LC-MS/MS QUANTITATION IN DRUG DISCOVERY & DEVELOPMENT

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Presentation Outline

• Bioanalysis and Metabolites in Drug Discovery and Development

• Case Study
  – Quantification of Phthalates in Urine

• Conclusion
What is Bioanalysis?

• Bioanalysis employed for the quantitative determination of drugs and their metabolites in biological fluids, plays a significant role in the evaluation and interpretation of bioequivalence, pharmacokinetic and toxicokinetic studies (Viswanathan c.t. et al. Workshop/conference Report, AAPS Journal 2007; 9 (1) Article 4)
Metabolites and Bioanalysis In Drug Development

• Identify and profile animal metabolites

In vitro

In vivo (cold/C¹⁴)

• Identify and profile human metabolites

In vitro

In vivo (cold/C¹⁴)

• Bioanalytical methods

P
Non-GLP method

M
GLP method

GLP: Good Laboratory Practice        P: Parent        M: Metabolite(s)

The cost to bring a drug to the market is about $1.3 billion (US FDA, 2004)
MS Instrumentation of Choice

• Bioanalysis
  – Triple Quadrupoles
    • Most of the Quantitative Assays are Performed in Multiple Reaction Monitoring (MRM) Mode

• Metabolite Identification
  – Ion Traps
  – Q-Tofs
  – Triple Quadrupoles
Bioanalysis in Drug Discovery Phase

Scope

Determine Concentrations/Exposures in Pharmacokinetic (PK), Pharmacodynamic (PD) Studies

Requirements and Challenges

– Significant Throughput for the Screening Across Different Chemical Series
– Generic Assays are Preferred/Automated Method Development
– No Stable Isotope Internal Standard
– Unknown Compound/Metabolite Stability
– No QC Samples to Verify Method Performance
Bioanalysis in Preclinical Phase

Scope

• Determine Concentrations/Exposures in Toxicokinetic Studies Using Validated Assays to Evaluate Drug Safety

• Requirements and Challenges
  – Full Validation Required
  – Stability in Matrix Investigated
  – Highly Regulated: Conducted According to Good Laboratory Practice (GLP) and Most Activities Should Follow Standard Operation Procedures (SOP)
  – More Work for Troubleshooting and Validation to Produce Rugged and Robust assay
  – Time Constraint: Validation Completed Before the Actual Study Starts
  – Long-Term Use for Routine Analysis
  – The Bioanalytical Assay Becomes a Part of Regulatory Submission (e.g. IND)
Bioanalysis in Clinical Phase

Scope

• Determine Concentrations/Exposures in Clinical /Bioequivalence Studies (Phase 1 - Phase 3)

• Requirements and Challenges
  – Assay Adjusted for Human Matrices (Plasma, Serum, Urine)
  – Ultra-Sensitive Assay May be Required
    • Novel Target is Pursued
    • Microdosing
  – Long-Term Usage is Desirable: Preferable to Use the Same Assay Through Phase 1 – Phase 3 clinical studies
  – Assay Flexibility with Regards to Concomitant Medications or Background Interferences
  – Fast Turn Around for First in Man Studies
Analytical Approaches for Bioanalysis

• **LC/Ultraviolet (UV)/Fluorescence**
  – Due to Low (if any) Specificity of Detectors Not Much in Use for Over a Decade

• **LC/MS**
  – Most of the Assays are Performed Using Triple Quadrupole Instruments Operating in Selected Reaction Monitoring (SRM) Mode

• **Ionization Techniques**
  – APCI
    • More appropriate for Poorly Ionized Analytes
    • Less Matrix Effect
  – ESI
    • With Biological Matrices ESI is Prone to Ionization Suppression/Enhancement
    • Sample Purification is Required
Liquid Chromatography in Bioanalysis

- Role of Chromatography for Bioanalysis Changed

- Baseline Separation is Not Required Due to High Selectivity of MRM
  - LC Remains an Important Method for
    - Concentration of Analytes During Injection or
    - Minimize Matrix Suppression

- Only in Selected Cases Separation of Analytes is Required
  - Low Level of Detection
  - Phase II Metabolites
Phase II Metabolites Interference
Phase II Metabolites Interference

Parent
XIC of +Q1: 376.0 to 376.5 amu from Sample 2

XIC of +Q1: 456 to 456.5 amu from Sample 2

N-Sulfate
XIC of +Q1: 418.0 to 418.5 amu from Sample 2

N-Acetyl

Intensity, cps

Time, min

Max. 5.7e7 cps.

