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Review

Clinical applications of tandem mass spectrometry: ten years of diagnosis and screening for inherited metabolic diseases

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Abstract

This paper reviews the clinical applications of tandem mass spectrometry (MS–MS) in diagnosis and screening for inherited metabolic diseases in the last 10 years. The broad-spectrum of diseases covered, specificity, ease of sample preparation, and high throughput provided by the MS–MS technology has led to the development of multi-disorder newborn screening programs in many countries for amino acid disorders, organic acidemias, and fatty acid oxidation defects. Issues related to sample acquisition, sample preparation, quantification of metabolites, and validation are discussed. Our current experience with the technique in screening is presented. The application of MS–MS in selective screening has revolutionized the field and made a major impact on the detection of certain disease classes such as the fatty acid oxidation defects. New specific and rapid MS–MS and LC–MS–MS methods for highly polar small molecules are supplementing or replacing some of the classical GC–MS methods for a multitude of metabolites and disorders. New exciting applications are appearing in fields of prenatal, postnatal, and even postmortem diagnosis. Examples for pitfalls in the technique are also presented.

Keywords: Reviews; Inherited metabolic diseases; Acylcarnitines; Acylglycines

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1. Introduction

Inherited metabolic disorders (IMDs) are a group of over 200 single gene disorders primarily inherited as autosomal recessive traits. Many of these disorders carry serious clinical consequences to the affected neonate or young infant, which include mild or severe irreversible mental retardation, physical handicaps, or even fatality. An accurate diagnosis of IMD is very important for reaching a rapid and favorable patient outcome. The application of tandem mass spectrometry (MS–MS) to the area of IMDs first appeared about 10 years ago. The application of this technology has had a significant impact on the field realized in the significant increase in the spectrum of diseases which can be screened, the development of multi-disease screening tests, the ever-increasing number of cases diagnosed among sick children (selective screening), the detection of diseases thought to be present only in certain ethnic groups, the saving of many lives, the acquisition of the technology by many clinical chemistry units or biochemical genetics laboratories, and the introduction of MS–MS in several newly developed or already existing newborn screening (NBS) programs around the world.

Organic acidemias caused by mitochondrial enzyme defects in the catabolism of branched-chain amino acids (AAs) and mitochondrial fatty acid oxidation defects constitute a group of >20 disorders in which acyl-Coenzyme A (acyl-CoA) esters accumulate in the mitochondria. In these disorders, L-carnitine (C0) plays a key role in removing the potentially toxic acylCoA esters through the formation of acylcarnitine (AC) esters and thereby releasing CoA and restoring mitochondrial homeostasis [1,2]. This results in increased concentrations of circulating ACs, increased excretion of ACs in urine, and secondary C0 deficiency. Therefore, metabolic profiling of free C0 and ACs in plasma, blood spots, or urine provides a powerful selective screening tool for these disorders.

Unlike AAs, for which existed many thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC), electrophoresis, and gas chromatography–mass chromatography (GC–MS) methods, ACs were difficult to analyze due to their polarity, zwitterionic nature, their relatively low concentration in body fluids, and the lack of a chromophore. Various spectrophotometric, radiochemical, or mass spectrometric methods were developed for this purpose, with some still in use [3–9]. However, these methods shared some of the drawbacks of lack of sensitivity or specificity, laborious sample preparation, and long analytical time.

The initial excitement about MS–MS in this area of research stemmed from its successful use in obtaining AC profiles from plasma and urine samples in a short analytical time of 2–3 min, with no chromatography. These profiles were highly specific and diagnostic for the recently recognized fatty acid oxidation defects (FOADs) and for organic acidemias due to disorders in branched-chain AA catabolism disorders. For the first few years of the last decade the group at Duke University pioneered the use of fast atom bombardment (FAB) MS–MS for profiling ACs from dried blood spots (DBSs), or plasma [10,11]. The ‘‘soft’’ ionization characteristics of FAB allowed the analysis of ACs either as their methyl or butyl esters. Thus, precursor-ion scans of m/z 85, the major fragment produced by collision-induced dissociation of all butylated ACs and the second major fragment of the butyl ester of C0 produced a mass spectrum that showed C0 and all

the known ACs, particularly if a blood spot rather than plasma is used, as the latter shows very little of the long-chain ACs (C12 to C18) [10]. The method was further extended to the analysis of neutral, acidic, and basic AAs from the same DBS extract and proved valuable in the diagnosis and screening of phenylketonuria (PKU), maples syrup urine disease (MSUD), and homocystinuria [12–14].

In 1994, we introduced the use of electrospray ionization (ESI) MS–MS as a more robust and more sensitive alternative technique to FAB-MS–MS for AC and AA profiling from DBSs [15,16]. We found that ESI-MS–MS produced better quality profiles for ACs, particularly an increased sensitivity for long-chain ACs. The ESI-MS–MS profiles (see Fig. 1A–C) were also free from artifacts that appear in the FAB-MS–MS profiles and are derived from glycerol adducts. We also found that ESI-MS–MS produced equally good profiles for neutral, acidic, and basic AAs (see Fig. 2A–C). The real advantage from the use of ESI-MS–MS rather than FAB-MS–MS was the high sample throughput achieved by ESI-MS–MS due to the ease of automation of sample introduction into the mass spectrometer, and the ''cleanliness'' of the technique as compared to FAB-MS–MS. Thus, we have shown that at least 1000 DBS extracts can be injected consecutively into the instrument before any need for ion source cleaning, a rather simple process in ESI as compared to cleaning a FAB ion source [17]. This high throughput for a multi-disorder test represented a very promising approach for the development of NBS programs for a large number of IMDs. It also promoted active research towards the development of new MS–MS methods for diagnosis of many other inherited diseases such as peroxisomal diseases, bile acids

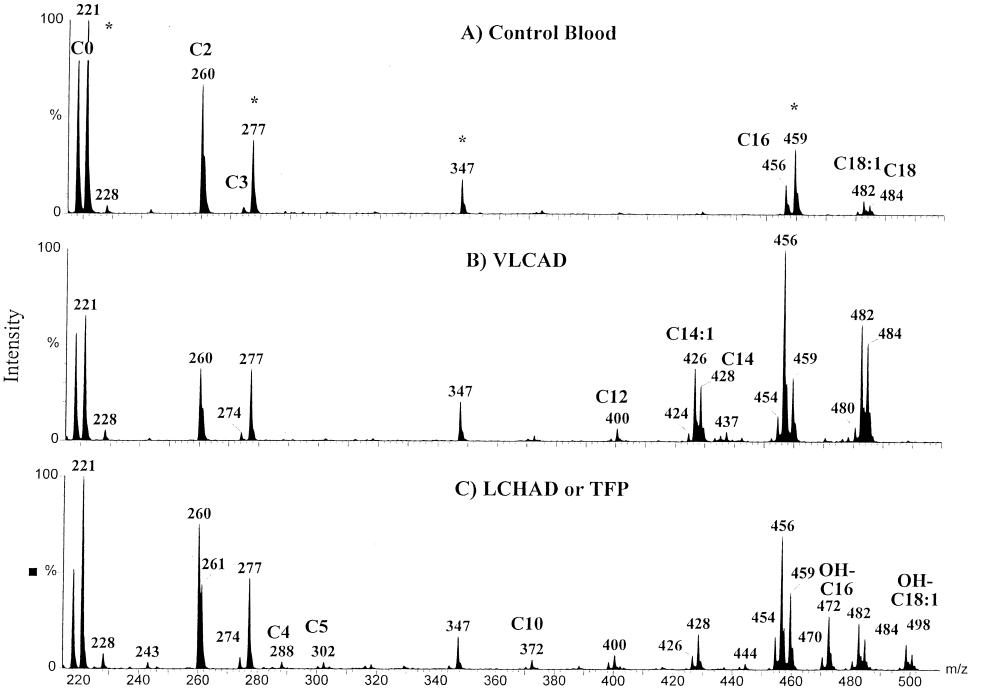

Fig. 1. Blood spot acylcarnitine profiles obtained by ESI-MS–MS analysis with precursor ion scanning of m/z 85. (A) Control. (B) VLCAD patient. (C) LCHAD (or TFP) patient. The peaks in the profiles are the molecular ions (M^+) of the acylcarnitine butyl esters. Their masses are as follows: free carnitine (C0, 218; $^2H_3$-isotope labeled), acetyl (C2, 260), propionyl (C3, 274; $^2H_3$-isotope labeled 277), butyryl or isobutyryl (C4, 288), isovaleryl or 2-methylbutyryl (C5, 302), 2-methyl-3-hydroxybutyryl or 3-hydroxyisovaleryl (OH-C5, 318), hexanoyl (C6, 316), octanoyl (C8, 344; $^2H_3$-isotope labeled 347), decanoyl (C10, 372), glutaryl (C4DC, 388), dodecanoyl (C12, 400), 3-methylglutaryl (C5DC, 402), tetradecanoyl (C14, 428), hexadecanoyl (C16, 456; $^2H_3$-isotope labeled 459), octadecanoyl (C18, 484). Their unsaturated analogs appear 2 rel. mass units lower in mass. *=Internal standard ions.

