Tandem mass spectrometry: the tool of choice for diagnosing inborn errors of metabolism?

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ABSTRACT

The early diagnosis of inborn errors of metabolism (IEM) by laboratory-based mass screening is a prime example of preventive medicine. However, several factors restrict the range of IEM that can be screened for, and the numbers of people to whom it can be made available. Mass screening in the United Kingdom is limited primarily to that for phenylketonuria and congenital hypothyroidism. Ideally, extension of mass screening of neonates for additional clinically significant IEM is a desirable strategy. Tandem mass spectrometry (TMS) is a powerful and effective diagnostic technique and has been proposed as a means to realise this aim. Its main advantages are improved accuracy, sensitivity and specificity over existing methods, and its suitability for cost-effective multidisease IEM mass screening. The evolution, principles and applications of TMS are described, and the practical and clinical implications of extending diagnostic services for IEM using TMS are discussed.

KEY WORDS: Metabolism, inborn errors.
Spectrum analysis, tandem mass.

Introduction

Inborn errors of metabolism (IEM) are inherited disorders caused by mutations of genes that govern the synthesis of key enzymes in metabolic pathways or essential proteins in transmembrane transport.[1] Early detection of IEM, notably phenylketonuria (PKU), by laboratory-based mass neonatal screening is a classic example of preventive medicine. Health benefits of early and accurate diagnosis of a range of IEM include the prevention of mental retardation, severe neurological disease, physical disablement and infant mortality. However, providing the laboratory services to achieve favourable outcomes in all cases poses diagnostic, ethical and practical challenges, as a vast range of genetic defects have been identified.[2] Data from a systematic review of the literature suggest that current strategies and methods for early diagnosis of a number of IEM fall short of a reasonable and achievable target.[1,3]

Appraisal of existing methodology

Currently, a range analytical methods is used in screening and diagnostic confirmatory tests (Table 1). Some of the technology employed in primary screening was first developed in the 1960s and 1970s[4-6] and may be considered outmoded when compared with modern standards of analysis. Most methods generally are applicable to a single IEM only and thus it is necessary to use different equipment for other IEM, resulting in staffing and cost implications. In addition, some methods are qualitative (e.g. thin-layer chromatography) or only semiquantitative (e.g. bacterial inhibition assays), and not easily automated or compatible with computerised data handling systems. Multitest automated immunoassay systems for congenital hypothyroidism, congenital adrenal hyperplasia, cystic fibrosis and Duchenne muscular dystrophy were developed in 1992 but are still not available commercially.[7]

A number of different methods are used currently to confirm the provisional diagnosis obtained in mass and selective screening. These include ion exchange amino acid analysis, which has a limited workload capacity. Diagnosis by DNA mutation techniques are technically demanding, time consuming and expensive. Although highly specific, diagnostic sensitivity is often low due to genetic and phenotypic heterogeneity that limits their use as a primary diagnostic tool. However, recent developments in automation and DNA array techniques show some promise.[8]

High sensitivity and specificity have been achieved with gas chromatography/mass spectrometry (GC/MS) methods, proving particularly useful in the diagnosis of numerous organic acidaemias.[9] However, their adaptation to screening is limited by long and laborious sample preparation procedures for derivatisation and by lengthy analytical times (typically one hour/sample). Consistent high-quality performance and interpretation requires considerable experience, and a significant risk of false-negative results has been reported in urine samples.[10] Thus, in view of the limitations described, alternative strategies and methods for more effective diagnostic services should be given serious consideration.

One possible option is to extend the number of conditions screened for in a mass-screening programme, using a single methodology and sample. Transition of tandem mass

