**Immunosuppressants**

James C. Ritchie, PhD  
*September 17, 2013*

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**DISCLOSE STATEMENT**

Speaker: James C. Ritchie, Ph.D.

Dr. Ritchie has disclosed the following financial relationships. Any real or apparent conflicts of interest related to the content of this presentation have been resolved.

- **Research / Educational Grants**
  - Beckman Coulter, Inc
  - Roche Diagnostics
  - Siemens Diagnostics
  - T2 Biosciences
  - Chromsystems

- **Federal Grants**
  - MH69056 - Emory / GSK NIMH Collaborative Mood Disorders Initiative
  - MH078105 - Early Experience, Stress and Neurobehavioral Development Center

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**Unapproved or Off Label Disclosures for James C. Ritchie, PhD**

James Ritchie has documented that his presentation involves comments or discussion of a validated Laboratory Developed Test (LDT), employing liquid chromatography and mass spectrometry for use in the clinical laboratory.
Emory Transplant Center

Region’s largest and only comprehensive organ and tissue transplant program.

In 2010 the Emory Center performed a total of 453 transplants:
- 160 Kidney
- 22 Heart
- 80 Liver
- 26 Pancreas
- 24 Lung
- 128 Stem Cell
- 13 Islet Cell

Classification of Immunosuppressant Drugs

<table>
<thead>
<tr>
<th>Category</th>
<th>Types</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome-binding drugs</td>
<td>Calcineurin inhibitors</td>
<td>Ciclosporine, Tacrolimus</td>
</tr>
<tr>
<td></td>
<td>mTOR inhibitors</td>
<td>Everolimus</td>
</tr>
<tr>
<td>20 metabolites</td>
<td>Inhibitors of de novo purine synthesis</td>
<td>Mycophenolic acid (MPA), Mycophenolate Mofetil (MMF), Azathioprine</td>
</tr>
<tr>
<td></td>
<td>Inhibitors of de novo pyrimidine synthesis</td>
<td>Leflunomide</td>
</tr>
<tr>
<td>Drug immunosuppression</td>
<td>Polyclonal antibodies</td>
<td>Anti-thymocyte gamma globulin, Thymoglobulin</td>
</tr>
<tr>
<td></td>
<td>Monoclonal antibodies</td>
<td>Anti-CD3 monoclonal antibody (OKT3), IL-2H (humanized), Belatacept, Basiliximab</td>
</tr>
<tr>
<td>Others</td>
<td>Deoxyaspergual, corticosteroids</td>
<td>FTY720, fingolimod</td>
</tr>
</tbody>
</table>

For the ISDs routinely measured today, it is still recommended that methods specific for the parent molecule be used.

2003 Immunosuppressant Testing

<table>
<thead>
<tr>
<th>Measurand</th>
<th>Volume / yr</th>
<th>Cost / Analysis</th>
<th>Cost / yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine</td>
<td>7586</td>
<td>$10.80</td>
<td>$81,950</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>13120</td>
<td>$12.00</td>
<td>$157,440</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>744</td>
<td>$57.45*</td>
<td>$42,743</td>
</tr>
<tr>
<td>TOTALS</td>
<td>21,452</td>
<td></td>
<td>$282,133</td>
</tr>
</tbody>
</table>

*Senta-Mayo Labs

- Rapamycin TAT = 1 to 4 days
- Expecting volume to increase substantially in future
- Each drug is a separate analysis, no opportunity for multiplexing
- Total cost to system over 7 years = $1,974,931
Immuoassays

Pros
- Very sensitive
- Lab friendly / automated
- Good precision
- FDA approved methods

Cons
- Cross reactivity issues
- Heterophile susceptibility
- Separate assay for each drug type
- Affected by hematocrit
- Require pretreatment


We Needed a Killer App!

- Measure Rapamycin
- Short TAT / same day results
- Moving manual procedures out of core lab
- High accuracy for parent drugs
- Multiplexed if possible
2003: What Were Other Labs Doing for IS Drugs?

