Simultaneous high throughput MSMS measurement of dried blood spot proteins, enzyme activities, and metabolites for diagnosis of IEM and haemoglobinopathies

Asian Pacific Conference of Chromatography & Mass Spectrometry 2010
14th - 16th January 2010

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WellChild Laboratory
King’s College, London
Evelina Children’s Hospital
Dried blood spot analysis
Newborn Screening
Guthrie bacterial inhibition assay
Dried blood spot analysis
Newborn Screening
Amino acid thin layer chromatography
Dried blood spot analysis

Electrospray (John Fenn)
Dried blood spot analysis
Mass Spectrometry-Mass Spectrometry
Triple quadrupole - schematic
Dried blood spot analysis
Newborn Screening
MSMS – amino acids (butylated)

PKU Positive
Dried blood spot analysis
Newborn Screening
MSMS - acylcarnitines (butylated)
Dried blood spot analysis
Newborn Screening
Sample preparation for MSMS

**Butylated**
- Punch out 3mm blood spot or pipette plasma
  - Add methanolic internal standard
  - Shake
  - Remove liquid to second plate and evaporate
    - Add butanolic HCl
    - Cover and heat at 65 C for 25 min
    - Evaporate to dryness
    - Reconstitute with running solvent
    - Cover, load and run on TMS

**Underivatised**
- Add methanolic internal standard
  - Shake
  - Remove liquid to second plate and evaporate
    - Cover, load and run on TMS
Dried blood spot analysis
Direct analysis - no butylation
Amino acid and acylcarnitine scans
Dried blood spot analysis
Direct analysis - no butylation
Phenylketonuria
Dried blood spot analysis
Direct analysis - no butylation
Medium chain acylCoA dehydrogenase deficiency
Dried blood spot analysis
Direct analysis - no butylation – MRM data acquisition
Phenylketonuria screening - control
Dried blood spot analysis
Direct analysis - no butylation – MRM data acquisition
Phenylketonuria screening - case
Dried blood spot analysis
Direct analysis - no butylation – MRM data acquisition
Phenylketonuria & MCADD screening - case
Dried blood spot analysis
Newborn Screening
MCADD
Dried blood spot analysis
Newborn Screening
MCADD

Prof Carol Dezateux & team at ICH
Participating laboratories/metabolic services
Birmingham, GOS, GSTFT, Leeds, Manchester, Sheffield
Screen for 2y, ascertain clinically presenting cases
for 4y to define costs & benefits of screening
Dried blood spot analysis
Newborn Screening
MCADD

UK Collaborative Study of Newborn Screening
Medium chain acyl CoA Dehydrogenase Deficiency

- Collaborating with the BIMDG, UKNSLN and Oxford University
- Funded by the Department of Health and National Screening Committee

Data collection: March 2004-March 2006/8
750,000 children screened – 75 cases detected
Economic case proven
Original 6 labs provided with continued funding
National coverage roll out completed in early 2009
Dried blood spot analysis

Newborn screening

Metabolites
direct analysis
PKU, MCADD

In the UK context an uneconomical use of technology
Dried blood spot analysis

Newborn screening

Rationalise
    Improve cost effectiveness of MSMS

Additional metabolites

Incorporation of other mandated tests
    Sickle cell disease/haemoglobinopathy screening
Dried blood spot analysis
NHS Sickle Cell and Thalassaemia screening programme

<table>
<thead>
<tr>
<th>variant</th>
<th>Wild Type AA</th>
<th>Variant AA</th>
<th>Position</th>
<th>Δ Mass</th>
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<td>HbC</td>
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<td>Gln</td>
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<td>Glu</td>
<td>Lys</td>
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HbS/HPFH, HbS/β-thalassaemia (β+, β0, δβ, Lepore)
**Strategy - Screen for sickle protein**
Dried blood spot analysis
Normal haemoglobin

Full scan – charge series
Protein mass reconstruction
Dried blood spot analysis
HbAS, HbSS
Dried blood spot analysis
Normal haemoglobin
full scan – charge series
Dried blood spot analysis
Screening for sickle protein by MS

Simplify

- Target a particular multiply charged species, i.e., 12, implies normal β-chain target MW is 1322.3 daltons
- Sickle mutation target MW is 1319.8
- The actual MWs, within limits, are irrelevant in screening because we know that the majority will be wild-type and we can standardise on that and then detect the sickle "shift", at charge 12, 2.5 daltons
Dried blood spot analysis
Screening for sickle protein by MS
Sample preparation and MS scans

3mm blood spot in deep well plate
Add 0.5 ml deionised water and mix
Inject 5μl into solvent stream
Solvent MeCN:water 50:50 with 0.025% formic acid

MS-API2000
Q3 scans (116), 2 experiments
MW 1315-1325 & 1215-1225
Inject to inject 75-90 secs
Dried blood spot analysis
Screening for sickle protein by MS

- HbA
- HbSS
- HbAS
Dried blood spot analysis
Screening for sickle protein by MS

Need to improve sensitivity and specificity

Proteomic sequence targeting – knowledge based

Tryptic digestion
Analyse peptides as small molecules
Fragmentation provides amino acid sequence
Use mutation informative MRM to detect
Scan for sequence

