Confirmation by Triple Quadrupole LC/MS/MS for HHS-compliant Workplace Urine Drug Testing

A complete applications solution for forensic toxicology laboratories

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Background – Workplace drug testing by LC/MS

• Workplace drug testing has required confirmation of initial drug tests since the beginning of Federally-regulated and military drug testing programs.

• GC/MS has been the 'gold standard' for this testing, with well-developed procedures and criteria for confirmation of a presumptive positive.

• US Dept of Health and Human Services (HHS) will allow the use of other chromatography-mass spectrometry techniques for confirmation beginning in October 2010: GC/MS/MS, LC/MS, and LC/MS/MS.

• Triple quadrupole (LC/MS/MS) is a well-accepted technique for high confidence identification and quantitation.
Other major changes to federally-regulated workplace drug testing, effective October 2010

• MDA, MDMA, MDEA added to amphetamines confirmation.
• Amphetamines and BE cutoffs have been lowered.
• 6-AM is confirmed after a positive screen result regardless of morphine concentration.
• Specific requirements are specified for amphetamines and opiates for demonstrating lack of interference by structurally-related compounds.
• At least 10 data points across a peak are required for all techniques.
"A specimen is positive when its confirmatory drug test is equal to or greater than the cutoff." (HHS Guidelines, Nov 2009)

<table>
<thead>
<tr>
<th>Target analyte</th>
<th>Confirmation cutoff (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamines</td>
<td>250</td>
</tr>
<tr>
<td>Amphetamine</td>
<td></td>
</tr>
<tr>
<td>Methamphetamine</td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td></td>
</tr>
<tr>
<td>MDA</td>
<td></td>
</tr>
<tr>
<td>MDMA</td>
<td></td>
</tr>
<tr>
<td>MDEA</td>
<td></td>
</tr>
<tr>
<td>Cocaine metabolite BE</td>
<td>100</td>
</tr>
<tr>
<td>Marijuana metabolite cTHC</td>
<td>15</td>
</tr>
<tr>
<td>Phencyclidine (PCP)</td>
<td>25</td>
</tr>
<tr>
<td>Opiates</td>
<td></td>
</tr>
<tr>
<td>Morphine</td>
<td>2000</td>
</tr>
<tr>
<td>Codeine</td>
<td>2000</td>
</tr>
<tr>
<td>6-acetylmorphine</td>
<td>10</td>
</tr>
</tbody>
</table>
SAMHSA-required elements of LC/MS/MS method validation for drug confirmation

1. Linearity of method across wide dynamic range
   - determine LOQ and ULOL

2. Determination of LOD

3. Method accuracy and precision:
   - measured at cutoff and above and below cutoff concentrations
   - at least five replicates per concentration

4. Demonstrated absence of carryover from high concentration samples

5. Measurement of potential interferences from structurally-related compounds
   (amphetamines and opiates)

6. Measurement of matrix effects (ion suppression), recovery and overall process efficiency

7. Documented optimization of instrument and method parameters

8. Parallel studies of NLCP PT samples and donor specimens when changing from GC/MS
Additional NLCP Validation Requirement for "New Technologies", September 2010: "Parallel Studies" for each drug class

1. Analyze 10 human urine specimens positive for the assay analyte(s) by GC/MS.
   (If positive donor specimens are used, use the previously obtained GC/MS values for the comparison.)
2. Analyze the same specimens using the new method.
3. Re-analyze PT samples from the 2 most recent NLCP PT cycles by GC/MS (request NCLP permission).
4. Analyze the same specimens using the new method.
5. Compare the results obtained by GC/MS with the results obtained by the new method, and resolve or explain any values that differ by more than 20%.
Agilent contributions to drug testing
("and now a word from our sponsor")

• Agilent (then Hewlett-Packard) provided the initial GC/MS confirmation solution to the drug-testing community.

• Agilent is the recognized leader in GC/MS, especially in the forensic toxicology community.

• Agilent now supplies several types of LC/MS systems to forensic toxicology laboratories.

• Agilent is the leading supplier of chromatography-mass spectrometry systems to the WADA doping control laboratories for Olympic and other international competition.

