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Review

Application of tandem mass spectrometry to biochemical genetics and newborn screening

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Abstract

Tandem mass spectrometry (MS/MS) has become a key technology in the fields of biochemical genetics and newborn screening. The development of electrospray ionisation (ESI) and associated automation of sample handling and data manipulation have allowed the introduction of expanded newborn screening for disorders which feature accumulation of acylcarnitines and certain amino acids in a number of programs worldwide. In addition, the technique has proven valuable in several areas of biochemical genetics including quantification of carnitine and acylcarnitines, in vitro studies of metabolic pathways (in particular β-oxidation), and diagnosis of peroxisomal and lysosomal disorders. This review covers some of the basic theory of MS/MS and focuses on the practical application of the technique in these two interrelated areas.

Keywords: Tandem mass spectrometry; Newborn screening

1. Introduction

Mass spectrometry (MS), in the form of gas chromatography-mass spectrometry (GC/MS) using electron impact or chemical ionisation, has played a key role in the discovery of inborn errors of organic acid [1] and fatty acid metabolism, [2] and is also widely used in the investigation of peroxisomal [3] and steroid disorders [4]. The technique requires the separation of components of the sample mixture by capillary gas chromatography and uses the unique fragmentation pattern (or mass spectrum) of the separated compounds in the mass spectrometer for confirmation of identity. It does have serious limitations, however, including laborious sample preparation to make compounds of interest amenable to separation by GC, and long analysis time.

The ability to analyse polar, nonvolatile biological molecules was realised with the introduction of "soft" ionisation techniques such as fast atom bombardment (FAB/MS), thermospray (TSP/MS), and most recently and importantly, electrospray ionisation (ESI/MS),

Abbreviations: CE, capillary electrophoresis; CID, collision-induced dissociation; ESI, electrospray ionisation; FAB, fast atom bombardment; GC, gas chromatography; HPLC, high-performance liquid chromatography; MCAD, medium chain acyl CoA dehydrogenase; MRM, multiple reaction monitoring; MS, mass spectrometry; MS/MS, tandem mass spectrometry; TSP, thermospray; VLCAD, very long chain acyl CoA dehydrogenase.

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allowing liquid chromatography to interface with mass spectrometry. In these techniques, the mass spectrometer is again acting as a sophisticated detector, capable of identifying the compounds presented to it but still requiring a separation technique.

Tandem mass spectrometry (MS/MS) was developed over 20 years ago [5] and was first applied to biochemical genetics soon afterwards [6]. Although ion trap instruments are capable of multiple MS/MS functions (MS^n), the technique is most commonly performed using a triple quadrupole mass spectrometer which, as the name suggests, comprises an ionisation source, three mass filters connected in tandem, and a conventional electron or photomultiplier detector (Fig. 1). Ions produced in the source are selected by the first quadrupole (MS1) for transmission to the second quadrupole, which is designated as the collision cell. In this region, the ions are accelerated and collide with molecules of an inert collision gas (usually argon) and undergo collision-induced dissociation (CID). The fragments produced are transmitted to the final quadrupole (MS2) where they are again selected for transmission to the detector. Ions transmitted by MS1 to the collision cell are called precursor ions (formerly referred to as "parent" ions), and the fragments produced from CID are product ions (formerly "daughter" ions). The MS/MS can be operated to scan for all fragments produced from a single precursor (product ion scan), all precursors producing a single product (precursor ion scan), or to scan both filters a fixed mass apart to select all precursors undergoing a loss of a common non-charged moiety (neutral loss scan). In addition to these scanning modes of operation, the instrument can monitor a number of fixed transitions in a manner analogous to selected ion monitoring in a single quadrupole instrument. This type of operation is often called multiple reaction monitoring (MRM) and is particularly useful to restrict the compounds being detected to those preselected, as opposed to the scanning modes which will detect all compounds undergoing the chosen transition.