<table>
<thead>
<tr>
<th>Reference Laboratories</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guest</td>
<td>HPLC and TDX (by request)</td>
</tr>
<tr>
<td>Mayo Labs</td>
<td>HPLC + LC-MS/MS</td>
</tr>
<tr>
<td>LabCorp</td>
<td>LC-MS/MS</td>
</tr>
<tr>
<td>ARUP</td>
<td>LC-MS/MS</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Academic Centers</th>
<th>Methodology</th>
</tr>
</thead>
<tbody>
<tr>
<td>MUSC</td>
<td>LC-MS/MS</td>
</tr>
<tr>
<td>UNC</td>
<td>LC-MS/MS</td>
</tr>
<tr>
<td>Univ. of Mich.</td>
<td>LC-MS/MS</td>
</tr>
<tr>
<td>Univ. Penn</td>
<td>HPLC (LC-MS for C2 protocols)</td>
</tr>
<tr>
<td>Univ. Washington (Seattle)</td>
<td>TDX</td>
</tr>
<tr>
<td>Children's</td>
<td></td>
</tr>
<tr>
<td>Duke</td>
<td>LC-MS/MS</td>
</tr>
</tbody>
</table>

**LC-MS/MS Analyses**

- Most methods are home brews
  - Resources:
    - Develop from scratch
    - Previously validated method from colleagues
    - Literature
    - Vendors
    - Most variation between labs related to differences in methods & standards
- CLIA – High Complexity
- Highly specific for parent compounds
- Capable of being multiplexed

**LC-MS/MS Proposal**

<table>
<thead>
<tr>
<th>Measurand</th>
<th>Volume / yr</th>
<th>Cost* / Analysis</th>
<th>Cost / yr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine</td>
<td>7588</td>
<td>$8.35</td>
<td>$68,184</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>13120</td>
<td>$6.35</td>
<td>$83,312</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>744</td>
<td>$6.35</td>
<td>$4,724</td>
</tr>
</tbody>
</table>

**TOTALS** 21,452 $136,220

* Cost includes reagents, labor, & instr. depreciation

- All drug TATs within 1 day
- Capable of expanding to meet increased need
- Runs are multiplexed
- Total cost to system over 7 years = $953,540
- Saving to system over 7 years = $1,021,391
- Provides opportunities to do other assays
Process for Developing a Clinical Method

<table>
<thead>
<tr>
<th>Make an Implementation Plan</th>
<th>Validate the Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Method Selection</td>
<td>• Imprecision</td>
</tr>
<tr>
<td>• Selection of Key Operator</td>
<td>• Recovery</td>
</tr>
<tr>
<td>• Outline/plan methods</td>
<td>• Linearity</td>
</tr>
<tr>
<td>• Acquisition of materials</td>
<td>• Sensitivity</td>
</tr>
<tr>
<td>• Set quality goals</td>
<td>• Specificity</td>
</tr>
<tr>
<td>• Validation</td>
<td>• Interferences</td>
</tr>
<tr>
<td>• Monitoring and statistics</td>
<td>• Specimen</td>
</tr>
<tr>
<td>• SOP preparation, staff</td>
<td>• Method Comparison</td>
</tr>
<tr>
<td>training</td>
<td>• Assay Calibration</td>
</tr>
</tbody>
</table>

Guidance Documents

• EU Directive 2002/657/EC
• FDA Guidance 118
• FDA Special Controls Guidance for Particular Drugs
• CAP Chemistry & Toxicology Checklist
• CLSI Guidance EP10-A3E
• SOFT and AAFS Guidelines
• WADA Identification Criteria
• New York State – Clinical Laboratory Standards of Practice

EP50 Mass Spectrometry in the Clinical Laboratory

• General overview of mass spectrometry and clinical applications
• General guidelines on analytical method development and validation
• Seeks to harmonize some of the international documents
Two New CLSI Documents in the Works

- C-57 Mass Spectrometry for Androgen and Estrogen Measurements in Serum
- C-60 Liquid Chromatography / Mass Spectrometry Methods.
- Should be available by end of year or early 2014.

Issues Unique to LC-MS/MS Analyses

- New Terminology
- Matrix Effects
- Differential ionization of Internal Standards or Calibrators
- In Source Transformation (fragmentation)
- Isobaric Compounds and Isomers
- Cross-Talk effects
- Chromatographic Resolution
- Carry-Over

Analytical Sensitivity

- Limit of Absence (LOA)
  - 20 replicates of zero calibrator or specimens without analyte run across multiple days
  - LOA = mean + 2SD or + 3 SD

- Limit of Detection
  - Measure specimens with levels (natural, diluted or spiked) that approximate the LOA, but are consistently detectable
  - LOD = mean of detectable concentration + 2SD or +3 SD of specimens
  - Also, noise x 3