• Today's topic: the Agilent applications organization has developed and validated a set of sample preparation and LC/MS/MS methods for the HHS (SAMHSA) October 2010 expanded workplace drug testing program.
Advantages of LC/MS/MS for Drug Testing

• No derivitization required for analytes
  – Lower reagent costs, lower labor costs = lower cost/sample
  – Faster turn-around
  – Less instrument maintenance (no aggressive reagents injected)
• Shorter runs than with GC/MS, using modern UPLC
• Potentially better sensitivity for some analytes (MS/MS) still with high specificity (ion ratios like GC/MS)
Objectives for LC/MS/MS Confirmation Methods for Drug Testing

• Utilize existing sample preparation methods already developed for GC/MS confirmation

• Provide reliable sensitivity and specificity comparable to or better than corresponding GC/MS methods

• Short cycle times to allow quick turnaround for confirmation testing

• Fast quantitative data analysis and reporting with GC/MS-like ion ratios and easy batch review

• Well-documented procedures that can be easily learned by GC/MS-trained personnel
Agilent LC/MS/MS drug confirmation methods: features and validation

• Methods meet or exceed HHS October 2010 requirements and guidelines.
• Methods use UPLC (sub-2µ particle, high-flow) column and fast gradients for best resolution and shortest cycle times.
• All methods use same column and only two mobile phase combinations; all five drug classes can be run on a single instrument without hardware or mobile phase changes.
• All methods use SPE sample preparation methods previously validated for HHS GC/MS confirmation, without derivitization.
• Methods use newer labeled internal standards with higher deuterium content for limiting analyte isotope "crosstalk", interferences.
• Methods have two qualifier ion ratios for all target analytes except AMP and METH (exceeds HHS requirements), and one qualifier ion for internal standards.
• Methods were validated in NLCP-certified workplace drug testing laboratory.
Features common to Agilent HHS methods

### Sample prep and LC separation

- Gravity-flow SPE columns from Biochemical Diagnostics (Varian SPE methods are available)
- Deuterated internal standards from Cerilliant Corporation
- Zorbax SB-C18 column, 3.0 x 50 mm RRHT (1.8μ), for speed and capacity
- Flow rates and fast gradients optimized for separation, peakshape, good retention (separation from void volume), MS sensitivity
- Mobile phase components:  
  - MeOH/20 mM AmFormate for cTHC  
  - MeOH/water 0.1% formic acid for all other methods

### MS/MS methods

- Utilize standard 6410 QQQ LC/MS/MS model or newer models
- Use high-flow ESI source for simplicity and robust operation (can use Agilent Jetstream ESI but not necessary for HHS cutoffs)
- 3 MRM transitions for target analytes (except 2 for AMP and METH), 2 qualifier ion ratios like GC/MS
- 2 MRM transitions, 1 ion ratio for IS's for additional confidence in results
- Customizable quantitation report with GC/MS-like content
Summaries and Performance of Methods
Amphetamines

Low calibrator, 25 ng/mL (10% of cutoff)
Dynamic MRM used for this method for optimum sensitivity
Potential amphetamine interferences* – method developed for separation from targets

*aphrentine
*phentermine
*pseudoephedrine
*PPA
*ephedrine
MDA
MDMA
MDEA
Pseudoephedrine
Ephedrine
PPA
Phentermine
Ephedrine
"For Forensic Use"
Amphetamines – typical calibration curves
25 – 10,000 ng/mL, \( r^2 \)'s 0.999 or better
Quadratic fit required for wide dynamic range found in real samples
Amphetamines – typical figures of merit from first of 5 validation batches

<table>
<thead>
<tr>
<th>Evaluation parameter</th>
<th>Accuracy</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument accuracy, precision (average % of target value, %RSD) n=5 injections</td>
<td>AMP 100.4</td>
<td>1.0</td>
</tr>
<tr>
<td>cutoff calib 250ng/mL</td>
<td>METH 102.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>MDA 98.7</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>MDMA 104.0</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>MDEA 101.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Linearity (accuracy across dynamic range) 25 – 10,000 ng/mL, 7 levels 10-4000% of</td>
<td>102.3</td>
<td>0.9</td>
</tr>
<tr>
<td>cutoff</td>
<td>(average of all 5 cpds)</td>
<td></td>
</tr>
<tr>
<td>Carryover: solvent blank and NegQC injections after 10,000 ng/mL calibration and</td>
<td>None detected (&lt; 0.05%)</td>
<td></td>
</tr>
<tr>
<td>linearity samples</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Amphetamines – interference studies

HHS guidelines require demonstrated absence of interference with detection and quantitation of amphetamines from the following compounds:

<table>
<thead>
<tr>
<th>Interferent</th>
<th>Level spiked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ephedrine</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>Pseudoephedrine</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>Phenylpropanolamine</td>
<td>1 mg/mL</td>
</tr>
<tr>
<td>Phentermine</td>
<td>50,000 ng/mL</td>
</tr>
</tbody>
</table>

Potential interferents are added to samples containing 0 and 100 ng/mL (40% of cutoff) of the target compounds.

No positives resulted from spiking drug-free urine with interferents.

