Improving the Detection of Inborn Errors of Metabolism Using LC-MSMS

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In general, #$@&%!?>*^$% deficiency =
• rare, serious, recessive genetic condition
• associated with $X (Y,Z …) levels
• symptoms sometimes not very specific
• efficient diagnosis is important for treatment and/or future prenatal diagnosis
• Multiplexing: one test, many biomarkers (38), many disorders (24)
• A screen is a screen: positives (≈0.5%) x 80,000 babies pa = 400 pa

Confirmatory testing → Second tier testing

Almost certainly medium chain acyl CoA dehydrogenase deficiency, methylmalonic acidemia, propionic acidemia, B12 deficiency, false positive.
Urine testing for high risk patients: symptoms of IEM or confirmation of newborn screening result

- Amino acids
  - paper electrophoresis
  - TLC
  - IEC
- Organic acids
  - GC-MS
- Purines & pyrimidines
  - HPLC
- Glycosaminoglycans electrophoresis
- MSMS Screen
  - Sialic acid
  - Bile acids
  - Creatine
  - Guanidinoacetic
  - Steroids
  - Acyl carnitines
Progress towards the ultimate urine test

Report
- Risk of type 2 diabetes
- Future lawyer
- Soccer fan
- 2 kids
- ....
Urine metabolic screening by MSMS
Current panel of 104 metabolites

1 to 40 μL of urine (related to creatinine), calibrators, QC

Mix with internal standards in MTP, dry down
Butylate, decant off from salts, dry down, recon in 50% ACN
Flow injection positive ESI-MSMS with 69 MRMs (2.3 mins per well)

Mix with internal standards in MTP
Flow injection negative ESI-MSMS with 59 MRMs (2.3 mins per well)

Spreadsheet analysis, abnormal levels flagged using absolute (μmol/mmol creatinine) or MoM cut-offs, qualitative reporting
**Negative ion mode: direct injection with MRMs**

- **TIC**

- **Ret time**

- **%**

- **m/z**

  - * = internal standards

  - laetic
  - 3-hydroxyisovaleric
  - 3-methylglutaconic
  - orotic
  - C5-glycine
  - uric
  - homovanillic
  - S-sulphocysteine
But what about........

SUPRESSION!!
Suppression effects in direct injection ESI-MSMS
Urine samples spiked at fixed concentrations

**Positive ion mode:**

Absolute response

**Negative ion mode:**

Ion ratio
Suppression effects in direct injection ESI-MSMS using surrogate internal standard

\[ [X] \propto \text{Area(IS)} \]

\[ \downarrow \text{Area(X)} \]

\[ \downarrow \text{Area(IS)} \]

Ret time →

\( m/z_1 \)

\( m/z_2 \)
Urine S-sulphocysteine (SSC) calibrations

Using $^{15}$N$_2$-uric as a “surrogate” internal standard

Interbatch
CV = 12%

Conventional SID using $^2$H$_2$-S-sulphocysteine internal standard

Interbatch
CV = 5.0%
Increased S-sulphocysteine and xanthine, lowish uric = molybdenum cofactor deficiency, subsequently confirmed as a MOCS1 gene defect
Confirmation of S-sulphocysteine with product ion spectra

Spiked control

Baby “Z” with neonatal seizures

Normal control

Confirmation of S-sulphocysteine with product ion spectra
Monitoring urine metabolite levels in baby “Z”
First MOCS1 patient treated with cPMP cofactor replacement therapy

Pediatrics (in press), collaboration with Dr Alex Veldman (Monash Medical Centre) & Prof Guenter Schwarz (U of Cologne)
Example:

- Biochemical basis for pyridoxine-dependent seizures published in 2006
- Caused by antiquitin deficiency, accumulating metabolites:

  Lysine

  \[ \xrightarrow{\text{\uparrow}} \]

  \[
  \begin{array}{c}
  \text{piperideine-6-carboxylic} \\
  \xrightarrow{\text{\uparrow}} \\
  \text{2-aminoadipic semialdehyde}
  \end{array}
  \]

- ESI-MSMS characteristics of metabolites established from patient samples, MRM conditions optimised
- Quantitation difficult, multiple of median (MoM) analysis used
- Pilot study showed urine piperideine-6-carboxylic dimer had good diagnostic sensitivity
- All urines tested for this disorder since Oct 2006
Urine blotter MSMS screening

Stability of metabolites on blotters at room temperature:

Assay imprecision (+/- 2 SDs)

- 9 days
- 79 days
Urine blotter MSMS screening of first Malaysian patient diagnosed with adenylosuccinate lyase deficiency: MoC chart