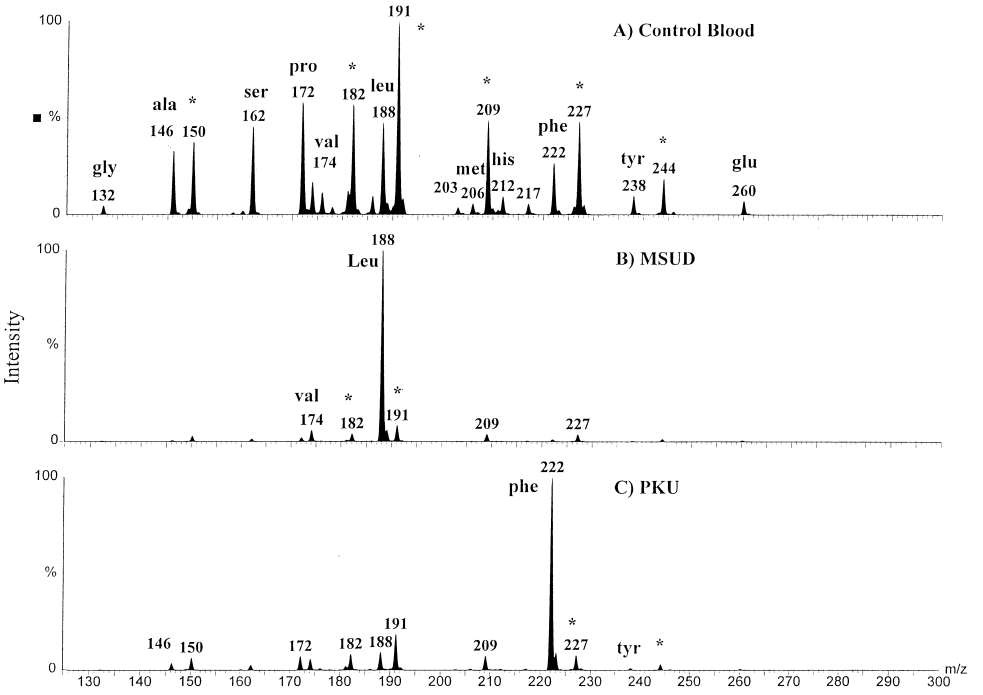

Fig. 2. Blood spots amino acid profiles obtained by ESI-MS–MS analysis with neutral loss scanning of 112 rel. mass units. (A) Control. (B) MSUD patient. (C) PKU patient. *=Internal standard ion. The peaks in the profiles are the protonated molecular ions (MH^+) of the amino acid butyl esters.

disorders, purine and pyrimidine metabolism disorders.

2. MS–MS-based newborn screening

2.1. Samples and sample preparation

There is no doubt that the DBS remains the most convenient, practical, and cheapest way of acquiring the sample for NBS. However, there are several issues related to the time of sample collection, method of collection, shipment of samples, and storage conditions that should be considered. Time of sample collection remains a chronic problem for NBS programs because of the universal problem of early discharge of mother and baby from the hospital. There is a general agreement among workers in the field that MS–MS analysis has increased the sensitivity for many analytes allowing the diagnosis in samples acquired in less than 24 h after birth. There is also increased specificity resulting in low false positives. However, this is tempered by situations where the rise of the diagnostic marker may be slow leading to false negative results. It appears that the best time for sample collection for a large number of disorders lies between 48 and 72 h. This however poses a significant logistical problem for most communities and could significantly increase the cost of sample acquisition, or result in a significant decrease in coverage of the newborn population.

DBS collection from a heel prick is supposedly simple, but yet we still find a lot of poor quality samples that may result in both false negative and false positive results. Therefore, adequate training and education of health professionals involved in this process should be taken in consideration when initiating NBS programs.

It should be noted also that some of the metabolites are affected by time lapse between the acquisition of blood spot and analysis. For example, methionine essentially disappears from the profile in about 1 week if samples are stored at room temperature or if slow regular mail delivery to the laboratory is used. Acetylcarnitine (C2) also degrades but to a lesser extent with time, while other ACs are relatively more stable. This degradation process is faster if the sample was not completely dry before placing in plastic bags for shipment. Adequate storage measures should be in effect for DBSs after analysis and some communities has developed the so-called ''biobanks'' for this purpose [18]. These samples are a valuable resource for retrospective studies, for repeat testing, and for quality assurance in case of false negative results from patients identified later in life.

Sample preparation for routine MS–MS analysis of blood spots is essentially based on the simple method developed by Millington et al. [10]. The method involves extraction of blood spots in methanolic solution of internals standards followed by evaporation of methanol and treating the residue with butanolic HCl at 65°C for 15 min to make the butyl esters. Heat-assisted evaporation is then carried out to remove excess reagent followed by reconstitution in an acetonitrile–water mixture and injection into the mass spectrometer. Slight variations to this procedure are required if plasma, serum, or urine sample are to be analyzed.

Some drawbacks do exist for this acidic esterification procedure such as degradation of some metabolites or internal standards as is the case with glutamine, which is deaminated to glutamic acid. Also, a significant amount of C2 is hydrolyzed during the acidic esterification to give free C0 [19].

In 1997 we introduced a high throughput method for sample preparation making use of the convenience of 96-well microplates [17]. This approach together with automated punching of blood spots and microplate mapping eliminated the tiresome process of labeling individual sample vials, writing lists of sample numbers, and reduced the inherent possibilities for sample confusion. Thus, two technicians can now prepare six microplates (576 samples) in a regular working day. However, the overall process at best remains semi-automated. Alternative approaches for sample derivatization should be sought for in order to achieve full automation.

2.2. Quantification of metabolites and choice of internal standards

The use of semi-quantitative isotope dilution MS–MS and appropriately labeled internal standards added during sample preparation allowed for reliable quantification of a large number of ACs and AAs. It should be noted however that the RSDs for the method are somewhat high (10–15%) due to the use of an estimated blood volume from the DBS punch which is affected by the type of filter paper used, how the sample was applied to paper, the hematocrit, and the dryness of paper [20].

The ideal situation is to have an internal standard for each analyte of interest. However, this is not possible yet as no standards are commercially available for some analytes. Deuterium-labeled, ^{13}C- and ^{15}N-labeled compounds are commercially available for most AAs but only for some ACs. However, it is important to consider the limited stability of some of these compounds as solids and in solution, and to take measures for appropriate storage conditions. This instability could be due to deuterium exchange as we had found on using tyrosine-ring-$^{2}H_{4}$ which led to overestimation of tyrosine in our samples and which we subsequently replaced with tyrosine-ring-$^{13}C_{6}$. Hydrolysis of some AC calibrators in solution and during sample preparation is also well known and should be corrected for, or the label should be placed on the C0 moiety backbone rather on the *N*-methyl group.

2.3. Instrumentation and data acquisition

A triple quadrupole mass spectrometer remains the only instrument capable of carrying out the different scan functions and scan speeds needed for MS–MS analysis of AAs and ACs. There are currently three different scan functions in use, precursor-ion scanning, neutral loss scanning, and product-ion scanning (for more details see Refs. [20,25]). The data can be acquired in two different ways; one is termed *class-specific analysis*, for example neutral loss scanning of 102 rel. mass units for acidic, and neutral AAs,

precursor-ion scanning of m/z 85 for ACs. The other way is termed *target compound analysis* (selected reaction monitoring; SRM, multiple reaction monitoring; MRM) where certain transitions of the precursor to product are monitored. Each of the two methods has advantages and disadvantages. SRM data allows for faster scanning speeds, better quality data, and shorter analytical time. It also allows for monitoring of target metabolites only in class of compounds, if decisions are made to restrict the screening method to certain diseases. On the other hand, class specific analysis is more comprehensive and can potentially lead to the finding of new diagnostically important analytes.

Careful calibration and appropriate resolution (0.6–0.7 m/z at half peak height) of the two mass spectrometers is necessary to ensure either lack of interference for some metabolites, or erroneous mass assignments of others. A case in point is the isobaric compounds mono-butyl derivative of C2 and the bis-butyl derivative of glutamic acid (m/z 260). On collision-induced dissociation (CID), glutamate yields a major fragment at m/z 84 while C2 yields a major fragment at m/z 85. If two signals are not well resolved there is an overestimation of C2 as a result of interference from mass 84 channel into mass 85 channel, and possibly vice versa. As the peak height ratio of propionylcarnitine/C2 (C3/C2) is an important parameter for detecting propionic acidemia (PPA), methylmalonic acidemia (MMA), and possibly multiple carboxylase deficiency (MCD), an overestimation of C2 may lead to a low C3/C2 ratio and thus to a false-negative results in milder forms of these disorders [22].

Most of the down time or poor quality data results form the autosampler connected to the mass spectrometer, as a result of partial or complete blockage of the injection port due to accumulation of filter paper lint, or from damage to the injection valve seal due to the large number of samples injected daily. Another important source of problems is the blockage the stainless steel capillary in the ESI source. The mass spectrometer itself causes very few problems and is easy to maintain. All of our work was carried out using Quattro instruments (Micromass, Altrincham, UK). Initially we used a Quattro I and currently we are using a QuattroLC instrument equipped with a Z-spray ESI source.

2.4. Validation of methods

The routine MS–MS analysis of DBSs went through 10 years of validation albeit at a small numbers of centers in the first 5 years. The initial phase involved retrospective clinical validation, i.e., the ability of the method to detect disease state in symptomatic cases undergoing treatment (selective screening). This was followed by retrospective studies on original neonatal DBSs of diagnosed cases to determine the sensitivity and specificity of the method as compared to classical methods such as the Guthrie bacterial inhibition test for PKU (retrospective neonatal screening). Most of this early work was carried out by FAB-MS–MS and proved the method as invaluable new resource to the field of diagnosis and screening for IMDs.