100 ng/mL QC samples with interferents added measured within 10% of target values.
Ion suppression/matrix effects

Use of deuterated internal standards helps compensate for matrix effects, however:

HHS guidelines and sound LC/MS method validation practices require the measurement of matrix effects on identification and quantitation.


Measurements compare responses (peak area of quant ion) for three samples:

1. Reconstitution solvent fortified with targets ('mobilephase')
2. Negative urine spiked pre-extraction, then extracted ('pre')
3. Negative urine extract spiked post-extraction ('post')

Method also measures recovery and overall process efficiency.
Ion suppression/matrix effects measurements: expressed as ratio of responses

Calculations

**Matrix effect** = post/mobilephase

< 100% ⇒ ion suppression

>100% ⇒ ion enhancement

**Recovery effect** = pre/post

**Process efficiency** = pre/mobilephase

Results for amphetamines using 10 different blank urines

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MatrixEff</th>
<th>Recovery</th>
<th>ProcessEff</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>103</td>
<td>72</td>
<td>74</td>
</tr>
<tr>
<td>METH</td>
<td>109</td>
<td>88</td>
<td>96</td>
</tr>
<tr>
<td>MDA</td>
<td>100</td>
<td>74</td>
<td>74</td>
</tr>
<tr>
<td>MDMA</td>
<td>102</td>
<td>88</td>
<td>90</td>
</tr>
<tr>
<td>MDEA</td>
<td>104</td>
<td>87</td>
<td>90</td>
</tr>
</tbody>
</table>

- No measurable ion suppression
- Slight ion enhancement for METH
Benzylecgonine, low calibrator, 10 ng/mL
3 transitions for BE, 2 for BE-d8 internal standard

For Forensic Use
Benzoylcegonine (BE) LC separation showing BE, cocaine, and other metabolites

Interference studies not required for this analyte, but LC separation developed for potential analysis of other metabolites for confirmation of cocaine use.
Benzoylcegonine – typical calibration curve
10 – 4,000 ng/mL, $r^2$'s 0.999 or better
Linear fit; 1/x weighting needed for best accuracy at low concentrations
## Benzylecgonine – typical figures of merit

<table>
<thead>
<tr>
<th>Evaluation parameter</th>
<th>Accuracy</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy, precision (avg % of target value, %RSD)</td>
<td>104.9</td>
<td>4.9</td>
</tr>
<tr>
<td>n=5 injections, Batch 1 of 5 cutoff calib 100 ng/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linearity (accuracy across dyn range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 – 4,000 ng/mL, 7 levels 10-4000% of cutoff</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 separate batches, each with own curve and QCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carryover: solvent blank and NegQC injections after 4,000 ng/mL calibration and linearity samples</td>
<td>None detected (&lt; 0.01%)</td>
<td></td>
</tr>
</tbody>
</table>
Benzoylcegonine Ion suppression/matrix effects

Calculations

Matrix effect = post/mobilephase

< 100% ⇒ ion suppression

>100% ⇒ ion enhancement

Recovery effect = pre/post

Process efficiency = pre/mobilephase

Results for BE
(10 different blank urines)

<table>
<thead>
<tr>
<th></th>
<th>MatrixEff</th>
<th>Recovery</th>
<th>ProcessEff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>97.0</td>
<td>62.1</td>
<td>60.1</td>
</tr>
<tr>
<td>StdDev</td>
<td>4.0</td>
<td>11.0</td>
<td>10.4</td>
</tr>
<tr>
<td>Range</td>
<td>90-102</td>
<td>55-72</td>
<td>53-70</td>
</tr>
</tbody>
</table>

• Little/no ion suppression apparent
• Recovery not as high as for Amps
Carboxy-THC (cTHC), 15 ng/mL cutoff calibrator
3 transitions for cTHC, 2 for cTHC-d9 internal standard

Negative ion detection gave better sensitivity than positive ion method.

Urine is base-hydrolyzed before SPE to cleave any cTHC-glucuronide.
cTHC – typical calibration curve
1.5 – 600 ng/mL, $r^2$'s 0.997 or better
Quadratic fit, $1/x^2$ weighting gave best accuracy across desired range
**cTHC – typical figures of merit from 5 validation batches**