4.79

4.56

50 6
Sample Preparation Techniques in Bioanalysis

• **Goal**
  – Increase Sensitivity and Selectivity
  – Minimize Ion Suppression
  – Concentrate Sample

• **Most Popular Techniques**
  – Protein Precipitation (PPT)
  – Liquid-Liquid Extraction (LLE)
  – Solid Phase Extraction (SPE)
    • On line
    • Off line
Case Study

Scope

• Develop Bioanalytical Method for Quantification of Urinary Phthalate Metabolites

Assay Requirements

– Urine
– Two Analytes
  • Total Monoethyl Phthalate (MEP) (Free and Glucuronidated)
  • Total Monobutyl Phthalate (MBP) (Free and Glucuronidated)
– Stable Isotope Labeled Internal Standards
– MEP and MBP Glucuronides are not available
– Lower Limit of Quantification LLOQ=5 ng/mL
– LC-MS/MS
Background

- People are routinely exposed to phthalates because of their wide use as industrial solvents and plasticizers.
- Diethyl and dibutyl phthalates are widely used in perfumes, cologne, soap, shampoo, nail polish and cosmetics.
- Some phthalates and their metabolites are responsible for reproductive and developmental toxicities in animals.
- Phthalate monoesters and their respective metabolites used as urinary or serum biomarkers of phthalate exposure.
Phthalate Metabolism

- In Humans, Phthalate diesters are metabolized to their respective monoesters which are partially glucuronidated.

- Excreted through Urine and Feces.

Phthalate Diester

- MEP: R=Ethyl
- MBP: R=Butyl

Monoester Glucuronide

β-Glucuronidase

Monoester
Challenges of the method

- Develop Hydrolysis procedure to determine total monoester amount
  - Free monoester+conjugated monoester
- Possible Matrix effect
- Needs to be pre-concentrated and purified before injection
  - Off line SPE
  - On-line SPE

On-line SPE Procedure Implemented

Silica based monolithic column for sample pre-concentration/purification

Column: Chromolith Flash RP-18e column (4.5x25mm, Merck KGaA, Germany)

Back-flush to Analytical column – MS

Column: Eclipse Plus, Phenyl Hexyl 2.1x150mm, 5um (Agilent)

Mobile Phase:

A (0.1% Acetic Acid in water);
B (0.1% Acetic Acid in Acetonitrile)

LC-MS Conditions

- Mass spectrometer: Triple Quadrupole (Micromass Quattro)
- Ionization: ESI-
- Mode: MRM

- Injection Volume
  - 50uL

<table>
<thead>
<tr>
<th>Compound</th>
<th>Transition monitored</th>
<th>Cone/Collision Energy</th>
<th>Retention Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEP</td>
<td>192.7→76.9</td>
<td>22/22</td>
<td>~3.43</td>
</tr>
<tr>
<td>MBP</td>
<td>220.9→76.9</td>
<td>22/18</td>
<td>~4.22</td>
</tr>
<tr>
<td>IS MEP</td>
<td>197.0→78.9</td>
<td>22/19</td>
<td>~3.43</td>
</tr>
<tr>
<td>IS MBP</td>
<td>224.7→78.9</td>
<td>25/20</td>
<td>~4.22</td>
</tr>
</tbody>
</table>
Selected Reaction Monitoring (MRM)

Transition: m/z 193 $\rightarrow$ m/z 77
ON-LINE SPE METHOD

• On-line SPE for pre-concentration and purification
  – Column: Chromolith Flash RP-18e column (4.5x25mm, Merck KGaA, Germany)
  – Mobile Phase:
    • A (0.1% Acetic Acid in water);
    • B (0.1% Acetic Acid in Acetonitrile)
SWITCHING VALVE POSITION 1

Inject (50 - 100uL)

Load on Trap Column while Analytical column is Equilibrating

1 Minute in Position 1
HPLC ANALYTICAL METHOD

- Column
  - Eclipse Plus, Phenyl Hexyl 2.1x150mm, 5um (Agilent)
- Mobile Phase:
  - A (0.1% Acetic Acid in water);
  - B (0.1% Acetic Acid in Acetonitrile)
Switching Valve
POSITION 2

Back Flush from Trap Column onto Analytical Column to Mass spectrometer

7 minutes in Position 2
5ng/mL Calibration Solution

5 ng/ml
PakV_100804_24708_14 Sm (Mn, 2x3)

MRM of 7 Channels ES-224.7 > 78.98 (13C4-MBP)
6.04e4 Area

220 92 76 9 (MBP)
41 8

MRM of 7 Channels ES-197.01 > 78.85 (13C4-MEP)
3.75e4 Area

192.7 > 76.97 (MEP)
1.46e3 Area
MEP/MBP Calibration Curves

- 5ng/mL - 1000ng/mL
- Injection volume: 50uL
- Some Amounts of MEP were Detected in Blank Urine (~30 – 100 ng/mL)
- Some Amounts of MBP were Detected in Blank Urine (~5 – 40 ng/mL)
Case Study: Summary

- On-line SPE Method Implemented
- 122 Urine Samples Analyzed in 3 Runs
- Wide Range of Concentrations Detected Confirming Human Exposure to Phthalates
  - MEP Concentration range: 5 – 2600 ng/mL
  - MBP Concentration range: <5 – 200 ng/mL
- This Method Could be Used for Other Studies which Require Sample Pre-concentration and Purification
Conclusions

• Bioanalysis is an Integral Part of PK/TK/PD Characterization of New Compounds from Discovery Through Various Stages of Drug Development Leading to the Market

• LC-MS Triple Quadrupoles are Instruments of Choice for Quantification

• Method Development is Challenging and Exciting Part of the Drug Development Process Since Unique Compounds are Used and New Targets Investigated

• The Choice of Instrumentation and its Characteristics is Important, but it is Essential to Understand Chemical Properties of the Compounds to be Successful!
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