ANALYSIS OF CARNITINE AND ACYLCARNITINES IN BIOLOGICAL FLUIDS AND APPLICATION TO A CLINICAL STUDY

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Laurence Vernez
aus Villars-Bramard, VD

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auf Antrag von:

Prof. Dr. Stephan Krähenbühl

Prof. Dr. Gérard Hopfgartner

Prof. Dr. Wolfgang Thormann

Basel, den 6. April 2004

Prof. Dr. Marcel Tanner
Dekan

POUR TOI QUI M’ATTENDS ENCORE

Un savant dans son laboratoire n'est pas seulement un technicien:

c'est aussi un enfant placé en face de phénomènes naturels qui

l'impressionnent comme un conte de fées

Marie Curie-Sklodowska, 1933

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RÉSUMÉ

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SUMMARY

RÉSUMÉ

La carnitine, un composé endogène présent dans la plupart des tissus animaux, est essentielle pour le transport des acides gras activés entre les organelles cellulaires et joue donc un rôle important dans le métabolisme des acides gras et dans la production d'énergie des cellules. La carnitine lie les acides gras et génère ainsi différentes acylcarnitines de longueurs de chaîne variables. La carnitine est également impliquée dans le maintien du réservoir cellulaire de coenzyme A libre et dans l'élimination d'acyl-CoAs potentiellement toxiques. Chez les mammifères, la carnitine est fournie pour deux tiers par un apport alimentaire et pour un tiers par biosynthèse à partir des acides aminés L-lysine et L-méthionine. Comme la carnitine est présente dans la plupart des tissus à des concentrations bien supérieures à celle du plasma, sa distribution depuis ses sites d'absorption et de synthèse à travers l'organisme est assurée par des systèmes de transport. Dans de nombreuses maladies métaboliques, le métabolisme de la carnitine est fortement perturbé, aboutissant à une redistribution du réservoir de carnitine et d'acylcarnitines. La détermination individuelle de chaque acylcarnitine dans les fluides biologiques est un procédé approprié pour diagnostiquer ces maladies. Le but de cette thèse était de développer des méthodes analytiques pour la détermination de la carnitine et des acylcarnitines dans les fluides biologiques. Finalement, une des méthodes développées a été utilisée pour le suivi d'une étude clinique.

Les connaissances actuelles sur la carnitine et les acylcarnitines, notamment les fonctions de la carnitine, sa biosynthèse et son homéostasie, sont passées en revue dans le premier chapitre. Des cas de déficiences en carnitine sont également présentés et une description des différentes méthodes analytiques utilisées pour la détermination de la carnitine et des acylcarnitines complète cette partie introductive.

Le chapitre 2 décrit une méthode d'électrophorèse capillaire développée pour déterminer le profil de la carnitine et des acylcarnitines à courte et moyenne chaîne, ceci après une extraction en phase solide sur une colonne de silice. Cet essai permet la séparation de la carnitine et de cinq acylcarnitines dans des solutions de standard, dans l'urine et dans des échantillons d'urine enrichie. Il a été validé pour la détermination de la carnitine et de l'acétylcarnitine dans des solutions de standard. Finalement, la carnitine a été quantifiée dans des échantillons d'urine et les résultats ont été comparés avec ceux obtenus par un essai radio-enzymatique.

Le chapitre 3 présente une méthode utilisant la chromatographie liquide à haute performance couplée à une détection par spectrométrie de masse en mode MS/MS (HPLC-MS/MS) pour la détermination de la carnitine et de huit différentes acylcarnitines, incluant les acylcarnitines à longue chaîne. Comme la détection est effectuée par spectrométrie de masse, une dérivatisation de la carnitine n'est pas nécessaire. Avant d'être injectés dans le système, les échantillons ont été soumis à une extraction en phase solide sur une colonne échangeuse de cations. La séparation à été obtenue en utilisant un réactif volatil de paires d'ions. La validation de la méthode pour la détermination de la carnitine dans des solutions de standard et des échantillons d'urine a été effectuée en utilisant un dérivé isotopique stable comme standard interne et de l'eau comme matrice de calibration. Les résultats obtenus pour la quantification de la carnitine dans des échantillons d'urine ont été comparés avec ceux issus d'une méthode radio-enzymatique. L'application à des échantillons d'urine provenant de patients souffrant de différentes aciduries organiques à permis de confirmer le diagnostic de ces maladies.

L'extension de la méthode HPLC-MS/MS à des échantillons de plasma, après des modifications mineures du protocole d'extraction, notamment la précipitation des protéines, est rapportée dans le chapitre 4. La butyrobetaine (précurseur direct de la carnitine lors de sa biosynthèse) qui, au contraire de l'urine, est présente dans le plasma, a pu être déterminée avec la même méthode d'analyse. La quantification de la carnitine, de l'acétylcarnitine, de la propionylcarnitine, de l'isovalérylcarnitine, de l'hexanoylcarnitine, de l'octanoylcarnitine et de la butyrobetaine a été validée pour des solutions de standard et des échantillons de plasma en utilisant une solution aqueuse à 4% d'albumine sérique de bœuf comme matrice de calibration. Un échantillon de sérum d'un patient souffrant d'acidurie méthylmalonique a pu être identifié comme caractéristique de cette maladie.

Une application pratique de cette méthode HPLC-MS/MS est présentée dans le chapitre 5. Une étude clinique à été menée avec sept patients atteints d'insuffisance rénale terminale et suivant un traitement de dialyse. La carnitine est efficacement éliminée au cours de la dialyse, provoquant ainsi une diminution des taux plasmatiques de carnitine et une augmentation relative de ceux des acylcarnitines. Le but de l'étude était d'identifier la composition plasmatique en carnitine et en acylcarnitines chez ces patients, tant en conditions de base qu'après administration de carnitine à la fin de chaque dialyse. Les cinétiques d'extraction de la carnitine et des acylcarnitines pendant la dialyse et leurs cinétiques après une administration intraveineuse post-dialyse de carnitine ont été étudiées. Une comparaison à été établie entre les conditions de base (sans substitution) et lorsque les patients étaient substitués avec deux dosages différents de carnitine. L'administration de

carnitine a permis de corriger les faibles taux plasmatiques de carnitine et d'augmenter l'extraction des acylcarnitines, suggérant qu'une administration de carnitine aux patients sous dialyse pourrait être bénéfique pour l'élimination de groupements acyls potentiellement toxiques.

ZUSAMMENFASSUNG

Carnitin, eine endogene Substanz die sich in den meisten Geweben der Säugetiere befindet, ist essentiell für den Transport von aktivierten Fettsäuren zwischen Zellorganellen, und spielt daher eine wichtige Rolle im Fettsäuremetabolismus und in der Energieproduktion einer Zelle. Carnitin bindet Fettsaüren und bildet Acylcarnitine mit unterschiedlichen Kettenlängen. Weitere wichtige Funktionen von Carnitin sind das Puffern des zellulären freie Coenzym A-Pools sowie die Beseitigung von potentiell toxischen AcylCoAs. In Säugetieren wird der Carnitinbedarf zu zwei Dritteln durch die Nahrung und zu einem Drittel durch Biosynthese aus den Aminosäuren L-Lysin und L-Methionin gedeckt. Da der Carnitingehalt in den meisten Geweben viel grösser ist als im Plasma, sichern Transportsysteme dessen Verteilung vom Absorptions- oder Syntheseort in den gesamten Körper. Bei verschiedenen metabolischen Störungen ist das Carnitinmetabolismus stark verändert, so dass der Carnitin- und Acylcarnitinpool verschoben ist. Die Bestimmung von einzelnen Acylcarnitinen in biologischen Flüssigkeiten ist ein wirksames diagnostisches Mittel zur Erkennung dieser Störungen. Ziel dieser Dissertationarbeit war es, analytische Methoden für die Bestimmung von Carnitin und Acylcarnitinen in verschiedenen biologischen Flüssigkeiten zu entwickeln. Als „follow up“ wurde einer der entwickelten Assays in einer klinischen Studie angewendet.

Das erste Kapitel bietet einen Überblick des gegenwärtigen Wissens über Carnitin und Acylcarnitin, inklusiv Carnitinfunktionen, -biosynthese und -homöostase. Fälle von Carnitinmangel werden besprochen und eine Beschreibung der verschiedenen zur Verfügung stehenden Analysemethoden für die Bestimmung von Carnitin und Acylcarnitinen ergänzt diese Einführung.

Kapitel 2 beschreibt die Entwicklung einer Methode zur Profilierung von Carnitin und kurz- sowie mittelkettigen Acylcarnitinen mittels Kapillarelektrophorese im Anschluss an eine Festphasenextraktion mittels einer Kieselguhrsäule. Diese Methode ermöglicht die Trennung von Carnitin und von fünf Acylcarnitinen in Standardlösungen, in Urin sowie in gespiktem Urin. Sie wurde für die Bestimmung von Carnitin und Acetylcarnitin in Standardlösungen charakterisiert. Carnitin wurde in Urinproben quantifiziert und die Resultate mit Konzentrationen verglichen, die durch den radioenzymatischen Assay erhalten wurden.

Im Kapitel 3 wird eine kombinierte Methode aus Hochleistungs-Flüssigchromatographie zur Trennung und aus Massenspektrometrie im MS/MS Modus zur anschliessenden Detektion (HPLC-MS/MS) für die Bestimmung von Carnitin und acht Acylcarnitinen, inklusiv langkettige Acylcarnitine, vorgestellt. Da die Detektion mittels Massenspektrometrie verwendet wird, ist

eine Derivatisierung des Carnitins nicht nötig. Die Proben wurden vor Injektion ins System zur verbesserten Festphasenextraktion auf einer Kationentauschersäule vorgelegt. Die Trennung wurde mittels eines Ionenpaarreagens ermöglicht. Die Validierung der Carnitinbestimmung in Standard- und Urinproben wurde mit einem stabilen Isotopenderivat als interner Standard und mit Wasser als Kalibrationsmatrix durchgeführt. Diese Resultate der Carnitinbestimmung in Urinproben wurden mit denen des radioenzymatischen Assay verglichen. Die Anwendung auf Urinproben von Patienten mit verschiedenen organischen Acidurien ermöglichte die Bestätigung der entsprechenden metabolischen Störungen.

Die Erweiterung dieser HPLC-MS/MS Methode auf Plasmaproben, nach kleinen Modifikationen im Extraktionsprotokoll, inklusiv Proteinfällung, wird im Kapitel 4 beschrieben. Butyrobetain, der direkte Carinitinvorläufer in der Biosynthese, der in Gegensatz zum Urin im Plasma vorkommt, konnte mit der gleichen Analysenmethode bestimmt werden. Die Quantifizierung von Carnitin, Acetylcarnitin, Propionylcarnitin, Isovalerylcarnitin, Hexanoylcarnitin, Octanoylcarnitin und Butyrobetain wurde in Standard- und Plasmaproben mittels einer wässerigen Lösung mit 4% bovinem Serumalbumin als Kalibrationmatrix validiert. Serum eines Patienten mit Methylmaloinsäure Acidurie wurde erfolgreich als spezifisch für diese Störung identifiziert.

Ein Beispiel für eine konkrete Anwendung der entwickelten HPLC-MS/MS Methode wird im Kapitel 5 dargestellt. Eine klinische Studie wurde mit sieben in der terminalen Phase einer renalen Krankheit leidenden und unter Hämodialyse stehenden Patienten durchgeführt. Carnitin wird während der Dialyse effizient entfernt, was zu verminderten Carnitinspiegeln und zu einer relativen Erhöhung der Acylcarnitine führt. Daher war das Ziel dieser Studie eine Untersuchung der Carnitin- und Acylcarnitinenplasmazusammensetzung bei diesen Patienten, jeweils unter Basisbedingungen sowie nach Carnitinverabreichung nach jeder Dialyse. Die Extraktionskinetik während der Dialyse und die Kinetik der intravenösen Carnitingabe nach einer Dialyse wurden studiert. Ein Vergleich der erhaltenen Spiegel wurde durchgeführt, wenn Daten ohne Carnitingabe und nach Verabreichung zweier unterschiedlicher Carnitindosierungen vorhanden waren. Es konnte gezeigt werden, dass durch die Carnitingabe verminderte Carnitinspiegel deutlich verbessert und die Extraktion von Acylcarnitinen erhöht werden konnten. Dieser Befund weist darauf hin, dass bei unter Dialyse stehenden Patienten Carnitinersatz für die Entfernung von potentiellen toxischen Acylgruppen nützlich sein könnte.

SUMMARY

Carnitine, an endogenous compound present in most mammalian tissues, is involved in the transport of activated fatty acids between cellular organelles and thus plays an important role in fatty acid metabolism and in cellular energy production. Carnitine binds fatty acids, generating various acylcarnitines with different chain lengths. Carnitine is also implicated in the maintenance of the cellular pool of free coenzyme A and in the elimination of potentially toxic acyl-CoA. In mammals, carnitine is provided for two thirds by dietary intake and for one third by biosynthesis from the amino acids L-lysine and L-methionine. Since carnitine is present in most body tissues at much higher concentrations than in plasma, transport systems ensure it's widespread distribution from sites of absorption and synthesis throughout the body. In many metabolic disorders, carnitine metabolism is greatly disturbed, leading to a redistribution of the carnitine and acylcarnitine pools. The determination of individual acylcarnitines in biological fluids is a powerful means to diagnose these disorders. It was the aim of this thesis work to develop analytical tools for the determination of carnitine and acylcarnitines in biological fluids. Finally, one developed assay was utilized for the follow up of a clinical study.

In chapter 1, the current knowledge about carnitine and acylcarnitines, including carnitine function, biosynthesis and homeostasis, are reviewed. Cases of carnitine deficiencies are discussed, and a description of the different available analytical methods used for carnitine and acylcarnitine determination completes this introduction part.

Chapter 2 describes a capillary electrophoresis method developed to profile carnitine, short- and medium-chain acylcarnitines, after a solid-phase extraction on a silica column. The assay enabled the separation of carnitine and five acylcarnitines in standard solutions, in urine and in spiked urines, and was characterized for carnitine and acetylcarnitine in standard solutions. Carnitine was quantified in urine samples and the results were compared with concentrations obtained using a radio-enzymatic assay.

Chapter 3 presents a high-performance liquid chromatography assay coupled with tandem mass spectrometry detection (HPLC-MS/MS) for the detection of carnitine and eight different acylcarnitines, including long-chain acylcarnitines. Samples were submitted to a solid-phase extraction on a cation-exchange column prior to injection in the system. Since the detection is performed with mass spectrometry, a derivatization of carnitine is not necessary. The separation was achieved using a volatile ion-pair reagent. The validation for the determination of carnitine in both standard and urine samples was performed using a stable

isotope derivative as the internal standard and water as a calibration matrix. The results obtained for the quantification of carnitine in urine samples were compared with those of a radio-enzymatic method. Application to urine samples from patients suffering from different organic acidurias enabled the diagnosis of these metabolic disorders.

The extension of the HPLC-MS/MS assay to plasma samples, after minor modifications in the extraction protocol, including protein precipitation, is reported in chapter 4. Butyrobetaine, the direct carnitine biosynthesis precursor is present in plasma, in contrast to urine, and could be analyzed during the same analysis. Quantification of carnitine, acetylcarnitine, propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine, octanoylcarnitine and butyrobetaine were validated for standard solutions and plasma samples using 4% bovine serum albumin solution in water as the calibration matrix. Serum from a patient suffering from methylmalonic aciduria was successfully identified as characteristic of this disorder.