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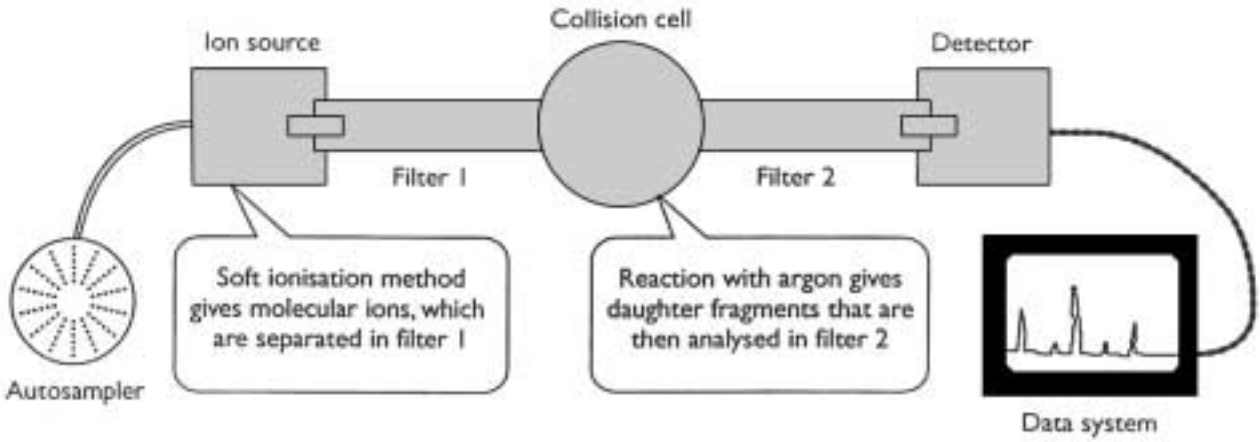
Fig. 1. Schematic diagram of a tandem mass spectrometer (from Ref. 1).



spectrometry (TMS or MS/MS) from a research and reference technique to a single analytical tool for screening and diagnosis is regarded as a highly significant development in this direction.[11-13] TMS can be applied to urine or plasma and has been adapted for dried capillary blood spots collected on filter paper – a preferred sample for mass screening due to its ease of collection, transport, stability and versatility.

Chromatography methods have been used successfully in the investigation of IEM for over 50 years; progressing through paper, thin layer, ion exchange, high-pressure liquid and gas/liquid systems. The discovery in 1966 of isovaleric acidaemia[14] – a defect of organic acid metabolism – was a significant landmark and paved the way for the discovery of many other organic acidaemias.[9,15] The discovery of medium chain acyl-CoA dehydrogenase deficiency (MCAD) – a defect in fatty acid oxidation,[16] often with severe clinical consequences – was a further incentive to move to a more versatile high-resolution technique.

Initially, in 1982, TMS was introduced into biochemistry for the metabolic profiling of urine carboxylic acids,[17] and used subsequently in the analysis of carnitine and its many acyl derivatives.[18] Over the next decade, TMS was applied to neonatal acylcarnitine profiling,[19] and the diagnosis of IEM of amino acid metabolism[20,21] and of organic acidaemias.[22] Most significantly, it was further demonstrated that acylcarnitine and amino acid profiles could be performed simultaneously by TMS using blood spot samples.[23]

General principles of tandem mass spectrometry

Mass spectrometry is a high-power analytical tool and has proved a definitive technique in structural studies of a wide range of biomolecules (e.g. proteins, nucleotides and drugs). Two mass analysers are used in TMS (Figure 1), eliminating the need for preliminary chromatographic separation. A mixture of compounds is ionised to produce a spectrum of 'daughter' ions that are characteristic of the precursor compounds.

Identification is based on the mass/charge ratio of the ions produced, and stable isotope-labelled internal standards are used for linear quantitation. 'Soft' ionisation techniques (e.g. fast atom bombardment, liquid secondary ionisation or electrospray methods) can be used to improve the analysis of polar, non-volatile, thermolabile and large molecules.[24] Electrospray ionisation is used most commonly due to its suitability for automation as a continuous flow system.

In combined amino acid and acylcarnitine profiling, blood spot discs are eluted into methanol and then isotope-labelled internal standards are added. Methanol is removed using dry nitrogen and the sample extract is esterified gently with acidified butanol to produce butyl esters, which are ionised.

The first mass analyser filters specific ions of a set mass range to a reaction or collision cell that contains an inert gas, and fragmentation occurs. In the collision cell, ionised molecules of the same type lose a common fragment. The second mass analyser filter is set up to transmit a single mass to the final detector.

The two mass analysers measure the mass of the parent molecules, either by detecting the loss of a characteristic neutral fragment (e.g. amino acids) or by monitoring a common ionised fragment lost by all the parent ions (e.g. acylcarnitine). The detected ions are measured by a signal recorder that can be used in various modes, either to examine profiles or focus on key spectral components at set mass/charge ratios.