<table>
<thead>
<tr>
<th>Evaluation parameter</th>
<th>Batch</th>
<th>Accuracy</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Instrument accuracy, precision:</strong> avg % of target value/RSD (n=5 injections, 125% cutoff QC at 18.75 ng/mL)</td>
<td>1</td>
<td>96.7</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td><strong>95.5</strong> (avg of 5 batches)</td>
<td><strong>3.0</strong></td>
</tr>
<tr>
<td><strong>Linearity</strong> (accuracy across dynamic range) 1.5 – 600 ng/mL, 7 levels 10-4000% of cutoff, 5 separate batches, each with own curve and QCs</td>
<td>1</td>
<td>98.1</td>
<td>6.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>98.9</td>
<td>6.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>100.3</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>100.1</td>
<td>8.1</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>100.6</td>
<td>5.6</td>
</tr>
<tr>
<td></td>
<td><strong>Avg</strong></td>
<td><strong>99.6</strong></td>
<td><strong>1.1</strong></td>
</tr>
<tr>
<td><strong>Carryover:</strong> solvent blank and NegQC injections after 600 ng/mL calibration and linearity samples</td>
<td></td>
<td>None detected (&lt; 0.01%)</td>
<td></td>
</tr>
</tbody>
</table>
cTHC Ion suppression/matrix effects

Calculations

Matrix effect = post/mobilephase

< 100% ⇒ ion suppression

>100% ⇒ ion enhancement

Recovery effect = pre/post

Process efficiency = pre/mobilephase

Results for cTHC
(10 different blank urines)

<table>
<thead>
<tr>
<th></th>
<th>MatrixEff</th>
<th>Recovery</th>
<th>ProcessEff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>102.3</td>
<td>63.2</td>
<td>64.7</td>
</tr>
<tr>
<td>StdDev</td>
<td>2.6</td>
<td>8.3</td>
<td>9.1</td>
</tr>
<tr>
<td>Range</td>
<td>99-107</td>
<td>52-79</td>
<td>54-80</td>
</tr>
</tbody>
</table>

• Little/no ion suppression apparent: good cleanup and good chromatography

• Recovery typical for cannabinoids
Phencyclidine (PCP), 25ng/mL cutoff calibrator
3 transitions for PCP, 2 for PCP-d5 internal standard

PCP quant ion

PCP qualifier 1

PCP qualifier 2

PCP-d5 quant ion

PCP-d5 qualifier
PCP – typical calibration curve
2.5 – 1000 ng/mL, $r^2$'s 0.9996 or better
Quadratic fit, 1/x weighting gave best accuracy across desired range
PCP – figures of merit from 5 validation batches

<table>
<thead>
<tr>
<th>Evaluation parameter</th>
<th>Batch</th>
<th>Accuracy</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Instrument <strong>accuracy</strong>, precision: avg % of target value/RSD (n=5 injections, QCs 40% and 125% of cutoff)</td>
<td>1</td>
<td>95.7 (10 ng/mL)</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>97.9 (31.25 ng/mL)</td>
<td>0.6</td>
</tr>
<tr>
<td><strong>Linearity</strong> (accuracy across dynamic range) 2.5 – 1000 ng/mL, 6 levels 10-4000% of cutoff, 5 separate batches, each with own curve and QCs</td>
<td>1</td>
<td>98.6/3.0</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>97.2/2.8</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>96.7/4.7</td>
<td>4.7</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>96.6/3.7</td>
<td>3.7</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>96.3/1.3</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>Avg</td>
<td><strong>97.1/0.9</strong></td>
<td><strong>0.9</strong></td>
</tr>
<tr>
<td><strong>Carryover</strong>: solvent blank and NegQC injections after 1000 ng/mL samples</td>
<td>None detected (&lt; 0.01%)</td>
<td>None detected (&lt; 0.01%)</td>
<td></td>
</tr>
</tbody>
</table>
PCP Ion suppression/matrix effects

Calculations

Matrix effect = post/mobilephase

< 100% ⇒ ion suppression

>100% ⇒ ion enhancement

Recovery effect = pre/post

Process efficiency = pre/mobilephase

Results for PCP
(10 different blank urines)

<table>
<thead>
<tr>
<th></th>
<th>MatrixEff</th>
<th>Recovery</th>
<th>ProcessEff</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average</td>
<td>109.4</td>
<td>81.8*</td>
<td>89.1</td>
</tr>
<tr>
<td>StdDev</td>
<td>5.3</td>
<td>17.1*</td>
<td>17.3*</td>
</tr>
<tr>
<td>Range</td>
<td>101-117</td>
<td>46*-98</td>
<td>53*-107</td>
</tr>
</tbody>
</table>

Some ion enhancement is suggested by the data.

*One odd pre-extraction spike skews these data. Quantitation is correct but area is low; area, not calculated concentration, is used in the ratio calculations.
Opiates – morphine, codeine and heroin metabolite 6-acetylmorphine (6-AM)

Analysis of these targets for HHS cutoffs in urine present a special challenge:

• MOR, COD cutoffs are 2000 ng/mL, 6-AM is 10 ng/mL.
• MOR and COD require acid or enzymatic hydrolysis to cleave glucuronide conjugates excreted in urine.
• 6-AM is remarkably labile and does not survive MOR/COD glucuronide hydrolysis.
• Most labs therefore analyze 6-AM using a separate extraction due to hydrolysis issues and large differences in concentration from MOR/COD.
HHS opiates analysis: One method or two?

