Therapeutic Drug Monitoring of Immunosuppressant Drugs by Liquid Chromatography-Tandem Mass Spectrometry

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Aims

• To critique LC-MS/MS and immunoassays

• To provide practical examples of using LC-MS/MS for the therapeutic drug monitoring of immunosuppressant drugs
Introduction

• The monitoring immunosuppressant drugs used for the prophylaxis of rejection after solid organ transplantation is routine clinical practice
Why Measure?

- Wide inter- and intra-patient variability in pharmacokinetics
Pharmacokinetic Variability of Tacrolimus in Paediatric Liver Transplant Recipients


[Graph showing trough concentrations and dose relationship with highlighted points at 2 ng/mL and 22 ng/mL]
Why Measure?

- Wide inter- and intra-patient variability in pharmacokinetics
- Potential for drug-drug interactions (CYP3A/Pgp) – induction or inhibition
- Compliance
- Narrow therapeutic window

Toxicity  Rejection
“Critical Dose Drugs”

- Cyclosporin
- Tacrolimus
- Sirolimus
- Everolimus
- Mycophenolic acid?
## Method Choices

<table>
<thead>
<tr>
<th>Cyclotacrolimus</th>
<th>Sirolimus</th>
<th>Everolimus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporin</td>
<td>Tacrolimus</td>
<td>Sirolimus</td>
</tr>
<tr>
<td>mFPIA</td>
<td>MEIA</td>
<td>MEIA</td>
</tr>
<tr>
<td>- TDx</td>
<td>- EMIT</td>
<td>- HPLC/UV</td>
</tr>
<tr>
<td>- AxSYM</td>
<td>- Cedia</td>
<td>- HPLC-MS</td>
</tr>
<tr>
<td>pFPIA</td>
<td>- HPLC-MS</td>
<td></td>
</tr>
<tr>
<td>EMIT</td>
<td></td>
<td>FPIA</td>
</tr>
<tr>
<td>mRIA</td>
<td></td>
<td>HPLC/UV</td>
</tr>
<tr>
<td>Cedia+</td>
<td></td>
<td>HPLC-MS</td>
</tr>
<tr>
<td>ACMIA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integra</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC/UV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC-MS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Immunoassays
Cyclosporin Metabolism

AM69        AM19          AM1c
AM4N69                        AM1c9
AM4N9 AM4N9 AM9 AM9 AM1 AM1
AM49 AM49 AM4N AM4N AM1A
CsA
AM4N9 AM9 CsA AM1 AM14N
AM69 AM19 AM1c
AM49 AM4N AM1A
Cross-reactivity with CsA-metabolites

(C0/trough samples referenced to HPLC)

<table>
<thead>
<tr>
<th></th>
<th>mean bias</th>
<th>range</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMIT</td>
<td>10%</td>
<td>-7 to 53%</td>
</tr>
<tr>
<td>mRIA (CycloTrac)</td>
<td>10%</td>
<td>(n.a.)</td>
</tr>
<tr>
<td>CEDIA*</td>
<td>18%</td>
<td>-3 to 81%</td>
</tr>
<tr>
<td>mFPIA (AxSYM)</td>
<td>29%</td>
<td>2 to 130%</td>
</tr>
<tr>
<td>mFPIA (TDx)</td>
<td>57%</td>
<td>20 to 174%</td>
</tr>
</tbody>
</table>

(*prior to current CEDIA Plus method)

Steimer, Clin Chem 45:371-81, 1999
Specificity of Various Methods

Specificity – Pooled sample (heart Tx) - Int. PT Scheme
Comparison of LC-MS/MS and MEIA – Sirolimus (n = 116)

Effect of Hematocrit on MEIA Results

- Tacrolimus and sirolimus
- The lower the hematocrit the higher the result
- False positive results (i.e. anaemic patients not receiving therapy but have measurable concentrations)

Morris RG and Taylor PJ. *Ther Drug Monit* 2003;25:259-60
Tacrolimus MEIA and Hematocrit

Sirolimus MEIA bias

MEIA Overestimation

% Difference

Time post-Transplant (months)

“It has recently come to the attention of Wyeth that one of the more commonly used immunoassay platforms, IMx®[2], may yield results with a negative bias relative to HPLC/MS/MS in some cases [4]. This may vary from one laboratory to another and may also be affected by whether fresh or frozen blood samples are utilized. The newer ARCHITECT® assay [3] performs on average as expected (a positive bias relative to HPLC/MS/MS) based on a proficiency testing scheme.”
“Therefore, switching between platforms, whether between immunoassay platforms or between immunoassay and HPLC, can produce differing results that may be clinically significant. As such, if different assays are used in monitoring a single patient without the knowledge of the Health Care Provider, the dose of Rapamune might be adjusted improperly with potential consequences, such as allograft rejection if drug exposure is too low or toxic side effects if exposure is too high.”
Summary