Because the first mass filter effectively removes the need for a separation technique prior to introduction of the sample, MS/MS can be used for analysis of complex mixtures with little or no sample clean up. The removal of the time-consuming step of resolving individual components by chromatography allows rapid analysis times, typically around 2–3 min per sample, and the use of stable isotope labelled internal standards allows accurate quantitative analysis. The methodology does have limitations, however: it cannot separate isomers and, in complex biological mixtures, the likelihood of encountering more than one compound with the same molecular weight and producing the same fragments is sometimes high. Therefore, several of the methods, which will be covered later, include a chromatographic separation prior to introduction to the MS/MS.

2. Carnitine and acylcarnitine analysis

Carnitine is a quaternary ammonium compound which has a key role in the transport of long chain fatty acids into the mitochondria. It also plays a role in detoxification by facilitating recycling of free coenzyme A via the carnitine acyltransferases which catalyse the formation of acylcarnitines from free carnitine and acyl CoA esters. Carnitine esters of short, medium, and long chain fatty acids are present in biological fluids and are found in increased concen-

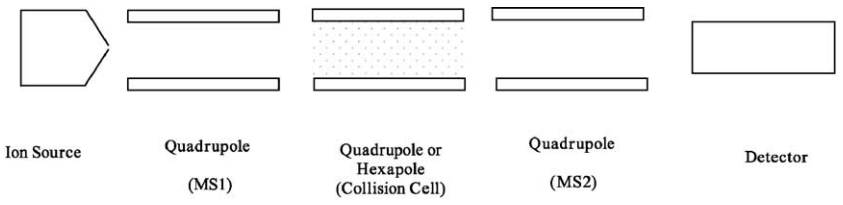

Fig. 1. Schematic representation of a triple quadrupole tandem mass spectrometer. Ions formed in the ion source are transferred to MS1, filtered and passed on to the collision cell where they are accelerated and collide with an inert gas causing collision-induced dissociation (CID). The product ions are filtered by MS2 before being passed to the detector.

trations in plasma and urine where metabolic defects result in accumulation of acyl CoA esters.

In the mid-1980s, the potential diagnostic value of analysis of acylcarnitines was demonstrated in propionic acidaemia, methylmalonic aciduria, isovaleric acidaemia, multiple carboxylase deficiency, 3-hydroxy-3-methylglutaric aciduria, methylacetoacetyl CoA thiolase deficiency, glutaric aciduria, medium chain acyl CoA dehydrogenase (MCAD) deficiency, and multiple acyl CoA dehydrogenase deficiency [7]. There followed a series of key publications from the group at Duke University using fast atom bombardment-tandem mass spectrometry (FAB-MS/MS) for analysis of acylcarnitine methyl esters in urine, plasma [8], and dried blood spots [9], identifying characteristic profiles in many disorders of fatty acid and organic acid metabolism as well as characterising the carnitine depletion associated with Valproate therapy [10]. The method pioneered at Duke utilises the fact that acylcarnitine methyl esters produce a characteristic fragment ion at m/z 99 upon collision-induced dissociation. Therefore, scanning for precursors of this fragment produces a profile showing free carnitine and acylcarnitines with little interference. By introduction of stable isotope labelled analogues of free carnitine and several acylcarnitine species, quantitation could be achieved using very small sample sizes and simple preparation [11].

The FAB-MS/MS method was refined to allow simultaneous analysis of amino acids by using butyl instead of methyl esters with acylcarnitine butyl esters yielding a common fragment of m/z 85 [12], but difficulties in automating sample introduction into the mass spectrometer limited the widespread application of FAB-MS/MS for acylcarnitine analysis.

The introduction of electrospray ionisation allowed introduction of the sample into a continuously flowing solvent stream, usually an acetonitrile/water mixture, with ionisation taking place at atmospheric pressure before the ions are transferred to the high-vacuum regions of the mass spectrometer. In positive ion mode, as operated for acylcarnitine analysis, the molecular ions of butyl or methyl esters carry a positive charge from the quaternary ammonium group. This technique is now the method of choice for analysis of free carnitine [13] and acylcarnitines in the fields of biochemical genetics and newborn screening (see below), and has proven to be robust and reliable in routine use.

