RESEARCH ENVISAGED AND PLAN OF WORK

The parent drug stability test guideline (Q1A) issued by the International Conference on Harmonization (ICH) requires that analytical test procedures for stability samples should be fully validated and the assays should be stability-indicating [1–4]. Further, it is suggested that stress studies should be carried out to establish the inherent stability characteristics of the molecule, for example the degradation pathways, leading to identification of degradation products and hence supporting the suitability of the analytical procedures proposed and followed by the Center for Drug Evaluation and Research (CDER) [5, 6].

A comprehensive LC-MS and ultra performance liquid chromatography (UPLC) study of the degradation behavior of febuxostat/piracetam under various ICH prescribed stress conditions has been lacking. So, the objective of the present research work is to carry out forced decomposition studies according to the ICH requirements and develop a selective and validated stability-indicating UPLC method. An integral aim of the study was to separate the degradation products.

2.1 Objectives of work for stress degradation studies

- To develop stability indicating assays methods (SIAMs) for determination of febuxostat/piracetam.
- To resolve the degradation products from mixture of stressed sample.
- To separate the degradation products formed under variety of conditions.
- To validate the developed method according to ICH guidelines.
2.1.1 Plan of work and execution

- Step I: Critical study of the drug structure to assess the likely decomposition routes.
- Step II: Collection of information on physicochemical properties.
- Step III: Stress (forced decomposition) studies.
  a) Hydrolysis (neutral pH)
  b) Acidic hydrolysis
  c) Alkaline hydrolysis
  d) Oxidation
  e) Photodegradation
  f) Thermal degradation
- Step IV: Preliminary separation studies on stressed samples.
- Step V: Final method development and optimization.
- Step VI: Separation of various degradants in a mixture of stressed solution by using HPLC and UPLC.
- Step VII: Validation of SIAMs
  1) System suitability parameters,
  2) Linearity,
  3) Range,
  4) Accuracy by recovery study,
  5) Precision,
  6) Robustness,
  7) LOD and LOQ.

2.2 In vitro metabolite identification and characterization

This project aims to ascertain the metabolites and metabolic pathways of drug molecules pathways by employing a combination of in vitro methods and LC-MS/MS analytical studies and identify the metabolites and metabolic pathways in silico. The ultimate goal of this work is to evaluate metabolite by using LC-MS/MS with the help of light sight software (optional).

The specific aims are:

(a) To develop and employ a method for preclinical in vitro testing of some drugs in liver microsomes.
(b) A well established way of prediction of drug metabolism is the use of *in silico* method: computer aided prediction of metabolism ie, so called metabolExpert & innovative light sight programs for screening possible metabolites (optional).

(c) The drugs metabolites formed in the incubations will be analyzed using LC-MS/MS.

(d) Stress degradation studies of same drug used for metabolite identification as per ICH guidelines.

2.2.1 Work plan (including detailed methodology and time schedule):

- Critical study of the drug structure to assess the likely metabolism routes.
- Collection of information on physicochemical properties.
- *Insilico* identification of metabolite by Metabolexpert.
- Screening of some drugs for metabolic stability.
- To utilize liver microsomes and trying to find the metabolites that most closely resemble to human metabolism.

  ✓ Dilution of microsomal solution to required protein concentration.
  ✓ Incubation of the test drugs with low amounts of microsomal protein to readily quantify the necessary metabolites.
  ✓ Carrying out control incubations should by excluding either the substrate, NADPH (or an NADPH generating system consisting of 1 mM NADP, 5 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase), or microsomes from the incubation mixture. Active and control groups should be prepared and incubated under the same conditions.
  ✓ Sampling at defined time intervals.
  ✓ Termination of the reaction by adding the sample to an equal volume of ice-cold extraction solvent (e.g., acetonitrile or dichloromethane) followed by centrifugation at 14,000 g for 20 min at 4°C.
  ✓ Analysis of supernatants either immediately or freezing the sample at −80°C until analysis.
  ✓ In depth analysis of obtained data and deciphering relevant conclusions.
Separation of various metabolites in a mixture of metabolizes solution by using HPLC.

