USES OF TANDEM MASS SPECTROMETRY AS A NOVEL TOOL FOR RESEARCH AND DIAGNOSIS OF INTERMEDIARY METABOLISM DISEASES

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ABSTRACT

The study of inherited inborn errors is becoming important as they have a fundamental role in public health. However the knowledge of these disorders is not common into the medical community, probably because of lacking technology for its diagnosis is one of the main problems the clinicians have to face. Tandem mass spectrometry is a novel technique used mainly in developed countries for the investigation of metabolic diseases and other disorders. The present review explain the way how a tandem mass spectrometer works and how the research in this field can be used for diagnosis of diseases related to intermediary metabolism.

Key words: Metabolic diseases, tandem mass spectrometry.

USOS DE LA ESPECTROMETRÍA DE MASAS EN TANDEM COMO UNA TÉCNICA NOVEDOSA PARA LA INVESTIGACIÓN Y DIAGNÓSTICO DE ENFERMEDADES DEL METABOLISMO INTERMEDIARIO

RESUMEN

El estudio de las enfermedades hereditarias se está volviendo importante, debido al papel fundamental de estas enfermedades en salud pública. Sin embargo, el conocimiento de estas alteraciones no es común entre la comunidad médica, probablemente debido a que la falta de tecnología para el diagnóstico de las mismas es uno de los principales problemas que los clínicos deben afrontar. La espectrometría de masas en tándem es una técnica novedosa utilizada principalmente en los países desarrollados, para la investigación de enfermedades metabólicas y otras alteraciones. La presente revisión explica la manera como funciona un espectrómetro de masas y cómo la investigación en este campo puede ser utilizada para el diagnóstico e investigación de las enfermedades relacionadas con el metabolismo intermediario.

Palabras clave: Enfermedades metabólicas, espectrometría de masas en tándem.

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INTRODUCTION

The first descriptions of the separation of ions, or charged molecules, by electrical charge and total atomic mass by magnetic and electrical fields, were published in the 1980. (1). Mass spectrometry (MS) is basically a sophisticated method for weighing molecules (2). The essential requirement to obtain a mass spectrum is to produce ions in a gas phase, accelerate them to a specific velocity using electric fields, project them into a suitable mass analyser that separates the entities by masses, and finally to detect each charged entity of particular masses sequentially in time (3). As mass spectrometers require charged, gaseous molecules for analysis, and biomolecules are large and polar, they are not easily transferred into the gas phase and ionised. After the discovery of methods that finally allowed gentle ionisation of large biomolecules, mass spectrometry has become a powerful tool in metabolite analysis and a fundamental technique in the emerging field of the inherited metabolic diseases.

The present review analyses the possibilities for MS technique for the diagnosis of diseases related to intermediary metabolism.

THE MASS SPECTROMETER

The three main sub-systems in the mass spectrometer device are: the ion source in which the ionisation of the organic molecules takes place; the mass analyser which separates the ions according to their mass/charge (*m/z*) values; and the detector where relative intensities (abundance) of the separated ions are determined. Very low pressures, i.e. high vacuum, in the region of 10^{-5} torr are used. This permits the ions to travel from the ion source to the detector virtually unimpeded with the minimal interaction with other gas phase molecules which might otherwise scatter or fragment the ions and cause a reduction in sensitivity (4).

IONISATION

During the ionisation step considerable amounts of energy are imparted to the initially formed molecular ions. The excess of energy causes some of the molecular ions to fragment. The mass spectrometer also measures the masses of all of the charged products of this fragmentation (the so-called fragment ions). A mass spectrum is a snapshot of the abundances of the molecular and fragment ions plotted against their masses. Such a mass spectrum acts as a characteristic molecular fingerprint for individual substances (5).

There are many ways of ionising molecules, a molecule (M) can be ionised by removal or addition of an electron to give species ($M^{+\cdot}$ and $M^{-\cdot}$ respectively) having a mass, which, for practical purposes, is identical to that of the original molecule. Optionally, the molecule may be ionised by addition or subtraction of other charged species X^+ or X^- to give ions $M+X^+$ or $M-X^-$. These ions are called quasi-molecular ions because they contain all or most of the original molecule but have masses that are different from that of the original. The original substance can be a salt (M^+X^-), in which instance, it is ionised already and the oppositely charged species need only to be separated prior to mass spectrometry (6).

