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Current Methods for Determination of *L*-Carnitine and Acylcarnitines

Monika Möder[1,*], **Andreas Kießling**[2], and **Heinz Löster**[3]

[1] Centre for Environmental Research Leipzig-Halle, Department of Analytical Chemistry, D-04318 Leipzig, Germany
[2] Heinrich Braun Clinic of Orthopaedics, Zwickau, Germany
[3] Institute of Laboratory Medicine, Clinical Chemistry and Molecular Diagnostics, University of Leipzig, Leipzig, Germany

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Summary. *L*-Carnitine as endogenous compound plays an important role within several metabolic pathways and a deficiency of *L*-carnitine can cause adverse effects in physiological and/or mental state of health and disease. The prevention of diseases related to carnitine deficiency requires, first of all, the exact determination of *L*-carnitine and its esters in biological material at pmol/cm^3 level. A series of analytical procedures based on biochemical assays as well as on physical methods are available today. Determination of free and total carnitine is sometimes sufficient for a clinical diagnosis, but in most cases, such as in newborn screening for genetic disorders, detailed qualitative and quantitative *L*-carnitine/acylcarnitine profiling is needed. Technological progress has also revolutionized the determination of carnitines. Today, comprehensive and diagnostically relevant information can be obtained by mass spectrometry. An overview is given of the technical and methodological developments in carnitine analysis and some applications, such as in neonatal screening, diabetes mellitus, and cardiomyopathy.

Keywords. Acylcarnitines; Analytical methods; *L*-Carnitine; Newborn screening.

Introduction

L-Carnitine, (3-hydroxy-4-trimethylammonium butyrate), is an essential factor in the fatty acid metabolism of mammalian organisms.

In humans, about 25% of *L*-carnitine is synthesized in liver, kidney, and brain from the amino acids lysine and methionine. Most of the carnitine in the body comes from dietary sources such as red meat and dairy products [1]. Most *L*-carnitine is located in tissue that uses fatty acids as their primary dietary fuel. In skeletal and cardiac muscle the *L*-carnitine level is about 20- to 50-fold higher than

* Corresponding author. E-mail: monika.moeder@ufz.de

in plasma [2]. The total carnitine pool of a healthy 70 kg human amounts to about 130 mmol (21 g), of that more than 98% are found in skeletal muscle, 1–1.5% in liver, and less than 0.5% in blood. The mean plasma concentration of free carnitine in healthy adults is about 40–60 μmol/dm^3 and total carnitine amounts to about 50–70 μmol/dm^3.

Primary carnitine deficiencies such as systemic and myopathic defects are rare hereditary disorders [3]. Typical hereditary carnitine deficiencies are caused by genetic defects in amino acid degradation (propionic aciduria) and lipid metabolism (medium chain acyl-CoA dehydrogenase deficiency).

A systemic disorder can be detected in infancy or early childhood due to a low serum *L*-carnitine level. A mutation in the gene coding for carnitine-acylcarnitine translocase or the OCTN2 transporter aetiologically causes a carnitine deficiency that results in poor intestinal absorption of dietary *L*-carnitine, its impaired reabsorption by the kidney and, consequently, in increased urinary loss of *L*-carnitine [4]. If unrecognized and untreated, these defects may result in life-threatening damage to liver, heart, or brain. Myopathic carnitine deficiency is less severe but more difficult to detect because serum *L*-carnitine is at a normal level. Symptoms include muscle pain and progressive muscle weakness [4].

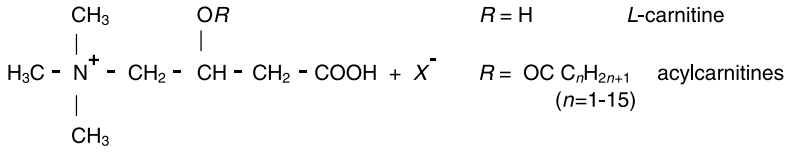
Secondary carnitine deficiencies are usually characterized by poor availability of free *L*-carnitine. Progressive loss of *L*-carnitine caused by hemodialysis or interaction with drugs (antiepileptic drugs containing valproate, the lipid regulator Lovastatin, antibiotics such as Ampicillin, or chemotherapeutics) may lead to carnitine deficiency, and in such cases supplementation with carnitine is beneficial [5–7]. During hemodialysis up to 70% of serum carnitine can be lost and a progressive decrease in carnitine is associated with long-term dialysis.