The concrete use of the developed HPLC-MS/MS method is illustrated in chapter 5. A clinical study was conducted with 7 patients suffering from end-stage renal disease undergoing long-term hemodialysis. As carnitine is efficiently removed during the hemodialysis session, leading to reduced carnitine levels with a relative increase of acylcarnitines, the aim of the study was to investigate the composition of the plasma carnitine and acylcarnitines pools in these patients, in baseline conditions and after they were supplemented with carnitine at the end of each hemodialysis session. Extraction kinetics during a hemodialysis session and kinetics of intravenous administration of carnitine after a hemodialysis session were studied. A comparison was established when patients were given either no supplement or one of two different dosages of carnitine. Carnitine supplementation corrected the hypocarnitinemia and yielded an increased extraction of acylcarnitines, suggesting that carnitine substitution in hemodialysis patients could be useful for the removal of potentially toxic acyl-groups.

List of abbreviations

ADAM	9-anthryldiazomethane
BBH	Butyrobetaine hydroxylase
CACT	Carnitine-acylcarnitine translocase
CAT	Carnitine acetyltranferase
CE	Capillary electrophoresis
CoA	Coenzyme A
CoASH	Free coenzyme A
COT	Carnitine octanoyltransferase
CPT I	Carnitine palmitoyltransferase I
CPT II	Carnitine palmitoyltransferase II
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
ESRD	End-stage renal disease
ESI	Electrospray ionization
FAB	Fast atom bombardment
(+)-FLEC	(+)-[1-(9-fluorenyl)-ethyl]-chloroformate
FMOC	9-fluorenyl-methylchloroformate
GC	Gas chromatography
HPLC	High-performance liquid chromatography
IVA	Isovaleric acidemia
LOD	Limit of detection
LOQ	Limit of quantification
MCAD	Medium-chain acylcarnitine dehydrogenase
MCD	Muscle carnitine deficiency
MMA	Methylmalonic aciduria
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
PCS	Palmitoyl-CoA synthetase
REA	Radioenzymatic assay
RSD	Relative standard deviation
SCD	Systemic carnitine deficiency
SDS	Sodium dodecyl sulfate
SPE	Solid-phase extraction
TMAO	Trimethylamine oxide
TML	Trimethyllysine

CHAPTER

1

INTRODUCTION

1.1 GENERAL ASPECTS

Carnitine, or 3-hydroxy-4-N-trimethylammino butyrate, is a ubiquitous molecule within mammalian tissue, which was first discovered in skeletal muscle extracts in the early twentieth century (Gulewitsch and Krimberg, 1905). Its chemical structure $C_7H_{15}NO_3$ was established in 1927 (Tomita and Sendju, 1927). Carnitine is a small (molecular weight = 161.2), water soluble, quaternary nitrogen-containing compound (Figure 1) that is present in both L- and D- forms, L-carnitine being the biologically active form. In the early 1950's carnitine was shown to be a growth factor for the mealworm *Tenebrio molitor* and the term vitamin B_T was assigned to carnitine (Carter et al., 1952).

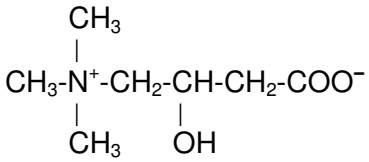


Figure 1: The structural formula of carnitine

Mammalians are able to synthesize carnitine from the amino acids L-methionine and L-lysine, but approximately two thirds of the human daily requirements come from an omnivorous diet. The main dietary source of carnitine is from eating fish and meat, particularly red meat, and to a lesser extent from dairy products (Rebouche and Engel, 1984). Vegetarian diets, although low in carnitine and associated with the lower plasma carnitine contents seen in strict vegetarians and lactoovovegetarians, are not a cause of carnitine deficiency as the carnitine concentrations remain within normal limits (Lombard et al., 1989).

Carnitine is most likely found in all animal species, in numerous organisms and plants (Fraenkel, 1954; Panter and Mudd, 1969; Kleber, 1997), in concentrations that vary to a great extent from one tissue to another one and from one species to another species. In man, a 70 kg healthy male's total carnitine body content is estimated to be around 21 grams (Brass, 1995). Carnitine distribution within the body reflects the needs of each organ towards this compound. Skeletal muscle and heart, that are consumers of carnitine, contain more

than 95% of the body total carnitine pool and carnitine reaches concentrations of 1570-3010 nmol/g tissue and 420-840 nmol/g tissue, respectively. Liver, the main site of carnitine synthesis, with levels between 570 to 1090 nmol/g tissue, is the next highest carnitine containing tissue of the body. The extracellular fluids, including plasma with carnitine levels between 36 and 56 µmol/L, contain only about 0.6 % of the total carnitine pool (Engel and Rebouche, 1984; Hoppel, 1991).

Carnitine is present as free carnitine and as esterified derivatives, or acylcarnitines, which are products of reactions catalyzed by carnitine acyltransferases that utilize acyl-CoA (Bremer, 1983; Sandor et al., 1987). Free carnitine is the major carnitine pool representative. The proportion of acylcarnitines, with acyl moiety ranging from the short-chain acetyl to the long-chain stearoyl (Choi et al., 1977), varies with nutritional conditions, exercise, and diseases states. Under normal metabolic conditions, acylcarnitines, which are mainly represented by acetylcarnitine, represent a mean value of 22% of the total carnitine pool in serum or plasma, 13% in muscle and liver and up to 60% in urine (Choi et al., 1977; Valkner and Bieber, 1982; Engel and Rebouche, 1984). Reported plasma concentrations in healthy humans are 29-50 µmol/L for free carnitine, 2.5-8.6 µmol/L for acetylcarnitine, 0.18-0.6 µmol/L for propionylcarnitine, 0.03-0.17 µmol/L for isovalerylcarnitine, 0.02-0.05 µmol/L for hexanoylcarnitine, 0.01-0.13 µmol/L for octanoylcarnitine and 2.2-4.9 µmol/L for the long-chain acylcarnitines (Hoppel, 1991; Minkler and Hoppel, 1993a; Costa et al., 1997; Inoue et al., 1999). The relative amounts of acylcarnitine are often expressed as a ratio of acylcarnitine to free carnitine. A ratio in plasma greater than 0.4 is indicative of carnitine insufficiency (Ahmad, 2001).

Some bacteria, like *Pseudomonas aeruginosa* and *Escherichia coli*, use carnitine under aerobic conditions as a unique carbon and nitrogen source or, under anaerobic conditions, metabolize carnitine to betaines with osmoprotectant properties (Larsen et al., 1987; Lucchesi et al., 1995; Kleber, 1997; Rebouche and Seim, 1998).

Carnitine has several direct and indirect roles in mammals and these essential functions are reflected by the mechanisms that have evolved to maintain a stable cellular/sub-cellular carnitine level: modest rate of endogenous synthesis, absorption from dietary sources, efficient conservation by the kidney and mechanisms present in most tissues that establish and maintain substantial concentration gradients between intracellular and extracellular carnitine pools (Rebouche and Seim, 1998).

Beside its main functions, described below, carnitine is also involved in membrane stabilization (Fritz and Arrigoni-Martelli, 1993), in the phospholipide metabolism in erythrocytes plasma membranes (Arduini et al., 1992) and may acts as an osmolyte (Peluso et al., 2000).

1.2 CARNITINE FUNCTIONS

In 1955, Friedman and Frankel showed that carnitine can be reversibly acetylated by acetyl-coenzyme A (acetyl-CoA) (Friedman and Fraenkel, 1955). In the same year, Fritz showed that carnitine stimulates fatty acid oxidation in liver homogenates, a fundamental source of cellular energy, particularly in cardiac and skeletal muscles (Fritz, 1955). These seminal studies led to the discovery that carnitine is implicated in the transport of activated long-chain fatty acids from the cytosol, across the mitochondrial membranes, towards the mitochondrial matrix, where β-oxidation takes place.

1.2.1 Mitochondrial long-chain fatty acid oxidation

Fatty acid metabolism occurs in the mitochondrial matrix, however, the mitochondrial inner membrane is impermeable to fatty acids. Carnitine, by binding fatty acids, is essential towards the transport of long-chain fatty acids into the mitochondrial matrix. This process involving several enzymes is depicted in Figure 2.
Cytosolic long-chain fatty acids are first activated by the palmitoyl-CoA synthetase (PCS), located in the outer leaflet of the outer mitochondrial membrane, to coenzyme A-derivatives (acyl-CoA). Acyl-CoA can cross the outer mitochondrial membrane but need to be converted to carnitine derivatives to be able to pass the inner mitochondrial membrane. For this purpose, the enzyme carnitine palmitoyltransferase I (CPT) I, located in the inner side of the outer mitochondrial membrane, transesterifies the acyl-CoA to the respective acylcarnitines (McGarry and Brown, 1997). In this reaction, which is the rate-limiting step in the β-oxidation of fatty acids and is inhibited by malonyl-CoA (Drynan et al., 1996), the acyl moiety of the long-chain fatty acids is transferred from CoA to the hydroxyl group of carnitine. The long-chain acylcarnitines are then transported into the mitochondrial matrix by a specific transporter, the carnitine-acylcarnitine translocase (CACT) (Pande, 1975), located in the inner mitochondrial membrane, which exchanges one long-chain acylcarnitine for one

carnitine (Pande and Parvin, 1980). Once in the mitochondrial matrix, the enzyme carnitine palmitoyltransferase II (CPT II), located in the inner leaflet of the inner mitochondrial matrix, back convert the long-chain acylcarnitines into the respective long-chain acyl-CoAs. The released acyl-CoAs can undergo β-oxidation and enter the respiratory chain within the matrix. The released carnitine can leave the mitochondrion via the above-mentioned translocase or be converted to acylcarnitines.

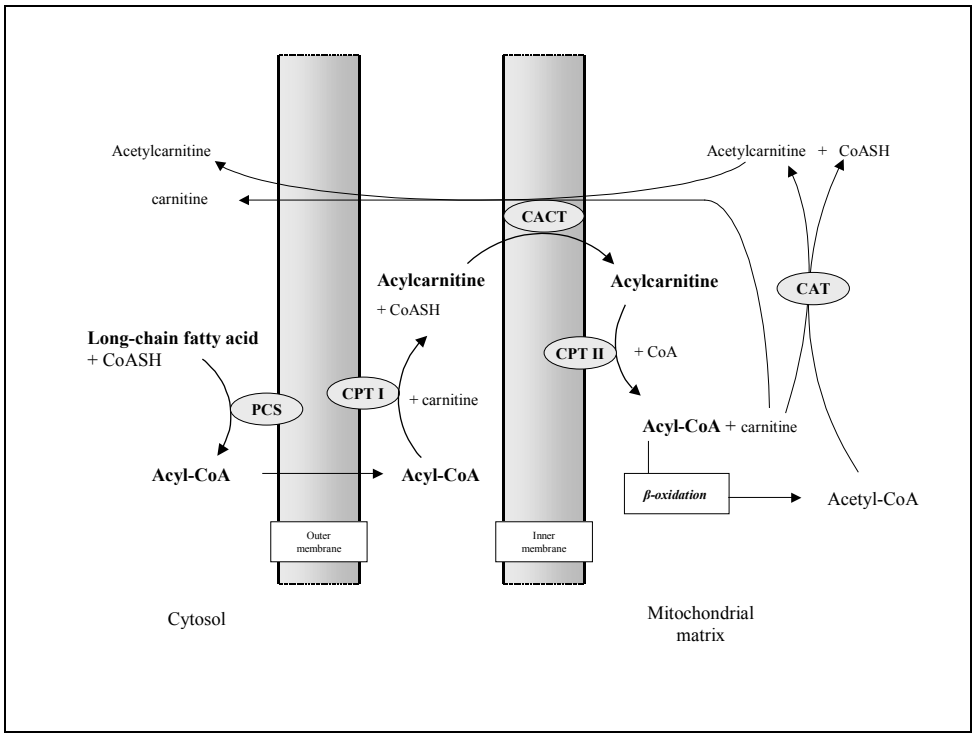


Figure 2: Schematic representation of mitochondrial long-chain fatty acid metabolism and regulation of intramitochondrial acyl-CoA/CoA ratio

Carnitine is the substrate for reactions catalyzed by carnitine acyltransferases that convert acyl-CoA to the corresponding acylcarnitine, according to the following reversible reaction:

$$\text{Acyl-CoA + carnitine} \leftrightarrow \text{Acylcarnitine + CoASH}$$

Three different groups of transferases distinguished by their substrate specificity, their cellular localization, their structure and reactivity with inhibitors have been described (Solberg, 1974; Bremer, 1983). Carnitine parmitoyltransferases (CPT I and CPT II) use long-chain acyl-groups as substrates and are found in mitochondria (Miyazawa et al., 1983). Carnitine octanoyltransferase (COT) utilizes medium-chain acyl moiety as substrate and is found in peroxisomes and microsomes (Miyazawa et al., 1983). Carnitine acetyltransferase

(CAT) uses short-chain acyl-groups as a substrate and its localization has been confirmed in mitochondria, peroxisomes and microsomes (Edwards et al., 1974; Kahonen, 1976). Through the action of acyltransferases, carnitine and acylcarnitines are rapidly interconvertible (Brass and Hoppel, 1980; Bremer, 1983).

1.2.2 Buffering of the mitochondrial acyl-CoA/CoA ratio

In the mitochondrial matrix, the enzyme CAT converts short-chain acyl-CoAs into the corresponding acylcarnitines, using mitochondrial carnitine and liberates free CoA (CoASH) (see Figure 2) (Brass and Hoppel, 1980; Bieber, 1988). Experiments conducted with blowfly flight muscle (Childress et al., 1967), rat heart (Pearson and Tubbs, 1967), sheep liver (Snoswell and Henderson, 1970), frog muscle (Alkonyi et al., 1975) and human skeletal muscle (Friolet et al., 1994) suggested that carnitine acts as a buffer for free CoA. Trough the action of CAT, carnitine and CoA pools are in close relationship. Brass and Hoppel showed that, in rat liver and under different metabolic conditions, the L-carnitine pool reflects the coenzyme A pool (Brass and Hoppel, 1980). This reversible exchange allows the cell to regulate its levels of free CoA using carnitine as a buffer, and, since carnitine is in most tissues in a much higher concentration than CoA, the extramitochondrial acetylcarnitine/carnitine ratio will prevent great fluctuations in the mitochondrial acetyl-CoA/CoA ratio by formation of acyl-CoA (Bremer, 1983; Ramsay and Arduini, 1993).

1.2.3 Removal of potentially toxic acyl-groups

Carnitine buffering effect can be extended to the regulation of poorly metabolized and potentially toxic acyl-groups, resulting either from xenobiotics (e.g. pivalic acid and valproate) (Arrigoni-Martelli and Caso, 2001) or from blockage of a normal metabolic pathway (e.g. propionic acid in propionic academia) (Rebouche and Seim, 1998). These acyl-groups are converted to CoA-derivatives, thus depleting the cellular pool of free CoA. The subsequent transesterification of these acyl-CoAs to the corresponding acylcarnitines, followed by their excretion in the urine (Melegh et al., 1987; Duran et al., 1990), replenish the free CoA pool, but lead to a carnitine deficiency that can be reversed through carnitine supplementation (Holme et al., 1992; Melegh et al., 1993).

1.2.4 Fatty acids oxidation in peroxisomes

Carnitine is involved in the transport of products resulting from the peroxisomal β-oxidation, from the peroxisome to the mitochondria. In contrast to the mitochondrial β-oxidation, the peroxisomal β-oxidation does not require carnitine since the peroxisomal membrane contains a direct carrier or a permease for acyl-CoA (Bremer, 1983), and is not as complete as it is in the mitochondria. Fatty acids, mainly very long-chain (C>22), are only degraded in shortened fatty acids, which then need to be transported into mitochondria for complete oxidation (Ishii et al., 1980; Osmundsen et al., 1991; Verhoeven et al., 1998). For this purpose, peroxisomes contain CAT and COT (but no CPT) which convert shortened-chain acyl-CoAs into shortened-chain acylcarnitines, which can be transported to mitochondria (Bieber et al., 1981).