When a molecular ion is formed, this may contain sufficient excess of internal energy to fragment by ejection of a neutral particle (N) with the formation of a fragment ion ($A^{+\cdot}$ or A^+). A neutral molecule gives a radical-cation as the molecular ion and the fragment ion may be either a cation or a radical cation (7).

If the fragment ion has sufficient excess of internal energy, then further decomposition may occur with the formation of new fragment ions (B^+, C^+, etc.) until there is insufficient excess of internal energy in any one ion for further reaction. Only the charged species may be accelerated out of the source and into the

mass analyser. Such a series of decompositions when elucidated from the mass spectrum is a fragmentation pathway.

The various fragmentation pathways together compose a fragmentation pattern characteristic of the compound under investigation. Hence, the mass spectrum is not simply the fragmentation pattern, but is the appearance of the fragmentation pattern at specified energies and time (8). The ability to analyse polar, non-volatile biological molecules was realised with the introduction of "soft" ionisation techniques such as fast atombombardment (FAB/MS), thermospray (TSI/MS), and most recently and importantly, electrospray ionisation (ESI/MS) which allows liquid chromatography to interface with mass spectrometry.

Fast atom bombardment (FAB) revolutionised mass spectrometry and has given rise to the field of biological mass spectrometry. The biological materials can be introduced into the ionising beam of neutral atoms in solution, and mixed with a relative involatile, viscous matrix such as glycerol, thioglycerol or *m*-nitrobenzyl alcohol. The mixture is then bombarded with atoms travelling at high velocity in vacuum and a very short-lived transient high temperature spike occurs, causing thermally induced bond breaking of sufficient length to allow ionisation fragmentation. The resulting collection of ions is then used to produce a mass-spectrum that contains considerable structural information in the mass spectrum (9). Positive and negative, and complementary mass spectra may be produced, but pseudomolecular species are produced as either protonated or deprotonated entities. Protonation occurs by abstraction of H^+ from the matrix and deprotonation by donation of H^+ to the matrix.

Another important desorption method which enables solid materials to be introduced into the mass spectrometer is the laser desorption ionisation (LD). The sample is coated on a suitable probe surface and laser beams (electromagnetic radiation) are focused, causing both ionisation and desorption (10). A modification named matrix-assisted laser desorption ionisation (MALDI) has been developed by Karas and Hillenkamp (11), and this, like ESI can be considered as a soft ionisation method.

To generate gas phase in MALDI, protonated molecules, and a large excess of matrix material is co precipitated with analyte molecules (that is, the molecules to be analysed) by pipetting a submicroliter volume of the mixture onto a metal substrate and allowing it to dry. The resulting solid is then irradiated by nanosecond laser pulses, usually from small nitrogen lasers with a wavelength of 337 nm. The matrix is typically a small organic molecule with an absorbance at the wavelength of the laser employed. Matrices differ in the amount of energy they impart to the biomolecules during desorption and ionisation and hence the degree of fragmentation (unimolecular decay) that they can cause (12). The precise nature of the ionisation process in MALDI is still largely unknown and the signal intensities depend on incorporation of the analytes into crystals, their likelihood of capturing and/or retaining a proton during the desorption process, and a number of other factors including suppression effects in some mixtures (13).

Electrospray ionisation (ESI) and thermospray ionisation (TSI) can also be named as soft ionisation techniques. The essential principle in these methods is that some form of atomisation, or nebulisation, produces a spray of charged liquid droplets. The species to be investigated are solvated on a charged droplet. As the solvent evaporates in the high vacuum region, the droplet size decreases and the charge eventually resides on the entity under study. ESI was developed by Fenn *et al.* (14). Liquid containing the analyte (large, highly polar biomolecules) is pumped at low microliter-per-minute flow rates (0.1-10

µl/min) through a capillary. Depending on the analytes a high positive or negative voltage (2-5 kV) is applied to the capillary. These droplets move through the atmosphere towards the entrance to the mass spectrometer and generate a cloud of charged analyte molecules (ions) (15). The mechanisms proposed to explain the ions formation are the coulomb fission mechanism which assumes that the increased charge density, due to solvent evaporation, causes large droplets to divide into smaller and smaller droplets, which eventually consist only of single ions (16); and the ion evaporation mechanism, which assumes that the increased charge density that results from solvent evaporation eventually causes coulombic repulsion to overcome the liquid's surface tension, resulting in a release of ions from droplets surface (17). The electric field can impart additional charge to droplets ensuring an excess of electronic charge. As the solvent evaporates from a droplet, whilst it traverses a desolvation chamber, its charge density increases substantially. These ions can then be efficiently collected by electronic lenses and directed through a skimmer into the mass spectrometer (18) see figure 1.