Diets that permanently lack *L*-carnitine may also increase the risk of secondary carnitine deficiency. In order to prevent carnitine deficiency in cases when infants are fed according to non-milk based formulas (*e.g.*, soy-based) over a prolonged period, the food has to be enriched with *L*-carnitine to achieve the level that is typically present in human milk (about 11 mg/dm^3).

Numerous disorders have been described that lead to disturbances in energy production and in intermediary metabolism in the organism [8, 9], which are characterized by the production and excretion of unusual acylcarnitines (“organoacidurias”) [10, 11].

Furthermore, carnitine facilitates the elimination of potentially toxic acyl compounds from the cells that may accumulate during certain pathological conditions. Consequently, the ratio of free *L*-carnitine and esterified carnitine is also a useful diagnostic indicator of the metabolic status. Therefore, determination of the qualitative pattern of acylcarnitines can be of diagnostic and therapeutic importance, but also the determination of free *L*-carnitine and total carnitine levels in various parts of an organism [12, 13].

The betaine structure of carnitine requires special analytical procedures for recording. The ionic nature of *L*-carnitine causes a high water solubility which decreases with increasing chain length of the ester group in the acylcarnitines. Therefore, the distribution of *L*-carnitine and acylcarnitines in various organs is defined by their function and their physico-chemical properties as well. The carnitine pattern and the concentration that has to be determined strongly depend on the matrix. Therefore, methods have been developed which make it possible



Scheme 1. Structures of *L*-carnitine and acylcarnitines

to determine carnitine in dried blood spots, plasma, dried urinary samples, crude urine, and tissue.

Analytical Methods of *L*-Carnitine and Acylcarnitine Determination

A broad spectrum of quite different analytical principles and methods are used for carnitine and acylcarnitine analysis. Methods that detect *L*-carnitine (free carnitine), and the sum of *L*-carnitine and acylcarnitines (total carnitine) are widely used in clinical laboratories. Current methods for the determination of free carnitine and its esters include spectrophotometric or radioenzymic assays in various modifications using [acetyl-1-^{14}C]CoA [14–19]. More sophisticated methods to determine *L*-carnitine and its individual carnitine esters employ chromatographic separation procedures, such as HPLC with UV-detection [20–24], GC-MS [25, 26], and capillary electrophoresis [27, 28], which are often connected with pre-derivatization. Nowadays, new developments in mass spectrometry allow a very fast and sensitive detection of carnitines, for instance in neonatal-screening programmes.

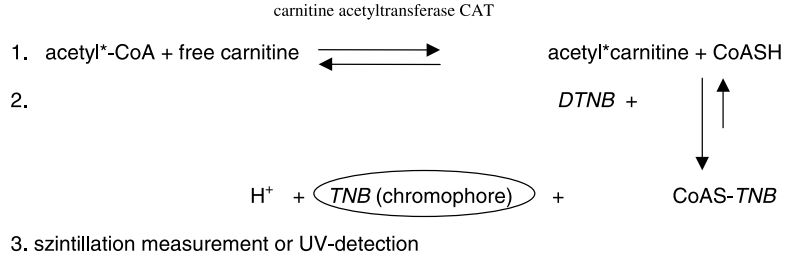
Enzymatic Assay

Quantitative carnitine analysis is commercially offered for urinary, plasma- and tissue-related samples. One standard procedure is based on assays that provide information on free and total carnitine contents.

In this procedure carnitine is assayed using carnitine acyltransferase, with and without alkaline hydrolysis of carnitine esters [19, 29]. The enzymatic method relies on the action of carnitine acetyltransferase which is catalyzed as shown in Scheme 2.

The enzymatic reaction (step 1 in Scheme 2) is a reversible process, therefore sulfhydryl-trapping reagents are added to prevent the reverse reaction and to stabilize the acetylcarnitine formed.