• Immunoassays are prone to non-specific anti-body cross-reactivity with metabolites that result in “variable” overestimation in drug concentration

• For tacrolimus and sirolimus, MEIA results are further confounded by hematocrit
LC-MS/MS
Mass Spectrometry – Ionization

- Cyclosporin, tacrolimus, sirolimus and everolimus are compounds that do not readily protonate in solution
- Preferentially form adducts with cations present in solution (Na\(^+\), K\(^+\), NH\(_4\)\(^+\))
- Addition of volatile buffers to the mobile phase (e.g. ammonium acetate)
Precursor Ion - Tacrolimus

\[
[M+NH_4]^+ \\
821.3
\]
Collision-Induced Dissociation of Tacrolimus

![Mass to charge ratio (m/z) vs. Intensity (x10^6 cps) graph]
Which Mode???

- Single ion monitoring (MS)
- Selected reactant monitoring (MS/MS)
Tacrolimus by SIM Versus SRM

SIM m/z 821

SRM m/z 821>768

3 µg/L

30 µg/L
Possible Sample Preparation

• Solid phase extraction
• Liquid-liquid extraction
Possible Sample Preparation

• Solid phase extraction
• Liquid-liquid extraction
• Protein precipitation - direct injection with fast gradient
Princess Alexandra Hospital Experience
Using a High-Throughput LC-MS/MS Method

Evaluation of a rapid micro-scale assay for tacrolimus by liquid chromatography–tandem mass spectrometry

BG Keevil¹, SJ McCann¹, DP Cooper² and MR Morris²

Sample Preparation

- Whole blood samples (25 µL) in 1 or 2 mL “96-round well plates”
  + 0.1 M zinc sulphate (100 µL)
  + acetonitrile (250 µL, containing ascomycin)

- Mix and centrifuge

- Inject supernatant (20 µL)
Chromatography

- Switch gradient: 50% organic to 100% organic composition
- Waters 10 x 2 mm C18 column
- Divert first 0.5 min to waste (built-in with software control)
Patient Sample (4.9 ng/mL)

**Cycle Time**

3 min/sample
Audit: November 2004 – August 2009

- 3,386 batches
- 67,160 samples (standards, controls and patient samples)
- 53 analytical columns (>1000 samples/column)
- 4 analysts
- 4 batch failures (3 analyst issue and 1 instrument) = 0.12% failure rate
Assay Performance
Linearity

- 1.0, 5.0, 10, 25 and 50 ng/mL
- 1551 calibration curves
- mean $r^2 = 0.999$

- Workflow: 1 x calibration curve in 1st Batch
  3 x quality controls in subsequent batches
### Accuracy and Imprecision (n = 3386)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Quality Controls (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>Accuracy (%)</td>
<td>98.3</td>
</tr>
<tr>
<td>Imprecision CV (%)</td>
<td>7.9</td>
</tr>
</tbody>
</table>
External Quality Control Results

$y = 1.0228x - 0.0236$

$R^2 = 0.9928$

$n = 89$
Maintenance – Sample Numbers?

- Daily: prime HPLC system
- Fortnightly: clean outer sample cone
- 2 Months: capillary replaced
- 6 monthly: clean whole ion source
- Yearly: preventative maintenance service
Can We Use This Approach for the Measurement of Other Immunosuppressant Drugs?
Can We Use This Approach for the Measurement of Other Immunosuppressant Drugs?

Possible Sample Preparation

- Solid phase extraction
- Liquid-liquid extraction
- Protein precipitation - direct injection with fast gradient
- 2-D Chromatography or column switching
Simultaneous determination of four immunosuppressants by means of high speed and robust on-line solid phase extraction–high performance liquid chromatography–tandem mass spectrometry☆

Therese Koal a,*, Michael Deters a, Bruno Casetta b, Volkhard Kaever a

Sample Preparation

- Blood (100 µL) + precipitation reagent (150 µL)
- Mix
- Centrifuge
- Transfer to 96-well
- Inject (100 µL)
Chromatography

- Extraction column – Poros 2.1 x 30 mm, 20 µm
- Analytical column – Phenyl hexyl 2 x 50 mm, 5 µm
- Pump A – 97% methanol/buffer
- Pump B – 50% methanol/water
Comments

- Selectivity can be achieved by using different columns (extraction and analytical) and mobile phase compositions

- High flow rate on extraction column enables rapid elution of unwanted material

- Off-line cleaning of extraction column is performed at a very high flow rate to allow quick equilibration
Representative Chromatograms