- Limit of Quantification (Functional Sensitivity)
  - Minimum concentration where concentration can be measured reliably
    - Imprecision CV < 20%
    - Noise x 10

- Goals: Must be below clinical needs
Theory of Electrospray (LC-MS)

Effect of Matrix on Analyte Response in ESI

Sample Preparation

- Matrix: Serum, whole blood, urine
- Pretreatment process
  - Protein Precipitation Protocols: Fast & easy, but associated with longer periods of ion suppression due to early eluting, low molecular weight matrix constituents.
  - Solid-Phase Extraction & Liquid-Liquid Extraction: Slower & more chance for error. Do have shorter periods of ion suppression.

Matrix effects

1. Prepare extracts on 5 random blood samples from patients not receiving an IS drug.
2. Prepare 2 extracts using water as matrix.
3. To 500 uL of each extract and 150 ng Cyclo A, 75 ng Cyclo D, 15 ng FK-506, 15 ng Rapa, and 20 ng Asco (all in 50uL).
4. Compare whole blood recoveries from water.

<table>
<thead>
<tr>
<th></th>
<th>FK-506 (Area)</th>
<th>Cyclo A (Area)</th>
<th>Rapa (Area)</th>
<th>Asco (Area)</th>
<th>Cyclo D (Area)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>6847</td>
<td>77289</td>
<td>15482</td>
<td>6832</td>
<td>18998</td>
</tr>
<tr>
<td>#1</td>
<td>6834</td>
<td>71467</td>
<td>15735</td>
<td>6878</td>
<td>20492</td>
</tr>
<tr>
<td>#2</td>
<td>4886</td>
<td>79005</td>
<td>15896</td>
<td>6210</td>
<td>27507</td>
</tr>
<tr>
<td>#3</td>
<td>3989</td>
<td>79308</td>
<td>15896</td>
<td>6525</td>
<td>20816</td>
</tr>
<tr>
<td>#4</td>
<td>4834</td>
<td>79093</td>
<td>15320</td>
<td>9223</td>
<td>32742</td>
</tr>
<tr>
<td>#5</td>
<td>4394</td>
<td>75969</td>
<td>14991</td>
<td>8241</td>
<td>38703</td>
</tr>
<tr>
<td>Recovery</td>
<td>4559</td>
<td>73796</td>
<td>1404</td>
<td>8581</td>
<td>33972</td>
</tr>
</tbody>
</table>

Recovery 125% 97% 111% 100% 92%

In Source Transformation:
- Fragmentation occurring after column separation but before collision cell
  - MAP G > MPA > MPA Fragment
  - MPA BE > MPA > MPA Fragment
- Can be a problem with endogenous drug metabolites
  - Check real patient samples in extended chromatographic runs

Isobaric Compounds & Isomers
- Extremely important when measuring endogenous analytes
- Mandates complete chromatographic resolution

Cross-Talk
- Occurs when several mass transitions with identical product ions are acquired over a short time interval
- If collision cell does not empty completely, spurious signals can be recorded in a subsequent trace
- Common when several metabolites of a single drug are detected with identical fragment ions
- Solution: Increase interscan delay
"Analyte co-elution is generally feasible due to the high selectivity of SRM/MRM experiments, if co-elution of isobaric analyte isomers or ion source fragmentation of the analyte metabolites can be ruled out."


Carryover

The AOI at 500 ng/ml causes a 331% Carry-Over.

<table>
<thead>
<tr>
<th></th>
<th>10 ng/ml</th>
<th>100 ng/ml</th>
<th>500 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma 1</td>
<td>537</td>
<td>451</td>
<td>517</td>
</tr>
<tr>
<td>Plasma 2</td>
<td>539</td>
<td>481</td>
<td></td>
</tr>
<tr>
<td>Analyte</td>
<td>108000</td>
<td>1010000</td>
<td>4700000</td>
</tr>
<tr>
<td>Plasma 3</td>
<td>985000</td>
<td>985000</td>
<td>4550000</td>
</tr>
<tr>
<td>Plasma 4</td>
<td>495</td>
<td>460</td>
<td>481</td>
</tr>
<tr>
<td>Plasma 5</td>
<td>464</td>
<td>831</td>
<td></td>
</tr>
<tr>
<td>Analyte</td>
<td>1005000</td>
<td>985000</td>
<td>4550000</td>
</tr>
<tr>
<td>Plasma 6</td>
<td>425</td>
<td>539</td>
<td></td>
</tr>
</tbody>
</table>

\[
\text{%Carryover} = \frac{(A_{avg} - B_{avg})}{B_{avg}} \times 100
\]

A = plasma pool 3, 5, 7
B = plasma pool 2, 4, 6, 8.