• A single 3.5-minute method was developed that would accommodate both high concentrations of MOR/COD and low concentrations of 6-AM; separates HHS interferents.

• 6-AM recovery even with mild enzyme hydrolysis was only 15-30%.

• Low 6-AM recovery would not allow reliable quantitation down to 1 ng/mL in same analysis as 20,000 ng/mL of MOR or COD without column overload for those analytes.

• Therefore: separate sample prep and analysis for 6-AM.

Cutoff calibrator

Morphine 2000 ng/mL
Codeine 2000 ng/mL
6-AM 10 ng/mL
Final procedures for HHS opiates

• Separate extractions for MOR/COD and 6-AM to allow glucuronide conjugate hydrolysis with glucuronidase enzyme.

• LC/MS/MS methods can then be optimum in injection volume for the disparate cutoffs and desired linear ranges.

• Original combined separation is used for simplicity
  - MOR/COD run time is still only 2.5 minutes.
  - 3.5-minute 6-AM method keeps longer retention for 6-AM for best sensitivity and to minimize matrix interferences and ion suppression

• Only samples which screen positive for 6-AM must be confirmed for 6-AM; typically a very small percentage for most labs.

• Additional optimization of glucuronidase conditions for improved 6-AM recovery might allow one consolidated sample prep.
HHS opiates, cutoff calibrators
3 transitions for targets, 2 for internal standards

- Morphine quant ion
  - Morphine qualifier 1
  - Morphine qualifier 2
  - Morphine-d6 quant ion
  - Morphine-d6 qualifier

- Codeine quant ion
  - Codeine qualifier 1
  - Codeine qualifier 2
  - Codeine-d6 quant ion
  - Codeine-d6 qualifier

- 6-AM quant ion
  - 6-AM qualifier 1
  - 6-AM qualifier 2
  - 6-AM-d6 quant ion
  - 6-AM-d6 qualifier
Opiates MOR, COD – typical calibration curves 200 – 20,000 ng/mL, \( r^2 \)'s 0.9995 or better

Quadratic fit, \( 1/x \) weighting gives best accuracy across the desired range
6-AM – typical calibration curve
1 – 400 ng/mL, r²'s 0.999 or better
Linear fit, 1/x weighting gives best accuracy across desired range

![Calibration Curve Graph](image_url)
**Morphine and codeine – figures of merit from 5 validation batches**

<table>
<thead>
<tr>
<th>Evaluation parameter</th>
<th>MOR</th>
<th>COD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Instrument accuracy, precision:</strong> avg % of target value/RSD (n=5 injections, QCs [ng/mL] as shown)</td>
<td>800</td>
<td>100.4/2.2</td>
</tr>
<tr>
<td>2500</td>
<td>101.1/1.1</td>
<td>107.6/0.4</td>
</tr>
<tr>
<td><strong>Linearity</strong> (accuracy across dyn range) 200 – 20,000 ng/mL, 7 levels 10-1000% of cutoff</td>
<td>1</td>
<td>100.8/2.1</td>
</tr>
<tr>
<td>2</td>
<td>100.3/4.6</td>
<td>99.4/3.9</td>
</tr>
<tr>
<td>3</td>
<td>99.7/2.9</td>
<td>100.8/5.0</td>
</tr>
<tr>
<td>4</td>
<td>99.2/1.6</td>
<td>100.5/6.4</td>
</tr>
<tr>
<td>5</td>
<td>101.8/3.0</td>
<td>103.0/4.1</td>
</tr>
<tr>
<td><strong>Avg</strong></td>
<td>100.4/1.0</td>
<td>101.0/1.3</td>
</tr>
<tr>
<td><strong>Carryover:</strong> solvent blank and NegQC injections after 20,000 ng/mL calibration and linearity samples</td>
<td>None detected (&lt; 0.01%)</td>
<td>None detected (&lt; 0.01%)</td>
</tr>
</tbody>
</table>
### 6-AM – figures of merit from 5 validation batches