1.3 CARNITINE BIOSYNTHESIS

Carnitine is synthesized from the amino acids L-methionine and L-lysine, after multiple steps involving four enzymes and several cofactors (see Figure 3). The entire biosynthetic pathway took a decade of research to be elucidated. In the early sixties it was shown that the injection of ^{14}C-butyrobetaine in rats resulted in apparition of radiolabeled carnitine in urine and tissues and that methyl-labeled methionine leads to the incorporation of radioactivity into the 4-N-methyl groups of carnitine (Wolf and Berger, 1961; Bremer, 1962). The biosynthetic origin of the carbon-chain and 4-nitrogen atom remained unclear for several years and, in 1971, it was shown by different investigators that lysine is the carbon chain and nitrogen atom donor in the mould *Neurospora crassa* (Horne et al., 1971; Horne and Broquist, 1973) and in rat (Tanphaichitr et al., 1971; Tanphaichitr and Broquist, 1973), with 6-N-trimethyllysine as intermediate.

In mammalians, free lysine is not methylated, unlike in *N. crassa*, but the three time methylation of lysine is a post-translational event that is catalyzed by a specific protein-dependant methyltransferase that uses S-adenosyl-L-methionine as methyl group donor (Paik and Kim, 1971; 1975). This results in the formation of a protein-linked 6-N-trimethyllysine (TML). TML is released from the protein-linkage by protein breakdown, which is considered to be the rate-limiting step in carnitine biosynthesis (Hoppel and Davis, 1986). As more than 65% of the whole-body protein-linked 6-N-trimethyllysine stock is located in skeletal muscle, skeletal muscle protein turnover is considered to be important for carnitine biosynthesis (Davis and Hoppel, 1983).

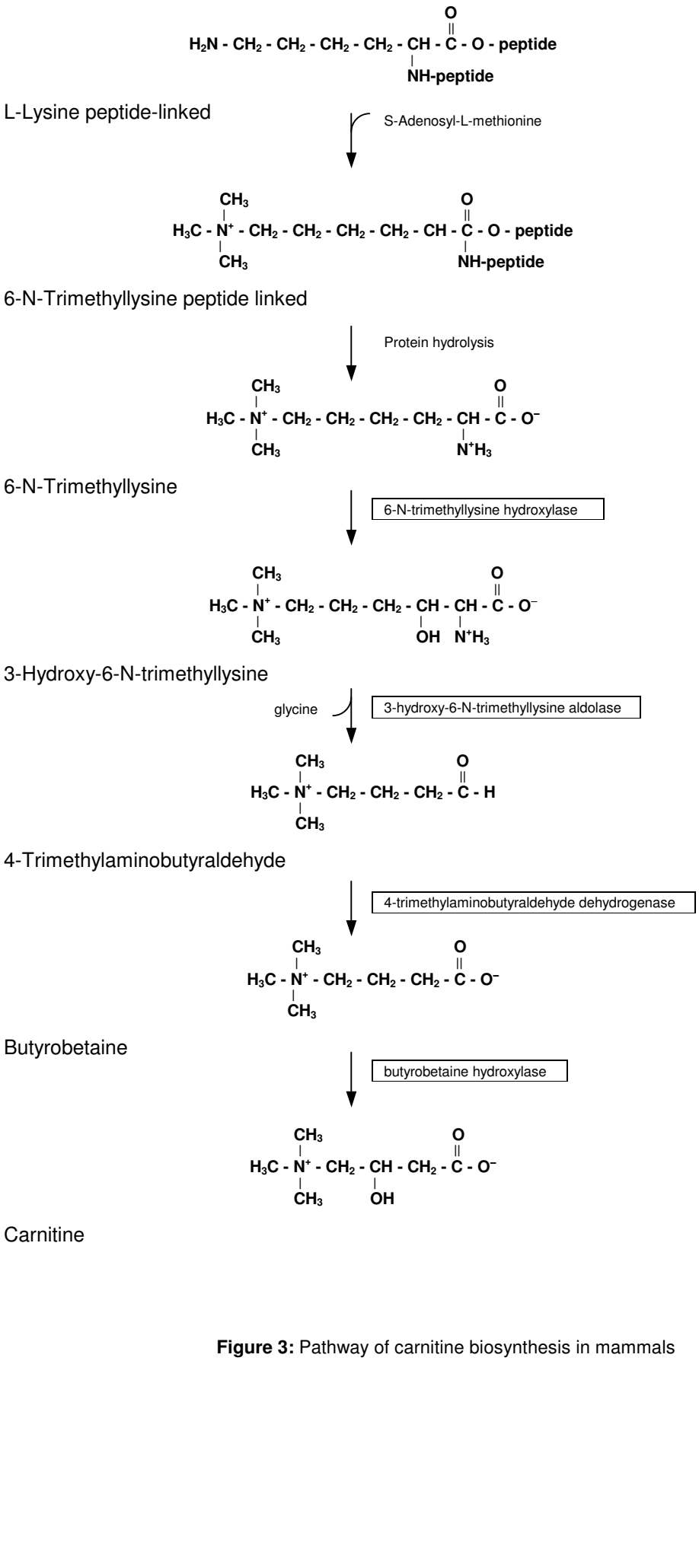


Figure 3: Pathway of carnitine biosynthesis in mammals

The next step is the hydroxylation of TML to 3-hydroxy-6-N-trimethyllysine. This convertion involves a 6-N-trimethyllysine hydroxylase, the only mitochondrial enzyme in the carnitine pathway, and requires α-ketoglutarate, ascorbate and Fe^{++} as cofactors (Hulse et al., 1978; Sachan and Hoppel, 1980). This enzyme is present in human especially in kidney, but also in liver, skeletal muscle, heart and brain, where its activity is about 4 times lower than in kidney (Rebouche and Engel, 1980; Stein and Englard, 1982).

The 3-hydroxy-6-N-trimethyllysine is cleaved by the 3-hydroxy-6-N-trimethyllysine aldolase in glycine and 4-trimethylaminobutyraldehyde. The enzyme needs pyroxidal phosphate as cofactor and its activity in human is the highest in liver (Rebouche and Engel, 1980).

Butyrobetaine results from the oxidation of 4-trimethylaminobutyraldehyde by help of the cytosolic enzyme 4-trimethylaminobutyraldehyde dehydrogenase with the transfer of a hydrogen ion to oxidized nicotinamide adenine dinucleotide (Hulse and Henderson, 1980). The activity of this enzyme in human tissues was found to be high in liver and kidney and very low in brain, heart and muscle (Rebouche and Engel, 1980).

According to the measured enzyme's activities in tissues, the biosynthesis steps from protein-linked lysine to butyrobetaine can be performed by most tissues (Rebouche, 1982), but skeletal muscle appears to be quantitatively the most important organ (Davis and Hoppel, 1983; Hoppel and Davis, 1986).

Finally, γ-butyrobetaine hydroxylase (BBH) catalyzes the stereospecific hydroxylation of butyrobetaine to L-carnitine (Englard et al., 1985). BBH is a cytosolic enzyme that requires α-ketoglutarate, ascorbate and Fe^{++} (Lindstedt and Lindstedt, 1970), and has a restricted and tissue specific expression. In humans, the enzyme is mainly located in liver and kidney, perhaps testis and also possibly brain (Rebouche and Engel, 1980). In rat and mouse, the BBH is only present in liver (Cox and Hoppel, 1974).

The availability of TML determines the rate of carnitine biosynthesis, which has been estimated to be about 1.2 µmol/kg body weight per day in humans (Rebouche, 1992). Carnitine biosynthesis accounts for one third to one half of the total carnitine sources when an omnivorous diet is consumed. If carnitine food intake is reduced, the biosynthesis of carnitine can account for more than 90% of the body requirements, so that a strict vegetarian diet is rarely a cause of carnitine deficiency presented in the clinic (Rebouche, 1992).

1.4 CARNITINE HOMEOSTASIS

Given the importance of carnitine for mammalian survival, its plasma and tissue levels need to be maintained within relatively narrow limits. Carnitine homeostasis is complex, reflecting its two sources of supply (diet and synthesis) and its wide distribution within the body.
The endogenous carnitine pool is maintained by a combination of absorption of carnitine from dietary sources, a modest rate of biosynthesis, an extensive but saturable tubular reabsorption from the glomerular filtrate and a distribution in most tissues ensured by transport and exchange systems that facilitate a high tissues to plasma concentration.

1.4.1 Absorption

In rats and humans, approximately 54-87% of dietary carnitine is absorbed (Rebouche and Chenard, 1991). Since carnitine is mainly present in animal products, carnitine's daily intake varies greatly with the dietary intake. By way of example, 2 to 12 µmol carnitine per kilogram of body weight are provided with an omnivorous diet and less than 0.1 µmol with a strict vegetarian one (Rebouche, 1992). By studying carnitine uptake into human intestinal mucosal biopsy specimens, Hamilton and colleagues observed that carnitine absorption results from a two-component system: a linear absorption, probably representing a passive diffusion, and a saturable system suggesting the existence of active transport (Hamilton et al., 1986). Rebouche and Chenard investigated the metabolic fate of dietary carnitine in humans by administrating an oral tracer dose of labeled carnitine and found that carnitine absorption is dependent on the intake amount (Rebouche and Chenard, 1991). Subjects on a low-carnitine diet presented a higher bioavailability than subjects under a high-carnitine diet. These data suggest that humans do not absorb all of the carnitine they consume and further support that a specific active transporter, one that can be saturated even with a normal dietary intake, might be involved. In a pharmacokinetic study conducted in healthy subjects with oral medicinal doses of 2 and 6 grams of carnitine, Harper et co-workers also found a dependency on intake amount in the bioavailability of carnitine (Harper et al., 1988). The bioavailability was 16% under the low dosage and 5% under the high dosage, which is considerably lower than the bioavailability of dietary carnitine. The authors did not find a statistically significant difference in the areas under the plasma carnitine concentration-time curves between the two dosages, and suggested that the absorption of carnitine was already saturated by the 2 grams dose. The significance of diffusion of carnitine as a mechanism of carnitine uptake is presumed to be small under physiological conditions, but may assume large importance during therapeutical administration of carnitine (Li et al., 1992).

Following oral administration of 2 or 6 grams carnitine in healthy humans, carnitine peak plasma concentrations were seen 2.5 to 9 hours after dosing (Harper et al., 1988). By studying the postabsorptive fate of injected radioactive-labeled carnitine in rat intestines, Gudjonsson and co-workers observed a slow appearance of carnitine in blood and suggested that carnitine enters an enterohepatic circulation with passive diffusion into bile (Gudjonsson et al., 1985). Uptake studies of carnitine by perfused rat liver showed that carnitine can be taken up by hepatocytes (Kispal et al., 1987). Bile from fed rats was shown to be highly esterified (up to 80%), one third of this as long-chain acylcarnitines, and the origin of these carnitine esters was associated with a hepatic carnitine metabolism (Hamilton and Hahn, 1987).

1.4.2 Tissue distribution - carnitine transporters

Carnitine is present in most parts of the body, mainly in skeletal muscle and heart. Since, as pointed out before, carnitine biosynthesis in human is restricted to liver and kidney, and two thirds of the carnitine needs are provided by diet, this implies the existence of a mechanism by which carnitine can be distributed from its place of synthesis or absorption to all tissues that rely upon carnitine for their energy supply. Given that carnitine concentration in tissues is 20 to 50 fold higher than in plasma, carnitine uptake from blood into tissues must take place via an active transport process against a concentration gradient.

Experiments of carnitine uptake by different tissues, either using cells or organelles, perfused tissues or directly in vivo, have shown the presence of sodium-dependant transporters with wide ranges of affinity according to the tissue studied. In liver, a low affinity transport for carnitine (high Km value) but a high affinity transport for butyrobetaine (the direct precursor to carnitine synthesis) was observed (Christiansen and Bremer, 1976; Kispal et al., 1987). This is in agreement with the central role of the liver to deliver carnitine to the body, while butyrobetaine enters easily to be converted into carnitine. Heart and skeletal muscles, that contain more than 95% of the carnitine pool and might be a storage site for carnitine, present both high and low affinity transport of carnitine (Martinuzzi et al., 1991; Georges et al., 2000). Kidney, which plays a key role in carnitine homeostasis, with its ability to reabsorb more than 90 % of carnitine, presents also a dual transport system of carnitine with a high affinity and a low affinity transporter (Stieger et al., 1995).

In recent studies, different carnitine transporters have been described. The first ones belong to the organic cation transporter family, that function primarily in the elimination of cationic

drugs and other xenobiotics, and include the high affinity transporter OCTN2, the low affinity transporter OCTN1 and the intermediate affinity transporter OCTN3 (Xuan et al., 2003). OCTN2 is strongly expressed in kidney and, to a lesser extent, in heart, placenta, intestine and liver, and also transports acetylcarnitine and propionylcarnitine. OCTN2 was also reported to be strongly expressed in skeletal muscle (Tamai et al., 1998), but experiments conducted in our laboratory demonstrated that it was absent in this tissue (Brooks, 2002). OCTN1 has a similar tissue expression profile as OCTN2 does, whereas studies conducted in mice revealed a predominant expression in testis for OCTN3 (Tamai et al., 2000), with a unique involvement in carnitine-dependent transport in peroxisomes shown in human (Lamhonwah et al., 2003). Another lower affinity transporter for carnitine and propionylcarnitine (but not for acetylcarnitine), $ATB^{0,+}$, is a member of the sodium- and chloride-coupled amino acid transporters family, and has been described to have a distinct tissue distribution, indeed a primary expression in intestinal tract, lung and mammary gland (Taylor, 2001).

i. Regulation of tissue distribution

Tissue distribution and uptake are at least partially controlled by hormones (Bremer, 1983). Glucagon and insulin deficiency were reported to reduce plasma carnitine in humans (Genuth and Hoppel, 1981). Sex hormones, and thus age, may also influence carnitine distribution.

Adult female rats have been reported to have lower plasma, heart and muscle carnitine concentrations and higher liver levels, thus a significantly higher liver/plasma carnitine concentration ratio than adults male rats (Borum, 1978). Nonetheless, the authors did not find these differences in rats younger than 50 days of life, suggesting an age-dependency for the observed tissue distribution. Plasma carnitine levels were showed to be lower in women than in men by about 20% to 25%, but carnitine levels of skeletal muscle presented no sex-differences (Cederblad, 1976; Opalka et al., 2001). A significant age-dependent decrease of carnitine in skeletal muscle was observed in men (but not in women) (Opalka et al., 2001). Opalka and colleagues found women increased their carnitine serum levels according to age, but not so in men, so that the differences between women and men seem to be minimal after menopause, further supporting the influences of sex hormones (Opalka et al., 2001).

ii. Kinetic of exogenous carnitine

Once carnitine reaches the systemic circulation, it is rapidly distributed into a central volume of distribution of approximately 0.2 L/kg, similar to extracellular fluid volume (Uematsu et al., 1988; Brass et al., 1994). Since carnitine removal from the central compartment occurs either

via irreversible elimination into the urine or via uptake by tissues, pharmacokinetic modeling using multi-compartmental behavior has been suggested to estimate the distribution of carnitine (Welling et al., 1979; Uematsu et al., 1988). For instance, Rebouche and Engel used a three-compartment open-system model consisting of extracellular fluid, cardiac and skeletal muscles and others tissues, particularly liver and kidney, to quantify the dynamic parameters of carnitine metabolism in the dog (Rebouche and Engel, 1983). This approach was judged to be applicable for the determination of carnitine fluxes into and out of muscle and other tissues, and the estimates of carnitine amount and turnover times obtained by this modeling agreed with experimentally measured values. The same model applied in healthy humans after intravenous administration of L-[methyl-^{3}H] carnitine, enabled the determination of turnover times for these different compartments (Rebouche and Engel, 1984). The turnover times were found to be 1.1 hour for extracellular fluids, representing the initial distribution volume, about 12 hours for the fast equilibrating tissues, more likely represented by kidney and liver, and 191 hours for the slow equilibrating tissues, that are likely represented by skeletal muscle and heart. The whole body turnover was found to be 66 days.