The application of quantitative acylcarnitine analysis in plasma during metabolic crisis using butyl esters and ESI-MS/MS has been shown to be highly sensitive and specific for the diagnosis of a large number of disorders, with reference values published for free carnitine and important acylcarnitine species [14,15]. It should be remembered, however, that accumulation of acylcarnitines depends upon flux through the pathway and disorders with mild or episodic presentation may not show abnormal results between episodes [15].

Quantitative acylcarnitine analysis has also been successfully used for postmortem diagnosis using bile fluid [16] and dried blood spots [17], and for prenatal diagnosis of organic acidaemias using amniotic fluid [18]. In several situations, absolute concentrations of individual acylcarnitine species may not be diagnostic, but the application of ratios of one species to another is more powerful.

The use of ESI does offer the ability to analyse acylcarnitines without prior derivatisation [19]. Since these compounds are zwitterions in the native state, ionisation may be enhanced by addition of a small amount of formic acid to the mobile phase as the positive ion is formed from adduction of a hydrogen ion. Analysis without prior derivatisation simplifies sample preparation and offers the added advantage of removing any potential errors caused by acid esterification-induced hydrolysis of short chain acylcarnitine species [20]. However, sensitivity is somewhat reduced when compared to butyl ester analysis especially for dicarboxylic acylcarnitines [21].

Whereas the accumulation of acylcarnitines in body fluids may require the metabolic pathway to be stressed by fasting or intercurrent illness, in vitro assays using cultured cells or peripheral blood cells can be used to identify disorders by employing stable isotope labelled substrates and analysis of accumulated labelled acylcarnitines. The use of labelled substrates identifies the metabolic source of the acylcarnitines and has been important in identifying a new defect of branched chain amino acid metabolism [22]. This method has also been applied to investigation of disorders of mitochondrial β oxidation [23–25] and peroxisomal defects [26]. The technique has been used on amniocytes for prenatal diagnosis of two fatty acid oxidation defects, MCAD, and very long chain acyl CoA dehydrogenase

(VLCAD) deficiency [27], but care in interpretation is required as some cell lines from patients with respiratory chain disorders produce profiles mimicking fatty acid defects [28].

3. Amino acid analysis

Disorders of amino acid metabolism were some of the earliest inborn errors of metabolism investigated on a large scale. Largely, this reflected the availability of techniques to separate and identify amino acids in body fluids. These techniques included paper, thin layer and ion exchange chromatography, and high voltage electrophoresis, all relying on the production of a coloured product [24] when reacted with ninhydrin. For quantitative analysis of plasma amino acids, ion exchange chromatography and post column ninhydrin detection as first described by Moore et al. [29] in 1958 remains the gold standard, but long analysis times (initially over 24 h and now typically 2–3 h per sample) are limiting. More rapid separation techniques such as gas chromatography [30], reverse phase HPLC [31], and capillary electrophoresis (CE) [32] are also widely used, but all require pre-analysis derivatisation and are still unsuitable for screening applications.

Mass spectrometry coupled to GC [33] has been used for the analysis of amino acids in plasma but with limited appeal, offering little advantage over other detection systems. Recently, ESI-MS has been coupled to CE for the analysis of amino acids in plasma from children [34] and offers the advantage of not requiring sample derivatisation prior to analysis.

The first report of MS/MS in the analysis of amino acids in body fluids was by Millington et al. [35] in 1991. Using butyl esters of amino acids, they observed the loss of a common fragment of butyl formate (102Da) upon CID. This transition was seen to be highly specific for α-amino acids so that scanning MS1 and MS2 with a constant mass difference of 102Da produces a spectrum of the molecular ions of the majority of the amino acids present in the sample mixture with no interference from signals derived from other compounds. Quantitation was achieved by addition of stable isotope analogues of the amino acids of interest.

