MSMS NEWBORN SCREENING - IS IT REALLY THAT SIMPLE?

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Abstract. The incorporation of tandem mass spectrometry (MSMS) into an existing newborn screening program is an evolving process. Limited worldwide experience has ensured that all stages of reliability testing need to be followed. These include a literature review to establish methodology and analytes/disorders for testing and a pilot screening project including assaying archival samples from subjects with proven target disorders. Algorithms used for analyte concentrations and the relationships of various analytes to one another for resample criteria need to be continually reassessed to maximise screening specificity, sensitivity and positive predictive value. Since 1st of April 1998, the NSW Newborn Screening Program has screened 320,848 babies using electrospray MSMS for selected amino acids and acyl carnitines. Screening for amino acids has led to requests for 415 repeat samples with 94 babies referred for further testing. Of these 73 had a disorder of amino acid metabolism, including 43 with persistent hyperphenylalaninemia (36 of whom had PKU, 2 had a pterin pathway defect, 5 HPAA). Screening for acyl carnitines has led to requests for 245 repeat samples with 63 babies referred for further investigation. Of these 44 had a diagnosed disorder, including 15 with medium chain acyl CoA dehydrogenase deficiency. Five babies with confirmed disorders detectable with MS/MS had negative test results. The cost of screening using MSMS was only $A0.50 more than the method for screening for PKU and homocystinuria alone (ie the bacterial inhibition assays) and has allowed detection of an additional 74 babies at least 48 of whom have a diagnosis for which early treatment seems clearly beneficial. MSMS has shown a sensitivity of 95.9% and specificity of 99.8% in our laboratory with a positive predictive value of 18%.

INTRODUCTION

A tandem mass spectrometer (MS/MS) is a complex instrument composed of two mass spectrometers in series (McLafferty, 1981). Triple quadrupole instruments are commonly used for multiple MS/MS functions on the same sample. They include an ionisation source (producing ions of derivatized compounds within a sample); three mass filters (quadrupoles) connected in tandem; and detectors. Parent (precursor) ions are introduced into the first quadrupole (MS1) and are selected according to their mass for passage into the second quadrupole, which acts as a collision cell. In the collision cell the ions are bombarded with inert gas molecules (argon) causing dissociation into smaller fragments (daughter or product ions) which flow into the third quadrupole (MS2) where they are selectively sent to the detector.

The MS/MS can be operated in multiple modes: (1) detection of all fragments in MS2 from a single precursor; (2) detection of all precursors producing a single fragment size in MS2; or (3) scanning MS1 and MS2 for molecular species that lose an uncharged common fragment. Using a specific operating mode, multiple reaction monitoring (MRM), instead of a full scanning mode, it is possible to specify which ions, and therefore which analytes, will be detected providing the required specificity for use as a screening tool. With the introduction of electrospray ionisation in a mass spectrometer, not only was liquid chromatography able to be interfaced with mass spectrometry but also automated sample introduction became possible, providing a technique suited to population screening. In the 1990s the use of tandem mass spectrometry for the screening of various organic acid, fatty acid oxidation defects and amino acid disorders was proposed (Millington *et al*, 1990; Chace *et al*, 1993; Rashed *et al*, 1995; Chace *et al*, 1995; Chace *et al*, 1996; Rashed *et al*, 1997; Rashed *et al*, 1999; Wiley *et al*, 1999).

In 1997, after assessing the available literature and determining appropriate analytes to be assayed in order to detect specific target disorders, archived samples from previously diagnosed patients were tested. Samples were also tested from a pilot population of approximately 25,000 babies; the data and appropriate cut-off levels established. MS/MS was then introduced as a screening tool.

MATERIALS AND METHODS

Blood samples were collected by heel prick onto S&S 903 paper (Schleicher and Schuell Inc, Keene, NH) when babies were 48 hours of age or older, and usually on

day 3 of life. Archived newborn screening samples from babies diagnosed with inborn errors of metabolism were retrieved from where they had been stored in a cellar at room temperature for up to 10 years. In the pilot program, 24,965 consecutively received samples from Dec 1997 until 31st March 1998 were analyzed after which routine screening ensued.