1.4.3 Metabolism

Carnitine has been shown to be extensively metabolized in microorganisms. Some bacterial species can degrade, under aerobic conditions, carnitine to dehydrocarnitine and finally to trimethylamine or, when carnitine is the unique source of carbon and nitrogen, to glycine (Rebouche and Seim, 1998). Under anaerobic conditions and in the presence of other carbon and nitrogen sources, enterobacteria, including *E. coli*, dehydrate carnitine to crotonobetaine that is further reduced to butyrobetaine (Seim et al., 1979).

Unlike microorganisms, mammals were shown to lack the enzymes to degrade carnitine (Rebouche et al., 1984; Seim et al., 1985). After oral administration of radioactive-labeled carnitine in rats, labeled trimethylamine N-oxide and butyrobetaine were found in urine and feces, respectively (Rebouche et al., 1984). These metabolites were not found after intravenous administration, nor in germ-free rats receiving oral carnitine (Seim et al., 1985), where all of the radioactivity was recovered in the form of carnitine. The authors concluded that carnitine degradation in the gastrointestinal tract of the rat is due to the indigenous flora. In similar experiments in which humans were administered orally a tracer dose of radioactive-labeled carnitine, the major metabolites were found to be labeled-trimethylamine oxide, eliminated by urine, and labeled-butyrobetaine, eliminated in feces (Rebouche and

Chenard, 1991). The non-absorbed carnitine was almost totally degraded and the formation of these metabolites in mammalians was attributed to the bacterial floral in the gastrointestinal tract.

Carnitine degradation in mammals is restricted to the non-absorbed carnitine in the intestinal tract, whereas absorbed or intravenously administered carnitine and endogenous carnitine are mostly eliminated in urine. After intravenous administration of 2 and 6 grams carnitine in healthy humans, 70 to 90% of the carnitine dose is recovered unchanged in the urine within 24 hours (Harper et al., 1988).

1.4.4 Elimination – role of kidney

By modulating the plasma carnitine concentration, the kidneys play a pivotal role in the establishment and maintenance of carnitine homeostasis in mammals. Carnitine, a water-soluble non protein-bound compound, is readily filtrated by the renal glomerulus and, to prevent excessive loss, is highly efficiently reabsorbed (Engel et al., 1981; Rebouche et al., 1993).

In healthy humans, 90 to 98 % of the approximately 5.8 mmol of the daily filtered carnitine is reabsorbed and only 100 to 300 µmol are excreted in urine (Maebashi et al., 1976; Leschke et al., 1984; Rebouche and Seim, 1998). Nonetheless, the rates of carnitine reabsorption and excretion are affected by several physiological and pathological factors. By way of example, excretion rate is increased by high-fat or high protein diets (Stadler et al., 1993), carnitine clearance is also increased during pregnancy (Cederblad et al., 1986) and reabsorption is impaired in Fanconi syndrome (Bernardini et al., 1985), a disease characterized by a generalized transport defect affecting tubular reabsorption of small molecules, including carnitine. At similar plasma carnitine concentrations, vegetarians excrete less carnitine than omnivores (Lombard et al., 1989). In a study where strict vegetarians were supplemented with carnitine, carnitine clearance was not affected but, at physiological plasma carnitine concentrations, carnitine reabsorption was significantly reduced and carnitine excretion increased. The authors concluded that the kidney adapts to dietary carnitine intake by reducing the efficiency of carnitine reabsorption (Rebouche et al., 1993).

Under normal homeostasis conditions, carnitine is eliminated by excretion in urine, in both free and esterified forms, mainly as acetylcarnitine (Chalmers et al., 1984). In urine, the acylcarnitines represent 56% of the total carnitine content, whereas in plasma, acylcarnitines

account for 22%. This higher acylcarnitine to carnitine ratio in urine in relation to plasma is suggested to be the result of a less efficient reabsorption of acylcarnitines or of a renal acylation of carnitine followed by leakage of the locally formed acylcarnitine product into urine (Wagner et al., 1986; Rebouche and Seim, 1998).

Carnitine is also excreted in milk. Its concentration in human milk was shown to increase from 39 to 63 µmol/L the first week post-partum and to be stabilized at 45 µmol/L after one month (Borum, 1981).

1.5 CARNITINE DEFICIENCY

Carnitine deficiency can be defined as a plasma or tissue carnitine concentration that is below the requirement for the normal function of the organism (Pons and De Vivo, 1995). Since carnitine needs depend on several other factors such as age, sex, diet, metabolic conditions and tissues dependency on fatty acid oxidation (Rebouche, 1992), the clinical consequence of hypocarnitinaemia is determined by the discrepancy between the carnitine level and the carnitine requirements. Clinical and biochemical data suggest that tissues carnitine content has to fall to less than 10-20% of normal before the biological effect can be clinically significant (Stanley, 1987), so that vegetarians, who have low plasma carnitine concentrations, do not present the symptoms of a carnitine deficiency (Lombard et al., 1989). Given that carnitine plays a key role, not only in fatty acid oxidation, and thus energy production, but also in cellular homeostasis of free and acyl-CoA, carnitine deficiency would be expected to produce a wide variety of disorders affecting the functions of several organs, including liver, skeletal muscle, heart and brain.

Since the first description of human muscle carnitine deficiency syndrome (Engel and Angelini, 1973), different forms of carnitine deficiency have been reported. According to their different etiologies, such as inherited disorders of metabolism or transport, contracted diseases (e.g. liver cirrhosis or HIV infection), therapeutic treatment for totally unrelated disorders (valproic acid treatment of epilepsy) or patient with end-stage renal disease (ESRD) undergoing hemodialysis, carnitine deficiencies are classified as primary and secondary carnitine deficiency. Carnitine deficiency syndromes are due to inherited and acquired diseases and carnitine deficiency is the consequence rather than the cause of the disease (Kerner and Hoppel, 1998).

The most severe clinical symptoms, which might be associated with carnitine deficiency, can often be reversed by an oral or intravenous supplementation with carnitine. Carnitine lacks toxicity at high dosage, is generally well tolerated and has limited and occasional adverse events, mostly gastro-intestinal, such as nausea, vomiting, gastritis and diarrhoea.

1.5.1 Primary carnitine deficiency

Primary carnitine deficiency is defined as a decrease in intracellular carnitine content, that is associated with impaired fatty acid oxidation, and with no other identifiable systemic illness that might deplete tissue carnitine stores (Roe and Coates, 1989). Depending on the tissue distribution of the low carnitine level, two different entities of primary carnitine deficiency syndromes have been described. A systemic form, characterized by low carnitine levels in plasma and in the affected tissue, and a muscular form, with a low carnitine concentration restricted to muscle (Engel and Angelini, 1973; Karpati et al., 1975).

i. Systemic carnitine deficiency (SCD)

Primary systemic carnitine deficiency is characterized by low plasma carnitine concentrations, low carnitine levels in the affected tissues (heart, skeletal muscle, liver), both usually below 10% of normal (Pons and De Vivo, 1995), and, as the main diagnostic factor, a higher than normal excretion of carnitine in the urine (Rodrigues Pereira et al., 1988). The defects in this disorder result from an impaired carnitine uptake into cells and are associated with a deficient renal carnitine transporter (Treem et al., 1988). Recently, point mutations in the gene encoding for the high affinity carnitine transporter OCTN2 have been identified in SCD patients (Nezu et al., 1999; Vaz et al., 1999), with a transmission displaying an autosomal recessive inheritance pattern (Tein et al., 1990; Lahjouji et al., 2001). The detection of SCD is done by the determination of carnitine uptake into cultured patient fibroblasts (Tein et al., 1990) and the diagnosis is conclusively made when the uptake is negligible.

SCD, which was first described in 1975 (Karpati et al., 1975), has an onset ranging from 1 month to 7 years, with a mean of 2 years, and has different types of clinical symptoms (Angelini et al., 1992). Progressive cardiomyopathy, the most common form of presentation, usually arises at an older age and the low carnitine levels in the heart leads to progressive congestive heart failure and death if carnitine substitution is not administered (Waber et al., 1982; Garavaglia et al., 1991; Stanley et al., 1991). Acute encephalopathy associated with hypoketotic hypoglycemia is more commonly seen in younger infants (Pons and De Vivo,

1995). Myopathy, manifested with hypotonia, mild motor delay or slowly progressive proximal weakness, is rarely present as an isolated form, but is commonly associated with cardiomyopathy or encephalopathy (Pons and De Vivo, 1995). The treatment consists of daily high doses of carnitine taken orally (100-200 mg/bodyweight), to ensure its absorption, possibly through passive diffusion and to reverse or attenuate the clinical symptoms in most patients, but without restoring totally the tissues carnitine stores (Angelini et al., 1992).

ii. Muscle carnitine deficiency (MCD)

This limited form of primary carnitine deficiency is characterized by severely reduced carnitine levels in skeletal muscle, only 20% of controls, but normal levels in plasma and liver, with no sign of renal carnitine leak (Angelini et al., 1992).

Although some patients have been shown to have fatty acid oxidation defects, for others no definitive biochemical defect has been discovered (Pons and De Vivo, 1995). As the plasma level is normal, it has been suggested that MCD might be caused by a defect in the low affinity muscle-specific carnitine transporter (Martinuzzi et al., 1991) and that this form of disease can be inherited as an autosomal recessive disorder, since parents also had low muscle carnitine levels (VanDyke et al., 1975). Studies in cultured myoblasts from a patient affected with MCD demonstrated normal carnitine uptake, but an increased carnitine efflux, resulting in significantly reduced intracellular carnitine content (Mesmer and Lo, 1990).

MCD can appear in the first year of life, but usually occurs at late adolescence (Angelini et al., 1987; Shapira et al., 1993). Affected patients suffer from progressive muscular weakness of varying degrees and some of them from lipid storage myopathy. Carnitine therapy has been beneficial on muscle strength in some patients only. Muscle carnitine content was increased with variable success, but carnitine stores were rarely replenished (Shapira et al., 1993).

1.5.2 Secondary carnitine deficiency

Secondary carnitine deficiency, manifested by decreased plasma or tissue carnitine is associated with a wide variety of genetic diseases, or acquired medical conditions or iatrogenic factors (Turnbull et al., 1984; Tanphaichitr and Leelahagul, 1993; Pons and De Vivo, 1995).

i. Metabolic disorders

Metabolic disorders are associated with impaired oxidation of acyl-CoA intermediates in the mitochondria and are the most characteristic causes of secondary carnitine deficiency. These disorders include fatty acid oxidation defects, which are the most frequent, but also amino acid oxidation defects, and are characterized by plasma and tissue carnitine levels of 25 to 50% of normal (Stanley, 1987). The block in the oxidation leads to the accumulation of acyl-CoA intermediates at or near the metabolic block. As mentioned earlier, transesterification of these acyl-CoAs with carnitine leads to the formation of acylcarnitines and to the release of free CoA (Brass and Hoppel, 1980; Bremer, 1983). Acylcarnitines are transported out of the mitochondria, out of the cell and finally excreted in the urine (Duran et al., 1990; Bohles et al., 1994). As a result, acylcarnitines in urine and blood reflect the accumulation of the corresponding acyl-CoA esters in the mitochondrial matrix and a characteristic increase of acylcarnitine to carnitine ratio is observed (Chalmers et al., 1984). Under normal conditions the plasma acylcarnitine to carnitine ratio is between 0.19 and 0.25, whereas ratios above 0.4 are considered abnormal (Penn and Schmidt-Sommerfeld, 1983; Golper et al., 1990; Ahmad, 2001). Determination of this ratio and of the acylcarnitine profile itself is a valuable means of screening for metabolic disorders (Bohles et al., 1994; Ahmad, 2001).

Fatty acid oxidation defects, that appear to be inherited in an autosomal recessive fashion, can be subdivided into defects of the carnitine cycle for the transport of the long-chain fatty acids into the mitochondria and defects of the β-oxidation cycle, that occur within the mitochondria (Pons and De Vivo, 1995). Defects of the enzymes involved in the carnitine cycle comprise carnitine-acylcarnitine translocase deficiency (Stanley et al., 1992), CPT I and CPT II deficiencies (Angelini et al., 1981; Trevisan et al., 1984). Deficiencies due to the enzymes involved in the β-oxidation spiral include the 4 acyl-CoA dehydrogenases: the short-chain (Turnbull et al., 1984), the medium-chain (MCAD) (Roe et al., 1986), the long-chain (Hale et al., 1985) and the very long-chain acyl-CoA dehydrogenases (Bertrand et al., 1993). MCAD deficiency is the most common enzyme deficiency of fatty acid oxidation found in the US, UK, and Northern Europe (Angelini et al., 1992) and is characterized by an increase of octanoylcarnitine, which can reach plasma concentrations between 1.8 and 28 µmol/L (Roe et al., 1985; Chace et al., 1997; Zytkovicz et al., 2001).

Block in the oxidation leading to accumulation of abnormal acyl-CoA intermediates at or near the metabolic block, associated with low plasma and tissue total carnitine levels, is also seen in branched-amino acid disorders (Bohles et al., 1994). Examples of the accumulated acylcarnitines in urine are isovalerylcarnitine in isovaleric academia (Roe et al., 1984b) and

propionylcarnitine in propionic acidaemia (Roe et al., 1984a) and methylmalonic acidemias (Minkler and Hoppel, 1993b).

Carnitine supplementation is given in patients with fatty acid oxidation disorders and other organic acidurias to correct the deficiency and to facilitate the removal of accumulating toxic acyl intermediates while regenerating mitochondrial free CoA. Increased excretion of disease-specific acylcarnitine derivatives in a dose-response relationship has been observed (Winter, 2003)

ii. Acquired medical conditions

Given the role of the liver in carnitine biosynthesis and the importance of the kidney in carnitine conservation, acquired metabolic conditions altering carnitine homeostasis might occur in patients with diseases of the liver (Krahenbuhl, 1996) or kidney (Leschke et al., 1984). Decreased carnitine biosynthesis may be associated with pre-maturity or chronic renal disease. Diminished carnitine intake may be associated with a low carnitine diet (vegetarians) or decreased reabsorption in malabsorption syndromes, such as cystic fibrosis, short-gut syndrome or celiac disease (Pons and De Vivo, 1995). Increased requirements and reduced body stores may accompany diverse clinical conditions, like pregnancy and extreme pre-maturity (Rebouche, 1992). Excessive renal loss of carnitine is seen in Fanconi syndrome and renal tubular acidosis (Bernardini et al., 1985; Tanphaichitr and Leelahagul, 1993; Pons and De Vivo, 1995).

iii. Drug induced

Several drugs are involved in secondary carnitine deficiency. The branched fatty acid valproic acid forms, like natural fatty acids, CoA and carnitine derivatives (Coude et al., 1983). While investigating the urine of children under chronic valproate therapy with mass spectrometry analysis, Millington et al. established the presence of valproylcarnitine in the samples (Millington et al., 1985). Chronic therapy with this anti-epileptic drug is associated with decreased plasma free carnitine levels and a slight increase of acylcarnitines (Matsuda et al., 1986; Coulter, 1991). Excretion of valproylcarnitine constitutes less than 10% of the total urinary acylcarnitine pools (Millington et al., 1985), whereas medium-chain acylcarnitines, namely hexanoylcarnitine and octanoylcarnitine, have been found to be excreted at the same levels as in MCAD deficient patients (Schmidt-Sommerfeld et al., 1992). Supplementation with carnitine has been shown to normalize plasma carnitine levels in patients under valproic treatment (Melegh et al., 1993).