Not all amino acids produce the strongest signal with a 102 neutral loss. Where the side chain contains a basic group, ammonia is lost first followed by butylformate resulting in a neutral loss of 119Da. Rashed et al. [36] used this function to quantify lysine, ornithine, arginine, glutamine, asparagine, and citrulline. Further refinement of sensitivity to glycine was achieved by observing neutral loss of 56Da [37], and to arginine by recording the most abundant transition, a loss of 161Da. A specific MS/MS method for argininosuccinate was described by Rashed et al. in which the tris-butyl derivative (which is formed under standard conditions) produces fragments at m/z 144 and 172, although a stable isotope internal standard is not commercially available for this amino acid and quantitation is not required for the diagnosis of argininosuccinate lyase deficiency [38].

The problem of isomers again limits MS/MS analysis and is especially important for amino acids with leucine, isoleucine, and hydroxyproline sharing a molecular ion of m/z 188, alanine and sarcosine m/z 146, proline and asparagine m/z 172, and glutamine and lysine m/z 186. Careful observation of the relative ratios of fragments produced can help to identify the isomer present and has been used successfully in our laboratory to distinguish between leucines (preferred loss 102) and hydroxyproline (preferred loss 120).

Unlike the situation with acylcarnitines where MS/MS is becoming the "gold standard method", tandem mass spectrometric analysis has not displaced traditional methods for amino acid monitoring. However, in newborn screening, combined MS/MS acylcarnitine and amino acid analysis has revolutionised the field.

4. Newborn screening

For over 30 years from the initiation of the first newborn screening program based upon Guthrie and Susi's [39] bacterial inhibition assay for phenylalanine in dried blood spots, each time a new disorder was added to a screening program, a new test, often with a completely different technology, was required. Thus, even the most comprehensive programs were limited to screening for a handful of disorders which fulfilled the criteria for a sensitive and specific test for a condition which occurs with reasonable frequency where early medical intervention can prevent or significantly reduce sequelae.

The potential of acylcarnitine analysis by MS/MS to be used for neonatal screening for a number of organic acid disorders was recognised early in the development of the technique [9]. There followed a number of reports on diagnosis of phenylketonuria [12], maple syrup urine disease [40], and homocystinuria [41] in newborn blood spots using amino acid analysis by fast atom bombardment tandem mass spectrometry. The true potential of the technique to perform population screening for a large number of disorders was realised when Rashed et al. [36] used electrospray ionisation to analyse butyl esters of amino acids and acylcarnitines and automated both sample introduction and data analysis. Further development by this group led to sample preparation in 96-well microtitre plates, and they were able to demonstrate reliable high-quality profiles from 500 to 1000 samples per day per instrument [37]. They reported the results of a limited pilot study over 3 years [38] during which they detected 20 cases comprising 10 different disorders in 27,624 samples tested.

Technical issues involved in routine use of the technology for newborn screening such as sample preparation, internal standards, mode of operation, and interpretation have been largely resolved [42]. There are now a number of newborn screening programs operating around the world using butyl ester formation and analysis of selected amino acids and acylcarnitines. The combination of information from all the published literature and our own experience of more than 325,000 babies screened in New South Wales, MS/MS has been used to detect 26 different disorders (Table 1) [43–51].

While false negative rates appear to be very low, some disorders have been missed by MS/MS newborn screening including glutaric aciduria Type I [52], tyrosinaemia Type I, nonketotic hyperglycinaemia, and Cobalamin C defect [47]. The problems of detection of glutaric aciduria type I relying on elevated glutarylcarnitine are similar to those found with urinary glutarate where diagnostic pitfalls are well known [53]. In the case of tyrosinaemia Type I, the elevated tyrosine may not appear until well after the newborn screening sample is taken.

The expanded newborn screening has also revealed some disorders to be more common than previously thought and possibly benign. One example is 3-methylcrotonyl CoA carboxylase deficiency which has been considered as a rare disorder but has been detected in several of the programs using MS/MS

Table 1
Disorders so far identified and marker compounds used in prospective newborn screening programs using tandem mass spectrometry. Acylcarnitines are represented by Cn where n = carbon chain length. C0 represents free carnitine, C5OH 3-hydroxyisovaleryl carnitine and C5DC glutaryl carnitine