The second phase involved analytical validation of the methodology in terms of recovery, accuracy, precision, and ruggedness. Chace and co-workers carried out several systematic studies to assess these issues [12–14]. Sample preparation as described above showed excellent recoveries (>90%) for most analytes. The use of isotope dilution mass spectrometry was shown to greatly reduce chemical interference because of its high molecular specificity and to significantly reduce errors due to instrument signal variability. The values obtained particularly for AAs compared well with values determined by other standard assays such as HPLC analysis of AAs. The detection and quantification limits were well below physiological levels, with exceptions for some ACs, which are circulating at very low levels. A step was taken recently by the National Institute of Health, USA towards standardization of MS–MS-based NBS by certifying batches of DBSs for AAs and making them available to different programs [21].

When ESI-MS–MS was introduced as an alternative technique the methodology had to be revalidated in exactly the same fashion, as was done for FAB-MS–MS. Surprisingly enough ESI-MS–MS a technique thought to be more useful for macromolecules proved to meet or exceed all the rigorous validation steps for such small molecules as AAs and ACs plus the added incentive of extremely high throughput resulting in full automation of sample analysis [16,17,22,23].

Another important aspect of validation related to data reduction is the use of software algorithms for data reduction and decision-making. The acquired raw data files contain a large number of signals that has to be processed and a relatively large number of calculations have to be carried out to obtain metabolite concentrations, or ratios of metabolites. The generated values have to be compared to cutoff values so that appropriate decisions can be made regarding a particular data file. This is a very laborious process and requires automation. These algorithms should be rigorously validated to avoid false negative results, which could be costly to the overall program.

We described the development of a software program for the automated processing of all data files to eliminate the labor-intensive and subjective process of manual data interpretation. We developed, tested, and validated a computer-assisted metabolic profiling algorithm (CAMPA), and introduced it into our clinical service in 1997 [17]. CAMPA processed the raw data, calculated all desired parameters, and flagged abnormal results. Retrospective experiments were carried out to validate the performance of CAMPA before its introduction into our daily service. The results indicated that the sensitivity of the algorithm was 100% and the weighted average cumulative specificity was 83.1%. Other similar algorithms were later made available either by the instrument manufacturers, or written in the laboratory by users [24].

2.5. Current experience with newborn screening

In a recent article we related our 3-year experience (1995–1998) for pilot prospective NBS using MS–MS in the Saudi population. We screened 27 624 blood spots from newborns and identified 20 cases yielding a frequency of 1:1381 [25]. No false-negative cases were identified. However, sample collection time was quite early and averaged 24.87 ± 16.4 h, which was a reason for concern. Several false-positive results were obtained. These were either eliminated by repeat analysis by MS–MS on the same or a second blood spot, by GC–MS analysis of urine for organic acids, and by MS–MS analysis of cerebrospinal fluid (CSF) for determination of glycine. In the study described we have identified three cases of PKU (one of which was a biopterin-dependent PKU), two MSUD, two argininosuccinic acidemia, two citrullinemia, one non-ketotic hyperglycinemia, two glutaric acidemia type-I (GA-I), four MMA, one PPA, two isovaleric acidemia (IVA), and two medium-chain acyl-CoA dehydrogenase deficiency (MCAD). Three metabolites contributed most to our falsely flagged samples. C3, or the C3/C2 ratio was high in many infants and this correlated with the early collection of blood spot. C5-Carnitine, which could be either isovalerylcarnitine diagnostic for IVA, or pivalylcarnitine. The latter interference results from the use of pivalic acid prodrug antibiotics given during pregnancy or given to the infant [2]. Glycine, was found borderline high in several samples, but was normal on repeat analysis of the same sample, or a second sample.

Several other prospective NBS studies were also described recently. Naylor and Chace described a 7-year study using both FAB-MS–MS and ESI-MS–MS in analyzing more than 700 000 samples from newborns from several states in the USA [26]. They prospectively diagnosed 163 cases, of which 86 were amino acid metabolism errors, 32 organic acidemias, and 45 fatty acid oxidation defects. According to these authors the presence of false positives with AC screening was not a serious problem with the exception of C3 where an appropriate cutoff value could not be established because maternal and newborn cobalamin deficiencies affect the levels of C3.

Wiley et al. described a 12-month experiment where they screened 137 120 blood spots using ESI-MS–MS. Samples were collected between 48 and 72 h. They detected 31 babies with an IMD, 17 PKU, one tetrahydrobiopterin deficiency, three hyperphenylalaninemia, one MSUD, one tyrosinemia type II, one congenital lactic acidosis, two MCAD, one short-chain acyl-CoA dehydrogenase deficiency (SCAD), one beta-ketothiolase (BKT), two vitamin B12 deficient babies of vegetarian mothers, and one GA-I [24].

Shigematsu et al. described a pilot study where 23 000 blood spots were screened by ESI-MS–MS in Japan [27]. One PPA was detected. These authors also indicated a false positive rate in the diagnosis of IVA due to the use of pivalic acid-containing antibiotics of 0.37%. Liebl et al. described their 1-year NBS experiment using MS–MS as well as other

screening tests. They screened 87 000 newborns and diagnosed 22 cases by MS–MS. Among these cases nine were PKU, and six MCAD [28].

MCAD appears to be the most common FAOD in the West and the prognosis and the quality of life is quite good for early detected and treated cases. Therefore, there is a significant interest in neonatal screening for this disease. Actually, several states in the USA are now screening for this disease by MS–MS. In this regard, Ziadeh et al. described a prospective neonatal screening for MCAD in Pennsylvania, PA, USA [29]. Their findings indicated a high incidence for the disease and unexpected mutation frequencies. Thus, from 80 371 newborns screened they found nine babies with MCAD (1/8390) plus two additional MCAD in high-risk babies. Molecular analysis showed 56% of the detected patients to be compound heterozygotes for the common A985G mutation together with a second mutation. This was in contrast to retrospective clinical studies, which have found only 20% to be compound heterozygotes. In another study a combined set of newborns from the above study and from newborns from North Carolina the frequency was found to be 1:17 706 [14]. These authors used FAB-MS–MS to validate the measurement of octanoylcarnitine (C8), the pathognomonic metabolite in MCAD as well as C6- and C10-carnitines using $^{2}H_{3}$-octanoylcarnitine as internal standard for all three analytes. Calibration curves using blood serially enriched with these three compounds were linear in the range of 0–10 μM. Good recovery, precision, and inter- and intra-assay variation were obtained. The effect of patient age on the concentration of AC was studied in 16 older patients outside the neonatal age. In these patients, the diagnostic AC (C6, C8, C10:1, and C10) were increased but to a lesser degree than observed in the neonatal period. This is probably due to progressive renal loss of C0. The authors determined however that a cutoff value of >0.3 μM and a ratio of C8/C10>2 to be predicative of MCAD in all age groups.

Based on a somewhat similar study in the UK using ESI-MS–MS Clayton et al. proposed a C8 concentration in the neonatal period (4 days to 2 weeks) of >1.2 μM with no increase in carnitine species $<$C6 or $>$C10 is diagnostic for MCAD [30]. For blood spots collected later than 2 weeks, they proposed a C8 concentration >1.0 μM, or a C8 concentration between 0.38 and 1.0 μM coupled with evidence of C0 depletion (free C0<20 μM) is predictive. They also proposed in such cases to check for the A985G mutation on the same blood spot. In cases with C8 of <1.0 μM and negative homozygosity for the A985G mutation, further tests are required to determine compound heterozygotes MCAD, such as *cis*-4-decenoic acid determination by GC–MS [31].

There appear to be a concern for false negative results from homocystinuria patients affected with CBS deficiency as none of the prospective studies described above reported any cases of homocystinuria. The current methodology detects homocystinuria by the finding of elevated methionine. The early discharge of babies from hospitals and the known slow increase of methionine in this condition may lead to false negative results. For this reason some centers has lowered their cutoff values for methionine to 1 mg/dl (67 μM), or even 0.7 mg/dl (47 μM) [26].

3. MS–MS-based selective screening

Although the main attraction of MS–MS in flow injection analysis mode (FIA), i.e., without chromatography remains the proven high throughput multidisorder detection applicable to NBS, the technique has found numerous new applications for selective screening and high-risk screening. The main incentive in this case was the short analytical time either due to the elimination or shortening of the chromatographic steps required for the analyte(s) of interest, elimination of interference, analysis of highly polar compounds, increase in assay precision, and simplification of sample preparation, while retaining a high level of sensitivity and specificity.

3.1. Postnatal diagnosis

The routine MS–MS method of butylated or methylated blood spots extracts or plasma extracts for profiling ACs and AAs has been in use for 10 years. Numerous retrospective studies and single case reports have been published highlighting MS–MS as a powerful tool for selective screening for a

large number of FOADs, organic acidemias, and aminoacidopathies [32–37]. Table 1 provides a list of diseases diagnosed by MS–MS analysis of blood spots. The list covers diseases detected in patients rather than NBS and is based on the authors' own experience and from reviewing the current literature.