The amount of charge on the droplets is equal to the amount of charge separation. This charge is sometimes called the excess charge, to differentiate it from the cations and anions in the droplet that are neutralised by counter-ions (19), because the neutralised charges are not likely to result in gas-phase ions. The maximum rate of production of vapour phase ions is equal to the rate of charge separation, and the amount of vapour phase ions produced cannot exceed the amount of excess charge introduced into the droplets. An electrochemical reaction keeps the flow of charge from metallic contact to the sample solution. In positive ion ESI the dominant reaction is oxidation whereas in negative ESI it is reduction (20). The ionisation process takes place at atmospheric pressure and is therefore very gentle (without fragmentation of analyte ions in the gas phase) and the molecules are transferred into the mass spectrometer with high efficiency for analysis.

To transfer the ions from the atmospheric pressure region to a low pressure region, of the mass analyser, differential pumping is generally employed, hence the mass spectrometer consists

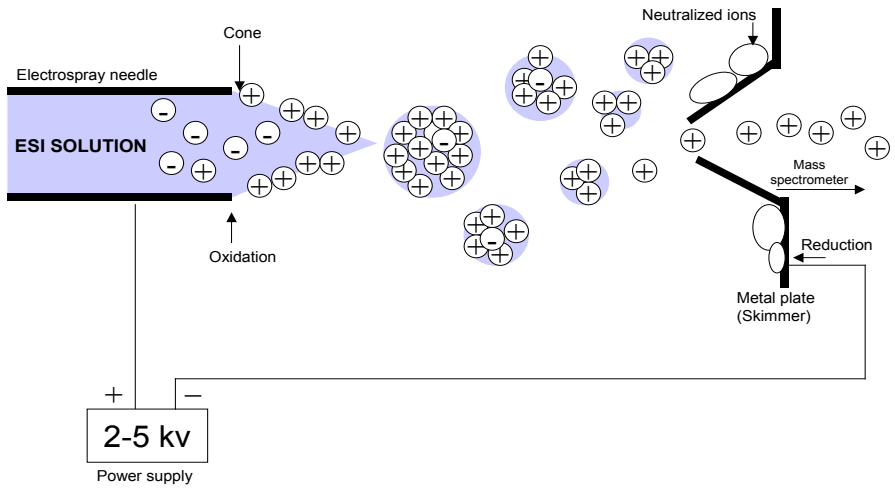


Figure 1. Electrospray ionisation mechanism. The resultant charged species are accelerated through differentially pumped regions where the remaining solvent is removed before entry into the mass spectrometer.

of a series of chambers at decreasing pressures separated by orifices through which ions must pass before reaching the mass analyser. To stabilise the spray, a nebuliser gas (known as sheath gas) or some other device is often employed. ESI that uses sheath gas is known as pneumatically assisted ESI (21). To drive neutral species away from the mass spectrometer orifice, some machines use a dry nitrogen "curtain" gas, hence by virtue of an electric field gradient, charged species are electrostatically attracted toward the orifice and thus penetrate the curtain.

Electrospray is typically performed in either the infusion mode, the nanoelectrospray format, or in combination with high-performance liquid chromatography. In the infusion mode, the sample is simply introduced into a continuous liquid stream (typically a mixture of organic and aqueous liquid (i.e. 70:30 acetonitrile: H_2O) via an injection valve (22). Flow rates are usually between 0.5 and several microliters per minute.

For ESI samples have to be substantially free of salt and detergent, and can be conveniently be cleaned up in a reversed-phase packing loop in the injector valve (5). Nanoelectrospray is a miniaturised version of electrospray that operates without pumps and at very low flow rates in the range of a few nanoliters per minute. It is performed in pulled glass capillaries with an inner diameter at the tip which permits the pass of about one microliter (23). A microliter volume of sample can be analysed for more than an hour at full signal strength, which allows complex sequencing experiments to be performed (24).