The sample preparation was adapted from the method of Millington (Millington *et al*, 1990) and was previously reported (Wiley *et al*, 1999). Changes were made in order to provide reliability as a screening tool. In summary, blood from a 3mm blood disc was eluted with shaking for 1 hour (the time required for optimum elution) in methanol containing isotopically labelled internal standards in 96 well polypropylene, deep well microtiter plates. The supernatant was decanted to a standard depth polypropylene plate and evaporated to dryness under warm (40°C) air. Analytes were derivatized using butanolic HCl heated to 60°C for 15 minutes before again being evaporated to dryness. The sample was then redissolved in solvent (50% v/v acetonitrile:water) for injection into the MSMS. Sample preparation required about 2 hours time for the average 400 samples, standards and controls analyzed daily. Total run time was 2.1 minutes per sample or less than 14 hours overnight per batch.

Calibration was performed as previously described (Wiley *et al*, 1999) using isotopically labelled standards for Ala, Cit, Leu, Met, Phe and Tyr and carnitine, C2, C8 and C16 (Cambridge Isotopes, Andover, MA). Nine external blood spot standards with varying levels of added amino acids and acyl carnitines were assayed in each batch and contained known spiked concentrations of each amino acid measured as well as carnitine, C2, C3, C5, C6, C8, C10 and C16. These external standards were used to create standard curves per analyte to determine quantitative levels of amino acids and acyl carnitines. A Quattro II (Micromass Ltd, UK), mass spectrometer equipped with a 215 Liquid Sampler (Gilson Instruments, Middleton, WI) and Series 1100 pump (Hewlett Packard, Palo Alto, CA) was used. The MS/MS was controlled by a Digital workstation using Windows NT operating system and Masslynx software (Micromass Ltd, UK) with spectra initially interpreted by Neolynx software (Micromass Ltd, UK). Amino acids were measured using constant neutral loss mode with the loss of an uncharged 102 Dalton fragment between MS1 and MS2 for all except citrulline (119 loss) and glycine (56 loss). Acyl carnitines were measured using precursor ion scan mode with a common fragment of 85 Daltons. Multiple reaction monitoring for specific fragmentation was used to control the analytes tested. Results were transferred to the Labmaster database (Wiley Associates Pty Ltd, Sydney, Australia) where each analytical result was checked against predefined reference ranges resulting in an analyte specific tests cascade if the result was outside the range. Various analytical ratios, eg phe/tyr or C8/C2, were checked against their predefined reference range and specific cascades were added if the ratio was outside the range. Loading the results of each of the 22 analytes per sample for 400 samples with algorithm checking and follow-up cascade for further testing required less than 15 minutes per day.

Samples with all results inside the cut-off values were reported as "no further tests required". If an analyte, was marginally abnormal, a repeat dried blood spot sample

Table 1. Cut-off values required for repeat sample collection for babies whose sample was collected on day 3 of life.

Class	Analyte	Cut-off value for resample (umol/l)
Amino Acid	Alanine	>900
	Citrulline	<4 or >75
	Glycine	>1100
	Leucine/Isoleucine	>500
	Methionine	>80
	Phenylalanine	>180
	Tyrosine	>500
Acyl carnitines	Carnitine	<5or >125
	Acetyl carnitine	< 10 or >200
	Propionyl carnitine	>10
	Butyryl carnitine	>2
	Isovaleryl carnitine	>2
	3-hydroxy isovaleryl carnitine	>1.5
	Hexanoyl carnitine	>1.0
	Octanoyl carnitine	>1.0
	Decanoyl carnitine	>1.5
	Decenoyl carnitine	>1.5
	Myristyl carnitine	>1.5
	Tetradecenoyl carnitine	>1.5
	Palmitoyl carnitine	>8.5
	Hydroxypalmitoyl carnitine	>0.1

was collected. If follow-up tests were abnormal or if the original sample was significantly abnormal, further investigation included a repeat dried blood spot sample, urine for a metabolic screen of organic acids and amino acids. Amino acid and/or acylcarnitine plasma studies were performed as a diagnostic workup.