In 1997 we carried out a study where we analyzed >1000 blood spots from our normal newborn population to establish reference ranges and cutoff values for AAs and ACs [17]. Thus, over 40 different parameters were calculated from each data file. These were concentration of some key metabolites, concentration ratios (e.g., phenylalanine/tyrosine ratio), or peak height ratios (e.g., C3/C2 peak height ratio). To determine cutoff values for these analytes (or parameters) we used the percentile method. Accordingly, the values obtained for each analyte were ranked, and the 0.5th and 99.5th percentiles were calculated. We compared these values with those obtained for a large data set obtained from affected individuals suffering from different disorders. Most of these diagnostic parameters showed a clear distinction between the abnormal values obtained from patients with metabolic disorders and the upper cutoff value from the population of unaffected newborns.

Table 1
Diseases detected by selective screening MS–MS of blood spots and plasma of patients

Fatty acid oxidation defects
CTD
CPT-I
CPT-II
Translocase
MAD
SCAD
Ethylmalonic acidemia
MCAD
VLCAD
LCHAD
TFP
Dienoyl-CoA reductase deficiency
Organic acidemias
MMA (different types)
Combined methylmalonic:homocystinuria
Propionic acidemia (acute neonatal and late onset)
MCD
BKT
3-Hydroxy-3-methylglutaryl-CoA lyase deficiency
Methylcrotonyl-CoA carboxylase deficiency (isolated)
IVA
GA-I
Malonic acidemia
Aminoacidopathies
PKU (classical and biopterin dependent)
MSUD
Homocystinuria (due to CBS deficiency)
Citrullinemia (acute neonatal and mild)
Argininosuccic acidemia (acute)
Tyrosinemia type-I
Tyrosinemia type-II
Methylenetetrahydrofolate reductase deficiency
Non-ketotic hyperglycinemia
Prolinemia type-II

3.1.1. Determination of free carnitine and acylcarnitines

Despite the convenience of DBS their use introduces some variation in the assay (see above). Therefore, some laboratories prefer to use plasma or serum that can be measured accurately. This is particularly important for follow-up of treatment. Vreken et al. described the development of a quantitative assay for plasma or serum analysis by FIA–ESI-MS–MS. They analyzed plasma samples from patients suffering from FOADs and organic acidemias ($n=103$) [23]. Abnormal AC profiles were obtained in all cases when the patients were in a metabolically decompensated state. Even in clinically stable patients abnormal AC profiles were obtained except in one case of SCAD, and in a mild case of glutaric acidemia type-II (multiple acyl-CoA dehydrogenase deficiency; MAD). These authors also presented the upper reference limit of concentrations of ACs or diagnostic ratios of different based on 95th percentile from a control group of 250 samples and compared it with the affected group. In all cases there was a clear discrimination between affected individuals and controls.

Measurements of free and total carnitine are important for the diagnosis and management of patients with either primary or secondary carnitine deficiency. The use of precursor ion scanning of m/z 85 analysis of blood spots and plasma (or serum) for the determination of carnitine and total carnitine suffers some drawbacks. There is partial hydrolysis of some of the short-chain ACs (particularly C2) during acid-catalyzed esterification or during the

heat-assisted evaporation of sample extracts before analysis to yield free C0, which causes an overestimation of free C0 [19]. There is also a difference in values for ACs between blood and plasma particularly with regards to long-chain ACs leading to differences in the values obtained for total carnitine. Furthermore, internal standards for many ACs are not commercially available and quantification of these species is based on approximation to the nearest internal standard in the spectrum possibly introducing errors in their estimation.

To overcome these difficulties Stevens et al. introduced recently a simplified assay for free and total carnitine in human plasma using FIA–ESI-MS–MS [38]. Two aliquots of plasma were used, one for determination of free C0 and the other for total carnitine after base-catalyzed hydrolysis. Both samples were analyzed without derivatization and data was acquired using precursor-ion scanning of *m/z* 85 in the range of *m/z* 160–210, which covers free C0 at *m/z* 162 and C2 at *m/z* 204. Analytical time was 2 min/sample and free C0 was quantified by isotope-dilution using the $^{2}H_{3}$-L-carnitine as internal standard (*m/z* 165) added during sample preparation. Excellent RSDs were obtained and calibration curves were linear across a wide physiological range.

3.1.2. Determination of amino acids

The routine MS–MS method does not detect homocysteine, or the disulfide homocystine. This is due to the known fact that most of homocysteine is bound to plasma protein and reduction is necessary for its release. A minor fraction is in the disulfide form either as homocystine or cysteine-homcysteine, both being highly polar AAs they do not circulate in blood and are rapidly excreted in urine. However, the routine method mentioned above does detect most if not all homocystinuria cases due cystathionine-β-synthetase deficiency (CBS) by the finding of a moderate to significant elevation of methionine. We use a rapid qualitative MS–MS method to screen sick patients who are either suspected clinically to have homocystinuria, or showed an isolated elevation of methionine in their blood spots. We use urine spotted on filter paper (1/8 in. punch; about 2 μl urine), followed by extraction with a mixture of 0.1 *M* HCl–methanol (1:9, v/v) for 30 min, evaporation, and derivatization to the butyl esters (see under Section 2.1) (1 in.=2.54 cm). The samples are then injected directly into the mass spectrometer in the FIA mode at a constant flow of 40 μl/min. A 2-min MRM scan function is carried out for three transitions, *m/z* 381→192 for homocystine bis-butyl ester (MH^+=381), *m/z* 367→190 for cysteine-homocysteine bis-butyl ester (MH^+=367), and *m/z* 335→190 for cystathionine bis-butyl ester (MH^+= 335). A dwell time of 0.1 s is used for each transition with a cone voltage of 28 V and collision energy of 13 eV. The mass chromatogram is averaged to yield a profile that shows all three ions (see Fig. 3A and B). So far, we have analyzed over 1000 urine spots by this method. In over 25 homocystinuria cases due to CBS deficiency, the ratios of ions *m/z* 381 (homocystine) and of *m/z* 367 (cysteine-homocysteine) to *m/z* 335 (cystathionine) were above 1, and vice versa for controls. In each case further confirmation was carried out by the established HPLC assay for total homocysteine in plasma [39].

Recently Magera et al. described an isotope dilution liquid chromatography tandem mass spectrometry (LC–MS–MS) method for the determination of total homocysteine in plasma and urine [40]. In this case, sample preparation involved reduction of disulfides by dithiothreitol in presence of d_8-homocystine used as internal standard, followed by protein precipitation, centrifugation, and analysis of the supernatant by LC–MS–MS. A short cyano column was used (3 cm×4.6 mm) at a flow-rate of 1 ml/min with the LC effluent split at 1:5 yielding an analytical time of 3 min per sample. The produced homocysteine was analyzed without derivatization using SRM for the transition of *m/z* 136→90 (MH^+ to MH^+-HCOOH) and the transition *m/z* 140→94 for the internal standard ($^{2}H_{4}$-homocysteine obtained after reduction). The calibration curves constructed in the range of 2.5 to 60 μmol/l were linear with excellent intra- and inter-assay RSD. The method correlated well with other established assays for homocysteine and gave a high throughput of 20 samples per h.

Almost simultaneously Gempel et al. described an LC–MS–MS method for determination of total homocysteine in blood spots. The method was essentially very similar to that of Magera et al.'s and showed good correlation with an established HPLC

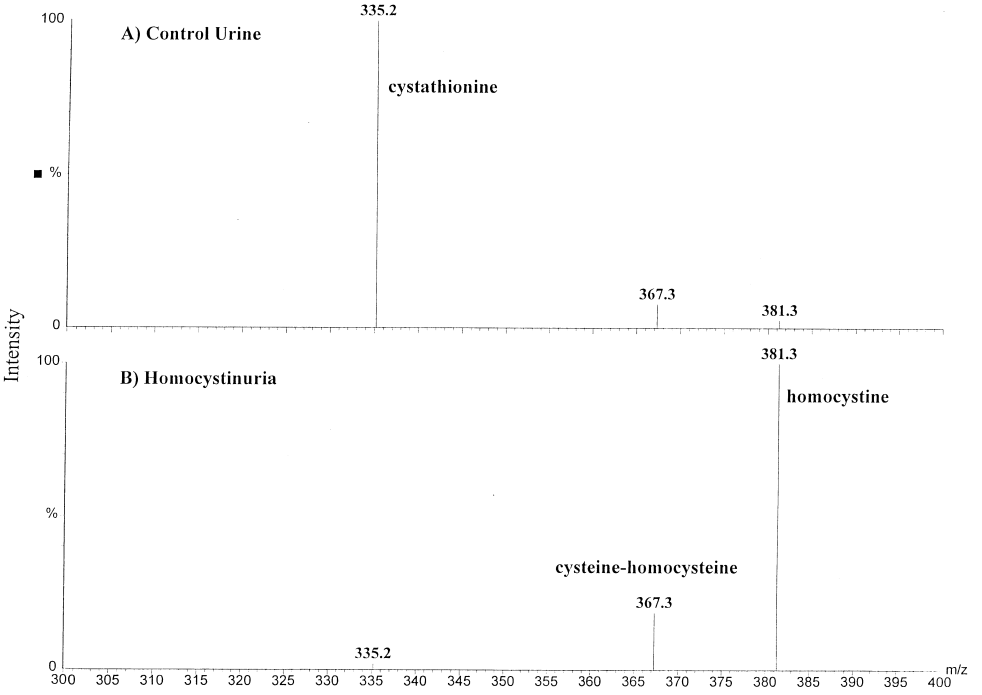

Fig. 3. Spectra obtained by averaging MRM chromatograms. (A) Control urine. (B) Homocystinuria patient. The masses in the profile are the protonated molecular ions of the butyl esters.

method and had the same throughput as that of Magera et al. [41].