Identification and characterization by LC-MS/MS using LightSight software (optional) that efficiently identify and confirm metabolites from data acquired on Applied Biosystems/MDS Sciex triple quadrupole and hybrid linear ion trap mass spectrometers.

*Figure 2.1* Generic studies for postulation of mass fragmentation pattern and for metabolite characterization

- **Insilico studies**
  - Predictive metabolite pattern ie. Metaboexpert
  - Application of “nitrogen rule”
  - Elucidation of sequence of fragmentation
  - Elucidation of molecular formula corresponding to loss.
  - Elucidation of sequence of fragmentation

- **MS^n studies**
  - Elucidation of molecular formula generation by product ion, precursor ion and neutral loss
  - Powerful *insilico* MS/MS tools for metabolite confirmation ie., lightsight software (optional).
2.3 Design of proposed research work

Drug molecules/ NCEs

<table>
<thead>
<tr>
<th>Absence of enzymes/Direct stress</th>
<th>Presence of enzymes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress Degradation study</td>
<td></td>
</tr>
<tr>
<td>Hydrolytic</td>
<td></td>
</tr>
<tr>
<td>- Acid (HCl)</td>
<td></td>
</tr>
<tr>
<td>- Base (NaOH)</td>
<td></td>
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<tr>
<td>- Neutral (Water)</td>
<td></td>
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<tr>
<td>Photolytic (sunlight, 2 days)</td>
<td></td>
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<tr>
<td>Thermal (hot air oven, 2 months)</td>
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<tr>
<td>Oxidative hydrolysis (H₂O₂)</td>
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<tr>
<td>Advantages</td>
<td></td>
</tr>
<tr>
<td>- Requires in regulatory (NDA)</td>
<td></td>
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<tr>
<td>- Degradation may be impurity</td>
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</tbody>
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Metabolite identification and characterization using human liver Microsomes

- Metabolic stability
- *In silico* studies (Metabol Expert)
- Separation of various metabolites in a mixture of metabolizes solution by using UPLC/HPLC.
- Identification and characterization by LC-MS/MS

Advantages

- The preclinical animal’s studies are expensive and time consuming. (No. of paper formalities, ethical permission)
- Species-species difference, a drug that is

2.4 Additional studies

- Effect of Morin on pharmacokinetics of Febuxostat/Piracetam in rats.
- Biological screening of the formed metabolites.
2.5 Drug profile

2.5.1 Febuxostat

- **Discoveried by**: Teijin Pharma Ltd, Tokyo, Japan, 2009

- **Structure**

- **Molecular Formula**: C\textsubscript{16}H\textsubscript{16}N\textsubscript{2}O\textsubscript{3}S
- **Molecular Weight**: 316.38 g/mol
- **Chemical Name**: 2-[3-cyano-4-(2-methylpropoxy) phenyl]-4- methylthiazole-5-carboxylic acid
- **Category**: Antigout
- **Description**: white crystalline powder
- **Solubility**: Soluble in dimethylsulfoxide; sparingly soluble in ethanol; soluble in methanol and acetonitrile; and practically insoluble in water.
- **Dissociation Constant**: 3.5
- **\( \lambda \text{ max} \)**: 315
- **Melting point**: 205°C to 208°C
2.5.2 Piracetam

- **Discovered by**: Dr. Corneliu E., Belgian Pharmaceutical Company, UCB, 1964

- **Structure**

- **Molecular Formula**: C₆H₁₀N₂O₂
- **Molecular Weight**: 142.16 g/mol
- **Chemical Name**: 2-Oxo-1-pyrrolidineacetamide
- **Category**: Nootropic
- **Description**: white powder
- **Solubility**: Soluble in dimethylsulfoxide; sparingly soluble in ethanol, methanol and acetonitrile; and freely soluble in water.
- **Dissociation Constant**: 3.9
- **λ max**: 209
- **Melting point**: 151°C to 152°C