In step 2 (Scheme 2), *DTNB* (3,3′-dithiobis(6-nitrobenzoic acid) is used to produce the chromophore *TNB* (3-thio-6-nitrobenzoic acid), which is the actually



Scheme 2. Enzymic reaction of free carnitine. If the acetyl-CoA is radioactively labelled (acetyl* = acetyl-^{14}C) a particularly sensitive detection of the formed acetyl*carnitine is possible

detectable compound in this carnitine determination procedure. Using this trapping, a significant back reaction with the available CoASH is probable, and acylcarnitines can become substrates used by CAT to form free carnitine, which may lead to exaggerated free carnitine levels and prevent a correct diagnosis.

Because this reaction permits only the determination of free carnitine, acylcarnitines have to be hydrolyzed (30 min at 56°C, *pH* 11) before they can be detected together with *L*-carnitine as total carnitine content. The difference of total carnitine minus free carnitine is the acylcarnitine amount of the sample.

Some advanced enzymatic operating procedures have been proposed to overcome such process-related ambiguities [30].

One such method determines free carnitine by flow injection into a bioreactor device equipped with immobilized carnitine-acetyltransferase, which minimizes the consumption of the rather expensive acetyl-CoA. This method is based on the reaction shown in Scheme 2. Free carnitine is determined spectrophotometrically after its reaction with carnitine-acetyltransferase to acetyl-CoA and dithiobenzoate. The calibration curve used for quantification is linear over a concentration range from 10 to 80 mg/dm^3 [31]. For validation purposes, the method was compared with standard protocols, *e.g.*, with an enzymatic UV test by Boehringer-Mannheim, which uses a multi-stage enzyme cascade involving adenosine monophosphate [32]. The sensitivity and reproducibility of the protocol specified guarantees exact carnitine concentration measurements even at μmol level. This automated, rapid, and economic method permits a high sample throughput that is required in monitoring breast milk substitutes fortified with *L*-carnitine or other dietary products with carnitine supplementation.

Spectrophotometric interferences caused by UV-absorbing compounds that are not carnitine-related, or by sulfhydryl groups that react with *DTNB*, can result in high blanks and/or false positive results [33]. Such problems can be avoided if highly substance-specific methods such as chromatographic, or even mass spectrometric techniques are included in the analysis. Furthermore, determination of free and total carnitines is not precise enough for diagnosing special metabolic disorders characterized by accumulation and excretion of individual carnitine esters [34]. Chromatographic and/or mass spectrometric techniques have to be applied as will be discussed in the following paragraphs.

Chromatographic Methods

Prior chromatographic analysis *L*-carnitine and its esters require a derivatization to reduce their polar properties and to enable an appropriate chromatographic separation and detection.

Only few applications of gas chromatographic (GC) methods have been described so far [25, 26, 35]. The transformation of carnitines into thermally stable, volatile derivatives is fairly time-consuming. However, gas chromatography coupled with a very selective and sensitive detection method, chemical ionization mass spectrometry, can compensate for such disadvantages. The use of labelled reference carnitine esters as internal standards allows exact identification and quantification of carnitines, also with a complex matrix [35].

High performance liquid chromatography (HPLC) permits screening for free and total carnitine, as well as complete quantitative acylcarnitine determination,

including the long-chain acylcarnitine profile. Analysis is performed using pre-column chemical derivatization, subsequent HPLC with ultra-violet (UV) as well as fluorescence detection. Minimum sample amounts needed for this analysis method are about 150 mm^3 of plasma, 350 mm^3 of urine or 20 mg of tissue. Carnitine is derivatized into its UV-absorbing 4′-bromophenacyl ester. During HPLC analysis, carnitine derivatives are well separated from other derivatives formed. For some applications such as carnitine detection in serum, this reaction is not complete enough. However, by adding diisopropylethylamine, this derivatization was successfully established for carnitine determination in urine [36]. This method uses 4′-phenacyl bromide dissolved in acetonitrile with 40% aqueous tetrabutylammonium hydroxide as reagent. This carnitine pre-treatment produced satisfying results for serum samples as well. Two hours of heating at 60°C is a just acceptable sample preparation time for large sample series. Carnitine derivatives are UV-detected at 260 nm. Hydrolysis of acylcarnitines as observed in the enzymatic assays was avoided and the recovery of carnitine by this detection method was about 97% [37].