Casetta et al. also described an LC–ionspray-MS–MS method for the rapid determination of 18 AAs in plasma and DBSs in less than 4 min [42]. Sample preparation was quite similar to the largely accepted method of extraction in methanolic solution using $^{2}H_{4}$-alanine and $^{2}H_{5}$-phenylalanine as internal standards, evaporation, and butylation. The butyl esters were injected onto a short cyano column interfaced with the mass spectrometer. The AAs were analyzed in MRM mode using specific transitions for each. The specificity offered by MS–MS analysis allowed quantification of several AAs in very short time windows despite significant co-elution. The method exhibited excellent recovery, linearity, and low RSDs. It also correlated well with values obtained from a standard AA analyzer. Furthermore, the method allowed for the quantification of the isobaric leucine and isoleucine, which is important for monitoring MSUD patients undergoing treatment. A particular problem was glutamine, which degraded to glutamate during the acidic esterification, and thus could not be accurately measured.

3.1.3. Analysis of acylglycines

Glycine conjugation is another detoxification pathway for some organic acids in several metabolic diseases. For some acyl-CoA groups this pathway is recognized to be quantitatively more important than conjugation with carnitine. For example, in IVA the formation of isovalerylglycine is preferable to the formation of isovalerylcarnitine due to the affinity of the substrate isovaleryl-CoA to conjugating enzyme, glycine-*N*-acylase [43,44]. Therefore, analysis of acylglyciness was found diagnostic for several diseases including the all too important and most common FAO disorder, MCAD. Actually, analysis of acylglycines by chemical ionization GC–MS developed by Rinaldo et al. has been recognized for many years as a key test for the confirmation of MCAD, independent of the clinical status of the patient [45]. In this case hexanoylglycine was found to be the most diagnostic metabolite.

In one of their groundbreaking articles Millington et al. investigated the use of FAB-MS–MS analysis of urinary acylglycines as a complementary test to blood AC profiling [11]. A small volume of urine was simply dried in presence of internal standards and treated with methanolic HCl to make the methyl esters of ACs and acylglycines. Upon CID, the acylglycines gave a common ion at m/z 90 and precursor-ion scanning of this ion gave a diagnostic profile for MCAD. The profile in this case showed elevated signals for the diagnostic metabolites hexanoylglycine and suberylglycine.

Recently, Bonafé et al. re-evaluated the analysis of urinary acylglycines by ESI-MS–MS while essentially using Millington's method of sample preparation and MS–MS conditions [46]. They presented a semiquantitative method for measuring acylglycines using several stable-isotope labeled derivatives. In MAD and MCAD acylglycines profiles were always informative. In MCAD, hexanoylglycine, phenylpropionylglycine, and suberylglycine were the main metabolites seen. Hexanoylglycine was always high irrespective of the clinical status while phenylpropionlyglycine and hydroxyoctanoylglycine were elevated during acute crises. In the neonatal form of MAD a C4-glycine (butyryl and isobutryl) and C5-glycine (methylbutyryl and isovaleryl) were the most prominent acylglycines. In IVA (5 samples), PPA (47 samples) and 3-methylcrotonyl-CoA carboxylase deficiency (MMC) (1 sample) the typical glycine conjugates in urine water clearly elevated. However, the profiles were either inconsistent or not informative in short-chain acyl-CoA dehydrogenase deficiency (SCAD), LCAD, VLCAD, Carnitine palmitoyltransferase deficiency type-II (CPT-II), MMA, and GAI.

We used the butyl esters of urinary acylglycines and ESI-MS–MS for confirmation of some of our cases such as MCC and IVA. An example is shown in Fig. 4A–C. MCC was suspected in a 4-month-old patient based on the AC profile obtained by MS–MS analysis of the patient blood spot shown in Fig. 4A

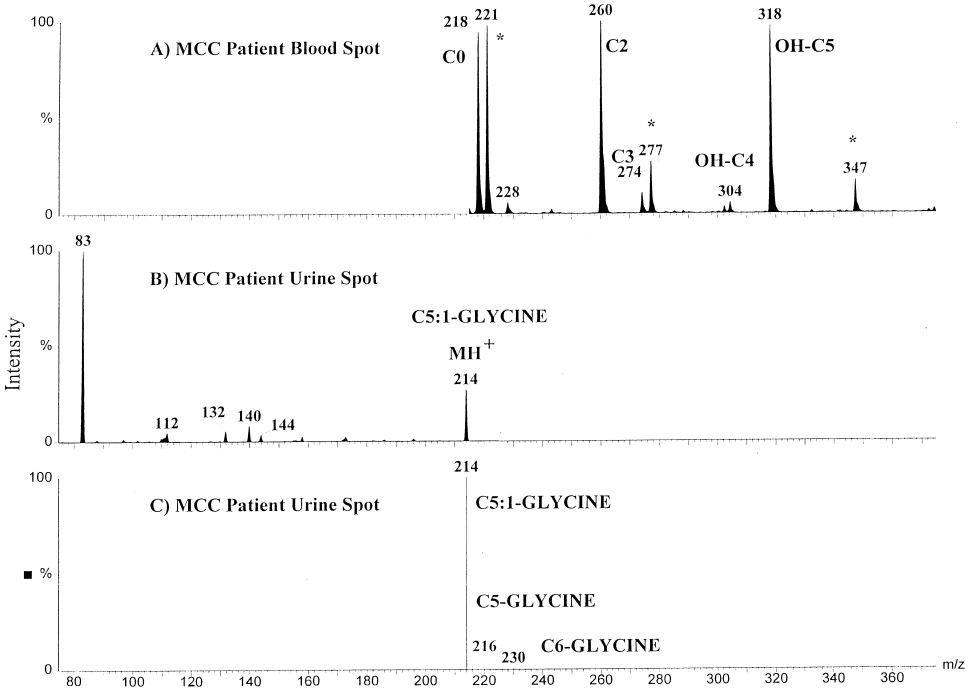

Fig. 4. MS–MS data for a patient with MCC. (A) Abnormal blood spot acylcarnitines profile. (B) Urine product-ion spectrum of m/z 214. (C) Urine MRIM data for four transitions, m/z 214→83, m/z 216→132, 230→132, and 264→132 for C5:1-glycine, C5-glycine, C6-glycine, phenylpropionylglycine, respectively.

that showed highly elevated ion at m/z 318 corresponding to a hydroxy-C5-carnitine. This particular ion is elevated to a different extent either by itself, or in combination with other diagnostic ions in diseases related to leucine and isoleucine catabolism disorders. In MCC (as hydroxyisovalerylcarnitine), in beta-ketothiolase (hydroxymethylbutrylcarnitine), in 3-hydroxy-3-methylglutaric acidemia (as hydroxyisovalerylcarnitine), in some MCD cases, and in some valproate-treated children. We examined the derivatized patient urine spot (~2 μl) by ESI-MS and observed a strong signal at m/z 214 possibly corresponding to a protonated C5:1-glycine (either methylcrotonylglycine or tiglylglycine) (data not shown). The product-ion spectrum of this ion is shown in Fig. 4B. The spectrum showed one major ion at m/z 83, which corresponds to the neutral loss of butylglycine ($H_2NCH_2COOC_4H_9$) thus retaining the positive charge on the molecule backbone. Urine samples from BKT patients known to excrete tiglylglycine (C5:1-glycine) gave the same results (data not shown). This is not the case in other butylglycines, for example isovalerylglycine (for IVA), hexanoylglycine, phenylpropionylglycine (for MCAD). These metabolites gave a major product ion at m/z 132 corresponding to the protonated butylglycine moiety. Therefore, we decided to use specific SRM functions for each metabolite (*target compound analysis*) rather than precursor-ion scans (*class-specific analysis*) (MCC). Thus we used four transitions, m/z 2l4→83, m/z 216→132, 230→132, and 264→132 for C5:1-glycine, C5-glycine, C6-glycine, phenylpropionylglycine, respectively. The mass spectrometer was scanned using a cone voltage of 25 V and collision energy of 12 eV. The averaged MRM chromatogram for our MCC patient urine yielded the spectrum shown in Fig. 4C which was dominated by a strong ion at m/z 214 corresponding to C5:1-glycine.

The method as described above by Bonafé et al. and by us lacked the specificity to pinpoint the defects BKT or MCC, even by two different FIA–MS–MS tests carried out on different biological matrices and GC–MS analysis in the electron impact mode was necessary for a firm diagnosis. This example highlights the important role of organic acid analysis by GC–MS as the disease-specific test in some situations.