Disorder	Marker metabolite in dried blood spots
Amino Acids	
Argininaemia	Arg
Argininosuccinic aciduria	Cit
Citrullinaemia type I	Cit
Citrullinaemia type II	Cit
Homocystinuria	Met
Hypermethioninaemia	Met
Maple syrup urine disease	total leucines, Val
Phenylketonuria and pterin defects	Phe
Tyrosinaemia type I	Tyr
Tyrosinaemia type II	Tyr
Organic Acids	
3-hydroxy-3-methylglutaryl CoA lyase deficiency	C5OH, 3methyl C5DC
3-methylacetoacetyl CoA thiolase deficiency	C5:1, C5OH
3-methylcrotonyl CoA carboxylase deficiency	C5OH
Glutaric acidaemia type I	C5DC
Isovaleric acidaemia	C5
Methylmalonic acidaemia	C3
Propionic acidaemia	C3
Fatty Acid Oxidation Defects	
Carnitine acylcarnitine translocase defect	C16, C18
Carnitine plasma membrane transporter defect	reduced C0
Carnitine palmitoyl transferase I	increased C0/C16 + C18 ratio
Carnitine palmitoyl transferase II	C16, C18
Medium chain acyl CoA dehydrogenase deficiency	C8, C10, C6, C10:1
Multiple acyl CoA dehydrogenase deficiency	C4, C5, C8, C12, C14, C5DC
Long chain 3-hydroxyacyl CoA dehydrogenase deficiency	C16OH
Short chain acyl CoA dehydrogenase deficiency	C4
Very long chain acyl CoA dehydrogenase deficiency	C14:1, C14, C16

[54]. In several of these cases, the mothers of the infants were found to have the defect but with very mild or no symptoms [55]. For short chain acyl-CoA dehydrogenase (SCAD) deficiency, the incidence again appears to be greater than previously thought, but the majority of patients detected remain asymptomatic [50].

For other conditions like MCAD deficiency, the mutational spectrum in newborns identified by MS/MS may not match that observed in clinically presenting patients [56,57]. For all the conditions theoretically capable of being detected from a blood spot by MS/MS, sensitivity depends upon the cutoffs chosen. Tandem mass spectrometry is only the first stage of making a diagnosis and follow up requires other biochemical genetics techniques. This is especially true where known interferences may cause false positives such as pivolylcarnitine mimicking isovalerylcarnitine in patients on pivalic acid containing antibiotics [58], or where one marker may indicate several different conditions, for example, 3-hydroxyisovaleryl carnitine.

Although the programs currently using tandem mass spectrometry are only analysing amino acids and acylcarnitines, MS/MS has been suggested for other metabolites which may be suitable for newborn screening. Screening for cholestatic hepatobiliary disease by analysis of conjugated bile acids in dried blood spots using negative ion electrospray without sample derivatisation was investigated by Mushtaq et al. [59]. Unfortunately, they concluded that there is too much overlap between bile acid concentrations in infants with cholestasis and those in control infants to make mass screening for cholestatic hepatobiliary disease a feasible option. Galactosaemia screening is included in a number of programs utilising manual fluorimetric or microbiological assays. Recently, a novel newborn screening method was published measuring hexose monophosphate and hence, indirectly, galactose-1-phosphate levels by MS/MS [60]. Although this method employs a simple sample preparation, the authors accept that it is not easy to see how it could be integrated into the current protocols used for amino acids and acylcarnitines, especially since it also has the disadvantage of operating the instrument in negative ion mode and rapid switching from positive ion to negative ion may limit the life of the instrument power source.

Other possible uses for MS/MS in newborn screening are being investigated. These include methods for thyroid and congenital adrenal hyperplasia testing. Even if successful methods are developed, these too may suffer the same problems when trying to integrate with the current protocols.