<table>
<thead>
<tr>
<th>Evaluation parameter</th>
<th>Accuracy</th>
<th>Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Instrument accuracy, precision:</strong> avg % of target value/RSD (n=5 injections, 40% and 125% QCs [ng/mL shown])</td>
<td>98.5</td>
<td>0.8</td>
</tr>
<tr>
<td>avg % of target value/RSD (n=5 injections, 40% and 125% QCs [ng/mL shown])</td>
<td>103.7</td>
<td>0.7</td>
</tr>
<tr>
<td><strong>Linearity (accuracy across dyn range)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 – 400 ng/mL, 7 levels 10-4000% of cutoff</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 separate batches, each with own curve and QCs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100.3</td>
<td>2.6</td>
</tr>
<tr>
<td>2</td>
<td>100.9</td>
<td>4.7</td>
</tr>
<tr>
<td>3</td>
<td>101.0</td>
<td>3.0</td>
</tr>
<tr>
<td>4</td>
<td>100.8</td>
<td>2.9</td>
</tr>
<tr>
<td>5</td>
<td>101.3</td>
<td>2.5</td>
</tr>
<tr>
<td><strong>Avg</strong></td>
<td><strong>100.9</strong></td>
<td><strong>0.4</strong></td>
</tr>
<tr>
<td><strong>Carryover: solvent blank and NegQC injections after 400 ng/mL calibration and linearity spls</strong></td>
<td>None detected (&lt; 0.01%)</td>
<td></td>
</tr>
</tbody>
</table>
Opiates – interference studies

HHS guidelines require demonstrated absence of interference with detection and quantitation for the following compounds:

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Interferent</th>
<th>Level spiked</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine/codeine</td>
<td>Hydromorphone, Oxymorphone, Hydrocodone, Oxycodone, Norcodeine</td>
<td>5000 ng/mL each</td>
</tr>
<tr>
<td>6-AM</td>
<td>All the above Plus MOR, COD</td>
<td>5000 ng/mL each</td>
</tr>
</tbody>
</table>

- Potential interferents are added to samples containing 0 and 40% of the cutoff concentration of the target compounds.
- No positives resulted from spiking drug-free urine with interferents.
- 40%-cutoff QCs with interferents added measured within 10% of target values.
Opiate analytes shown with SAMHSA interferents* and other opiates

- Morphine
- Hydromorphone*
- Oxymorphone*
- Dihydrocodeine
- Codeine
- Hydrocodone*
- Oxycodone*
- 6-Acetylmorphine

*For Forensic Use
Opiates Ion Suppression/Matrix Effects

Calculations

Matrix effect = post/mobilephase

< 100% ⇒ ion suppression

>100% ⇒ ion enhancement

Recovery effect = pre/post

Process efficiency = pre/mobilephase

Results for opiates
(10 different blank urines)

<table>
<thead>
<tr>
<th>Analyte</th>
<th>MatrixEff</th>
<th>Recovery</th>
<th>ProcessEff</th>
</tr>
</thead>
<tbody>
<tr>
<td>morphine</td>
<td>98</td>
<td>53</td>
<td>52</td>
</tr>
<tr>
<td>codeine</td>
<td>101</td>
<td>80</td>
<td>81</td>
</tr>
<tr>
<td>6-AM</td>
<td>84</td>
<td>89</td>
<td>74</td>
</tr>
</tbody>
</table>

• No measurable ion suppression for MOR and COD in spite of fast LC separation.

• Measurable but acceptable ion suppression for 6-AM.

• Lower recovery for morphine compared to the other analytes.
One lab's experience: GC/MS vs. LC/MS/MS confirmation for HHS-compliant drug testing

• South Bend Medical Foundation collaboration with Agilent
• Paul Moorman, Technical Manager, Toxicology (today's speaker)
• Dr. Prentiss Jones, Toxicology Director
  – NLCP-certified workplace drug testing laboratory
  – Clinical laboratory supporting region hospitals
  – Evaluating Agilent-developed QQQ methods using their Agilent 6410 QQQ LC/MS/MS
  – Utilized existing SPE sample prep methods already validated for GC/MS confirmation
  – Followed NLCP guidelines for validation of confirmation methods, using spiked control urines
  – Hosted Agilent applications chemists for methods training
Types of analyses

• Workplace urine drug testing (NLCP Category 2):
  EIA, ADH (Ethanol), SVT, GC-FID (Ethanol), GC/MS

• LC/MS/MS:
  Ethyl glucuronide (EtG, for alcohol abstinence)
SBMF LC/MS/MS Workload and Ionization Modes

• EtG [ESI] - as needed
• Drugs confirmation [ESI] (not yet implemented)
• Each assay uses a different column: simplified by use of two column compartments with 6-port and Column Selection valves
### SBMF LC/MS/MS Workload

Use of two valves and two column compartments avoids frequent removal and reinstallation of LC columns, allows automatic method changeover.