DERIVATISATION

In order to have an analyte amenable to analysis by ESI–MS/MS, it must already be in ionic form in the solution phase, or be chargeable through adduct formation, eletrochemical reactions, or gas-phase reactions. Derivatisation reactions can enhance the ESI response of an analyte by either making it more easily charged (25) or by increasing its surface activity (Sysoev *et al.*, 2004). Analytes most responsive to ESI analysis will have significant nonpolar regions, which give them a high affinity for the surface of the ESI droplet.

Ideally, derivatisation should proceed rapidly and quantitatively to one product and the reagents for derivatisation must affect molecules in a predictable and reproducible way and it is important that derivatisation does not cause an inordinate increase in relative molecular mass. A derivative is therefore required that provides particularly stable and abundant ions, preferably of a high mass (18).

Derivatisation is used to convert a neutral molecule, M, into some ionic form, by the addition of various functional groups (26) or by the introduction of electrochemically reactive functional groups into the molecular structure (27). The signal-to-noise ratio for the analyte can be improved because the adduct mass is shifted out of the low-mass region of the mass spectrum region that is typically complicated by the presence of solvent clusters and contaminants (28).

Further increase of sensitivity was obtained by the analysis of carnitine and acylcarnitines as methyl esters by MS/MS through a precursors of *m/z* 99 scan (29). The use of the MS/MS improved the detection limit from 50 nmol/ml to about 1 nmol/ml for individual acylcarnitines (30). This improvement was achieved primarily because of the drastic reduction in chemical interference that takes place entirely within the tandem mass spectrometer. However, the derivatisation of carnitine using butanolic HCl (31) showed that the prominent collision-induced fragment at *m/z* 103, seen also by MS/MS of the isotopically labelled form, enabled the application of a precursor ion scan function to analyse the carnitine specifically and essentially without any sample preparation. Then butylation rather

than methylation was employed to optimise the analysis of carnitine and acylcarnitines and other compounds by MS/MS as these butyl esters are well suited for analysis by this technique since they already carry a positive charge and accordingly, no additives are needed in the mobile phase.

ANALYSERS

Essentially, all mass spectrometers separate ions by measuring the mass-to-charge ratio (m/z) of analytes. Three different principles may be applied to achieve mass separation: separation on the basis of time-of-flight (TOF-MS), separation by quadrupole electric fields generated by metal rods (quadrupole-MS), or separation by selective ejection of ions from a three-dimensional trapping field (ion trap of Fourier transform ion cyclotron MS).

The quadrupole device, is generally constructed using four solid cylindrical rods, of circular cross-section, to which are applied both direct current (DC) and radiofrequency (RF) voltages. Both the fixed (DC) and oscillating (RF) fields cause the ions to undergo complicated motion in the x-y plane. This, together with the component of motion in the z-direction, results in the ions following complicated trajectories through the quadrupole filter. For a given set of field conditions only certain trajectories are stable, allowing ions of specific masses to be transmitted through to the collector/detector. Ions, whose mass determines that they travel along unstable trajectories, do not get transmitted, hence the use of the term filter. By careful control of the field conditions, ions of different masses can be successively filtered and transmitted (32). The ions that have just sufficient energy to fragment some time after leaving the ion source, but before arriving at the detector are called "metastable ions". The excess of internal energy imparted to these metastable ions during ionisation is sufficient to give them a rate of decomposition such that the latter occurs during the ion flight-time. In ordinary quadrupole mass filters, metastable ions cannot be studied because they are not distinguished from normal ions. Hence instruments have been designed with a series, usually three, quadrupole in which ion fragmentation can occur between quadrupoles (see figure 2) The first analyser is used to transmit selected ions but the second is not used for mass separation; but for ion fron ion-fragmentation and resultant product ions transmission to the next quadrupole, which performs the final mass analysis (33).