3.1.4. Analysis of organic acids

We introduced recently another LC–ESI-MS–MS application in selective screening for the determination of the configuration of 2-hydroxyglutaric acid (2-HG) in urine of patients with 2-hydroxyglutaric aciduria [47]. 2-HG is a chiral polar aliphatic dicarboxylic acid that exists in two configurations, D-2-HG and L-2-HG. The two enantiomers are intermediary metabolites normally excreted in very small amounts in mammalian urine. D-2-hydroxyglutaric aciduria and L-2-hydroxyglutaric aciduria are two distinct metabolic disorders with different phenotypes. The discovery of either defect is usually accomplished through routine urinary organic acid analysis by GC–MS. In both cases the electron impact mass spectra obtained are identical with standard 2-HG. The determination of the absolute configuration of 2-HG is necessary for accurate diagnosis. For this purpose we developed an enantiomeric chiral separation method using a ristocetin A glycopeptide antibiotic silica gel bonded column (25 cm×4.6 mm). A 0.1-ml volume of urine was diluted with mobile phase (5 m*M* triethylamine acetate, pH 7–methanol, 9:1, v/v), filtered and injected onto the column interfaced to the mass spectrometer ion source through a T-shaped splitter at a flow-rate of 0.5 ml/min with a 1:8.5 split ratio. We used negative ion ESI-MS–MS in the MRM mode to monitor three transitions of MH^- to product ions (147→129, 147→85, and 147→57). We later found that 100% triethylamine acetate, pH 5 at a flow-rate of 1 ml/min (1:17 split) provided better resolution of the two enantiomers (Fig. 5A and B). Under both these conditions L-2HG eluted before D-2HG.

3.1.5. Analysis of purine and pyrimidines

Another recent and exciting application of ESI-MS–MS was in the selective screening for inherited disorders of purine and pyrimidine metabolism. These disorders have a wide variety of clinical presentations that are often non-specific, and the severity of the cases ranges from fatal to asymptomatic. Therefore, the development of a simple, rapid, and specific screening method for these diseases was highly desirable. Ito et al. described an LC–ESI-MS–MS assay using urine soaked filter paper [48]. Sample preparation was simple and essentially in-

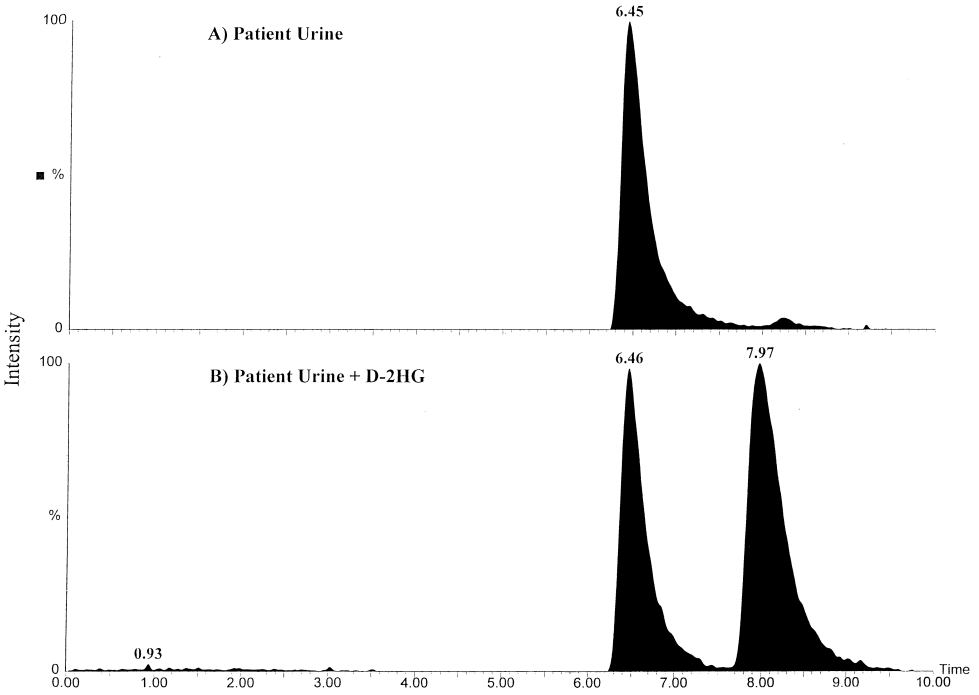

Fig. 5. LC–MS–MS negative ion chromatograms obtained in the MRM mode of analysis using a ristocetin A column. (A) Urine from an L-2-hydroxyglutaric aciduria patient. (B) Urine sample from same patient spiked with standard D-2-HG.

volved extraction of filter paper with an aqueous methanolic solution containing stable isotope labeled standards of uracil, thymine, orotic acid, thymidine, uridine, and others. The extracts were dried, reconstituted in mobile phase and injected a narrow bore octadecylsilane column (250×2.1 mm) at a flow-rate of 0.3 ml/min. Specific MRM transitions were used for a large number of analytes and HPLC was necessary for separation of isomeric compounds. Analytical time was 15 min/sample. The mean recovery of all analytes was 87 to 102% and RSDs were from 1.6 to 13.3%. The method was evaluated with urine samples from patients with established dihydropyrimidine dehydrogenase deficiency (elevated uracil, thymine, and 5-hydroxyuracil), molybdenum cofactor deficiency (elevated xanthine), ornithine transcarbamylase deficiency (elevated orotic and uracil), purine nucleoside phosphorylase deficiency (elevated inosine and guansine), and adenylosuccinase deficiency (elevated succinyladenosine).

3.1.6. Analysis of bile acids and very-long-chain fatty acids

Another area for application of MS–MS analysis in this decade was in the analysis of bile acids and their conjugates, which is important for the diagnosis of several bile acids metabolism disorders as well as for peroxisomal diseases. A number of inherited metabolic diseases have been characterized in which the normal conversion of cholesterol to the primary 24-carbon bile acids is disrupted. Examples include Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, peroxisomal bifunctional protein deficiency, 3-oxoacyl-CoA thiolase deficiency, and cholestatic hepatobiliary disease [49–53].

GC–MS methods for the analysis of bile acids in urine samples are the most sophisticated and informative methods but are technically demanding and time consuming [54]. In the 1980s several authors demonstrated the usefulness of FAB-MS for the rapid detection of bile acids in human fluids with

minimum sample preparation [53–55]. FAB-MS operated in the negative ion mode showed intense ions for abnormal bile salts in urine extracts. However, the information provided was limited to the presence of the pseudo molecular ion $[M-H]^-$ of these compounds, interferences form the matrix, and lack of adequate sensitivity in some situations.

Tomer et al. using pure standards were the first to study the use of negative ion FAB-MS–MS as an alternative approach for determination of bile salts and their conjugates [56]. Evans et al. studied the use of negative thermospray-LC–MS and thermospray-LC–MS–MS of bile acids and their glycine conjugates and indicated more specificity by MS–MS [57]. Libert et al. using negative ion FAB-MS–MS for the analysis of serum or urine extracts introduced the first clinical application of this methodology in patients with liver diseases and peroxisomal diseases. They reported better specificity by MS–MS and foresaw that the method might become the first line of investigation of peroxisomal disorders [58].

Warrack and DiDonato were the first to introduce the use of ionspray mass spectrometry (ISP-MS) and ISP-LC–MS of bile acids and their conjugates. The method demonstrated high sensitivity than those reported for FAB-MS but no clinical applications were shown [59]. Later, Roda et al. used negative ion ESI-LC–MS–MS to separate and detect a large number of free bile acids, their glycine and taurine conjugates at the picogram level in human serum and hamster bile [60].

Mills et al. recently described the use of a negative ion ESI-MS–MS isotope dilution method for the measurement of taurotrihydroxycholanoates and glycodihydroxycholanoates in blood spots with the purpose of mass screening for cholestasis [61]. They used precursor-ion scanning for m/z 74 to detect glycine conjugates and precursor-ion scanning for m/z 80 to detect taurine conjugates. The use of deutrated internal standards allowed the construction of calibration curves for glycochenodeoxycholic acid, glycocholic acid, taurochenodeoxycholic acid, and taurocholic acids. In another study they examined the feasibility of using this method for screening for cholestatic hepatobiliary disease and extrahepatic biliary atresia [62]. They studied 218 children with cholestatic hepatobiliary disease and compared the total bile concentration (four bile acid conjugates) to 708 blood spots from normal. Unfortunately, although significant differences in mean bile acid concentrations were found, a small but important overlap existed between the population distributions of unaffected neonates and those with cholestatic hepatobiliary disease. The separation was greater between neonates with extrahepatic biliary atresia and normal neonates, but even here the overlap was too great to make screening by this method alone a feasible option.

Bootsma et al. presented a method for rapid selective screening of peroxisomal diseases from plasma samples [63]. Sample preparation was quite simple. They used negative ion ESI-MS–MS and the same internal standards used by Mills et al. [61]. However, in this case LC–MS–MS rather than FIA–MS–MS was utilized with a short narrow bore column (5 cm×2 mm) interfaced to the ESI ion source with a flow-rate of 200 μl/min. Glycine conjugates were detected by MRM using specific transitions with a mass difference of m/z 74 and the transitions with the mass difference of m/z 80 for the taurine conjugates. The C_{29} dicarboxylic bile acid was detected using the specific transition m/z (507→463). The method allowed measuring absolute concentrations of taurine- and glycine-conjugated cholic acid, and those of chenodeoxycholic acid.