<table>
<thead>
<tr>
<th>Assay</th>
<th>6-port valve</th>
<th>Column selection valve</th>
</tr>
</thead>
<tbody>
<tr>
<td>EtG</td>
<td>position 2</td>
<td>position 3</td>
</tr>
<tr>
<td>Drugs</td>
<td>position 2</td>
<td>position 4</td>
</tr>
</tbody>
</table>
Possible Advantages of LC/MS/MS for Drug Confirmation (revisited)

1. No derivatization required
   - Some labor savings
   - Some reagent savings
   - Avoid incubation time
   - No degradation of GC columns due to aggressive derivitization reagents
LC/MS/MS Possible Advantages

2. Analysis cycle times (inj-to-inj) may be shorter than for GC/MS.

SBMF examples:

<table>
<thead>
<tr>
<th>Assay</th>
<th>GC/MS</th>
<th>LC/MS/MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMPS</td>
<td>13 minutes</td>
<td>8 minutes</td>
</tr>
<tr>
<td>BE</td>
<td>9 minutes</td>
<td>8 minutes</td>
</tr>
<tr>
<td>cTHC</td>
<td>8 minutes</td>
<td>7 minutes</td>
</tr>
<tr>
<td>PCP</td>
<td>11.5 minutes</td>
<td>8 minutes</td>
</tr>
<tr>
<td>OPI</td>
<td>12 minutes</td>
<td>8 minutes</td>
</tr>
<tr>
<td>6-AM</td>
<td>14 minutes</td>
<td>8 minutes</td>
</tr>
</tbody>
</table>
3. LC/MS/MS in lab may provide opportunities to expand test menu or to perform current tests with more convenience (e.g., non-regulated expanded opioids panel at SBMF).
LC/MS/MS Possible Disadvantages

• Although well-established in forensic tox labs, LC/MS/MS is relatively new to the workplace drug testing environment. There will be a learning curve for the labs, inspectors, and, eventually, the legal system.

• An LC/MS/MS system requires a significantly greater capital investment than a GC/MS system.
Example of Matrix Study Sample Prep: Amphetamines

• Ten different blank urines
• Amphetamines spiked at 100 ng/mL (40% of cut-off, 5 drugs)
• 5 Deuterated Internal standards added at 250 ng/mL to all samples
• Compare drug responses for
  • spiked MP (X% MeOH/water, matching t=0 composition)
  • extract of urine, spiked pre-extraction
  • extract of urine, spiked post-extraction
## Example of Matrix Study Sample Prep

<table>
<thead>
<tr>
<th>Sample</th>
<th>MP</th>
<th>Pre-extract</th>
<th>Post-extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>-------</td>
<td>1.0 mL</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>Methanol</td>
<td>-------</td>
<td>------</td>
<td>25 uL</td>
</tr>
<tr>
<td>Drug mix in methanol 10,000 ng/mL</td>
<td>-------</td>
<td>10 uL</td>
<td>------</td>
</tr>
<tr>
<td>I.S. mix in methanol 16,667 ng/mL</td>
<td>-------</td>
<td>15 uL</td>
<td>------</td>
</tr>
<tr>
<td>(i.e. 1:6 dilution of 100,000 ng/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[-----------------perform extraction------------------]

<table>
<thead>
<tr>
<th>Sample</th>
<th>MP</th>
<th>Pre-extract</th>
<th>Post-extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>475 uL</td>
<td>475 uL</td>
<td>475 uL</td>
</tr>
<tr>
<td>Methanol</td>
<td>-------</td>
<td>25 uL</td>
<td>------</td>
</tr>
<tr>
<td>Drug mix in methanol 10,000 ng/mL</td>
<td>10 uL</td>
<td>------</td>
<td>10 uL</td>
</tr>
<tr>
<td>I.S. mix in methanol 16.667 ng/mL</td>
<td>15 uL</td>
<td>------</td>
<td>15 uL</td>
</tr>
</tbody>
</table>

NOTE: One vial of spiked MP may be enough for the study.

Recommend re-injecting spiked MP with each pair of pre- and post- extraction samples, and a solvent blank between sets of 3.

Recommend use of positive displacement pipettes (e.g., Drummond ®)
Summary of Agilent HHS-compliant workplace drug confirmation methods

• All methods use the same UPLC column

• Methods can use standard or SL (600 bar) versions of 1200 LC

• Methods developed on standard 6410 QQQ with ESI; can also use more sensitive 6430 and 6460 models but not necessary.