4′-Bromophenacyl trifluoromethylsulfonate is better suited for serum analysis because the derivatives are formed within a few minutes in the presence of magnesium oxide. However, this reagent is often impure and the derivatization yield is low [38–40]. Despite some remaining problems, HPLC with pre-derivatization and UV or fluorescence detection is a suitable and economic tool in carnitine analysis [41, 42]. Especially in the preparatory phase, HPLC methods are labour-intensive and information on unknown compounds is difficult to obtain. As far as the highest information potential is concerned, mass spectrometry belongs to the most powerful tools in carnitine analysis.

Mass Spectrometry in Carnitine Analysis

Since conventional MS techniques like GC-MS are of limited use due to the ionic nature and the lack of volatility of carnitine compounds, some alternative MS approaches were developed. Fast atom bombardment (FAB) MS [43–48], desorption chemical ionization (DCI) MS [46], atmospheric pressure ionization (API) MS [49, 50], and matrix-assisted laser desorption ionization (MALDI) MS [50] require a minimum of sample preparation. These methods provide information on acylcarnitine profiles as well as on concentrations of the individual compounds even without any pre-separation. Thermospray-ionization MS [51] in combination with HPLC using a resin-based stationary phase was shown to be suitable for the determination of quaternary ammonium drugs structurally related to carnitine. The reported sensitivity was similar to that of radioenzymatic assays. Nevertheless, interpretation of MS spectra of complex biological mixtures is sometimes difficult and suffers to a certain extent from matrix interference and signal suppression caused by accompanying substances. Examples for negative influence of biological matrices on the results of acylcarnitine electrospray ionization (ESI) MS analysis were reported by *Kelly et al*. [48]. Indeed, the selectivity and sensitivity of mass spectrometric analysis can be improved by MS–MS techniques, but exactly defined target analytes are required. This means, MS–MS techniques used for quantification entail certain knowledge about the analytes under study.

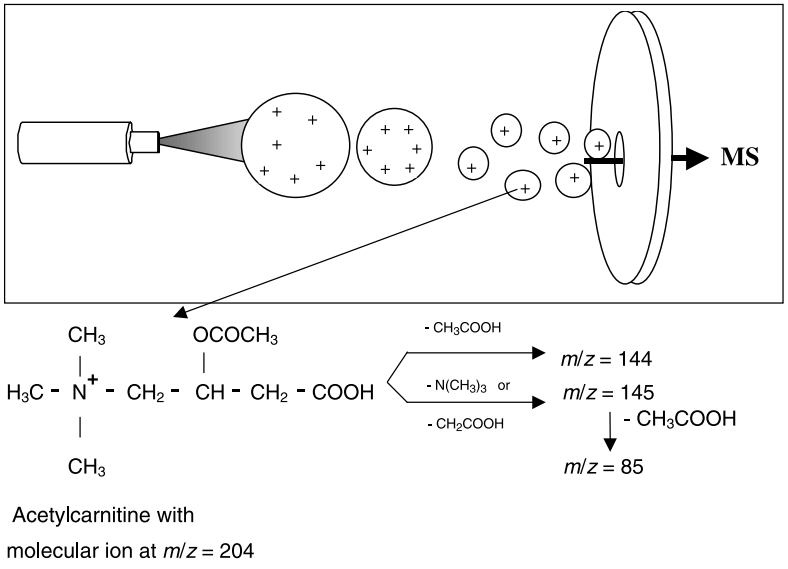


Fig. 1. Schematic electrospray ionization process and some characteristic fragments formed by collision induced dissociation of acetylcarnitines used as examples for their fragmentation

Electrospray ionization MS is the most suitable and applied mass spectrometric technique in carnitine analysis [52–57]. ESI-MS, an atmospheric pressure ionization method, is characterized by a high voltage-supported spray that permits the evaporization of solvent molecules from pre-formed or already present ions as in the case of carnitine. Because carnitines exist as zwitterions (positive and negative charges within the molecule) in solutions, their analysis in positive ESI mode is more sensitive if a small amount of acid is added to the spray eluent.