Treatments with cisplatin, a cytostatique, or pivalate pro-drugs are associated with an increase in renal carnitine excretion (Holme et al., 1992; Heuberger et al., 1998; Brass et al., 2003).

Possible implication of the carnitine transporter OCTN2 in secondary carnitine deficiency due to the therapeutical use of several drugs has been investigated. Ohashi and co-workers showed that cationic drugs, such as quinidine, verapamil and valproate, are transported by OCTN2 and that quinidine, verapamil and cephaloridine, a zwitterionic drug, have inhibitory effect on OCTN2-mediated carnitine transport (Ohashi et al., 1999). Wu and colleagues demonstrated that the cationic drugs desipramine and verapamil produce a strong inhibition of OCTN2, whereas cimetidine presented a weaker inhibition (Wu et al., 1999). Beta-lactam antibiotics having a quaternary nitrogen, like cephaloridine, cefepime and ceflupreman, were found to inhibit carnitine transport by OCTN2, in contrast to beta-lactam, without quaternary nitrogen, which exhibited no interaction (Ganapathy et al., 2000). OCTN2 has been shown to be strongly inhibited by several drugs known to produce carnitine deficiency. The most potent blockers were the antibiotic emetine and the ion channel blockers quinidine and verapamil (Wagner et al., 2000). The authors did not find a significant inhibition of carnitine transport by valproic acid, suggesting that the deficiency induced by valproate therapy is due to a different mode of action.

iv. Hemodialysis treatment

Carnitine homeostasis is grossly disturbed in patients with chronic renal failure (Chen and Lincoln, 1977; Leschke et al., 1984; Guarnieri et al., 1987; Wanner et al., 1987). The normal route of carnitine renal excretion is lost, which in turn leads, as kidney function further deteriorates, to an increase in the plasma content of carnitine and its esters (Chen and Lincoln, 1977). Uremic patients have increased plasma levels of both free and total carnitine, with a marked relative rise of esterified carnitine translated by a high acylcarnitine to carnitine ratio (Rodriguez-Segade et al., 1986b; Wanner et al., 1987). However, significant elevations in plasma carnitine concentrations (>100 µmol/L) appear only when creatinine clearance decreases below 20 mL/min (Leschke et al., 1984).

In ESRD patients undergoing hemodialysis, the small water-soluble molecules that are carnitine and acetylcarnitine are efficiently removed from blood through the dialysis membrane (Moorthy et al., 1983; Panzetta et al., 1985). In a study conducted with 12 patients undergoing long-term hemodialysis, mean extraction coefficients of carnitine and acetylcarnitine were reported to be 0.74 and 0.71, respectively (Evans et al., 2000). The

acylcarnitine to carnitine ratio was found to be abnormally high before the hemodialysis and even higher after the hemodialysis, with values up to 1.9 (Guarnieri et al., 1987; Wanner et al., 1987). After termination of hemodialysis, a hyperbolic rise in plasma carnitine, which reaches pre-dialysis values within 44 to 48 hours, is observed (Bartel et al., 1981).

Subsequent to chronic hemodialysis therapy, plasma free carnitine was found to decrease gradually in accordance with months on hemodialysis, whereas the acylcarnitine to carnitine ratio increased in the same time (Sakurauchi et al., 1998). Despite abnormal carnitine fractions, the total carnitine plasma concentrations were reported, depending on the study, to be normal (Moorthy et al., 1983; Golper et al., 1990), but also decreased (Rodriguez-Segade et al., 1986a; b) or elevated (Wanner et al., 1987). Investigations performed on skeletal muscle of patients under hemodialysis demonstrated a decrease of the free and total carnitine content by about 50% and 10%, respectively, when compared to control values (Bellinghieri et al., 1983; Golper et al., 1990), and appeared to be more severe in the longer-term hemodialysis patients (Savica et al., 1983). Nonetheless, plasma and muscle carnitine levels could so far not be correlated (Debska et al., 2000). It has been suggested that to correct the low carnitine plasma levels, a net movement out of skeletal muscle into plasma would occur during and after hemodialysis, leading to deplete tissues stores, a depleted state that would persist as the carnitine uptake by muscle is a very slow process (Moorthy et al., 1983; Panzetta et al., 1985).
Even though the dialysis membrane irreversibly eliminates carnitine, weekly carnitine losses have been reported to be similar (Leschke et al., 1983), lower (Rumpf et al., 1983) or higher (Bohmer et al., 1978) than the renal losses in healthy individuals. It has been suggested that a reduced carnitine biosynthesis, as kidney is not functional anymore, a reduced dietary intake or an increased requirement might be, together with hemodialysis treatment, responsible for the carnitine deficiency observed in hemodialysis patients (Leschke et al., 1983; Guarnieri et al., 1987). However, based on a comparison of hemodialysis and renal clearances of carnitine, Evans and colleagues considered that a healthy kidney serves to conserve carnitine, on the basis of an entire week, more efficiently than does hemodialysis (Evans and Fornasini, 2003).

Patients under long-term hemodialysis suffer from many complications, which are similar to those observed with carnitine deficiency. Examples of such dialysis-associated symptoms include skeletal muscle myopathies and intradialytic cramps (Sakurauchi et al., 1998), impaired exercise performance (Hiatt et al., 1992), cardiac dysfunctions, responsible for as many as 50% of deaths in hemodialysis patients (Foley et al., 1998) and intradialytic complications, such as hypotensive episodes (Riley et al., 1997).

Carnitine supplementation has been evaluated in several studies, administered either intravenously at the end of each hemodialysis session at typical doses of 10-20 mg/kg (Wanner et al., 1987; Ahmad et al., 1990; Golper et al., 1990; Brass et al., 2001; Vesela et al., 2001) or orally at higher dosage of 2-4 g/day (Bellinghieri et al., 1983; Sakurauchi et al., 1998) due to the poor bioavailability of carnitine.

Treatment is clearly associated with significant increases in plasma concentrations of free carnitine and total carnitine (Golper et al., 1990). Although the acyl to free carnitine ratio decreases during the treatment, it is still markedly abnormal, even after 6 months of supplementation (Golper et al., 1990). Because renal elimination is not functional, the full dose is accessible to tissues, so that muscle carnitine content could be increased in hemodialysis patients receiving carnitine (Brass, 1995), in contrast to healthy persons treated with carnitine (Brass et al., 1994; Wachter et al., 2002). The magnitude of the increase appeared to be related to the duration of the treatment (Brass, 1995). A three fold increase in muscular carnitine concentration in hemodialysis patients has been observed following intravenous supplementation of 2 grams carnitine 3 times weekly for 6 months (Siami et al., 1991). Carnitine therapy clearly corrects the hypocarnitinemia found in hemodialysis patients and the supraphysiological carnitine concentrations achieved might be effective at removing metabolic intermediates from the active intramitochondrial coenzyme A pool (Brass et al., 2001).

The increased carnitine plasma and tissue levels reached under carnitine supplementation in hemodialysis patients have been shown to have positive effects in some patients on the following dialysis-related symptoms: the cardiac ejection fraction could be increased (van Es et al., 1992), the skeletal muscle mass and the exercise capacity were improved (Siami et al., 1991; Hiatt et al., 1992), the incidence of intradialytic hypotension and muscle cramps were decreased (Ahmad et al., 1990), chronic anemia was improved either by increasing the hematocrit in non erythropoietin treated patients or by enhancing the response to erythropoietin and thus decreasing the erythropoietin requirement (Labonia, 1995; Kletzmayr et al., 1999; Ahmad, 2001; Vesela et al., 2001) and the global well-being of hemodialysis patients was better (Sakurauchi et al., 1998).

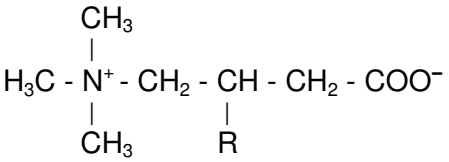
In December 1999, the American Food and Drugs Administration granted the approval for the use of carnitine for the prevention and treatment of carnitine deficiency in ESRD patients who are undergoing hemodialysis (Schreiber, 2002; Bellinghieri et al., 2003). However, despite the fact that several clinical syndromes associated with hemodialysis appear to respond to intravenous as well as to oral supplementation with carnitine (Brass et al., 2001), the approval was specific for the intravenous administration. Indeed, carnitine should not be administered orally at high dosage for long periods of time in patients with severely

compromised renal function due to the fact that major metabolites formed following oral administration will accumulate since they cannot be efficiently remove by the kidneys. As already mentioned, unabsorbed carnitine is degraded by intestinal bacteria to butyrobetaine, which is eliminated in the feces, and to trimethylamine (TMA) (Rebouche and Chenard, 1991; Rebouche and Seim, 1998). TMA is efficiently absorbed through the intestinal mucosa, further metabolized in the liver to trimethylamine-oxide (TMAO) and eliminated by renal excretion (Rebouche and Chenard, 1991). As TMAO is only partially dialyzed, it is not normally eliminated in patients with reduced renal function undergoing hemodialysis (Schreiber, 2002). When it reaches high concentration in the plasma, TMAO can be back converted to TMA, which is potentially toxic (Simenhoff et al., 1977b; Schreiber, 2002). Furthermore, TMA accumulation is not desirable since it increases the amount of nitrogenous waste to be removed in the dialysis procedure. In addition, the inefficient removal of TMA may result in the development of "fishy odor" syndrome (Simenhoff et al., 1977a; Rehman, 1999).

Due to the lack of homogeneity in individual patient responses to carnitine therapy and the inability for the clinicians to predict who will benefit from carnitine supplementation, routine carnitine administration to all hemodialysis patients cannot be recommended (Ahmad, 2001; Brass et al., 2001). In 2003, the American National Consensus Conference published recommendations for the use of carnitine in hemodialysis patients (Eknoyan et al., 2003). Four clinical conditions were considered as indications for treatment with carnitine: anaemia, intradialytic hypotension, cardiomyopathy and muscle weakness. A low plasma carnitine level associated with high acylcarnitine to carnitine ratio is not essential for diagnosis, nor is it predictive of clinical response to carnitine therapy, but normal values would exclude carnitine-related disorders as a cause of the observed clinical symptoms. The recommended dosage is 20 mg/kg body weight, intravenously following each hemodialysis session.
Owing to the lack of a relationship between plasma carnitine concentrations towards the patient's symptoms, monitoring plasma free carnitine or the acyl to free carnitine ratio during the therapy is not justified, but the clinical response to treatment should be evaluated at 3-months intervals. The therapy should be discontinued if no clinical improvement had occurred within 9-12 months (Eknoyan et al., 2003).

1.6 CARNITINE AND ACYLCARNITINES DETERMINATION

Numerous analytical methods have been described to determine carnitine and acylcarnitines (Figure 4) in foods, chemical and pharmaceutical processes, biological fluids and tissues (Lowes and Rose, 1989; Marzo and Curti, 1997; Rashed, 2001). In the first instance, techniques enabled the determination of carnitine only (Marquis and Fritz, 1964; Cederblad and Lindstedt, 1972), before further developments enabled the determination of various carnitine derivatives (i.e. acylcarnitine) (Millington et al., 1989; Minkler and Hoppel, 1993a) and the analytical resolution of D- and L-carnitine isomers (De Witt et al., 1994; Mardones et al., 1999a). Indeed, the detection of acylcarnitines is of great interest towards interpreting the state of the carnitine pool, which is of key importance towards the diagnosis of several metabolic disorders. Similarly the determination of carnitine's enantiomeric purity is important prior to its administration in human for therapeutic purposes, as the D-isomer has toxic effect on physiological processes. For example, D-carnitine has been shown to produce a competitive inhibition of the carnitine acetyltransferase, leading to depletion of the body L-carnitine storage (Jung et al., 1993), to inhibit the intestinal L-carnitine uptake (Gross and Henderson, 1984), to inhibit in a concentration-dependant manner L-carnitine transport in rat kidney brush-border-membrane vesicles (Stieger et al., 1995) and to inhibit OCTN2-mediated carnitine transport (Tamai et al., 1998; Wu et al., 1999).



R	***Name***	***R***	***Name***
OH	Carnitine	$OCO(CH_2)_4CH_3$	Hexanoylcarnitine
H	Butyrobetaine	$OCO(CH_2)_6CH_3$	Octanoylcarnitine
$OCOCH_3$	Acetylcarnitine	$OCO(CH_2)_{12}CH_3$	Myristoylcarnitine
$OCOCH_2CH_3$	Propionylcarnitine	$OCO(CH_2)_{14}CH_3$	Palmitoylcarnitine
$OCOC(CH_3)$	Isovalerylcarnitine	$OCO(CH_2)_{16}CH_3$	Stearoylcarnitine

Figure 4: Chemical structures of carnitine, butyrobetaine and acylcarnitines

The first carnitine assay described used *Tenebrio molitor* larvae and was based on larvae growth in the presence of the testing material compared with its growth in the presence of a known amount of carnitine. Because of its lack of precision, specificity and sensitivity, this bioassay was not adequate to quantify carnitine in biological fluids and tissues (Fraenkel, 1954).
Chemical methods, based upon the formation of colored complexes between the quaternary ammonium group of carnitine and chromophoric compounds, such as bromophenol blue, were developed in the late 1950s. These colorimetric assays, although an advancement upon the larval approach, still had specificity problems and sensitivity limitations (Friedman, 1958).

A significant improvement in carnitine determination was made with the enzymatic assay developed by Marquis and Fritz (Marquis and Fritz, 1964). The methodology is based upon the conversion of the L-isomer of carnitine to acetylcarnitine by the reversible enzyme carnitine acetyltransferase using acetyl-CoA as substrate:

$$\text{L-carnitine + acetyl-CoA} \overset{\text{CAT}}{\leftrightarrow} \text{acetylcarnitine + CoASH}$$

The sulfhydryl groups of the liberated free CoA further react with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) producing a thiophenolate ion which absorbs at 412 nm. This spectrophotometric method, developed for rat tissues, needed blank tissue to compensate for sulfhydryl groups present in biological tissues and had a vastly improved specificity and sensitivity for carnitine.

1.6.1 Radioenzymatic assay

The enzymatic assay developed by Marquis and Fritz was further improved by using [^{14}C]-acetyl-CoA, which leads to labeled acetylcarnitine (Cederblad and Lindstedt, 1972). The un-reacted, labeled acetyl-CoA is separated from the labeled newly formed acetylcarnitine by passing the reaction mixture through an anion-exchange column. Because the reaction has a low equilibrium constant and needs to be forced to the formation of acetylcarnitine to get linear standard curves, labeled acetyl-CoA has to be in excess and the released CoASH needs to be trapped. DTNB was used to render the reaction irreversible (Bohmer et al., 1974) but, as it was found to slowly inactivate CAT, it was substituted with sodium tetrathionate (McGarry and Foster, 1976) or with N-ethylmaleimide (Parvin and Pande, 1977). Parvin and Pande demonstrated that N-ethylmaleimide is more efficient in preventing

the reversal of the CAT reaction because it removes free CoA more rapidly than sodium tetrathionate does (Parvin and Pande, 1977).

This assay allows the specific determination of L-carnitine only, but, as acylcarnitines are easily hydrolysable in alkaline solutions to carnitine, an appropriate sample preparation allows the determination of the different acylcarnitine groups. When samples are treated with perchloric acid, two fractions are obtained. The first one, recovered in the supernatant and called the acid-soluble fraction, contains free carnitine and short-chain acylcarnitines having a chain length up to 10 carbons. The second one, named acid-insoluble fraction, is recovered in the pellet and contains acylcarnitines with chain length longer than 10 carbons (Hoppel, 1991). Linearities were reported to be 5-50 µmol/L for free carnitine and 5-100 µmol/L for total carnitine, limit of detection was 1 µmol/L for carnitine, and the inter-day variations for free and total carnitine in plasma and in urine samples were below 2.7 %. Although the enzymatic assays performed on each fraction, with and without previous hydrolysis, enables the determination of free carnitine, short- and medium-chain acylcarnitines, long-chain acylcarnitines and total carnitine, the assay is not able to identify individual acylcarnitines.