Collaboration with Mrs Chen Bee Chin, Pediatric Institute, Kuala Lumpur Hospital, Malaysia
New diagnoses for the biochemical genetics lab:
congenital adrenal hyperplasia: steroid 21-hydroxylase deficiency

Urine pregnanetriol glucuronide (multiples of batch median)

- No notes
- Vomiting
On-line solid phase extraction: urine steroids and bile acids

Apply sample, aqueous wash

Elute with methanol

Steroid sulphates

Bile acids (taurine)

Steroid glucuronides

Bile acids (glycine)

1 x 20 mm C18 column, SPE mode, 0.05 mL/min
On-line solid phase extraction: urine steroid glucuronides

17-hydroxyprogesterone metabolites

Pregnanetriolone

Pregnanetriol

Cortisol metabolites

Patient

control

Parents of 75ES-1.93e4

Parents of 75ES-1.93e4

1 x 20 mm C18 column, SPE mode, 0.05 mL/min
LC-MS confirmation: urine steroids

EICs for pregnanetriol glucuronide $m/z = -511.3$

EICs for pregnanetriolone glucuronide $m/z = -525.3$

Patient

control

Scan ES: 511.3

Scan ES: 525.3

2.1 x 100 mm x 3 μm C18 column, methanol gradient, 0.15 mL/min
On-line solid phase extraction: doing it cheap
Chromatography on disposable column
Spiked urine, butyl esters

PU01, STRATA-X ONLINE CLEAN UP

ONLINE03

C10 carnitine

ONLINE03

C6 carnitine

ONLINE03

C2 carnitine

ONLINE03

3-O-methylDOPA

ONLINE03

creatinine

ONLINE03

homocystine

1 x 20 mm Strata-X homemade column, fast methanol gradient, 0.05 mL/min

17-Sep-2009

3: MRM of 19 Channels ES+
372.2 > 85
4.35e5

3: MRM of 19 Channels ES+
316.2 > 85
1.03e6

3: MRM of 19 Channels ES+
260.2 > 85
9.86e6

4: MRM of 12 Channels ES+
268.2 > 166
2.79e4

4: MRM of 12 Channels ES+
188.1 > 90
1.30e7

2: MRM of 31 Channels ES+
389.2 > 196.1
1.34e5
Virtually all amino and organic acid disorders detected

Other disorders (unlikely to be detected by conventional amino/organic acid screening):
• Adenylosuccinase deficiency (2)
• Ureidopropionase deficiency (1)
• Generalised peroxisomal biogenesis defects
• D-bifunctional protein deficiency (2)
• Smith-Lemli-Opitz syndrome (3)
• Guanidinoacetate methyl transferase deficiency (1)
• Creatine transporter defect (1)
• Isolated xanthine oxidase (1)
• Cerebrotendinous xanthomatosis
• Antiquitin deficiency (10)
• Aspartylglucosaminuria
• Hawkinsuria
• 3-hydroxyisobutyryl CoA hydrolase deficiency
Developing a quantitative platform

Single (UP)LC-MSMS, multi MRM/window method for:

- Amino acids including leucine/isoleucine/allo-isoleucine, total homocysteine
- Selected organic acids (methyl malonic, 3-hydroxybutyric, orotic, succinyl acetone)
- Free and acyl carnitines
- Creatine and guanidinoacetic
- Neurotransmitters

For urine, plasma, CSF and dried blood spot samples (second tier test for NBS)
Chloroformates: a useful derivatisation method

Aqueous propyl chloroformate/propanol/pyridine

ca 30 seconds, room temperature

PCF derivative
LC-MSMS of PCF derivatives: control urine

- **5-hydroxytryptophan**
- **3-O-methylDOPA**
- **pyroglutamic**
- **pipecolic**
- **glycine**
Improving value

\[
\text{Value} = \frac{\text{quality}}{\text{cost}}
\]

Expanded newborn screening model
Biochemical genetics model

Penny pincher model
Zoe  Mary  Erin+?  Lan  Maggie  Michiel  James
Nick  Mary  Ivan  Joe  Manal  Thanh

the VCGR team!
Urine piperideine-6-carboxylic dimer

Multiple of batch median

99th% ile = 2.9
Suppression effects in LC-ESI-MSMS using surrogate internal standard

\[ \text{Area(IS)} \propto \text{Area(X)} \]

\[ \text{Ret time} \rightarrow \]

\[ m/z_1 \]

\[ m/z_2 \]
• There are hundreds of other compounds eluting in your LC-MSMS run
• Some of them are clinically significant
• Run a standard, work out LC-MSMS parameters, plug in the MRM
• Try it on patient samples and see (but check the ethics first!)
• Impress your boss!