Immunosuppressant Drugs by LC/MS/MS – Protocol (circa 2004)

Microfuge tube protocol:
1. In a 1.5mL Eppendorf tube accurately pipette:
   50µL lysis Solution A (0.4M ZnSO4)
   200µL whole blood (Calibrators, QCs or patient samples)
2. Briefly vortex mix samples (5-10 sec)
3. Add 500µL of Precipitating Solution (25ng/mL Ascomycin + 100 ng/mL CycloD in acetonitrile)
4. Vortex mix for approximately 1 minute or until the entire sample is thoroughly mixed
5. Centrifuge for 5 minutes at 14,500rpm
6. Cut top off of micro-centrifuge vial. Place vial into HPLC autosampler
7. Inject 10µL on the LC-MS/MS system. Use C-18 column (4X3mm – Phenomenex)
Immunosuppressant Drugs by LC/MS/MS - Protocol

<table>
<thead>
<tr>
<th>Compound</th>
<th>Precursor Ion</th>
<th>Daughter Ion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine A</td>
<td>1220</td>
<td>1203</td>
</tr>
<tr>
<td>Tacrolimus</td>
<td>821.5</td>
<td>768.5</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>931.6</td>
<td>864.5</td>
</tr>
<tr>
<td>Ascomycin</td>
<td>809.5</td>
<td>756.4</td>
</tr>
<tr>
<td>Cyclosporine D</td>
<td>1234.1</td>
<td>1217.2</td>
</tr>
</tbody>
</table>
### Immunosuppressant Drugs by LC/MS/MS - Limits

<table>
<thead>
<tr>
<th>Compound</th>
<th>LOD (ng/mL)</th>
<th>LOQ (ng/mL)</th>
<th>Linearity (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tacrolimus</td>
<td>0.1</td>
<td>0.6</td>
<td>40</td>
</tr>
<tr>
<td>Cyclosporine A</td>
<td>3.0</td>
<td>10</td>
<td>1000</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>0.3</td>
<td>0.6</td>
<td>40</td>
</tr>
</tbody>
</table>

### Immunosuppressant Drugs by LC/MS/MS - Imprecision

<table>
<thead>
<tr>
<th></th>
<th>Tacrolimus</th>
<th>Cyclosporine A</th>
<th>Rapamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration (ng/mL)</td>
<td>4</td>
<td>90</td>
<td>4</td>
</tr>
<tr>
<td>%CV</td>
<td>7</td>
<td>10</td>
<td>13</td>
</tr>
</tbody>
</table>

| %CV          | 2          | 7              | 9         |

### AMR & CRR

<table>
<thead>
<tr>
<th></th>
<th>AMR (ng/mL)</th>
<th>CRR (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine A</td>
<td>100 - 1000</td>
<td>10 - 2000</td>
</tr>
<tr>
<td>FK-506</td>
<td>0.3 - 40</td>
<td>0.3 - 80</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>0.6 - 40</td>
<td>0.6 - 80</td>
</tr>
</tbody>
</table>
**LC/MS/MS - Throughput**

- Run time = 2 mins/sample
- Cycle time = 3.5 mins.
- One run contains 18 samples + 4 stds + 3 controls.
- A run takes 87.5 mins.
- 5 runs can be performed in 7.3 hours.
- This means a maximum of 90 specimens can be analyzed per shift per instrument.

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**Emory Medical Laboratories Immunosuppressant Monitoring**

- Average monthly workload for 2004:
  - Tacrolimus – 1322
  - Cyclosporine A – 732
  - Sirolimus – 426

  This equals 2480 samples a month or 83 samples per day

- Analysis performed in the Special Chemistry Section, 7 days per week, dayshift only.