Kerner and Bieber proposed a radioisotopic-exchange method, which enables the determination of picomole amounts of individual short-chain acylcarnitines of up to 5 carbon atoms (Kerner and Bieber, 1983). A sample containing a pool of carnitine and short-chain acylcarnitines is incubated with radioactive-labeled carnitine in the presence of CoASH and CAT. As the reaction catalyzed by CAT is easily reversible, an isotopic equilibrium is attained, resulting in general labeling of carnitine esters. The produced radioactive-labeled carnitine and acylcarnitines are then separated by thin layer chromatography or high-performance liquid chromatography and the radioactivity of each fraction counted.

Dugan and colleagues exploited the enzymatic conversion by CAT, in the presence of CoASH, of urinary short-chain carnitine esters to short-chain acyl-CoAs followed by high-performance liquid chromatography separation (Dugan et al., 1987). CoA esters were quantified by integration of the area under the 254 nm absorption peaks. Limitation of this assay is the presence of high free carnitine concentrations because the rate of conversion of short-chain acylcarnitines appeared to be reduced.

1.6.2 Gas chromatography

Since carnitine and its esters are highly polar, non-volatile compounds, derivatization to generate volatile analogues is required prior to gas chromatography (GC) analysis. Determination by GC, as described in the methods below, achieves usual detection limits for acylcarnitines in the low µmol/L ranges.

Carnitine and its esters are unstable above 100^0C and give rise to desmethyl moiety, crotonoylbetaine or oxybutyrolactone ring plus trimethylamine. Lewin and coworkers exploited this thermal instability to analyzed carnitine by GC (Lewin et al., 1975). In the presence of NaOH and $NaBH_4$, carnitine was decomposed at 160^0C to oxybutyrolactone and then to 4-butyrolactone, which was analyzed as an expression of carnitine.

Procedures for the determination of soluble short-chain acylcarnitines, relying upon alkaline hydrolysis of the acylcarnitines and subsequent determination of the released fatty acids, have been reported (Choi and Bieber, 1977; Bieber and Lewin, 1981). The main drawback of this method is the ambiguity surrounding the origin of the acyl residues in the biological matrix, since the liberated fatty acids may arise from acyl-containing compounds other than acylcarnitines (Lowes and Rose, 1989). Sample pre-treatment using extraction on a strong cation-exchange column could shrink the uncertainty about the origin of the released fatty acids to cationic hydrolysable-acyl-containing molecules (Kumps et al., 1994).

However, GC techniques are potentially very useful owing to the possibility of being coupled to mass spectrometry (MS) detection, providing not only high sensitivity and selectivity, but also structural information. For instance, after derivatization of acylcarnitines to cyclic volatile lactones, which kept fatty acid units still bound, GC-MS was applied to the qualitative determination of acylcarnitines in urine (Lowes and Rose, 1990) and to the quantitative determination in plasma (Costa et al., 1997). Both methods allowed the diagnosis of metabolic disorders. The same derivatization technique was recently used to achieve enantiomeric separation of D- and L-carnitine on a cyclodextrin chiral stationary phase (Di Tullio et al., 2002). A two step derivatization, involving esterification to propyl esters followed by nucleophile-assisted thermolytic N-demethylation on the GC column, was used to convert the zwitterionic acylcarnitines to their volatile acyl N-demethylcarnitine propyl esters, which were then monitored by MS detection (Huang et al., 1991).

1.6.3 High-performance liquid chromatography

High-performance liquid chromatography (HPLC) using reversed-phase columns is particularly useful to evaluate carnitine and the different acylcarnitines. Because carnitine and acylcarnitines have only a weak and non-specific chromophoric absorption at λ_{max} = 210 nm due to the carboxyl groups, a pre-column derivatization is required so that the usual UV and fluorescence detectors can be coupled with liquid chromatography separation. The use of different chromophores reacting either with the carboxyl group or with the hydroxyl group of carnitine has been described (see below). In the latter case, acylcarnitine profiling will be impossible, as the fatty acid moiety has to be released to free the hydroxyl group for derivatization.
HPLC has however been used for the fractionation of un-derivatized acylcarnitines using a step gradient with increasing flow and methanol percentage. Four different fractions, detected by non-specific UV-absorption at 210 nm, containing respectively carnitine and acetylcarnitine, short-chain acylcarnitines, medium-chain acylcarnitines, and long-chain acylcarnitines, respectively, were collected and subsequent quantification using radioenzymatic assay was performed. The assay was applied to rat liver tissues (Hoppel et al., 1986).

Formation of bromophenacyl esters by reaction with 4'-bromophenacyl trifluoromethane sulfonate, known to react with carboxylic acids (Ingalls et al., 1984), is the most used derivatization process for carnitine and acylcarnitines determined via UV detection at 254 or 260 nm. Using this derivative reagent coupled with a separation in a reversed-phase column, Minkler and co-workers proposed several methods for carnitine and acylcarnitines analysis. The typical linear ranges are 5-1000 µmol/L for carnitine and 2.5-50 µmol/L for acylcarnitines, and the limits of detection around 1 µmol/L (Minkler et al., 1984; Minkler et al., 1990; Minkler and Hoppel, 1992; 1993a; b; Minkler et al., 1995).

Using isocratic conditions, carnitine, butyrobetaine and betaine standards could be analyzed as 4'-bromophenacyl ester derivatives in a 100 x 0.8 cm C_{18} column within 12 minutes (Minkler et al., 1984).
The separation of carnitine and 11 acylcarnitines in an identical column was achieved within 60 minutes when using a tertiary gradient containing varying proportions of water, acetonitrile, tetrahydrofuran, triethylamine, potassium phosphate, and phosphoric acid (Minkler et al., 1990). This assay was applied to urine, spiked urine and urine from patients suffering from different metabolic disorders. Isovalerylcarnitine was quantified in the urine of

a patient with isovaleric academia using heptanoylcarnitine as an internal standard (Minkler et al., 1990).

The use of a quaternary gradient, containing varying proportions of water, acetonitrile, triethylamine and phosphoric acid, enabled to analyze free and total carnitine within 9 minutes in a 150 x 3.9 mm C_8 column (Minkler and Hoppel, 1992). Standards curves were established using 4-(N,N-dimethyl-N-ethylammonio)-3-hydroxybutanoate (e-carnitine) as the internal standard. Quantification in urine samples was demonstrated to give values that are not different from the results obtained using a radioenzymatic assay. By using a shorter column (100 x 4.6 mm C_8 column) and modifying the gradient, carnitine and 5 short- and medium-chain acylcarnitines could be separated in a reduced time of 12 minutes. This procedure was applied for the quantification in urine (Minkler and Hoppel, 1993b) and plasma samples (Minkler and Hoppel, 1993a) from healthy controls and from patients diagnosed with different metabolic disorders, and in human muscle biopsy samples (Minkler et al., 1995). Day-to-day variabilities determined in urine samples from patients suffering from different metabolic disorders were reported to be below 10% for carnitine and short-chain acylcarnitines, but up to 26% for medium-chain acylcarnitines (Minkler and Hoppel, 1993b). Day-to-day variabilities for plasma samples were below 10% for carnitine, 25% for acetylcarnitine and 33% for propionylcarnitine (Minkler and Hoppel, 1993a). This method was also demonstrated to be suitable, using a 300 x 3.9 mm C_{18} column, to determine butyrobetaine in plasma, urine and liver tissues from rat, but a minor modification of one mobile phase constitution was required (Krahenbuhl et al., 1992).

Other derivatizing reagents used for the determination of carnitine and acylcarnitines with UV detection include phenacyl bromide, to form phenacylcarnitine absorbing at 245 nm (van Kempen and Odle, 1992) and 4'-bromophenacylbromide (Poorthuis et al., 1993).

Fluorescence detection has been used after pre-column derivatization utilizing different fluorescent reagents. Following derivatization of carnitine and acetylcarnitine with a fluorescent trifluoromethanesulfonate as the derivatizing agent, fluorometric detection showed a 500-fold increase in sensitivity when compared to UV-detection of 4'-bromophenacyl trifluoromethanesulfonate derivatives, thus enabling the detection of acetylcarnitine in dilute human muscle homogenate samples (Minkler et al., 1995). Derivatization with 9-anthryldiazomethane (ADAM) and separation in a silica column with isocratic elution enabled the determination of carnitine in solution and pharmaceutically produced tablets (Yoshida et al., 1988). Acylcarnitine's profile was obtained in urine from healthy human subjects and patients with propionic acidemia, after derivatization with 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1*H*)-quinoxalinone. Carnitine and short-chain acylcarnitines were determined using different chromatographic conditions (column and

gradient) than the ones for medium-and long-chain acylcarnitines, requiring up to 80 minutes separation time, and cyclopentanoylcarnitine was used as internal standard (Kamimori et al., 1994). Determination of carnitine, acetylcarnitine and propionylcarnitine in plasma samples after derivatization with 1-aminoanthracene, with a separation performed in 20 minutes in a 250 x 4.6 mm C_{18} column under isocratic elution, was reported to get improved quantification limits of 5 µmol/L for carnitine, 1 µmol/L for acetylcarnitine and 0.25 µmol/L for propionylcarnitine and intra- and inter-day variabilities below 17% and accuracies of 10% (Longo et al., 1996). Derivatization with 2-(4-hydrazinocarbonylphenyl)-4,5-diphenylimidazole and separation in a reversed-phase column under gradient elution conditions yielded in detection limits ranging from 0.2 and 2.0 µmol/L for carnitine, short- and medium-chain acylcarnitines in plasma (Kuroda and al., 1996).
When compared to UV detection, fluorescence detection facilitates enhanced detection and quantification limits. This might be useful for the determination of acylcarnitines, which are present at lower concentrations than carnitine within the body. However the chromatographic conditions appear to be more complicated and the analysis time would be longer.

HPLC was also used to determine the enantiomeric purity of D- and L-carnitine and carnitine esters in pharmaceutical preparations. Two different techniques, a direct and an indirect, were used to detect the presence of the D-isomer up to 0.05% in a L-isomer preparation. The direct method involves the use of a chiral stationary phase, commercially available (Hirota et al., 1994) or laboratory-made (D'Acquarica et al., 1999), to separate the D- and L-isomers. Detection was performed either by UV or fluorescence after an appropriated derivatization (Hirota et al., 1994), or by evaporative light scattering, thus without requiring derivatization (D'Acquarica et al., 1999). The indirect method relies on the determination of diastereoisomers formed after reaction with a chiral reagent. Diastereoisomers were formed by derivatization with a chiral amino compound that has a chromophore for UV detection (Kagawa et al., 1999) or a chiral fluorescent compound (Freimuller and Altorfer, 2002), and subsequently separated on a reversed-phase column with isocratic elution.

1.6.4 Capillary electrophoresis

Because carnitine is a zwitterionic compound, presenting a permanent positive charge under acidic conditions (pKa = 3.8), it represents a good candidate for capillary electrophoresis (CE) analysis, where separation is based on the migration ability of charged molecules, according to their charge to mass ratio when an electric field is applied.

CE uses the same detectors as HPLC and the same reagents were used to derivatize free and acyl carnitine. After derivatization with the fluorescent ADAM, carnitine and 10 acylcarnitines, up to palmitoylcarnitine, were separated in a 77 cm x 50 µm I.D. fused-silica capillary within 45 minutes using a running buffer containing methanol, as the formed derivatives were poorly soluble in water, phosphoric acid and sodium dodecyl sulfate (SDS) (Kiessig and Vogt, 1997). A CE method, after derivatization with 4'-bromophenacyl trifluoromethane sulfonate, enabling the separation of carnitine and 5 acylcarnitines in less than 8 minutes in a 40 cm x 50 µm I.D. fused-silica was developed in our laboratory (Vernez et al., 2000) (see Chapter 2). The assay used a binary buffer system containing phosphate/phosphoric acid and SDS, had limits of detection for carnitine and acetylcarnitine of 3 µmol/L and was applicable to urine samples.
Indirect UV detection has been investigated on un-derivatized carnitine to overcome the non-UV absorbance of carnitine (Mardones et al., 1999b). Using quinine as background signal in a sulphate buffer, carnitine and 5 acylcarnitines were separated within 20 minutes in a 57 cm x 75 µm I.D. fused-silica. The achieved detection limits, ranging from 1.0 to 7.3 µmol/L, depending on the compound, are not so good when compared to direct UV-detection preceded with a derivatization step, and accuracies were reported to be below 12%.

Determination of the enantiomeric purity of D- and L-carnitine, in ratio up to 1:200 (Vogt et al., 1995), by CE was carried out with direct and indirect methods. In contrast to HPLC where a chiral stationary phase was used, determination by direct method using CE involved an achiral stationary phase in the presence of a chiral selector to separate the stereoisomers. After derivatization with the fluorescent 9-fluorenylmethyl chloroformate, reacting on the carboxyl group of carnitine, the stereoisomers were separated in a fused-silica capillary using a β-cyclodextrine (Mardones et al., 1999a) or a γ-cyclodextrine (Vogt et al., 1995) as the chiral selector. Indirect determination was achieved after derivatization with (+) or (-)-1-(9-fluorenyl)ethyl chloroformate on the carboxyl groups and the formed diastereoisomers were separated using a phosphate buffer (De Witt et al., 1994; Vogt et al., 1995).

More recently, CE was used coupled with mass spectrometry to determine free and acylcarnitines in urine and plasma samples from healthy humans. (Heinig and Henion, 1999). Un-derivatized carnitine and 11 acylcarnitines were separated in less than 10 minutes in a 80 cm x 75 µm I.D. fused-silica capillary using an optimized mixed organic-aqueous buffer containing formic acid and ammonium acetate. As mass spectrometry detection is used, a baseline separation is not required, hence a reduced sample time, and better sensitivity is achieved. Limits of detection ranged for the different compounds between 0.1 and 1.2

µmol/L. Intra- and inter-day variabilities and accuracies performed on plasma samples were below 15%.

Capillary electrophoresis generates separations with high resolution, in short analysis time and with the consumption of only small amounts of solvent. Nevertheless, organic solvents often need to be added to the electrolytes buffer in order to solubilize the more apolar derivatized carnitine and acylcarnitines. Because organic solvents tend to evaporate over time, the electrolytes buffer needs to be frequently renewed, which renders, according to our experience, analytical automation problematic.

1.6.5 Mass spectrometry

Given that a clinical diagnosis relies on the unambiguous identification of body fluid components, mass spectrometry (MS), due to its ability to provide structural information, is an attractive technique used on it's own or as a detector coupled with a separation technique.