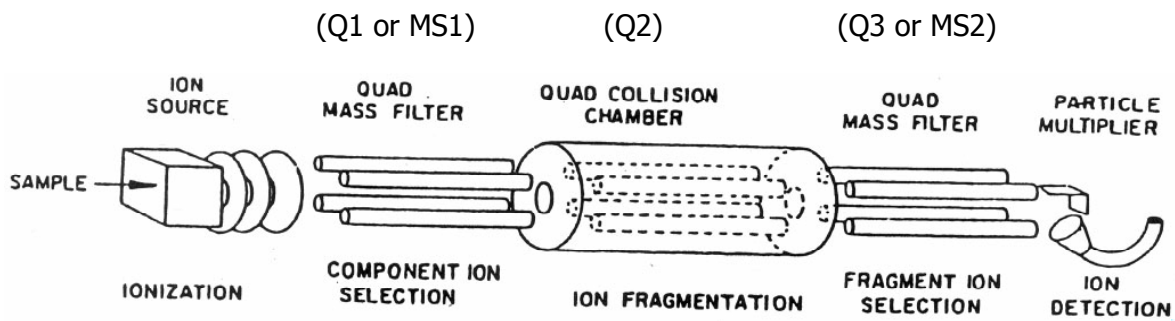


Figure 2. Tandem mass spectrometer. The machine is divided in five parts: the ion source where the ionisation takes place; the first mass analyser (Q1 or MS1) for component ion selection; the quad (quadrupole) collition chamber (Q2) where ion fragmentation by CID is performed; the second mass analyser (Q3 or MS2) for fragment ion selection; and the particle multiplier for ion detection.

TANDEM MASS SPECTROMETRY

The ions formed can be induced to fragment further by the addition of more internal energy after they leave the source, then a particular peak can be selected for further investigation. The ions comprising this peak are made to undergo further fragmentation, usually by the method known as collisionally induced dissociation (CID) (34). The ions are allowed to interact, and collide with atoms or molecules of an inert gas (helium, neon, argon, or nitrogen). Energy transferred to the ions under investigation can be distributed in a variety of ways (35).

Some of the transferred energy will remain as translational (involved in direction changes, scattering etc.) whilst some will be distributed into vibrational modes of the chemical bonds of the ion. It is the latter energy that, if sufficient, can cause further degradation, the products of which can be analysed in another mass analyser. If the gas pressure is too high, all ions will be scattered by collision and none will get through the cell, hence successful CID depends on having a high enough gas pressure so that multiple collisions produce sufficient fragment ions for the following analyser to detect. Two collisional activation regimes are in use, one at low kinetic energies corresponding to acceleration of ions through 0-100 eV and one at high kinetic energy, to accelerate the ions through more than 1 KeV. The former is employed in quadrupole instruments. The amount of internal energy deposited in the ion is mostly rotational and vibrational. CID is very useful with a soft ionisation technique like ESI, which is likely to yield only molecular or quasi-molecular ions (36). The molecular ion can be selected and further fragmented in collision cell with further analysis in the following analyser. This enables mixture analysis. When quadrupole mass filters are used for mass selection and analysis, the first and third quadrupoles are operated as normally for mass selection and analysis with RF and DC, and the second quadrupole (sometimes hexapole), or activation region is used only with RF voltage so as to act as both a collision region and a means by which to reduce ion scatter after collision (figure 2).

As with metastable ions in a fragmentation process where $M^+ \rightarrow A^+$, M^+ and A^+ may be referred to as parent and daughter ions respectively. This means that there are several types of scan. The daughter scan mode may select a parent ion characteristic of the analyte with the first mass analyser, fragmenting it by CID, and scanning the second mass analyser to obtain a daughter spectrum.

In a parent ion scan (figure 3), a specific daughter ion is selected with the second mass analyser, and the first mass analyser is scanned over a specific mass range, selecting parent ions of different *m/z* that fragment to yield the specific daughter ion. This approach is useful for a class of compounds that fragment to yield a common substructure (for example acylcarnitines).

A third scan mode, known as neutral loss scan, can also be used. In this method the precursor and the product ions are separated in mass by a predetermined amount. For example, if after mass spectrometric fragmentation of molecular ions ($M^{+\cdot}$) the fragment ions A^+, B^+, C^+ are obtained, and the difference between ions M^+, A^+ and B^+, C^+ were 30 mass units each, then setting the two MS analysers to detect mass losses of 30, M^+ and A^+ would be linked as would B^+ and C^+. Both mass analysers are scanned with a constant difference in mass. The resulting neutral loss spectrum contains the daughter ions, which arise from the loss of a specific neutral fragment from the parent ions. This scan function is useful for screening for a class of compounds characterised by a common fragmentation pathway (for example neutral amino acids). For trace analysis, selected reaction monitoring (SRM or MRM) is used (figure 3), where a limited number of parent ion-daughter ion pairs are monitored for each analyte, improving the sensitivity (37).