The principle of ion formation during electrospraying is shown in Fig. 1, as well as some typical mass spectrometric fragmentations of carnitines. During ionization the pre-formed positively charged ions in the spray lose their solvation sheath assisted by high voltage (4–5 kV). The reduction of the solvent molecules concentrates the ions at the outer shell of the droplet, which finally results in a *Coulomb* explosion of the highly charged microdroplet. The ions are released and accelerated into the high vacuum region of the mass spectrometer for mass analysis.

The fragmentations specified in Fig. 1 produce the target ions used for identification as well as for quantification of *L*-carnitine and acylcarnitines. In case of particular dysfunctions in metabolism or inherited carnitine deficiencies, individual acylcarnitine levels are increased in blood or urine. Their higher concentrations appear as conspicuous ions in the mass spectra of the acylcarnitine pattern as shown in Table 1.

The detection of these substance-characteristic ions allows a selective determination of carnitine even if a complex matrix is present. Component separation by chromatographic methods, which include time-consuming derivatization, is not stringently needed when ESI-MS in SIM mode is applied. Selected ion monitoring (SIM), which is also known as selected ion recording (SIR) or multiple ion recording (MIR), can already be performed with reasonably priced single quadrupole mass spectrometers [60]. Limits of detection range from 4–20 $pmol/cm^3$ for

Table 1. Target ions indicative for metabolic dysfunctions because of their elevated abundance in the mass spectrum (examples in Fig. 3 [58, 59])

Dysfunction	Elevated excretion of acylcarnitines	Indicator ions for detection in SIM (or MIR) mode	Indicator ions (as butylester for precursor ion scan analysis of $m/z = 85$)
Medium-chain acyl-CoA dehydrogenase deficiency	C6 carnitine	260	316
	C8 carnitine	288	344
	C10, C10:1 carnitines	316	372
Long-chain acyl-CoA dehydrogenase deficiency	C12 carnitine	344	400
	C14 carnitine	372	428
	C12:1 carnitine	342	398
	C14:1 carnitine	370	426
3-Hydroxy-3-methylglutaric aciduria (HMG-CoA Lyase deficiency)	3-hydroxyisovaleryl carnitine	318	402
Methylmalonic aciduria (Methylmalonyl-CoA mutase deficiency)	C3 carnitine, methylmalonylcarnitine	218	274
Propionic aciduria (Propionyl-CoA carboxylase deficiency)	C3 carnitine	218	274
Isovaleriane acidemia (Isovaleryl-CoA dehydrogenase deficiency)	isovalerylcarnitine	246	302

L-carnitine to hexadecanoylcarnitine. Improved sensitivity and selectivity can be reached with mass spectrometers designed as triple stage quadrupole, ion trap, or combinations of quadrupole and ion trap systems. All these MS configurations permit substance-specific MS–MS experiments [61, 62]. Remaining matrix-related influences are suppressed and the improved sensitivity guarantees low limits of detection between $0.02\,\mathrm{pmol/cm^3}$ (octanoylcarnitine) and $10.5\,\mathrm{pmol/dm^3}$ for free carnitine [56]. Nowadays, ESI-MS–MS devices belong to the standard equipment of clinical laboratories, especially for newborn screening and therapy monitoring.

MS–MS Techniques

In tandem mass spectrometry or MS–MS analysis the first mass analyzer is used to separate an ion (precursor) from the complete ion mixture produced during the ionization process (*e.g.*, ESI). The separated species are passed through a collision cell filled with argon gas. The ions decay under the conditions in the collision cell (collision induced dissociation), and the fragments produced can subsequently be analyzed for their mass weights by the second mass analyzer. The resulting mass spectrum contains all fragments of a pre-selected ion and provides important structural information (product ion scan). By selecting two compound-specific ions,