RESULTS

A problem for some programs contemplating use of MS/MS is a lack of birth prevalence data. Fortunately, the NSW Newborn Screening Program is associated with the NSW Biochemical Genetics Service, which is a population-matched service with over 20 years of data for the various disorders. However, for each target disorder there are very few proven cases to assay retrospectively making cut-off determinations difficult. A further complication is that the various analyte concentrations may be affected by storage. Results from the pilot project were evaluated with retesting on the original sample if initial results were outside the 99 percentile confidence interval. Population data and data from retrospective samples with proven disorders were used to establish cut-off levels for each analyte tested. These cut-off levels were under constant review as more population results were obtained and the values currently used are listed in Table 1. Individual analyte cut-off values were coupled with ratios of various analytes to increase specificity and sensitivity. Tables 2 and 3 give the data for amino acid and acyl-carnitine testing respectively.

The overall sensitivity (affected persons with a positive result) was 95.9% with 5 missed cases. Two of the missed cases (glutaric aciduria and very long chain acyl CoA dehydrogenase deficiency) were positive on the initial screen but the repeat sample gave results below the cut-off even for age-matched controls. The VLCAD patient showed inconclusive urine metabolic screen results.

The overall specificity (healthy persons with a negative result) was 99.8% with less than a 0.2% resample rate. The cut-off levels for resample were deliberately set low. Problems associated included the following: (1) proper concentration of marker analytes is often not known in neonates and in dried blood spots; and (2) there is an overlap between affected individuals and the normal ranges. Various isomers are not distinguished by tandem mass spectrometry further complicating the situation. For example, leucine and isoleucine and hydroxyproline all have the same ion fragments of interest in MS1 and MS2 under standard operating conditions.

The number of true cases among samples (positive predictive value) was 18%. A major problem, however, was the possibility of detecting disorders that are probably benign but have elevations of the same analyte(s) as the target disorders. Conditions detected that are probably benign include hydroxyprolinemia, methionine adenosyl transferase deficiency, short chain acyl CoA dehydrogenase deficiency and methylcrotonyl CoA carboxylase deficiency.

DISCUSSION

The reliability of MS/MS needs to be established

Table 2. Diagnosis of amino acid disorders.

Babies screened	320,848
- Resamples requested	415
Confirmed diagnoses	
- Phenylketonuria	36
- BH4 deficiency	2
- Hyperphenylalaninemia	5
- Hydroxyprolinemia	17
- other amino acid disorders	11
- secondary (B12 deficiency, hepatitis)	2
Missed cases	
- Tyrosinemia type I	1
- Non ketotic hyperglycinemia	1

Table 3. Diagnosis of acyl carnitine defects.

Babies screened	320,848
- Resamples requested	245
Confirmed diagnoses	
- Organic acidurias	12
- MCAD deficiency	15
- Other fatty acid oxidation defects	7
- Maternal fatty acid oxidation defects	7
- secondary (B12 deficiency)	3
Missed cases	
- Cobalamin C deficiency	1
- Glutaric aciduria	1
- very long chain acyl CoA dehydrogenase deficiency	1

before incorporating it into an existing program. An average analytical batch required 14 hours of unattended machine operation overnight. Many small problems were associated with the complexity of the instrumentation. Instrument failures occurred in 38 of 875 analytical runs, 24 of which were during the pilot program. In the initial runs, failures were usually due to fused silica inlet tubing blockages. This was resolved by conversion to peek tubing. Blockages also occurred in the rheodyne and in the source capillary. Many were resolved by enclosing the autosampler, thus minimizing the introduction of air borne contaminants. In order to minimize problems, MS/MS equipment has been subjected to rigorous maintenance schedules.

Computerisation is essential for high throughput. Software upgrades, however, caused handshaking problems with the controlling software for the autosampler or liquid chromatography pump. These were resolved either by patching the software the same day or temporarily reloading the previous version. Therefore, no significant delay in sample processing resulted. In general, initial screening results were available one working day after sample receipt for more than 95% of the samples.

Assessing costs associated with introduction of tandem mass spectrometry can be very complex since clinical costs for follow-up are offset by savings due to early diagnosis. Costs for consumables, instrumentation (including replacement, maintenance and depreciation) and staffing are easier to assess. In the NSW Newborn Screening Program the total cost of addition of MS/MS testing to an already existing newborn screening program was Aus$0.93. This cost was actually less than 50 cents since the MS/MS replaced other assays for measuring phenylalanine and methionine.

CONCLUSION

Tandem mass spectrometry has been shown to reliably detect many disorders that are potentially damaging or lethal without early treatment, however; it detects some disorders with lower sensitivity. Criteria can be set for a very low false positive rate. Therefore, MS/MS is really that simple – most of the time!

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