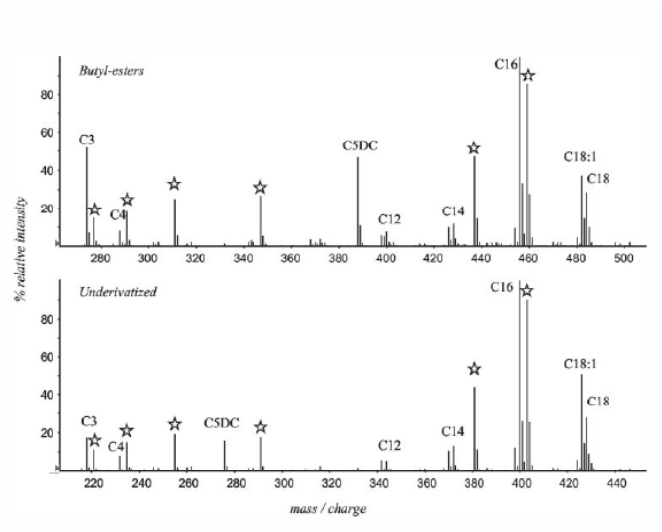


Figure 3. Pre 85 MS/MS scans of acylcarnitines in a newborn confirmed to have Glutaric aciduria type I. Were glutarylcarnitine (C5DC) is accumulated. Top, blood specimen prepared by butyl esterification, C5DC is detected at m/z 388. Bottom, blood specimen prepared without derivatization. C5DC is detected at m/z 276.

DETECTORS

The detector is made by a surface on which the ions impinge and the charge being neutralised either by collection or donation of electrons. The resultant current is converted into a signal recorded on a chart or processed by computer. The total ion current is the sum of all the currents carried by all the ions.

The main methods for detecting ions and generating an electric current proportional to their abundance are a) by directing the ions sequentially in the mass analyser to a point where there is a single-channel detector, such as in Faraday cup or various focal plane detectors, scintillation counting, and photomultiplier, and electron multiplier (as used in quadrupole mass filter or time-of-flight analyser), and b) where ions are dispersed simultaneously to a plane and detected by a focal-plane detector, such as array detectors (as used in magnetic sector instruments) (38).

DISORDERS INVOLVING ENERGY METABOLISM

This group consists of inborn errors of intermediary metabolism with symptoms due at least partlyt o a deficiency in energy production or utilization resulting form a defect in the liver, myocardium, muscle, or brain. Included in this group are disorders presenting as a predominant hypoglycaemia like glycogenosis, gluconeogenesis defects and hyperinsulinism, the fatty acid oxidation disorders, and the congenital lactic acidaemias (deficiencies of pyruvate carboxylase, pyruvate dehydrogenase, Krebs cycle and mitochondrial respiratory chain disorders). Symptoms common to this group include failure to thrive, severe hypoglycaemia, hyperlactacidaemia, severe generalized hypotonia, myopathy, cardiomyopathy, cardiac failure, arrhythmias, conduction defects, circulatory collapse, sudden infant death, dysmorphia and malformations. Most of the disorders presenting with hypoglycaemia are at least partly amenable to treatment while congenital lactic acidaemias are in general not treatable.

USES OF TANDEM MASS SPECTROMETRY FOR THE STUDY OF INHERITED INBORN ERRORS OF INTERMEDIARY METABOLISM

The number of inherited disorders of intermediary metabolism that can be identified through MS/MS has increased more the fivefold in a single decade. The metabolites which can be analysed by MS/MS are as follow:

Carnitine, acylcarnitines and amino acids. Since carnitine is a vehicle by which the acyl groups can leave the mitochondria and there is an equilibrium between acylcarnitines and their respective CoA thioesters, the analysis of carnitine and acylcarnitines in blood is approximately equivalent to the analysis of acyl-CoAs in the mitochondria. The concept of an acylcarnitine profile rather than a urine organic acid profile was therefore indicated as a potentially more valuable diagnostic tool for the disorders of branched-chain amino acid and FA catabolism (39).