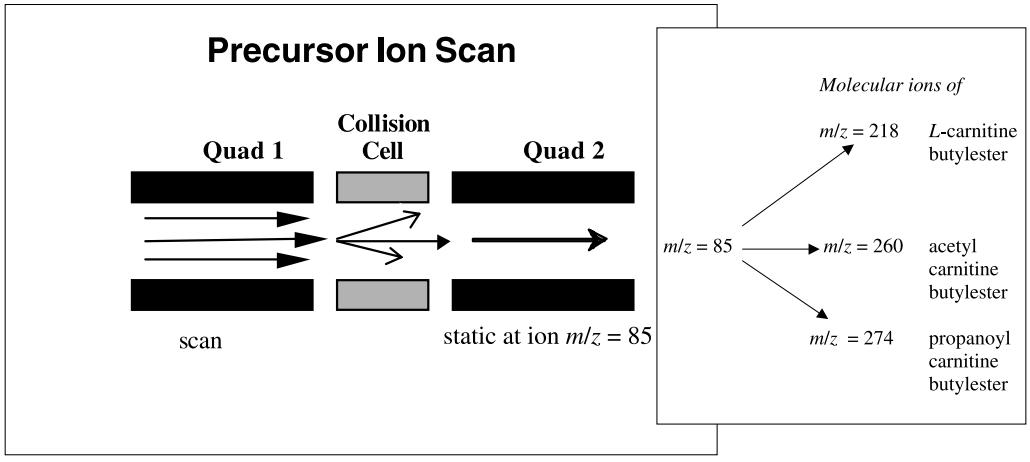
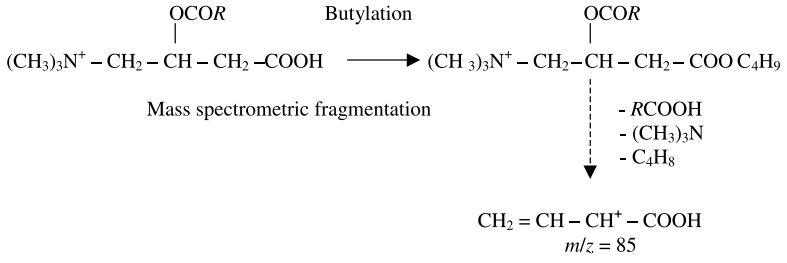


Fig. 2. Both quadrupol analyzers operate in linked scan mode to isolate the precursors of the preselected fragment ion $m/z = 85$; in the right picture the precursor scan mode of $m/z = 85$ is applied for the determination of carnitine and acylcarnitine butylesters



Scheme 3. Derivatization of the carnitines and the mass spectrometric fragmentation of the related butylesters which form an abundant ion at $m/z = 85$ under Collision Induced Dissociation (CID) [56, 57]

mostly the molecular ion ($m/z = 204$, Fig. 1) and a further characteristic fragment ion (*e.g.*, $m/z = 145$, Fig. 1), it is possible to use both ions for highly sensitive quantification of an individual compound by multiple reaction monitoring (MRM). Such single experiments need only microseconds so that the time-limiting step is the sample preparation or, if required, HPLC separation. The most widely used MS–MS mode in carnitine and acylcarnitine analysis is the precursor ion scan (Fig. 2). In order to enhance the sensitivity of the method, a butylation of the carnitines is recommended prior to MS–MS runs [63, 64]. The derivatization is described in Scheme 3. Butanolic HCl is added to the dried extract of a blood spot and heated for 15 min at 60°C. After evaporation to dryness, acetonitrile or methanol is added and the sample can be injected into the mass spectrometer (flow injection analysis). The sample introduction is carried out by a loop of 10 or 20 mm^3 without any HPLC separation. When analysis renounces HPLC separation then isomeric acylcarnitines such as *n*-butyryl and isobutyrylcarnitine can not be distinguished. However, in most cases isobutyrylcarnitine is more likely because it is accumulated as a decomposition product of the ascorbic acid-metabolism.