In a related approach, Johnson described a rapid screening procedure for the diagnosis of peroxisomal disorders via the analysis of very-long-chain fatty acids (VLCFAs) in small volumes of plasma and from blood spots using the dimethylaminoethyl esters and positive ion ESI-MS–MS analysis [64]. All the VLCFA-containing lipid species were converted to free VLCFAs by heating plasma at 100°C in 10% HCl in acetonitrile for 45 min. The free acids were then converted into their corresponding acid chlorides using oxalyl chloride followed by cold esterification by *N*,*N*-dimethylethanolamine. The derivatized acids yield strong protonated molecular ions in ESI-MS and few fragments upon CID analysis. MRM profiles were obtained for the diagnostically important acids, C20:0, C22:0, C24:0, and C26:0 using the transitions resulting from the neutral loss of 45 rel. mass units (loss of HCOOH). Using trideutrated internal standards for the four mentioned metabolites allowed quantification of these species. Calibration curves showed good correlation but there

was an overestimation of VLCFAs by this method as compared to standard GC–MS methods. In plasma the diagnostic ratios of C26/C22 and C24/C22 had good precision and adequate separation between normal and affected levels. As for C20 there was significant interference as the method measured both arachidic acid and phytanic acid as one. In blood spots C24/C22 ratio did not differentiate between peroxisomal disease patients and control population. The author advocated the use of this method for screening purposes, and that those positive results should be confirmed by GC or GC–MS.

3.2. Postmortem diagnosis

One example of postmortem is our use of FIA–ESI-MS–MS in the analysis of bile filter paper spots obtained post-mortem from infants of sudden infant death syndrome (SIDS) [65]. In this case the bile spots extracts were prepared exactly as blood spots. Qualitative profiles of AC methyl and butyl esters were obtained by ESI-MS–MS analysis by monitoring precursor-ion scans of the common fragment at m/z 99 (methyl-) or m/z 85 (*n*-butyl). Two SIDS cases suspected of FOADs due to appearance of fatty liver, undetectable glucose in liver tissue, or increased palmitoleic acid gave bile AC profiles strongly suggestive of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHAD). The profiles were similar to those previously described in plasma. A third case diagnosed as GA-I by GC–MS analysis of postmortem urine gave a profile dominated by the signal corresponding to glutarylcarnitine, a pathognomonic marker for the disease. The use of both methyl and butyl esters with two different CID fragments served to increase our confidence in the nature of the diagnostic ions in the profile (see later). Boles et al. described recently a larger retrospective study where 418 cases of SIDS were examined. Several investigations were carried out on postmortem liver tissue but bile was available in only 32 cases. The study detected 14 cases of FOADs, two MCAD, four MAD, four cases with either very-long chain acyl-CoA dehydrogenase deficiency or LCHAD, and four cases predicted with carnitine transport defect (CTD) [66].

3.3. Prenatal diagnosis

Another important field of application of MS–MS was in prenatal diagnosis for branched-chain catabolism disorders. Shigematsu et al. described the use of FAB-MS–MS in the prenatal diagnosis of IVA from amniotic fluid in a 32-week-old fetus [67]. Using the methyl ester derivatives and precursor-ion scans of m/z 99 they found elevated C5-carnitine (isovalerylcarnitine) as compared to an age-matched control. They also carried out precursor-ion scans of m/z 90 for acylglycines and found significantly elevated C5-glycine as compared to controls. Van Hove et al. described a retrospective prenatal study on three terminated PPA based on methylcitrate determination by GC–MS. They carried out an isotope-dilution assay for measuring C3 in the amniotic fluid using FAB-MS–MS [68]. They found that C3 was elevated by a factor of 5 as compared to 25 control amniotic fluid samples ranging in gestational age from 12 to 17.5 weeks. They proposed that AC analysis is a valid and rapid method for screening pregnancies at risk for PPA.

Shigematsu et al. carried out a larger retrospective study using ESI-MS–MS on a larger set of stored amniotic fluid samples in at risk pregnancies for several organic acidemias versus controls, at early gestational age (11–20 weeks). They reported that the concentration of single AC marker is not sufficient for a valid discrimination. Instead they used concentration ratios of C3/C4 (butyryl- and isobutyrylcarnitine) for PPA and MMA, C5/C3 ratio for IVA, C5-dioylcarnitine (glutarylcarnitine)/C3 ratio for GA-I, C5/C3 ratio for MAD. The cutoff values for the latter disease seemed not to be reliable enough for diagnosis [69].

In another exciting application Nada et al. described a prenatal diagnosis study using cultured amniocytes loaded with (16,16,16-$^{2}H_{3}$-palmitic acid and L-carnitine [70]). After a 96-h incubation at 37°C the media was separated and $^{2}H_{9}$-octanoylcarnitine ($^{2}H_{9}$-C8) and $^{2}H_{9}$-isovalerylcarnitine ($^{2}H_{9}$-C5) were added as internal standards. The mixture was then prepared for AC analysis by FAB-MS–MS as described before. Concentrations of C8 and longer-chain ACs were measured relative to the concentration of the internal standard $^{2}H_{9}$-C8 and expressed

as nmol/mg protein/96 h. In control amniocytes incubation the AC profile showed the products of the labeled substrate at odd masses corresponding to C12, C10, C8, C6, and C4, and thus clearly distinguished from the natural metabolites, which appeared at even masses. In the amniocytes with A985G homozygosity the profile showed significant elevation of C8, C10 as well as a mild increase in C6. Analysis following the incubation of cells from the pregnancy at risk for an unspecified metabolic disorder revealed elevated amounts of long-chain ACs. The most prominent species were the substrate itself and C14. The latter was undetectable in control incubations. Measurements of the enzymatic activities of the acyl-CoA dehydrogenases in both the mitochondrial membrane and the soluble fraction of the amniocytes homogenate showed a severely reduced activity of VLCAD.

This MS–MS-based prenatal diagnosis of MCAD and VLCAD deficiencies suggested that prenatal diagnosis of other FAODs could be also accomplished, regardless of the underlying mutation. There is no doubt that MS–MS has now an important role to play in prenatal diagnosis for metabolic diseases. However, it remains to be seen whether it can become the only mean for such diagnosis or that more classical and complementary techniques such as GC–MS, enzyme assays, or molecular analysis still has to be carried out for a more definitive diagnosis. It is this author view that more than one test will and should remain a common practice for such a serious question (affected or unaffected?). However, MS–MS may become the same analytical tool used for these different tests as shown above for the rapid analysis of amniotic fluid for ACs followed by a loading study using cultured amniocytes.

3.4. In vitro loading studies

Fibroblasts are frequently utilized to determine impairment of fatty acid oxidation pathways and pinpoint the exact enzymatic defect. The most commonly used methods are the $[^{14}C]CO_2$ and $[^{3}H]H_2O$ release assays. The cells are incubated in medium containing ^{14}C-labeled or ^{3}H-labeled fatty acids (e.g., [9,10-^{3}H]palmitic acid or [9, 10-^{3}H]myristic acid), followed by quantification of released $[^{14}C]CO_2$ or $[^{3}H][H_2O]$. However, these global assays lack diagnostic specificity and are not sufficiently robust. Kler et al. developed methods in which the actual β-oxidation intermediates were analyzed rather than just the end products. Thus, isolated mitochondria or permeabilized cells were incubated with radiolabeled palmitoyl-CoA, followed by resolution of the acyl-CoA esters using radio-HPLC. Later studies led to the development of a technique in which both the acyl-CoA and AC were resolved by HPLC [71,72].

A novel method was developed by Nada et al. in 1995 which involved specific AC analysis by MS–MS following incubations of intact fibroblasts or lymphpblastoid cells with deutrated long-chain fatty acids such as ([17,7,18,18-$^{2}H_4$]linoleic acid) in the presence of L-carnitine [73]. Deuterium-labeled ACs, as well as unlabeled ACs (from branched AA metabolism) were detected and quantified. The profiles were often very characteristic leading to immediate identification of the underlying enzyme defect [74,75]. MS–MS analysis eliminated the need for purification, isolation, and separation of intermediates. This method was successfully used for the delineation of the metabolic defect in VLCAD, TFP, LCHAD, MCAD, SCAD, carnitine: acylcarnitine translocase, CPT-II, but not for CPT-I [76,77]. The method was further extended to the study of mitochondrial branched-chain amino acid pathways, and resulted in the identification of a new disorder affecting valine metabolism [78].

4. Pitfalls in neonatal and selective screening by MS–MS

Many of the reports appearing in the literature highlight the power of MS–MS in routine analysis of AAs and ACs in selective screening as compared to traditional techniques such as GC–MS where false negative results are occasionally obtained when the patient is in a compensated metabolic state. These reports also boast higher sensitivity and specificity of MS–MS in NBS as compared to classical screening methods, e.g., for PKU. There is no doubt that all of this data is legitimate but the fact remains that no single technique is uniquely perfect and there are

always trade-off between sensitivity and specificity of the test.

From an analytical point of view our current MS–MS approach aims at detecting a large number of diseases in a single test through measuring a large number of metabolites (or parameters). Small analytical errors for each parameter may lead to a large cumulative error, and thus to a relatively large number of falsely flagged data files. This may lead to the use of wider cutoff ranges in order to decrease the recall rate and thus to false negative results. There is also the problem of using cutoff values obtained from normal newborns to selectively screen for older patients due to the difficulty in acquiring age-matched controls. Furthermore, in selective screening there often is a lack of information on drugs given to patient that may interfere with the assay.

From a clinical point of view, the situation is further complicated by the fact that not all biochemical abnormalities reflect a disease state, e.g., asymptomatic MCAD patients and hyperphenylalaninemia cases, and there exists cases where a symptomatic patient may be not be excreting the pathognomonic metabolites as is the case in a small number of GA-I patients. The impressive record achieved by the MS–MS method in both selective screening and NBS and the occasional lack of adequate communications between the laboratory and the clinic has increased the tendency of some physicians to regard MS–MS analysis as the ultimate test, and to use it to rule out an inherited metabolic disease in their patient disregarding important tests such as the routine organic acid analysis by GC–MS, or more specific GC–MS tests.