• Simple mobile phase components used for all methods
  – MeOH in place of ACN for cost and availability reasons
  – MeOH/ 20 mM ammonium formate for cTHC in negative ion
  – MeOH/water w/ 0.1% formic acid for remaining methods

• Run times are 4 min or less (cycle time/re-equilibration depends on LC configuration)

• Could use alternating column regeneration to save 2-3 minutes per sample for high-volume labs (add10-port valve and second pump).
## Confirmation Method Summary

<table>
<thead>
<tr>
<th>Analytes</th>
<th>HHS Cutoff ng/mL</th>
<th>Linear range(^1) ng/mL</th>
<th>Run time(^2) min</th>
<th>Cycle time(^3) min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amphetamines</td>
<td>250</td>
<td>25 – 10,000</td>
<td>4.5</td>
<td>8</td>
</tr>
<tr>
<td>Benzoylecgonine</td>
<td>100</td>
<td>10 - 4000</td>
<td>4.5</td>
<td>8</td>
</tr>
<tr>
<td>cTHC</td>
<td>15</td>
<td>1.5 - 600</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>PCP</td>
<td>25</td>
<td>2.5 - 1000</td>
<td>3.5</td>
<td>8</td>
</tr>
<tr>
<td>Opiates: morphine, codeine</td>
<td>2000</td>
<td>200 – 20,000</td>
<td>2.5</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>1 - 400</td>
<td>3.5</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^1\) Accurate to within 10% of target value, and all ion ratios within ± 20% rel. of cutoff calibrator

\(^2\) LC/MS run time; includes conservative column cleanup step for each method

\(^3\) Injection-to-injection time at SBMF, includes column cleanup and re-equilibration times
Design objectives achieved for Agilent LC/MS/MS Confirmation Solutions

• Minimize number of columns (1) and mobile phases (2) for all 5 drug classes.

• Fast, robust methods for high throughput, using Agilent Zorbax RRHT sub-2µ column

• Use only single ionization mode (ESI) for all drug classes

• Methods meet or exceed all NLCP guidelines (accuracy, precision, linearity, carryover, ion ratios, etc.)

• Methods compatible with existing sample prep used for GC/MS

• Carry out successful method validations in existing NLCP lab familiar with both GC/MS and LC/MS/MS

• Develop complete, step-by-step SOPs which include reagents, sample prep, and instrument operation
HHS-compliant LC/MS/MS Urine Confirmation resources available in "application bundle"

• Standard Operating Procedures which include:
  – specifications and suppliers of all reagents and supplies
  – step-by-step sample preparation procedures (SPE, hydrolysis where req'd)
  – step-by-step instrument setup and batch analysis procedures
  – step-by-step MassHunter Quant review and reporting procedure

• Electronic versions of acquisition and quantitation methods

• Custom MassHunter Quant report template providing HHS-required information for each sample

• Zorbax RRHT column used for methods

• On-site applications consulting for startup of one method, and training beyond standard operator course

• Note: SAMHSA guidelines require method validation and documentation of method and parameter optimization/verification in each laboratory.
The Agilent Advantage for LC/MS/MS workplace drug confirmation methods

Complete: includes sample preparation, LC separation, acquisition and data analysis methods, custom DrugQuant report

Fast and robust methods, validated in NLCP-certified laboratory using gravity-flow SPE already in use for GC/MS confirmation.

Excellent quality of validation data demonstrates the straightforward transition from GC/MS to LC/MS/MS confirmation for Agilent-trained staff using the 6400 series LC/MS/MS.

Analyte list is expandable to additional drugs using MS/MS parameters available from Agilent.

Single-vendor service, applications, and columns support for LC, MS, workstation and software.
Literature references for SAMHSA2010 LC/MS/MS Confirmation using Agilent QQQQ Systems

Same samples analyzed by GC/MS at 5 HHS-certified labs and by Agilent LC/MS/MS at RTI

Journal of Analytical Toxicology, Vol 33, 398-408 (October 2009)

A Comparison of the Validity of Gas Chromatography–Mass Spectrometry and Liquid Chromatography–Tandem Mass Spectrometry Analysis of Urine Samples for Morphine, Codeine, 6-Acetylmorphine, and Benzoylecgonine

Peter R. Stout*, Nichole D. Bynum, John M. Mitchell, Michael R. Baylor, and Jeri D. Ropero-Miller
Research Triangle Institute International, Center for Forensic Sciences, Research Triangle Park, North Carolina

Journal of Analytical Toxicology, Vol 34, 430-433 (October 2010)

A Comparison of the Validity of Gas Chromatography–Mass Spectrometry and Liquid Chromatography–Tandem Mass Spectrometry Analysis of Urine Samples II: [Amphetamine, Methamphetamine, MDA, MDMA, MDEA, PCP and cTHC]

Peter R. Stout*, Nichole D. Bynum, John M. Mitchell, Michael R. Baylor, and Jeri D. Ropero-Miller
Research Triangle Institute International, Center for Forensic Sciences, Research Triangle Park, North Carolina
Time for questions

“Mr. Osborne, may I be excused? My brain is full.”