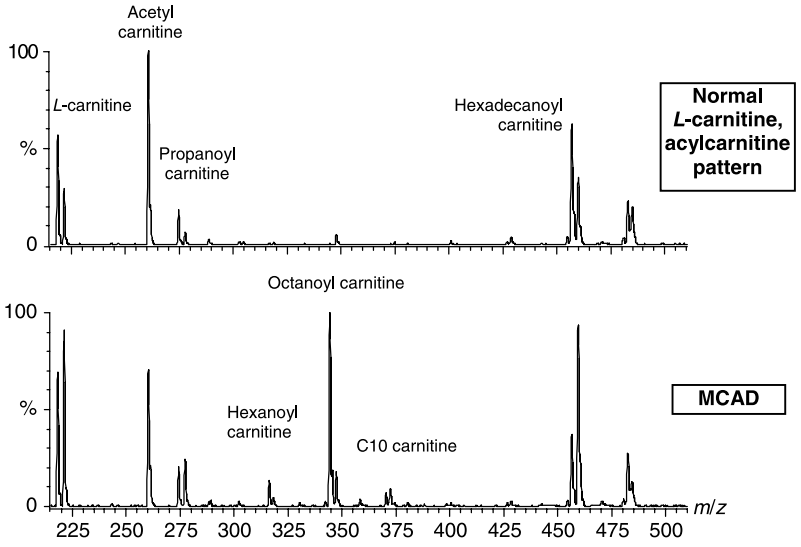


Fig. 3. Mass spectra of the carnitine pattern of a healthy person (upper spectrum) and of a patient with medium chain acylcarnitine deficiency (MCAD, lower spectrum) [57]; spectra also contain the signals of partially D-labelled internal standards needed for quantification [65]

Acylcarnitine profiling in plasma by ESI-MS–MS has become indispensable for the diagnosis of a series of fatty acid oxidation deficiencies and specific defects related to membrane transport processes.

The elevated amounts of particular acylcarnitines can be detected very quickly and with great reliability, thus therapy can be initiated or at least recommended immediately. The comparison of ESI-MS–MS spectra (precursor ion scan of $m/z = 85$) of a healthy patient and a patient who suffers from medium-chain acylcarnitine deficiency is shown in Fig. 3. Markedly elevated amounts of hexanoyl, octanoyl, decanoyl, and decenoylcarnitines indicate a MCA deficiency.

The technology and methodology for acylcarnitine profiling in blood spots by MS–MS are currently being scrutinized, and recommendations are made for utilization and standardization for national neonatal screening programs and other applications.

(SPME) ESI-MS

A special version of acylcarnitine determination is the combination of solid phase microextraction (SPME) and ESI-MS. If very low concentrations of acylcarnitines from plasma or urinary samples have to be analyzed, pre-concentration of carnitines is favourable. SPME is a solvent-free microextraction technique developed by *Pawliszyn et al.* [66] for the simple and fast enrichment of trace analytes from aqueous and gaseous samples. Due to the high water solubility of *L*-carnitine and short-chain acylcarnitines, their extraction yields obtained by SPME are fairly low, thus underestimating their concentrations is possible. However, detection of long-chain acylcarnitines (C10–C16) is improved by enrichment factors which are between 1.2 and 2. The advantages of SPME are easy handling as well as fast and solvent-free extraction and separation from accompanying biological

matter. Lipophilic acylcarnitines or other bioactive and diagnostically relevant compounds such as drugs can be favorably extracted and analyzed from complex samples [67].

Mass Spectrometry as a Diagnostic Tool in Clinical Chemistry

The following examples include only selected applications of mass spectrometry in clinical laboratories, especially with focus on L-carnitine and acylcarnitine determination. Currently, LC-MS–MS belongs to the most widespread analytical techniques in medical, pharmaceutical, or biochemical research and monitoring.

Newborn Screening

The first method used for a population screening for phenylketonuria (PKU) was a bacterial inhibition assay developed by *Guthrie* in the early 1960s. Phenylalanine was determined in blood spots of newborns because it was recognized that early reaction on PKU would result in a considerable decrease in morbidity of infants. Consequently, the detection of genetic disorders at an early, pre-symptomatic life stage can avoid disruption of health and growth.

Over the last years clinical laboratories that search for metabolic disorders have introduced tandem mass spectrometry into their neonatal screening programme. Mass spectrometry is rapidly replacing conventional assays. As multi-component methodology MS–MS is also more sensitive, specific, reliable, and comprehensive than traditional assays. Excellent reviews on the application of mass spectrometry in clinical diagnosis such as newborn screening have been given previously [68–71].