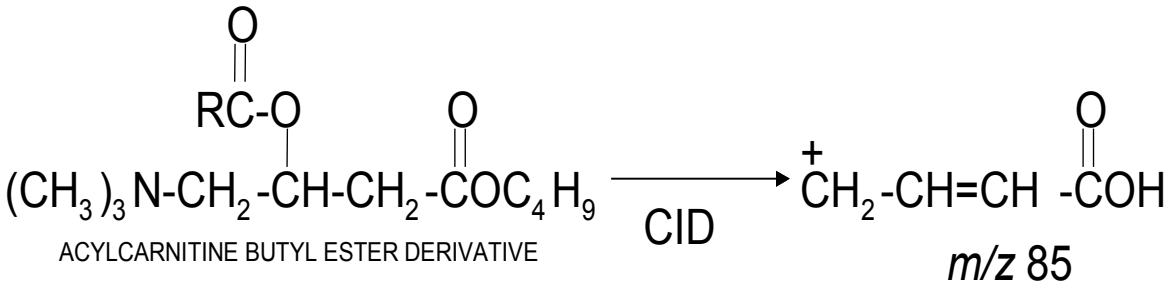
Acylcarnitine identification in body fluids using MS/MS was developed in the late 1980s and represents a valuable tool for the diagnosis of some long-chain FA oxidation defects which are difficult to diagnose by classical chromatographic method. The method has the potential to screen effectively for at least a dozen other disorders (40,41,42,43).

Some authors suggest that a plasma acylcarnitine profile should be performed in all patients presenting an acute episode of hypoketotic hypoglycaemia, Reye syndrome, hypertrophic cardiomyopathy, pericardial effusion, cardiac failure or rapid unexpected death in the neonatal period or during infancy, also heart beat disorders during neonatal period, hypotonia with unexplained failure to thrive, retinitis pigmentosa or even muscle pain triggered by exercise (44). The measurement of acylcarnitines using MS/MS has been reported in whole blood (45), plasma (46), urine (47), amniotic fluid (48), and bile (49).

Carnitine and acylcarnitines contain a quaternary ammonium functional group, making them preformed positive ions (cations) that are polar and non-volatile (50). Ions produced in the source are selected by MS1 for transmission to the collision cell. The fragments produced after CID are transmitted to MS2 where they are again selected for transmission to the detector. Ions transmitted by MS1 to the collision cell are called precursor ions (commonly referred as "parent" ions), and the fragments produced from CID are product ions (known as "daughter" ions). During the derivatisation process butyl esters of acylcarnitines are formed. These butyl esters are well suited for analysis by MS/MS since they already carry a positive charge and accordingly no additives are needed in the mobile phase. Both butyl esters derivatives and underivatised carnitine and acylcarnitines share a common product ion upon CID, which is singly charged with a mass of 85 Da, corresponding to $+CH_2$-CH=CH-COOH. This fragment results from the loss of elements of both $(CH_3)_3N$ and C_4H_8 and the side chain acyl group as RCOOH (figure 4).

Amino acids (AA). Analyses of AAs using tandem mass spectrometry started specifically for phenylalanine (51), physiological AAs share a common structural feature, a formic acid chemical group, which enables their selective analysis by MS/MS. After butylation and exposure to CID, butylformate (102 Da) is lost, because butylformate is a molecule (uncharged) rather than an ion, the product ion detected in MS2 is equal to the alfa-AA minus butylformate. Phenylalanine, tyrosine, leucine, alanine, valine, and methionine share this mode of fragmentation and can be analysed by MS/MS.

Many of the intermediates in organic acid metabolism are acyl-CoA intermediates that undergo β-oxidation. As a result, in certain organic acid disorders, the concentration of acyl-CoAs will be substantially elevated, resulting in high concentrations of associated acylcarnitines, which can be detected by MS/MS.