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**RAPA – Assay Comparison (both LC/MS/MS)**

Demming Regression:
- Emory = 1.006 Mayo + 0.05
- $R = 0.9903$
- $N = 24$
- Average Bias = 0.10
**FK-506 Assay Comparisons**

Demming Regression:

\[
\text{LC/MS/MS} = 1.022 \times \text{IMx} - 0.117 \\
R = 0.9380 \\
N = 39 \\
\text{Average Bias} = 0.064
\]

**Patient Sample Comparison**

*Note:* The patient samples were from 39 patients at 11 different centers.

**Metabolic Pathway of Cyclosporine A**

AM1, AM9, AM4n are the primary metabolites found in human blood and urine. Other metabolites result from further metabolism of the primary metabolites.
Cross Reactivity of Cyclosporine Major Metabolites

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Concentration Range (ng/mL)</th>
<th>Siemens ACMIA</th>
<th>Siemens ADVIA</th>
<th>Microgenics CEDIA</th>
<th>Roche EMIT</th>
<th>Syva EMIT</th>
<th>Abbott MEIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>AM1 (M17)</td>
<td>10–20 80–150</td>
<td>1.8%</td>
<td>@1000 ng/mL</td>
<td>&lt;5%</td>
<td>4.4%</td>
<td>@1000 ng/mL</td>
<td>&lt;AS @500 ng/mL</td>
</tr>
<tr>
<td>AM9 (M1)</td>
<td>5–10 50–75</td>
<td>2.1%</td>
<td>@1000 ng/mL</td>
<td>15%</td>
<td>20.0%</td>
<td>@1000 ng/mL</td>
<td>13% @667 ng/mL</td>
</tr>
<tr>
<td>AM4N (M21)</td>
<td>3–5 5–25</td>
<td>6.0%</td>
<td>@1000 ng/mL</td>
<td>&lt;5%</td>
<td>16.0%</td>
<td>@1000 ng/mL</td>
<td>5.1% @500 ng/mL</td>
</tr>
</tbody>
</table>

Maynard S. Clin Lab News 2011; 36(8)

Figures show the inter-institutional precision of each method and the number of participants by method.
Common Problems

• Whole blood assays require extraction.
• Need for Standard Reference Materials (SRM) and Clinical Reference Materials (CRM)
• Reference methods are also needed to harmonize methods
• Lack of full automation

Extraction & Connectivity

• Current manual extractions are similar for both IA and LC-MS/MS
• Run Times:
  – IA = 12 to 18 mins/sample
  – LC-MS/MS = 2 to 4 mins/sample
• Most systems now accept bar code from sample labels to build work lists.
• Interfaces to connect to lab computer systems now available on many instruments.

Standardization & Harmonization

• Standards:
  – Each IA manufacturer prepares their own
  – Many LC-MS/MS users prepare their own
  – Commercial STDS are available from 3 manufacturers
• Procedures:
  – Each IA vendor has specific extraction procedure(s), antibodies, buffers, & incubations
  – Most LC-MS/MS procedures are LDTs using different volumes, extractions, ionization modes, etc.
**Trends in Immunosuppressant Monitoring**

- Decrease dose of IS drug to as low as possible to maintain graft
- Most of current IS drugs exhibit kidney cytotoxicity to some degree
- Discontinuing use of Cyclosporine A
- Rapamycin used sparingly
- IS drugs are finding expanded use in autoimmune diseases.
- Should we be monitoring these patients?

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**Analysis from Dried Blood Spots**

- Uses ~30 µL of whole blood (1 spot)
- Dried cards are stable for four weeks
- Cards could be mailed in
- Would allow for more frequent sampling & better dosing

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**Conclusions**

A. Both IAs & LC-MS methods can be used for TDM of IS drugs
B. LC-MS methods offer advantages of specificity and multiplexing
C. SRM, CRMs and a Certified Reference Method are sorely needed for each of the IS drugs routinely measured no matter what analytical technique is used.
D. Caution must be observed when comparing results between laboratories measuring IS drugs due to method differences
Status of LC-MS/MS Assays at EML Today:

**Clinically in Use**
- Cyclosporine
- Rapamycin
- Tacrolimus
- Mycophenolic acid
- Everolimus
- Busulfan
- Antidepressants (14)
- Antipsychotics (12)
- 25 (OH) Vitamin D
- Testosterone

**Research**
- Aragatoban
- Lenalidomide
- Levamisole
- Bile Accids
- Iodothalomate
- Flumazenil

**In Development**
- Glucocorticoids
- Metanephrines
- Benzodiazepines
- Pain Medications
Thank you!!

Questions ???
jritchi@emory.edu