Potential for single stage screening and unequivocal confirmation
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Tryptic digestion predictable

Human $\beta$-globin
15 peptides, T1-T15

Position of mutations known

Sickle mutation in T1
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Full scan tryptic digest

Sickle mutation in T1
  position, 6 glutamic acid to valine

<table>
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<tr>
<th></th>
<th>VHLTPEEK</th>
<th>MW 951.5</th>
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<tr>
<td>Sickle</td>
<td>VHLTPEVK</td>
<td>MW 921.5</td>
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Singly charged peptides  m/z 952.5  922.5
Doubly charged peptides  m/z 476.8  461.8

API4000, inject 2µl, usual solvent, flow rate 75µl/min
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Isolate doubly charged peptide ion in Q1, fragment, Q3 product ion scan

y series, N-terminal AA sequence
VHLTPE(V)EK, HLTP(V)EK (y7), LTPE(V)EK (y6), TPE(V)EK (y5), PE(V)EK (y4), E(V)EK (y3), EK (y2), K

b series, C-terminal AA sequence
VHLTPE(V)EK, VHLTPE(V)E (b7), VHLTPE(V) (b6), VHLTP (b5), VHLT (b4), VHL (b3), VH (b2), V
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Proteomic approach to haemoglobinopathies

Blood spots digested with trypsin

Tryptic peptides analysed like metabolites

Product ion scans – peptide sequence

MRM – mutation detection
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS
Dried blood spot analysis
NHS Sickle Cell and Thalassaemia screening programme

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</table>

HbS/HPFH, HbS/β-thalassaemia (β+, β0, δβ, Lepore)
*new peptide
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Targeted sequences for haemoglobins:
A, S, (MRM), C, DPunjab, OArab, E (surviving ion)

200 blood samples analysed:
AA 52       AS 57       AC 44       SS 14
SC 16       AE 10      ADPunjab 2  CC 1
DDPunjab 1  EE 1      AOArab 1   OArabOArab 1

Sensitivity 100%       Specificity 100%

British Journal of Haematology, 130:635-643
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

2007-2008, DOH funded technical evaluation of MSMS

Leeds, St James’s site chosen
isoelectric focussing - Resolve haemoglobin test kit
(PerkinElmer Life Sciences, Waltham, USA)
c.40,000 p.a.
extra blood spot punch, 2-6 96 well plates/day
overnight courier to WellChild laboratory

Study completed – July 2008
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Sample preparation
3.2mm blood spots digested for 30min at 37°C with trypsin reagent
diluted in mobile phase (acetonitrile: water, 50:50, with 0.025% formic acid)
2µl inject, flow injection, flow rate 80µl/min
Acquisition time 60s

API4000 or API4000 QTRAP
<table>
<thead>
<tr>
<th>Tryptic peptide</th>
<th>Target Peptide Ion (m/z)</th>
<th>Target fragment ion (m/z)</th>
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<td>Wild Type (Beta) T1</td>
<td>476.9</td>
<td>y4 502.3</td>
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<td>HbS T1</td>
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<td>HbC T1</td>
<td>694.5</td>
<td>b4 451.3</td>
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Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Normal neonate

Sickle trait
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Normal neonate

HbD\textsuperscript{Punjab} heterozygote
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Normal neonate

HbE heterozygote
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

HbF (gamma) neonate

HbF (gamma) adult
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

40,054 newborn dried blood spots from Leeds screened

T1 S peptide 199
  HbS/HbF 9, HBSC 3, HbS trait 187
  Incidence of sickle peptide 1:201, SCD 1:3338

T1 C peptide 39 (not including the 3 HbSC)
T13 D^{punjab} peptide 49
T13 O^{arab} peptide 0
T3 E peptide 47

Total 334 1:120
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

Real-time confirmation of peptide sequence

Collaboration with Applied Biosystems|MDS Analytical Technologies
Approximately 5000 samples analysed using API4000 QTRAP

Information dependent acquisition (IDA)
Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

<table>
<thead>
<tr>
<th>Name</th>
<th>Formula</th>
<th>Molecular weight (amu)</th>
<th>Fit</th>
<th>RevFit</th>
<th>Purity</th>
<th>CE</th>
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<tr>
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<td>77.291</td>
<td>62.310</td>
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Dried blood spot analysis
Screening for clinically significant haemoglobinopathies by MSMS

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Dried blood spot analysis

Newborn screening by MSMS

Improving efficiency

Integration of metabolite, PKU and MCADD, screening by MSMS with protein, sickle cell disease/haemoglobinopathy, screening by MSMS dramatically improves MSMS resource utilisation and can be highly cost effective

Enzymology?
Dried blood spot analysis
biotinidase deficiency

Blank

Control
Dried blood spot analysis
biotinidase deficiency

Substrate concentration

Incubation time
Dried blood spot analysis
biotinidase deficiency – optimised substrate/product ratio