5. Other biochemical genetics applications of MS/MS

Although the focus of tandem mass spectrometry in biochemical genetics has been directed to acylcarnitine and amino acid assays, other applications have been investigated. Diagnosis of peroxisomal disorders is usually performed by demonstrating increased very long chain fatty acids in plasma by gas chromatography/mass spectrometry. Johnson [61] developed an MS/MS method using dimethylaminoethyl esters, applying it to the diagnosis of peroxisomal defects using as little as 5 μl of plasma or dried blood spot samples [62]. He subsequently improved sensitivity markedly by forming alkyldimethylaminoethyl ester iodides [63]. Analysis time is greatly improved over conventional methods, but sample preparation is still quite involved. An adjunct to very long chain fatty acid analysis is red cell plasmalogen analysis. Vreken et al. [64] published a method for ESI-MS/MS analysis of plasmenylethanolamines which allows rapid estimation of plasmalogen status.

Biochemical diagnosis of Smith–Lemli–Opitz syndrome is achieved by estimation of 7-dehydrocholesterol and 8-dehydrocholesterol in plasma, again most often using GC/MS techniques. A recent publication using a novel mono-(dimethylaminoethyl) succinyl (MDMAES) ester formation and analysis by MS/MS has shown that free sterols can be measured following direct hexane extraction of plasma without base hydrolysis, with a total sample analysis time of less than 1 h [65].

Disorders of purine and pyrimidine metabolism have traditionally been investigated by analysis of nucleosides and bases in biological fluids by high-performance liquid chromatography (HPLC) with ultraviolet detection [66]. Relatively long separation times and reliance on wavelength ratios to aid identity are drawbacks of this technique. Ito et al. [67] have recently published a method utilising reverse phase

HPLC and MS/MS with considerably reduced analysis time. The method demonstrated expected abnormalities in urine samples from patients with purine nucleoside phosphorylase deficiency, ornithine transcarbamylase deficiency, molybdenum cofactor deficiency, adenylosuccinase deficiency, and dihydropyrimidine dehydrogenase deficiency.

Another area of metabolism where LC-MS/MS has been used to provide rapid analysis is porphyrias. The method described by Ford et al. [68] gives rapid quantitation of porphobilinogen in urine by positive ion MS/MS following solid phase extraction and a short HPLC run. The authors note that because it uses a stable isotope internal standard and does not require derivatisation, the method offers improved specificity over traditional techniques.

Research is continuing into determination of oligosaccharides and glycolipids by MS/MS in lysosomal storage disorders. Characterisation of ganglioside storage material in mucopolysaccharidosis type IIIA [69] and urinary sulphatides in metachromatic leukodystrophy [70] has been published. Again, there is potential for newborn screening applications, but these are more likely to be in the form of follow up tests with initial screening based on immunoassays. Using solid phase extraction and ESI-MS/MS, Whitfield et al. [71] have demonstrated elevated glycophospholipids in a number of tissues from patients with Gaucher disease, Fabry disease, Krabbe disease, and metachromatic leukodystrophy. A novel use of electrospray mass spectrometry in this field is its use to determine activities of acid sphingomyelinase and galactocerebroside beta-galactosidase by monitoring the change in molecular mass of the substrate [72]. In this instance, no collision-induced dissociation is required, but the analysis can be performed on the same instrument as the other techniques described.

Tandem mass spectrometry has also been used for analysis of other small molecules of interest to biochemical geneticists including methylmalonate (for methylmalonic acidaemia and vitamin B12 deficiency) [73], homocysteine (cystathionine β synthase and vitamin B12 deficiency) [74], *S*-adenosylmethionine and *S*-adenosylhomocysteine (methylenetetrahydrofolate reductase deficiency, cobalamin defects, and methionine adenosyltransferase II deficiency) [75], and guanidinoacetate and creatine (guanidinoacetate methyltransferase deficiency) [76].

6. Conclusions

Tandem mass spectrometry has emerged rapidly over the last decade as a key technique in the fields of biochemical genetics and newborn screening. Although the capital cost of equipment is high, its short analysis times and resultant high throughput make it an attractive platform for development of new assays, and the list is likely to expand. One of its most important features is the ability to detect and quantify a very wide range of different metabolites from a single sample preparation and injection. In this respect, the very first report of MS/MS for metabolic profiling of urinary carboxylic acids in 1982 [6] may well be revisited, and the first "emergency" investigation performed in a child with suspected metabolic disease may be a simple dilution and injection of urine into a tandem mass spectrometer.

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