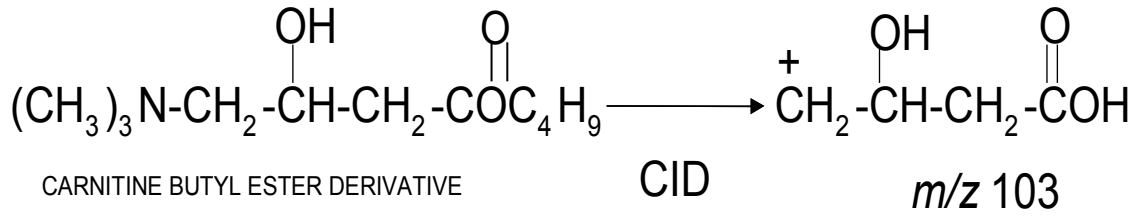


Figure 4. Schematic representation of the specific fragmentation in the tandem mass spectrometer of acylcarnitines and carnitine butyl ester derivatives**.**

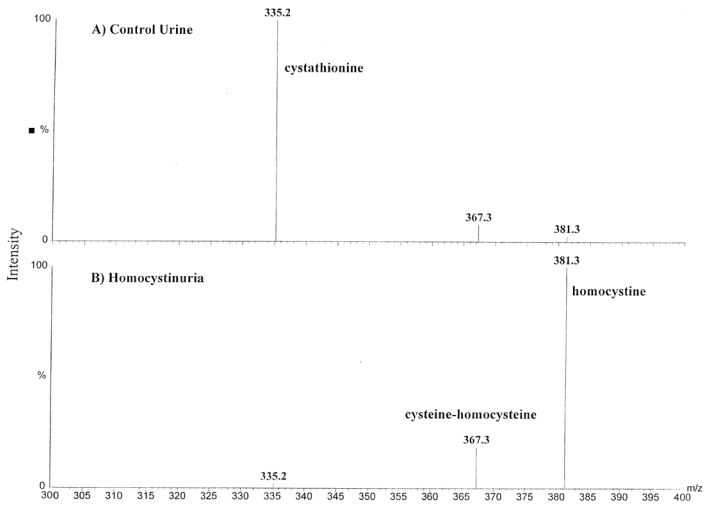


Figure 5. Spectra obtained by averaging MRM chromatograms. (A) Control urine. (B) Homocystinuria patient. The masses in the profile are the protonated molecular ions of the butyl esters.

CONCLUSION

The analysis for amino acids and acylcarnitines using MS/MS in blood obtained from newborns for screening procedures, has provided a bridge between the clinicians and the diagnosis for intermediary disorders. The diagnosis using the technique includes several number of diseases (table 1) using different body fluids (figures 3 and 5) from patients at any period of life, for those reasons tandem mass spectrometry constitutes a novel tool for research and diagnosis of intermediary metabolism diseases.

Table 1. Diseases (enzymatic deficiencies) detected by MS–MS analysis of blood spots and plasma of patients

Fatty acid oxidation defects	Organic acidemias	Aminoacidopathies
OCTN2	MMA (different types)	PKU (classical and biopterin dependent)
CPT-I	Combined methylmalonic: homocystinuria	MSUD
CPT-II	Propionic acidemia (acute neonatal and late onset)	Homocystinuria
CACT	3-Hydroxy-3-methylglutaryl-CoA lyase deficiency	Citrullinemia (acute neonatal and mild)
MAD	Methylcrotonyl-CoA carboxylase deficiency (isolated)	Argininosuccic acidemia (acute)
SCAD	IVA	Tyrosinemia type-I
Ethylmalonic acidemia	GA-I	Tyrosinemia type-II
MCAD	Malonic acidemia	Methylenetetrahydrofolate reductase deficiency
VLCAD		Non-ketotic hyperglycinemia
LCHAD		Prolinemia type-II
TFP		
Dienoyl-CoA reductase deficiency		

Abbreviations: CPT I, Carnitine palmitoyl transferase I; CPT II, Carnitine palmitoyl transferase II; CACT, carnitine acylcarnitine translocase; MAD, multiple Acyl-CoA dehydrogenase; SCAD, Short-Chain Acyl-CoA dehydrogenase; MCAD, Medium-Chain Acyl-CoA dehydrogenase; VLCAD, very long-Chain Acyl-CoA dehydrogenase; LCHAD, Medium-Chain Acyl-CoA dehydrogenase; TFP, Trifunctional protein; MMA, methyl malonic aciduria; IVA, Isovaleric academia; GA-I, glutaric academia I; PKU, phenylketonuria; MSUD, maple syrup urine disease.

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