The facility to analyze polar, thermal labile and non-volatile molecules, such as carnitine and acylcarnitines, was realized with the introduction of mild ionization techniques, such as fast atom bombardment (FAB), thermospray and more recently electrospray ionization (ESI) that allowed liquid chromatography to interface with mass spectrometry.
Millington and co-workers pioneered mass spectrometry methods to assay carnitine and acylcarnitines, the most successful being the combination of fast atom bombardment ionization with tandem quadrupole mass spectrometry (FAB-MS/MS) for analysis of carnitine and acylcarnitines as methyl derivatives, after esterification on their carboxylic group, in urine and plasma samples (Millington et al., 1989) and dried blood spots (Millington et al., 1990; Millington et al., 1991). The methods utilizes the fact that acylcarnitine methyl esters produce a characteristic fragment ion at m/z 99, upon collision-induced dissociation, derived from the loss of both the quaternary ammonium function (as trimethylamine) and the acyl moiety, and that carnitine and acylcarnitines exhibit excellent mass spectra from a FAB ion source (Millington et al., 1989). The esterification blocks the acidic function, thereby the formation of protonated molecules is enhanced and the sensitivity of detection is improved. Scanning in the range of m/z 200-500 in the first quadrupole for precursors of this fragment, the third quadrupole being set at m/z 99, produces a profile showing free carnitine and acylcarnitines. Quantitation could be achieved by introduction of stable isotope labeled analogues of carnitine and acylcarnitines (Kodo et al., 1990). When using butylation instead of methylation

for the derivatization procedure, acylcarnitine butyl esters produce a common fragment at m/z 85, and amino acids can be determined simultaneously (Chace et al., 1993; Chace et al., 1997). The widespread application of FAB-MS/MS for acylcarnitine analysis was limited by the difficulties in automating sample introduction into the mass spectrometer.

The development of electrospray ionization allowed introduction of the sample into a continuously flowing solvent stream and offered a more robust and more sensitive alternative to FAB-MS/MS for the profiling of acylcarnitines (Rashed et al., 1994). ESI-MS/MS of carnitine and acylcarnitine methyl or butyl esters has been successfully applied in newborn screening (Rashed et al., 1995b; Rashed et al., 1997), selective screening (Van Hove et al., 1993; Chace et al., 1997), post-mortem diagnosis using bile samples (Rashed et al., 1995a) and for prenatal diagnosis (Nada et al., 1996; Shigematsu et al., 1996) for many inborn errors of metabolism.
The presence of carnitine biosynthesis precursors, trimethyllysine and butyrobetaine, in serum samples has been determined by ESI-MS/MS (Terada et al., 1999). However, although butyrobetaine could be successfully measured after methylation, the analysis of methylated trimethyllysine suffered from homoarginine interference, which could be overcome by propylation and acetylation of the sample instead of methylation.

In the field of screening for metabolic disorders, quantitative analysis is performed using stable isotope labeled analogues and yield highly sensitive and reproducible results. Each metabolic disorder is associated with primary and secondary markers and for each marker a cut-off value is defined. Any value above this threshold is strongly indicative for the presence of the suspected disorder. The setting of appropriate cut-off values would minimize false negatives without producing an excessive number of false positives. As carnitine and acylcarnitine concentrations are variable according to the weight at birth, full-term baby or premature baby, and age at collection time, optimal discrimination may require determination of cut-off points for these distinct subpopulations (Zytkovicz et al., 2001).
Rather than using only absolute concentrations of acylcarnitines, diagnosis for metabolic disorders relies often on semi-quantitative data based on the ratio between two acylcarnitines or between an acylcarnitine and an internal standard. For instance, Rashed and coworkers defined isovaleryl academia by an isovalerylcarnitine to acetylcarnitine ratio higher than 0.09 (Rashed et al., 1997). Chace and colleagues considered an octanoylcarnitine concentration above 0.3 µmol/L combined with an octanoyl- to acetylcarnitine ratio above 0.1 as strongly indicative of MCAD deficiency (Chace et al., 1997) and that the use of a ratio between two acylcarnitines, instead of the concentrations of one

acylcarnitine, could reduced false-positive diagnosis due to general increase in the short-chain acylcarnitines (Chace et al., 2001b).

Inaccuracies in the measurement of free carnitine (Johnson, 1999) and octanoylcarnitine (Chace et al., 2001a) from blood spots by ESI-MS/MS were reported.
Standard procedure to derivatize carnitine and acylcarnitine to butyl esters implies heating of the dried extract in the presence of butanolic HCl at 65^0C for 15 minutes (Chace et al., 1995; Rashed et al., 1995b). If incomplete butylation occurs, under milder conditions for instance, a loss of selectivity due to interference from un-derivatized acylcarnitines that give the same product ion than butyl esters at m/z 85 can be expected (Chace, 2001). Prolonged butylation was demonstrated to produce acylcarnitine hydrolysis, resulting in decreased signal intensities for acylcarnitine ions and corresponding increase of the carnitine ion peak (Johnson, 1999). Thus, the free carnitine concentrations should be viewed with caution in samples with high concentrations of acylcarnitines. Determination of un-derivatized free carnitine in healthy newborns was found to give 35% lower concentrations than the one obtained after a standard butylation procedure (Schulze et al., 2003). This suggests that it would be preferable to perform carnitine ESI-MS/MS analysis without derivatization, a process that is realizable (Stevens et al., 2000), but in which sensitivity is somewhat reduced for dicarboxylic acylcarnitines when compared to butyl ester analysis (Manning et al., 2001).
The use of racemic D,L- octanoylcarnitine to establish a calibration curve for blood spot analysis was shown to produce overestimation of the octanoylcarnitine content (Chace et al., 2001a). The authors found that the racemic mixture gave smaller signals than L-octanoylcarnitine alone, and attributed it to a specific hydrolysis of the D- isomer during the sample preparation.

Analysis of free and acyl carnitine butyl esters by ESI-MS/MS for the screening of errors of metabolism are mostly performed on dried blood spot samples. However, quantitative determination on plasma or serum samples (Vreken et al., 1999), where a clear discrimination could be made between controls and affected individuals, was reported to be as reliable. The use of butylated urine samples was reported to be suitable for diagnostic purposes with analogous criteria than the one used for dried blood spots (Mueller et al., 2003). Nonetheless, as urine was not submitted to extraction, influences of the matrix on signal and ionization suppression had to be overcome by diluting urine samples 10 fold with methanol.

ESI-MS/MS presents excellent sensitivity and specificity. Starting from a drop of blood, plasma or urine, the whole content of carnitine and its acyl esters can be depicted. Typical

linearity ranges obtained for the analysis of the butylated compounds are 5-100 µmol/L for carnitine, 2.5-50 µmol/L for acetylcarnitine and 0.2-10 µmol/L for the other acylcarnitines. Detection limits are between 0.02 and 0.2 µmol/L (depending on the analyzed acylcarnitine), what represent an improvement when compared to the detection limits obtained with HPLC-UV determination. Reported coefficients of variation for the determination of carnitine and acylcarnitine as butyl or methyl esters in blood spot, plasma or serum samples are below 14% for intra-day assays and below 19% for inter-day assays (Chace et al., 1997; Inoue et al., 1999; Chace et al., 2001b; Fingerhut et al., 2001). Similar coefficients of variation were reported for the determination of carnitine as butyl ester in urine (Mueller et al., 2003).

Unfortunately, tandem mass spectrometry is unable to distinguish isomeric compounds as they have the same precursors and product ions. Based on their differential relative abundance of the product ions, valeryl- and isovalerylcarnitine could nonetheless be differentiated (Gaskell et al., 1986). A distinction was obtained for the isomeric valproylcarnitine and octanoylcarnitine with a prior chromatographic separation (Millington et al., 1985). Furthermore, as tandem mass spectrometry is performed on biological samples with little sample clean up, the procedure suffers from limitations due to interferences that can arise in a biological matrix.
Nonetheless, because of the automated sample introduction, ESI-MS/MS brought a high-throughput method of newborn screening and is used in many laboratories as the method of choice for the analysis of carnitine and acylcarnitines and screening for metabolic disorders, such as fatty acid oxidation defects or organic acidemias. It represents, however, only the first stage of making a diagnostic. As the sensitivity depends upon the chosen cut-off, confirmation of the diagnosis or follow up require other biochemical/molecular genetic techniques (Chace et al., 2001c; Carpenter and Wiley, 2002; Shigematsu et al., 2003).

HPLC-MS/MS determination of carnitine, acetylcarnitine and butyrobetaine has been obtained unintentionally, when a reversed-phase assay using an ion-pairing reagent was set up to determine acetylcholine concentrations in rat brain (Zhu et al., 2000). The chromatogram obtained showed three unexpected extra peaks. As acetylcholine is a quaternary ammonium compound, it was supposed that the three peaks were belonging to the same class of compounds and subsequent MS/MS experiments yielded to the identification of carnitine, acetylcarnitine and butyrobetaine.
HPLC-MS/MS has been used to investigate carnitine biosynthesis precursors in urine samples, previously derivatized to methyl esters, using step gradient ion-pair mobile phase (Vaz et al., 2002). Under these conditions, carnitine, butyrobetaine, trimethyllysine and hydroxy-trimethyllysine were resolved within 13 minutes. Quantification was performed with

stable isotope dilution. The limits of detection from 0.05 to 0.1 µmol/L are similar to those obtained by ESI-MS/MS. The imprecision for the determination of carnitine and butyrobetaine was below 5% for intra-day assays and below 9% for inter-day assays (Vaz et al., 2002).
In our laboratory, we set up an HPLC-MS/MS method to determine carnitine and acylcarnitines in urine and plasma samples. In contrast to most published methods, the analytes were not derivatized and the mass spectrometer used was an ion-trap machine. The quantification limits achieved were between 0.25 and 5 µmol/L depending on the compound (see Chapters 3 and 4) (Vernez et al., 2003).

1.6.6 Carnitine extraction

Carnitine determination in pharmaceuticals can be performed directly on an aliquot of the dissolved tablets or on an aliquot of the injection or drinkable solution. When carnitine and acylcarnitines have to be determined in biological fluids or tissues, a previous appropriate extraction procedure is often required. Solid-phase extraction (SPE) on disposable cartridges is the most commonly used extraction method for the determination of carnitine and acylcarnitines. Several procedures have been described, following a previous protein precipitation step with acetonitrile and/or methanol, for both plasma and serum analysis.

SPE on silica column was used to extract carnitine and acylcarnitines from 100 µL plasma, after protein precipitation with acetonitrile/methanol (3:1; v/v), or 25 µL urine and elution was performed with 4 mL 1% acetic acid in methanol (Minkler and Hoppel, 1993a; b). The reported recoveries were 50% for carnitine, 63-68% for short-chain acylcarnitine and 58-67% for medium-chain acylcarnitines in urine (Minkler and Hoppel, 1993b). In plasma, the mean recoveries were 64% for carnitine, 64-67% for short-chain acylcarnitines and 62-78% for medium-chain acylcarnitines (Minkler and Hoppel, 1993a). The use of a more acidic solvent, methanol/water/acetic acid (50:45:5), to elute carnitine and acylcarnitines from a silica column enabled to reduce the required elution volume to 2 mL and to increase the recoveries (Heinig and Henion, 1999). Extraction performed on 2 mL urine showed mean recoveries of 88% for carnitine, 85-97% for short-chain acylcarnitines and 79-83% for medium-chain acylcarnitines. The same protocol applied to 300 µL plasma gave mean recoveries of 75% for carnitine, 76-106% for short-chain acylcarnitines and 91% for medium-chain acylcarnitines.
Extraction from 200 µL plasma on a reversed-phase C_{18} column with methanol elution yielded recoveries of 88% for acetylcarnitine, 85% for propionylcarnitne and 80% for

octanoylcarnitine (Millington et al., 1989). When a C_{18} column was combined with an anion-exchange resin, recoveries obtained for the extraction of 200 µL urine were 52% for acetylcarnitine, 68% for propionylcarnitine and 52% for octanoylcarnitine. The association of an anion-exchange resin with a cation-exchange column was shown to increase the recoveries of low molecular weight acylcarnitine in urine samples (99% for acetylcarnitine and 98% for propionylcarnitine) (Millington et al., 1989).
Strong cation-exchange columns have been used on acidified urine samples, together with ammoniac (Kumps et al., 1994) or with pyridine (Kamimori et al., 1994) elution. Elution with pyridine gave analytical recoveries higher than 96% for carnitine, short- and medium-chain acylcarnitines, whereas long-chain acylcarnitines showed analytical recoveries between 70-76% (Kamimori et al., 1994). Ammoniac elution gave extraction recoveries between 90 and 100% for the different acylcarnitines (Kumps et al., 1994).
Anion-exchange columns have also been used and mean extraction recoveries from 100 µL plasmas were reported to be between 82-85% for carnitine, 93-96% for acetylcarnitine and 85-91% for propionylcarnitine, depending on the concentration tested (Longo et al., 1996).

No matter which SPE column is used, a similar tendency can be observed. Recoveries of carnitine and medium-chain acylcarnitines are often of the same magnitude, whereas recovery of short-chain acylcarnitines is often increased. The use of specific strong cation-exchange columns appears to enable increased recoveries for the extraction of carnitine and acycarnitines.

Liquid-liquid extraction has been proposed to extract the more hydrophobic and protein-bound long-chain acylcarnitines from plasma samples. Extraction from 500 µL plasma with 500 µL ethyl acetate gave recoveries of 66% for palmitoylcarnitine and 47% for stearoylcarnitine (Heinig and Henion, 1999).

A simple liquid extraction is also usually applied for ESI-MS/MS analysis of dried blood spots on filter paper (Chace et al., 1995; Rashed et al., 1995b; Chace et al., 1997; Fingerhut et al., 2001). Carnitine and acylcarnitines are extracted from the spots, corresponding from 3 to 15 µL whole blood, with 150 to 400 µL methanol. Reported mean recoveries are 93% for carnitine, 86-101% for acetylcarnitine, 92-126% medium-chain acylcarnitine and 83-97% for long-chain acylcarnitines, depending of the concentrations taken into consideration (Chace et al., 1997; Fingerhut et al., 2001). Extraction of 20 µL serum with 1 mL methanol gave similar recoveries of 93% for carnitine and 77% for butyrobetaine (Inoue et al., 1999).

1.7 AIMS OF THE THESIS

The major objective of this thesis was to develop analytical tools for the quantitative determination of endogenous carnitine and acylcarnitines in biological samples.

To obtain the profile of each individual acylcarnitine, in order to be able to detect metabolic disorders, for instance, a separation technique is required. HPLC and CE have been successfully used, coupled with UV or fluorescent detection. An HPLC method after derivatization with 4'-bromophenacyl trifluoromethane sulfonate, based on the assay described by Minkler and Hoppel (Minkler and Hoppel, 1993b), was in use in our laboratory. Briefly, samples were extracted by solid phase extraction on a silica column and HPLC separation took place in a 150 x 4.6 mm C_8 reversed-phase column using a quaternary gradient over 45 minutes. Limitations of this method were the relative long analysis time and the use of large volumes of solvent as the flow was set at 1 mL/min.

Since capillary electrophoresis is adapted to the analysis of charged molecules, such as carnitine, and as derivatization with 4'-bromophenacyl trifluoromethane sulfonate enables UV-detection, the combination of these two techniques were exploited to set up a new method for carnitine and short- and medium-chain acylcarnitines in urine. The objective was to get shorter analysis time and reduced consumption of solvent.

When our laboratory received the opportunity to acquire an ion trap mass spectrometer, which in itself dictates a restriction in the use of compatible solvents, the method we had developed for CE could not be directly transposed, namely, because the SDS used in the running buffer is not MS friendly. Additionally, CE analysis proved to be difficult to automate as the running buffer has to be renewed every two analyses. Finally, since MS detection, owing to its sensitivity, enables the use of small HPLC columns and thus reduced solvent volumes, we focused on the development of an HPLC-MS/MS assay. The major advantage brought by MS detection is that a derivatization step becomes unnecessary, and the assay could be extended to the detection of long-chain acylcarnitines.
HPLC-MS/MS was first developed to analyze urine samples and then its application was extended to plasma, where the precursor to carnitine biosynthesis, butyrobetaine, could also be determined.

