td>
<td>13.93</td>
<td>12.33</td>
<td>16.90</td>
<td>21.54</td>
<td>17.01</td>
</tr>
<tr>
<td>M4</td>
<td>508.1933</td>
<td></td>
<td>37.631</td>
<td>6.32</td>
<td>0.51</td>
<td>4.84</td>
<td>1.91</td>
<td>11.20</td>
</tr>
<tr>
<td>M5</td>
<td>450.1739</td>
<td></td>
<td>34.295</td>
<td>8.13</td>
<td>7.11</td>
<td>14.80</td>
<td>17.30</td>
<td>11.42</td>
</tr>
<tr>
<td>M6</td>
<td>406.1441</td>
<td></td>
<td>32.385</td>
<td>9.40</td>
<td>8.33</td>
<td>8.13</td>
<td>7.74</td>
<td>13.38</td>
</tr>
<tr>
<td>M7</td>
<td>568.2393</td>
<td></td>
<td>49.980</td>
<td>4.72</td>
<td>8.91</td>
<td>0.72</td>
<td>0.96</td>
<td>0.71</td>
</tr>
<tr>
<td>M8</td>
<td>554.2202</td>
<td></td>
<td>48.301</td>
<td>4.97</td>
<td>27.80</td>
<td>3.34</td>
<td>0.46</td>
<td>1.10</td>
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<tr>
<td>M9</td>
<td>568.2332</td>
<td></td>
<td>44.712</td>
<td>0.06</td>
<td>10.22</td>
<td>0.04</td>
<td>0.13</td>
<td>0.09</td>
</tr>
<tr>
<td>M10</td>
<td>510.1942</td>
<td>Aromatic Hydroxylation + Side chain cleavage and carboxylation</td>
<td>37.634</td>
<td>0.61</td>
<td>11.83</td>
<td>0.91</td>
<td>1.24</td>
<td>0.90</td>
</tr>
<tr>
<td>M11</td>
<td>524.1930</td>
<td></td>
<td>33.161</td>
<td>0.05</td>
<td>0.74</td>
<td>0.91</td>
<td>1.24</td>
<td>0.09</td>
</tr>
<tr>
<td>M12</td>
<td>466.1694</td>
<td></td>
<td>36.214</td>
<td>0.11</td>
<td>1.37</td>
<td>0.91</td>
<td>0.13</td>
<td>0.11</td>
</tr>
<tr>
<td>M13</td>
<td>478.1686</td>
<td></td>
<td>31.110</td>
<td>0.10</td>
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<td>0.00</td>
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<td>M14</td>
<td>422.1376</td>
<td></td>
<td>31.911</td>
<td>0.05</td>
<td>0.35</td>
<td>0.02</td>
<td>0.05</td>
<td>0.11</td>
</tr>
</tbody>
</table>

The comprehensive approach of single extraction using a single analytical platform was worked up for the Test compound.
The metabolites identified, based on the theoretical interpretation in support of MS/MS and Elemental composition. Preliminary data obtained after microsomal incubation suggested the formation of metabolites M1 to M14 and a proposed metabolic pathway for the NCE is shown in Figure 8.

Figure 8: The Proposed metabolic pathway of Test Compound by Simple & Aromatic oxidation

Metabolite profiling being one of the main drives in the drug discovery process to optimize pharmacokinetic properties and to increase the success rate of drugs. The work up has built an in vitro methodology for identification of the probable metabolites, and the metabolites observed were through the oxidative cleavage of side chain and Aromatic hydroxylation. The main annotation for identification of the metabolites is through the MS/MS fragmentation and Elemental composition apart from the isotopic pattern observed due to presence of Chlorine atom.

The metabolites observed upon incubation in hepatic microsomes of various species like Human (HLM), Rat (RLM), Mouse (mLM), Dog (DLM) and Monkey (MoLM) for 60 minutes gave the information such as - the metabolism of parent to M1 is high in Human, Monkey and Rat, Aromatic metabolites formed are less in comparison to the normal ones and the compound is showing sustained levels of the parent in mice, however the data have to be confirmed by in vivo experimentation.

Note: The inferences were based on Mass Spec as the UV signal was weak for the compound, and the mass spec response might be quite different for different metabolites and parent compound.

CONCLUSIONS

The metabolite identification study performed in various hepatic liver microsomes has clearly shown that the metabolic pathway for the Test compound is through microsomal oxidation.

This in vitro work up using Test compound demonstrates that the compound undergoes direct and aromatic oxidativemetabolism under in vitro conditions. It offers a good substrate for the hepaticmicrosomal enzymes, under specific experimental conditions. While the observed sustained level of concentration for the Test compound is probably due to its structural chemistry involved in the drug
design. This built-in construction shall be taken into consideration during the interpretation of the results obtained. Thus the inferences drawn in this manuscript act as a valuable tool in obtaining the necessary SAR and to re-design the molecule. This customized approach represents a significant efficiency and selectivity improvement over traditional methods, and can be used to understand the drug’s biotransformation under the influence of microsomal enzymes.

CONFLICTS OF INTEREST

The authors report no conflict of interest and want to convey that this experiment doesn’t restrict the scope of further evaluation, and the inferences drawn were purely based on the test procedure adopted under academia.

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