Blank

Control
Dried blood spot analysis
porphobilinogen (PBG) synthase activity

Blank

Control
Dried blood spot analysis
PBG synthase activity

Substrate concentration

Incubation time
Dried blood spot analysis
porphobilinogen (PBG) synthase activity
optimised substrate/product ratio

Blank

Control
Dried blood spot analysis

Rationalisation

The phenotype multiplex

Single 3.2mm blood spot
Incubate with enzyme substrates for 60min at 37°C
Incubate with trypsin for 30min at 37°C
Stop reaction – final volume 1.125ml
Inject 2μl
MSMS (API4000) acquisition 60sec

Simultaneous measurement of enzyme activities, haemoglobinopathies, and metabolites
Dried blood spot analysis
Phenylketonuria

DBS neonate
phenylalanine, d\textsubscript{5}-phenylalanine

DBS phenylketonuria at diagnosis
Dried blood spot analysis
Medium chain acylCoA dehydrogenase deficiency

DBS neonate
octanoylcarnitine
d$_3$-octanoylcarnitine

DBS MCADD at diagnosis
Dried blood spot analysis
Sickle screening

DBS neonate

sickle peptide. wild-type beta T1

DBS sickle trait
Dried blood spot analysis
Biotinidase deficiency

DBS neonate

substrate/product measurement

DBS biotinidase deficiency
Dried blood spot analysis
Type 1 tyrosinaemia (porphobilinogen synthase activity)

DBS neonate

substrate/product measurement

DBS 100µmol/l succinylacetone added
Dried blood spot analysis

The phenotype multiplex

Additionally tested

All current haemoglobinopathy transitions
Maple syrup urine disease (leucine)
Glutaric aciduria type 1 (glutaryl carnitine)
Very long chain CoA dehydrogenase deficiency (tetradecenoylcarnitine)
MethylmalonylCoA mutase deficiency (propionylcarnitine)

All on a single 3.2mm blood spot
2h sample preparation
60sec MSMS time
Dried blood spot analysis

Application to newborn screening in UK unlikely in the near future

Application to routine IEM screening

Include chromatography
Dried blood spot analysis
MSMS amino acid analyser
Plasma amino acids – normal

Aspartic acid
Glutamic acid
Glutamine
Valine
Leucine/(allo)isoleucine
Tyrosine
Phenylalanine
Glycine
Alanine
Threonine
Serine
Tryptophan
Citrulline
Argininosuccinic acid
Argininosuccinic anhydride
Ornithine
Lysine
Arginine
Dried blood spot analysis
MSMS amino acid analyser
Plasma amino acids – citrullinaemia

Aspartic acid
Glutamic acid
Glutamine
Valine
Leucine/(allo)isoleucine
Tyrosine
Phenylalanine
Glycine
Alanine
Threonine
Serine
Tryptophan
Citrulline
Argininosuccinic acid
Argininosuccinic anhydride
Ornithine
Lysine
Arginine
Dried blood spot analysis
AADC, pyridoxine, pyridoxal phosphate deficiencies

DBS adult

3-O-methyl-DOPA
(2 transitions)

DBS patient with AADC
Dried blood spot analysis
Ornithine transcarbamylase deficiency

DBS adult

orotic acid

$^{15}$N$_2$-orotic acid

DBS adult + 25µmol/l orotic acid
Dried blood spot analysis
Argininosuccinate synthase deficiency (citrullinaemia)

Plasma control

orotic acid
$^{15}$N$_2$-orotic acid

Acute sample
Argininosuccinate synthase deficiency
Dried blood spot analysis
Homocystinuria

DBS adult

total homocysteine
d$_4$-homocysteine

DBS homocystinuria
Dried blood spot analysis
Caeruloplasmin

4 transitions confirming caeruloplasmin

2 quantitative transitions

βT1 wild type, HbS, HbC
Plasma tryptic peptides for clinical protein analysis
MSMS protein analyser

Albumin T6 internal standard
Retinol binding protein
Transferrin
Caeruloplasmin
α1-antitrypsin
Apolipoprotein B
Apolipoprotein E
Complement C3
Haemopexin
Prothrombin
Plasminogen
Fibrinogen
Anti-thrombin III
Alpha-fetoprotein
Dried blood spot analysis

Multiplexed assay system for simultaneous measurement of clinically diagnostic metabolites, proteins, and enzymes on a 3.2mm blood spot, requiring <1min of MSMS instrument time

Plasma, red cells, lymphocytes, fast chromatography, negative ionisation

“Expanded” newborn screening

Rationalised routine inborn errors of metabolism screening

Routine clinical diagnostics………..
Acknowledgements

Charles Turner

Sue Bird, GSTT metabolic team
Carol Dezateux and ICH MCADD team
Yvonne Daniel (GSTT), Lisa Farrar and Michaela Sutcliffe (Leeds)
NHS Sickle Cell & Thalassaemia Screening Programme
Lisa Sapp, Applied Biosystems

WellChild

Guy’s & St Thomas’ Charity
Evelina Children’s Hospital Appeal

Guy’s and St Thomas’ NHS Foundation Trust