Appraisal of tandem mass spectrometry

The tandem mass spectrometer is claimed to be the 'ultimate' analytical detector because of its very high accuracy, selectivity, precision, versatility and robust nature.[25] Screening and diagnostic methods for IEM need to be specific and sensitive, in order to achieve accurate diagnoses and acceptably low false-positive or -negative rates. Two retrospective TMS studies of PKU demonstrate that all proven positives were confirmed, with no false-negative and a significant reduction in false-positive results.[26,27]

TMS can be used to detect many IEM and 10 different types[23] have been diagnosed in relatively small-scale studies of newborn populations. Larger studies of PKU and other amino acid, fatty acid oxidation and organic acid metabolism disorders have shown a combined incidence of 1 in 4700.[25] While this is a useful indicator of incidence, it will vary considerably with the population studied. Unlike GC/MS,

Table 1. Existing laboratory methods for the diagnosis of inborn errors of metabolism

Inborn error of metabolism	Sample	Analytes	Methods
Phenylketonuria	Blood spot/plasma	Phenylalanine	BIA,FA,TLC,AAA
Maple syrup urine disease	Blood spot/plasma	Branched chain amino acids	BIA,TLC,AAA
Homocystinuria	Blood spot/plasma	Homocystine, methionine	BIA,TLC,AAA
Tyrosinaemia type 1	Blood spot/plasma	Tyrosine	BIA,FA,AAA
Urea cycle disorders	Urine	Citrulline	TLC,AAA
Organic acidaemias	Urine	Organic acids	GC/MS
Medium-chain acyl CoA	Urine	Acyl carnitines	GC/MS
Dehydrogenase defect	Blood spot	Mutations	DNAA
Peroxisomal disorders	Plasma	Very long chain fatty acids	GC/MS
Galactosaemia	Blood spot	GPUT,galactose/metabolites	RE,BIA,FA
Congenital adrenal hyperplasia	Blood spot	17 Hydroxyprogesterone	FIA,IRMA
Familial hypercholesterolaemia	Blood spot	Cholesterol/lipoproteins	CA,RID
Biotinidase deficiency	Blood spot	Biotinidase	CA
Cystic fibrosis	Blood spot	Immunoreactive trypsin	FIA
Cystic fibrosis	Blood spot	Mutations	DNAA
Duchenne muscular dystrophy	Blood spot	Creatine kinase	FIA,FA
Duchenne muscular dystrophy	Whole blood	Mutations	DNAA

BIA: Bacterial inhibition assay,

FA: Fluorometric analysis TLC: Thin-layer chromatography,

AAA: Amino acid analysis, GC/MS: Gas chromatography/mass spectrometry,

DNAA: DNA analysis, RE: Radioenzymatic assay, FIA: Fluoroimmunoassay,

IRMA: Immunoradiometric assay, CA: Colorimetric assay, RID: Radial immunodiffusion

sample preparation for TMS is simple, rapid and can be performed in batches using 96-well microplates. This allows up to 600 blood spot samples to be prepared and, with automatic sample introduction, processed in 24 h.

The high throughput and combined incidence figures are conducive to cost-effective newborn screening. Most attention has been given to the diagnosis of PKU and MCAD, and, using the selective scanning function, blood spot acylcarnitines, phenylalanine and tyrosine also can be quantitated from the same sample.[23] Significantly, TMS can combine screening, diagnostic and treatment monitoring functions for many IEM without the need for other equipment.

Although TMS is analytically superior to other IEM screening and diagnostic methods, it is not without limitations or problems. In order to provide and maintain reliable, high-quality performance, attention to each phase of the system – from sample preparation to interpretation of results – is required.[25] Problems may be encountered while using filter paper blood samples, due to the release of fibres into the system and the use of a poor collection technique which may 'overload' the punched disc (quantitation is based on an assumed average volume of blood per disc). In addition, the preparation and validation of reference and quality assurance materials for bloodspot assays may pose difficulties. Some amino acids (e.g. homocystine and methionine) are unstable and delays in delivery for analysis hamper their clinical interpretation.

Elution and derivatisation must be controlled carefully for precise quantitation. To keep the TMS equipment performing optimally, daily system 'tuning' and monitoring is required to achieve consistently high throughput, before recalibration or further maintenance. Result processing is critical and suitable data processing systems are necessary. Scan functions and chosen mass/charge ratios must be selected carefully to avoid overlap with other metabolite derivatives or sources of interference (e.g. antibiotics containing pivalic acid[28]).

Clinical significance of the spectra and concentrations is the most demanding phase of TMS analysis. Similar acylcarnitine profiles may occur in some carnitine disorders, for example, and quite different clinical outcomes create significant diagnostic difficulties.[29] Appropriate cut-off concentrations must be established by statistical analysis and clinical follow-up to avoid an excessive number of false results. Computer-assisted metabolic profiling algorithms that include preset cut-off decision limits have been developed for the automatic flagging of abnormal profiles.[30] There are several important IEM (e.g. cystic fibrosis, congenital adrenal hyperplasia and Duchenne muscular dystrophy) for which TMS is unsuitable and these will require separate instrumentation and methodology.