Various examples relating our experience with pitfalls in selective screening are presented here. In two patients from two different families with tyrosinemia type-I the diagnosis was delayed due to a repeated finding of normal tyrosine level by MS–MS. Later, consultation with the laboratory has led to succinylacetone analysis by selected ion monitoring GC–MS, which confirmed the diagnosis. A different problem was encountered in the high-risk screening for MSUD due to placing the newborn on special diet immediately after birth. Two cases from two different families were presumed normal based on daily MS–MS analysis of DBS for valine and leucine (+isoleucine). In one case, these AAs remained normal for 4 days, and in the other case the values were normal for 10 days. Both cases were discharged from the hospital on regular diet to crash at home few hours later. An alternative approach adopted by some physicians in our institution is to place the child on regular diet, collect a 24 h DBS for MS–MS analysis and then place the child on special formula until the results are available.

Another interesting case we encountered recently was a normal blood AC profile with normal free C0, normal free to total carnitine ratio. However, GC–MS analysis of urine showed an abnormal organic acids profile with highly elevated glutaric and 3-hydroxyglutaric acids indicating GA-I. This goes in the face of a large number of cases of GA-I (>30) diagnosed by the finding of elevated glutarylcarnitine by MS–MS analysis of DBS in our patient population. The reverse of this situation was also found as we encountered numerous cases in renal patients where the blood AC profile was flagged abnormal due to elevation of the signal at m/z 388 that corresponds to glutarylcarnitine while the urine organic acid profile was normal. We observed in most of these that there are other signals at m/z 342, m/z 358, m/z 402, some of these ions are commonly found in urine AC profiles, the exact nature of which remains to be determined.

We encountered another problem when we increased our upper cutoff value for the ratio of C3/C2 from 0.4 (99.5 percentile) to 0.51 (99.9 percentile). This resulted in missing several cases of methylmalonic acidemias as determined by GC–MS analysis of urine. Even the 99.5% percentile value was not sensitive enough in two other cases. Decreasing this cutoff value to 0.3 resulted in finding two more cases but caused a considerable number of falsely flagged data files. This C3/C2 parameter or the absolute concentration of C3 appears to be the most problematic in AC profiling as also expressed by other workers in the field [26].

As mentioned above Vreken et al. reported on the finding of a normal plasma AC profile in a case of SCAD, and in a mild form of MAD [23]. We, on the other hand encountered several liver disease patients where their AC profiles showed mild to moderate elevation of metabolites diagnostic for MAD, but with normal organic acid profiles.

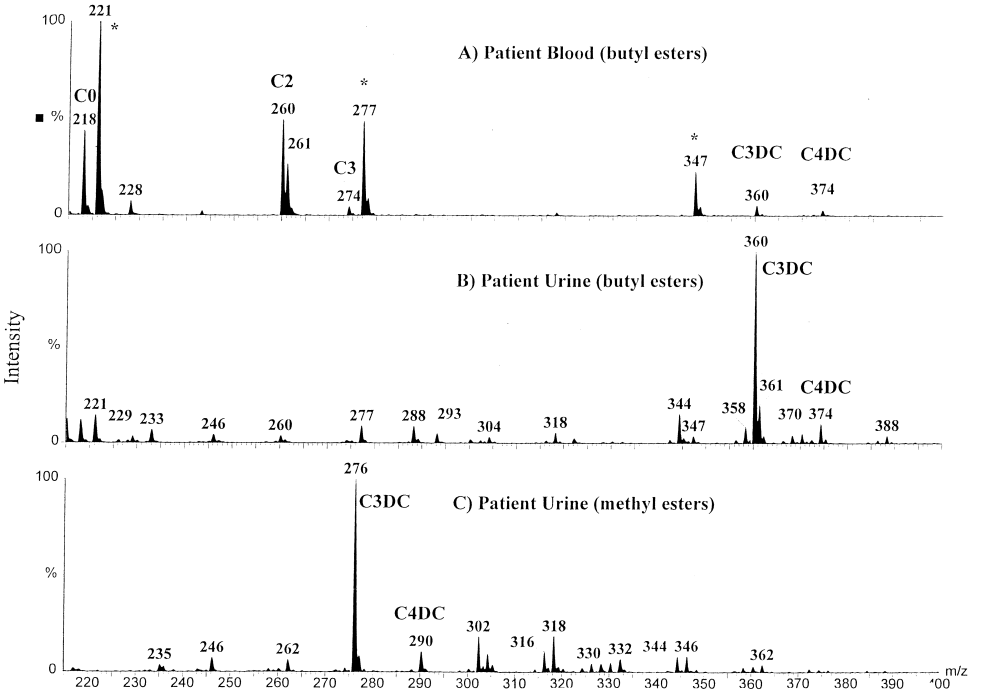

Fig. 6. MS–MS data for a patient with malonic aciduria. (A) Abnormal blood spot acylcarnitine profile showing slight elevation of m/z 360 (malonylcarnitine; C3DC). (B) Abnormal urine spot acylcarnitine profile for the butyl esters. (C) Abnormal urine spot acylcarnitine profile for the methyl esters.

We also missed two cases of malonic aciduria in 12- and 8-year-old siblings simply because our algorithm (CAMPA) did not include malonylcarnitine as a signal to be looked for, or quantified. GC–MS analysis of urine revealed grossly elevated malonic acid (normally not detected) and moderately elevated methylmalonic acid. On re-examining the blood AC profiles visually we found a slight but distinctly elevated signal at m/z 360 corresponding to the bis-butyl ester of malonylcarnitine (Fig. 6A). Analysis of urine by MS–MS showed the ion at m/z 360 as the abundant one (Fig. 6B). Further confirmation of the nature of this carnitine ester was obtained by preparing the corresponding methyl esters where the signal in both blood and urine shifted to m/z 276 (Fig. 6C).

5. Conclusions and perspectives

In the last 10 years tremendous progress was made in the field of diagnosis and screening for IMD through the use of MS–MS. Numerous centers around the world expanded their screening program to include MS–MS analysis or are currently evaluating the technology through pilot studies. Furthermore, some centers replaced classical NBS methods with MS–MS. The cost-effectiveness and proven record of the methodology in the last 10 years has overcome the initial resistance by some health authorities in many countries. However, there remains some unresolved issues such as time of collection of samples, and its logistics, as well as lack of consensus on the effectiveness of treatment for some of the disorders detected, and therefore on the number of diseases to be screened.

The situation is clearer in case of selective screening or high-risk screening. In dealing with a sick infant suspected with IMD every possible test should be carried out to pinpoint the defect. The active research in developing FIA–MS–MS or LC–MS–MS methodologies for diagnosing different diseases has led to many valuable methods, many of which are accepted as confirmatory tests, and there is more

to come. However, the workers in the field should not neglect ''older'' tests such as GC–MS and they should strive to correlate their MS–MS findings from different biological matrices with other biochemical findings, with enzyme data and molecular data, and most certainly with clinical data.

6. Nomenclature

2-HG	2-Hydroxyglutaric acid
AA	Amino acid
AC	Acylcarnitine
BKT	Beta-ketothiolase deficiency
C0	L-Carnitine
C10	Decanoylcarnitine
C10:1	Decenoylcarnitine
C2	Acetylcarnitine
C3	Propionylcarnitine
C5	C5-Carnitine
C6	Hexanoylcarnitine
C8	Octanoylcarnitine
CAMPA	Computer-assisted metabolic profiling algorithm
CBS	Cystathionine-β-synthetase
CID	Collision-induced dissociation
CPT-I	Carnitine palmitoyltransferase deficiency type-I
CPT-II	Carnitine palmitoyltransferase deficiency type-II
CSF	Cerebrospinal fluid
CTD	Carnitine transport defect
DBS	Dried blood spot
ESI	Electrospray ionization
FAB	Fast atom bombardment
FIA	Flow injection analysis
FOAD	Fatty acid oxidation defect
GA-I	Glutaric acidemia type-I
GC–MS	Gas chromatography–mass spectrometry
HPLC	High-performance liquid chromatography
IMD	Inherited metabolic disease
ISP-MS	Ionspray mass spectrometry
LCHAD	Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency
LC–MS–MS	Liquid chromatography–tandem mass spectrometry
MAD	Glutaric acidemia type-II
MCAD	Medium-chain acyl-CoA dehydrogenase deficiency
MCC	3-Methylcrotonyl-CoA carboxylase deficiency
MCD	Multiple carboxylase deficiency
MMA	Methylmalonic acidemia
MRM	Multiple reaction monitoring
MS–MS	Tandem mass spectrometry
MSUD	Maple syrup urine disease
NBS	Newborn screening
PKU	Phenylketonuria
PPA	Propionic acidemia
RSD	Relative standard deviation
SCAD	Short-chain acyl-CoA dehydrogenase deficiency
SIDS	Sudden infant death syndrome
SRM	Selected reaction monitoring
TFP	Trifunctional protein deficiency
TLC	Thin-layer chromatography
VLCAD	Very-long-chain acyl-CoA dehydrogenase deficiency
VLCFA	Very-long-chain fatty acid

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