There is strong scientific support for MS–MS-based newborn screening for fatty acid oxidation disorders [68], but only very few states have established national screening programmes. For instance, only 13 US states mandate medium-chain acyl-CoA dehydrogenase (MCAD) screening by MS–MS, and only in 5 states a screening for very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) is required. These studies were able to identify MCAD deficiency in about 1 of 10000 newborn infants [72] and MS–MS screening has proved its value as an excellent method for the detection of MCAD [73–76].

Apart from acylcarnitine profiling, a series of other indicator compounds detected by tandem MS can be of assistance in detecting metabolic diseases. Elevated occurrence of individual amino acids such as phenylalanine are characteristic for PKU, tyrosine for tyrosinaemia, citrulline for a deficiency of argininosuccinate synthase (urea cycle defect), or fatty acids and acylcarnitine for MCAD deficiency [57, 69]. However, usually more than one criterion must be fulfilled for the final indication of a metabolic disorder. Normally, mass spectrometric results can be confirmed by additional biochemical methods based on mutational and enzymatic investigations [77].

Mass Spectrometric Investigations in Diabetes Mellitus and Cardiomyopathy Patients

Flow injection analysis electrospray ionization mass spectrometry was used to determine the typical urinary carnitine profile of diabetes mellitus patients. The typical

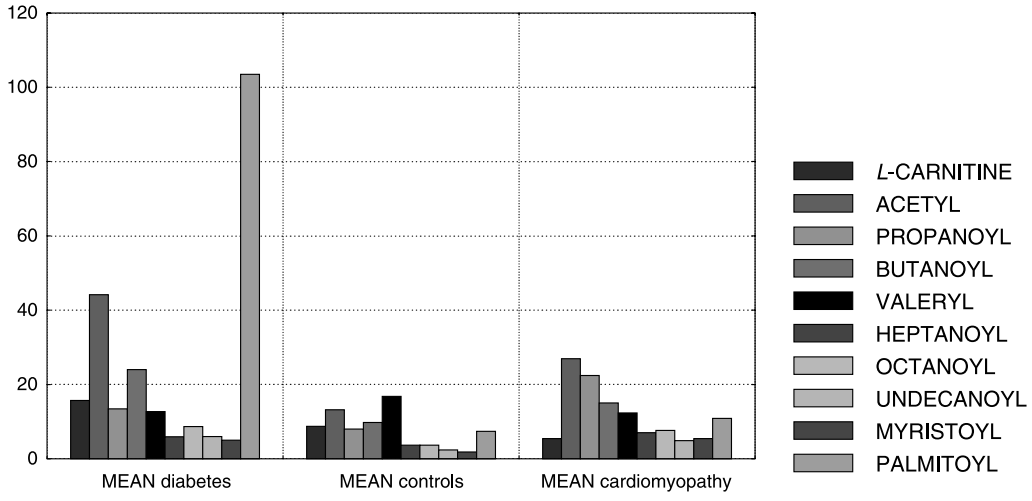


Fig. 4. Comparison of mean carnitine concentrations (ng/cm^3) of diabetes mellitus and cardiomyopathy patients as well as in healthy probands (controls)

acylcarnitine pattern of healthy probands showed a predominant role of *L*-carnitine and acetylcarnitines, whereas medium-chain and long-chain acylcarnitines occurred in lower concentrations in urine. Although the number of vegetarians in the control group was not representative (4 of 14), it appears that they have slightly elevated urinary levels of butanoyl, valeroyl, and palmitoylcarnitine [58, 60].

The corresponding acylcarnitine profiles of patients suffering from diabetes mellitus were characterized by increased concentrations of long-chain acylcarnitines and a decreased *L*-carnitine level.

Contrary to these findings, the urinary acylcarnitine pattern of cardiomyopathy patients is characterized by increased acetyl, propanoyl, and butanoylcarnitine excretion [58] (Fig. 4).

Cluster analyses were able to clearly distinguish all groups of probands from each other just by their typical urinary carnitine profiles. These results emphasize the enormous value and prospects of carnitine profiling by mass spectrometry. Carnitine analysis can support the diagnosis of various metabolic diseases in which fatty acid oxydation plays an essential role.

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