931.6/864.8

931.6/882.9
## Analytical Performance

Table 3

<p>| Method performance parameters determined for spiked blood samples according to Guidance for Industry [29] |
|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|--------------------------------------------------|</p>
<table>
<thead>
<tr>
<th></th>
<th>Cyclosporin A (CyA)</th>
<th>Tacrolimus (TRL)</th>
<th>Sirolimus (SRL)</th>
<th>Everolimus (RAD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD (ng ml(^{-1}))</td>
<td>1.3</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>LLOQ (ng ml(^{-1}))</td>
<td>10.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Linearity(^a) (R(^2))</td>
<td>0.9999</td>
<td>0.9997</td>
<td>0.9998</td>
<td>0.9998</td>
</tr>
<tr>
<td>Recovery(^b) (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration 1</td>
<td>103</td>
<td>91</td>
<td>92</td>
<td>94</td>
</tr>
<tr>
<td>Concentration 2</td>
<td>98</td>
<td>88</td>
<td>91</td>
<td>92</td>
</tr>
<tr>
<td>R.S.D. (%) intra-day(^c)</td>
<td>4.6</td>
<td>10.2</td>
<td>9.3</td>
<td>10.3</td>
</tr>
<tr>
<td>R.S.D. at LLOQ (%) intra-day(^d)</td>
<td>4.4</td>
<td>12.5</td>
<td>11.0</td>
<td>11.5</td>
</tr>
<tr>
<td>R.S.D. (%) inter-day(^e)</td>
<td>12.3</td>
<td>13.5</td>
<td>12.3</td>
<td>10.9</td>
</tr>
<tr>
<td>Accuracy (%)(^f)</td>
<td>99.2</td>
<td>96.9</td>
<td>98.9</td>
<td>103.1</td>
</tr>
</tbody>
</table>
What Does LC-MS/MS Mean to the Clinician and Ultimately the Patient?
Follow-up of a patient after transplantation: Tapering of tacrolimus dosage

Dr Michael Vogeser, M.D.
Institute of Clinical Chemistry, University Hospital Munich, Germany.
Follow-up of a patient after transplantation: Tapering of tacrolimus dosage

Dr Michael Vogeser, M.D.
Institute of Clinical Chemistry, University Hospital Munich, Germany.
Gold Standard?
Sirolimus - External Proficiency Scheme

CV (%)

- Other (n = 7)

- S109C

www.bioanalytics.co.uk
Sirolimus - External Proficiency Scheme

CV (%)

- Other (n = 7) : 8.1
- Abbott Imx (n = 66) : 12.2
- HPLC/MS (n = 86) : 18.3

www.bioanalytics.co.uk
Harmonization

• With the development of slightly different variations of a particular test used by various laboratories there is a greater likelihood of different results
$ Cost $
Economic Barrier

- There is a *perceived* cost barrier for HPLC-MS
  
- A triple quadrupole instrument requires an initial capital investment of greater than $US150 000
  
- This compares poorly with immunoassays that are supplied by the manufacturer on a reagent-rental basis
## Cost of Tacrolimus Measurement

(Prof M Oellerich, Germany)

<table>
<thead>
<tr>
<th></th>
<th>LC-MS/MS</th>
<th>MEIA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number of samples</strong></td>
<td>n = 3</td>
<td>n = 3</td>
</tr>
<tr>
<td>Direct costs (€)</td>
<td>5.10</td>
<td>29.98</td>
</tr>
<tr>
<td>Technician time (€)</td>
<td>11.10</td>
<td>7.40</td>
</tr>
<tr>
<td><strong>Total costs (€)</strong></td>
<td>16.20</td>
<td>37.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21.07</td>
</tr>
</tbody>
</table>
Discussion
Disadvantages of LC-MS/MS (1)

- High initial capital costs for equipment
- Special technical expertise required
- No automated systems available
Disadvantages of LC-MS/MS (2)

- Lack of standardisation (calibration, methodology)
- Limited availability outside regular laboratory hours
- Necessity to re-evaluate current therapeutic ranges established with immunoassays
Advantages of LC-MS/MS (1)

- High selectivity, accuracy and precision
- Broad calibration range
- More rational dosage individualisation based on the parent drug
Advantages of LC-MS/MS (2)

• Simultaneous quantification

• Direct costs per test including technician time at least 50% lower compared to immunoassays

• Independence from commercial immunoassays
Method Requirements

• “The accuracy, precision, and specificity, of the analytical systems are fundamental to the efficient use of TDM in clinical practice.”
• “Cost effective”
• “Reasonably easy to perform”

Conclusions

• For immunosuppressant drug monitoring, LC-MS/MS is superior in analytical performance compared to immunoassays.

• LC-MS/MS provides a “total” solution to the analytical problem of measuring immunosuppressant drugs and is worlds’ best practice.
Final Thought

• It is hoped that by providing the clinician with high quality pharmacokinetic data using LC-MS/MS that this will lead to better patient outcomes