The way forward for tandem mass spectrometry?

In the UK, neonatal mass screening is limited to PKU and congenital hypothyroidism and is provided by a large number of reference laboratories distributed throughout the

country. Capillary heel-prick blood spots are collected, ideally between the 7th and 10th day, through the NHS network of midwives and health visitors. This combined screen, introduced in 1985, has proved effective in the diagnosis of both these disorders.[1,3]

TMS provides the opportunity to replace the PKU methodology with a more accurate technique and, at the same time, extend the range of IEM that can be diagnosed from the one sample. The main questions raised by this approach relate to the strategies that need to be adopted to best achieve these aims, and any clinical implications that may arise. The capital outlay for equipment is considerable (c. £200 000), with a limited number of commercial manufacturers to choose from.

The technique was pioneered in the USA at Duke University, North Carolina, and is linked with a commercial laboratory, NEOGEN, based in Pittsburgh, Pennsylvania. Most of the experience with TMS has been gained in the USA, where early testing and early healthcare discharge procedures are more prevalent and there is no equivalent to the NHS network for sample collection. TMS equipment is now in use in at least five major centres in the UK, resulting in a centralised 'core' of knowledge and experience in this country. Leading exponents of TMS emphasise the importance of selecting systems that are fit for diagnostic purpose, and the need for practical expertise and sufficient clinical experience in the interpretation of spectra and calculated results.[25]

Possible strategies include a comprehensive programme of validation by current users, followed by a complete rationalisation of services to a few reference laboratories. Alternatively, TMS may be introduced into other screening laboratories that have a cost-effective and defined workload (e.g. 100 000/annum). It must be appreciated, however, that these laboratories would also need to provide a 'fast track' service for MCAD and organic acidaemias in some critically ill neonates, and provide screening for congenital hypothyroidism at the same site to avoid sample transfer and sharing difficulties.

Either option will bring radical changes to the organisation and management of IEM screening services in the UK, and implementation will be a highly significant issue.[31] There is little doubt that the UK Department of Health will be the ultimate authority in the decision-making process. The evidence presented should show clearly that TMS screening is both more effective and practically feasible as a national screening service than existing methods. Risk factors include the finances and resources needed to implement this change. It is rather disconcerting that TMS has not been adopted more widely in the USA, and Levy[11] tends to place the blame for this on the inflexibility of administration systems and precautionary attitudes that cannot adapt to progress.

The high sensitivity of TMS will continue to reveal 'new' profiles, the diagnostic significance (if any) of which may not be apparent and may create significant ethical and clinical dilemmas. The temptation to pursue these findings may prove irresistible.

From a systematic review of the world literature[3] it would appear that the choice of diagnostic target is critical for the effective implementation of TMS. The current state of knowledge of IEM does not inspire confidence that we have even enough data on existing conditions. TMS will improve the accuracy of diagnosis; however, this is only the start of the process and there is particular concern over the lack of effective treatment for many IEM.

Advocates of TMS may argue that a correct diagnosis, even without effective treatment, is beneficial in prenatal diagnosis and genetic counselling. The formal quality of the literature on treatment is poor; many IEM are rare and show wide variation in geographical distribution, consequently systematic studies and trials are difficult to perform[3,32]

Finally, we must be mindful of the problems faced by clinicians in the treatment and general management of IEM. The stress and anxiety for the family concerned cannot be underestimated and clinicians and their support teams (e.g. dietitians and genetic counsellors) must have sufficient experience and reliable information in order to deal effectively with all types of situation. In addition, it must be appreciated that IEM with effective treatment are a cumulative and long-standing 'burden' on their clinical care time. Extending the range of IEM that can be diagnosed is problematic in view of the relatively limited number of paediatric clinicians who have special interest and experience in this area. It should also be appreciated that GPs are very much involved in clinical support and management.

Perhaps the time is now right for a strategic review of IEM screening services in the UK, providing an opportunity to improve general management, coordination and regulation, and be able to define areas of responsibility with a vision of the future. Unification of methodology and regulation may help to facilitate this process. Consultation with representatives of all staff involved, from screening to clinical, is necessary for the effective management of change.[31]

TMS presents an opportunity for a strategic change in practice for the diagnosis of a wider range of IEM. Validation procedures will be required initially and comprehensive practical experience and expertise achieved by laboratories using TMS. Additional clinical support must be available in order to maximise the healthcare benefits for babies born with IEM and for their families. □

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