Different extraction procedures were considered in order to optimize the recovery of carnitine and acylcarnitines. For this purpose, many solid phase extraction materials and elution

buffers were tested as well as liquid-liquid extraction, especially for the determination of long-chain acylcarnitines.
In order to achieve the potential of the newly developed assay, it was applied to the follow up of a clinical study, where carnitine, acylcarnitines and butyrobetaine plasma content were investigated in patients with end-stage renal disease undergoing long-term hemodialysis. Profiles of these compounds, extraction kinetics during a hemodialysis session and kinetics following intravenous administration of carnitine after a hemodialysis session were considered and comparisons were established before and after patients were supplemented with two different dosages of carnitine at the end of each hemodialysis session.

The different projects investigated during my thesis were the topic of four publications. These publications are presented in chapters 2 to 5 in the form they have been published (chapter 2, 3 and 4) or submitted (chapter 5) to publication. To facilitate the reading, all the references were brought together at the end of this script.

CHAPTER

2

ANALYSIS OF CARNITINE AND ACYLCARNITINES IN URINE BY CAPILLARY ELECTROPHORESIS

Laurence Vernez, Wolfgang Thormann, Stephan Krähenbühl

Department of Clinical Pharmacology, University of Berne, Berne, Switzerland

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2.1 SUMMARY

A capillary electrophoresis method is described for the simultaneous analysis of carnitine and short-chain acylcarnitines in aqueous standard solutions and urine samples. Samples were worked up using silica gel extraction and derivatization with 4'-bromophenacyl trifluoromethanesulfonate. Separation was performed in less than 8 minutes using a binary buffer system containing phosphate/phosphoric acid and sodium dodecyl sulfate. 3-(2, 2, 2-trimethylhydrazinium)-propionate (mildronate) was used as an internal standard. The method was developed with aqueous standard solutions and then applied successfully to spiked and unspiked human urine samples. The limit of detection for both carnitine and acetylcarnitine is 3 µmol/L.

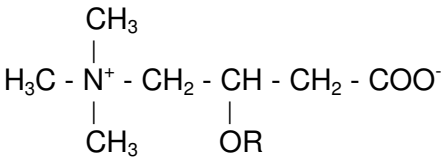
2.2 INTRODUCTION

Carnitine is an endogenous compound, which is either biosynthesized (from the amino acids L-lysine and L-methionine) or taken up by the food (mainly in meat and milk) (Carter et al., 1995). Carnitine is essential for the transport of fatty acids into the mitochondrial matrix, where they are metabolized (β-oxidation for energy production). Fatty acids bind carnitine via an ester binding on the hydroxyl group in position 3 of carnitine (see Figure 1), thereby generating various acylcarnitines with different chain lengths. Carnitine is also important to maintain the cellular pool of free coenzyme A (Friolet et al., 1994). Free (non-esterified) carnitine and acetyl-carnitine represent the major constituents of the body fluids and tissue carnitine pools in normal subjects. Under pathological conditions, other acylcarnitines can accumulate, for instance in inherited or acquired organic acidurias such as propionic or isovaleric acidemia. Profiling of the acylcarnitines in body fluids is therefore useful for diagnostic purposes.

Existing analytical methods are problematic in that they either do not allow differentiation between acylcarnitines (radioenzymatic method) or are time-consuming [high-performance liquid chromatography (HPLC method)]. The radioenzymatic assay is strictly enantioselective for L-carnitine, but it allows only the determination of the free carnitine, short-chain acylcarnitine (difference between total acid-soluble and free carnitine) and long-chain acylcarnitines (Hoppel, 1991). HPLC methods have been developed to obtain a profile of acylcarnitines (Minkler and Hoppel, 1993a; b; De Witt et al., 1994; Vogt et al., 1995; Longo et al., 1996). As carnitine has no specific UV absorption, it has to be derivatized for detection. Different chromophores have been described in the literature, reacting either with the

carboxyl or the hydroxyl group of carnitine. Examples of such derivatizing agents include 1-aminoanthracene (Longo et al., 1996), (+)-[1-(9-fluorenyl)-ethyl]-chloroformate [(+)-FLEC] (De Witt et al., 1994) and 9-fluorenyl-methylchloroformate (FMOC) (Vogt et al., 1995). Minkler and Hoppel used 4'-bromophenacyl trifluoromethanesulfonate which reacts with the carboxyl group of carnitine and can be detected at 260 nm (Minkler and Hoppel, 1993a; b).

Capillary electrophoresis (CE) may have several advantages in comparison to HPLC for the separation of carnitine and acylcarnitines. The separation time is generally shorter and the amount of solvent used smaller, rendering the analysis faster and less expensive. Accordingly, CE procedures for the separation of carnitine and acylcarnitine standard solutions have been described (De Witt et al., 1994; Vogt et al., 1995; Vogt and Kiessig, 1996; Kiessig and Vogt, 1997; Mardones et al., 1999a; b). Most recently, also a CE-mass spectrometry (MS) method for the separation of carnitine/acylcarnitines in biological fluids has been published, but MS is not widely available (Heinig and Henion, 1999). In the present paper we describe a CE method to separate acylcarnitines up to C_8 in standard solutions and in urine, after derivatization of the carboxyl group with 4'-bromophenacyl trifluoromethanesulfonate.



R	***Name***	***R***	***Name***
H	Carnitine	$COC(CH_3)_3$	Isovalerylcarnitine
$COCH_3$	Acetylcarnitine	$CO(CH_2)_4CH_3$	Hexanoylcarnitine
$COCH_2CH_3$	Propionylcarnitine	$CO(CH_2)_6CH_3$	Octanoylcarnitine

Figure 1: Chemical structures of carnitine and short-chain acylcarnitines analyzed in this study.

2.3 EXPERIMENTAL

2.3.1 Chemicals

L-Carnitine was from Fluka (Buchs, Switzerland), and acetylcarnitine, hexanoylcarnitine, octanoylcarnitine and sodium dodecyl sulfate (SDS) from Sigma (St. Louis, MO, USA). Propionylcarnitine and isovalerylcarnitine were a gift of Sigma-Tau (Zofingen, Switzerland). Potassium hydroxide, potassium dihydrogensulfate, sodium dihydrogenphosphate dihydrate, silica gel and acetic acid were purchased from Merck (Darmstadt, Germany). Phosphoric acid was from Fluka (Buchs, Switzerland). N,N-diisopropylethylamine was from Aldrich (Buchs, Switzerland). Methanol and acetonitrile were HPLC grade from Biosolve (The Netherlands). 3-(2, 2, 2-trimethylhydrazinium)-propionate (mildronate) was synthesized using the methods of Le Berre and Porte (Le Berre and Porte, 1978) and Kosower and Patton (Kosower and Patton, 1961) with some modifications. 3-(2, 2, 2-trimethylhydrazinium)-propionate was used as an internal standard. The derivatizing agent 4'-bromophenacyl trifluoromethanesulfonate was synthesized as described elsewhere (Ingalls et al., 1984; Minkler and Hoppel, 1992).

2.3.2 Standard solutions

A stock 100 mmol/L L-carnitine solution was prepared in water, standardized by a spectrophotometric method (Marquis and Fritz, 1964), stored in aliquots at -20 ^{O}C and diluted with water to the desired concentration just before use. Stock 20 mmol/L solutions of all other acylcarnitines were prepared in water (not standardized), stored in aliquots at -20 ^{O}C and diluted with water to the desired concentration as needed. A 50 µmol/L internal standard solution (mildronate) was prepared in water and stored at -20 ^{O}C.

2.3.3 Sample preparation and derivatization

Carnitine, acylcarnitines and total carnitine were extracted and derivatized according to Minkler et al. (Minkler et al., 1995) with minor modifications. Extraction was performed from 50 µL human urine (undiluted or after dilution up to 10 times) or aqueous standard mixtures. A 50-µL volume of aqueous mildronate solution (50 µmol/L) were used as internal standard. The samples were dried with an evaporator under reduced pressure at 35 °C. The derivatized and dried samples were dissolved with 50 µL acetonitrile-water (80:20, v/v) and

injected into the CE system. If necessary, samples were diluted further with the same solvent.

2.3.4 Capillary electrophoresis conditions

Electrophoresis was carried out on a BioFocus 3000 CE system (Biorad, Hercules, CA, USA) equipped with a multi-wavelength detector operating at 260 nm. The separation took place in a 40 cm (35.4 cm to detector) x 50 µm I.D fused-silica capillary thermostated at 20 ^{O}C. A voltage of 18 kV was applied in a constant and cationic mode for a current of about 19 µA. Samples were hydro-dynamically injected with pressure for 2 p.s.i. * s (1 p.s.i. =6894.76 Pa).

The capillary was conditioned each morning during 5 min with 0.1 mol/L NaOH, then during 5 min with water and finally for 30 min with the running buffer. Between runs, a 2 min wash with the running buffer was performed. At the end of the day, the capillary was rinsed for 5 min with 0.1 mol/L NaOH and for another 15 min with water. The optimized running buffer (optimized for separation of carnitine and acylcarnitines) was 20 mmol/L NaH_2PO_4, 20 mol/L H_3PO_4 and 20 mmol/L SDS in acetonitrile-water (50:50, v/v). Fresh buffer was prepared each day from a 2 mol/L salt aqueous solution and a 1 mol/L SDS aqueous solution. Both inlet and outlet vials were refreshed every two analyses.

2.3.5 Radioenzymatic analysis of urine samples

Urine samples of healthy persons were assayed for free and for total carnitine with the radioenzymatic method as described by Hoppel (Hoppel, 1991).

2.4 RESULTS AND DISCUSSION

2.4.1 Choice of the internal standard

The internal standard has to have a similar behavior in the analytical system as the analytes but should not be present in biological samples. Mildronate (Figure 2), an inhibitor of γ-butyrobetaine hydroxylase, the enzyme catalyzing the last step in carnitine biosynthesis, was chosen due to its structural similarity to carnitine (Tsoko et al., 1995; Galland et al., 1998).

Pilot studies showed that extraction and derivatization properties of mildronate did not differ from carnitine.

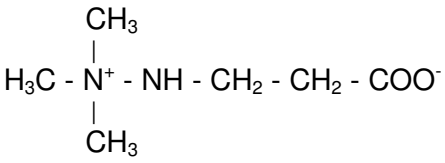


Figure 2: Chemical structure of 3-(2, 2, 2-trimethylhydrazinium)-propionate (mildronate) which was used as an internal standard

2.4.2 Separation of acylcarnitines

Carnitine and acylcarnitines are zwitterionic compounds, which are positively charged in acidic solutions (Figure 1). The derivatization with 4'-bromophenacyl trifluoromethanesulfonate is performed on the carboxyl group of carnitine/acylcarnitines and introduces a permanent positive charge (the charge of the nitrogen in position 4 of carnitine, see Figure 1). Therefore, a positive voltage polarity (anode at the inlet) was chosen. As all analyzed compounds have the same charge, they were expected to be separated mainly according to their molecular mass: the small internal standard shows the fastest migration, followed by carnitine and then by the acylcarnitines in the order of increasing chain length of the acyl group. The molecular masses of the analytes are quite close, however, requiring the use of SDS to widen the separation window. Due to decreased hydrophilicity after introduction of an apolar group with derivatization, an organic solvent had to be added to the running buffer to solubilize all compounds. Non-aqueous or binary running buffers do not only allow analysis of more hydrophobic compounds by solubilization, but also can introduce different selectivity (Sarmini and Kenndler, 1997; Valko et al., 1997). Furthermore, the interaction between analytes and silanol groups appears to be favorably modified.

Acetonitrile was chosen as an organic solvent, as it had proved its efficiency in solubilizing and separating carnitine and acylcarnitines by HPLC (Minkler and Hoppel, 1993b). Different concentrations of acetonitrile in the buffer (initially 10 mmol/L NaH_2PO_4, 10 mmol/L H_3PO_4 and 10 mmol/L SDS) were tested. With 30% (v/v) acetonitrile in water, the solubility of the analytes was insufficient, resulting in very broad peaks. Fifty and 80% (v/v) acetonitrile in starting buffer showed similar separation profiles of carnitine and acylcarnitine standards. A concentration of 50% (v/v) acetonitrile in starting buffer was chosen as a compromise between solubility of carnitine/acylcarnitines and prevention of buffer evaporation during the analysis.

The SDS concentration was varied between 10 to 30 mmol/L. In the presence of 10 mmol/L SDS, peaks were not well separated, whereas in the presence of 30 mmol/L SDS, the migration time increased dramatically. A concentration of 20 mmol/L SDS resulted in an acceptable resolution of the analytes with a short running time. Finally, the phosphate/phosphoric acid concentration of the starting buffer was adjusted to 20 mmol/L in order to sharpen the peaks.

A typical profile of a standard solution of short-chain acylcarnitines is shown in Figure 3. All peaks were attributed to the acylcarnitine indicated by injecting each standard alone. As expected, the migration order corresponds to the order of the molecular masses. The peak attribution was confirmed by the disappearance of the corresponding acylcarnitine peaks and the simultaneous increase in the carnitine peak after hydrolysis, by which all acylcarnitines are converted into carnitine (Figure 4). Figure 3 demonstrates that the five acylcarnitines chosen can be separated from carnitine and internal standard in less than 8 minutes. The separation is acceptable for all analytes assayed except isovalerylcarnitine, which could be resolved hardly from an interfering peak of the derivatizing agent.

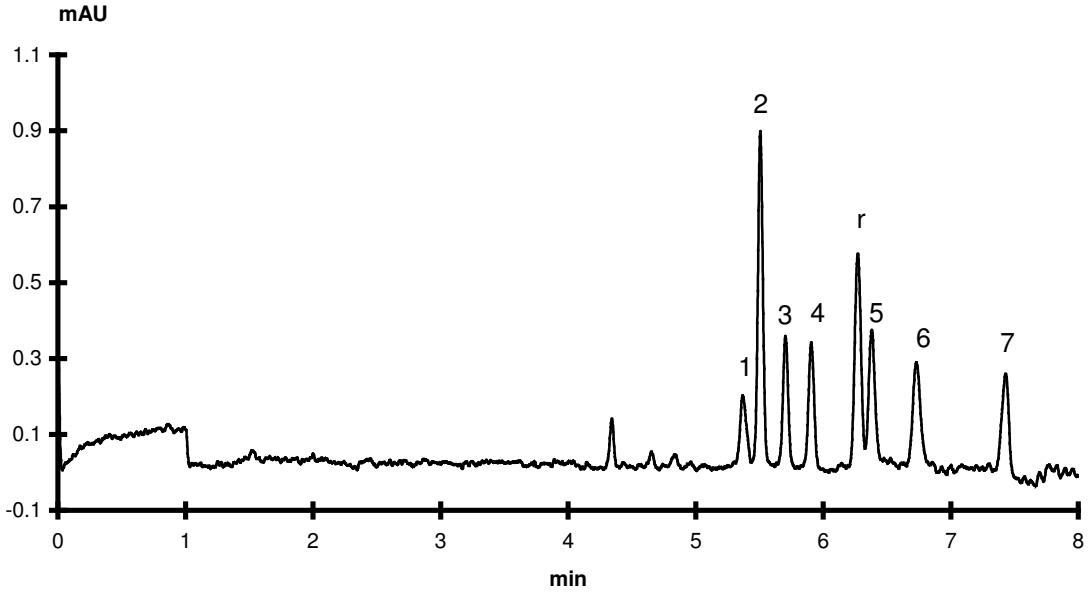


Figure 3: Typical electropherogram of carnitine and short-chain acylcarnitines standards. The concentrations are 100 µmol/L for carnitine/acylcarnitines and 50 µmol/L for the internal standard. The final preparation was diluted 10 times before injection. Capillary electrophoresis conditions are given in the Experimental section. The peaks are: 1 : internal standard, 2 : carnitine, 3 : acetylcarnitine, 4 : propionylcarnitine, 5 : isovalerylcarnitine, 6 : hexanoylcarnitine, 7 : octanoylcarnitine and r : peak from the derivatizing agent.

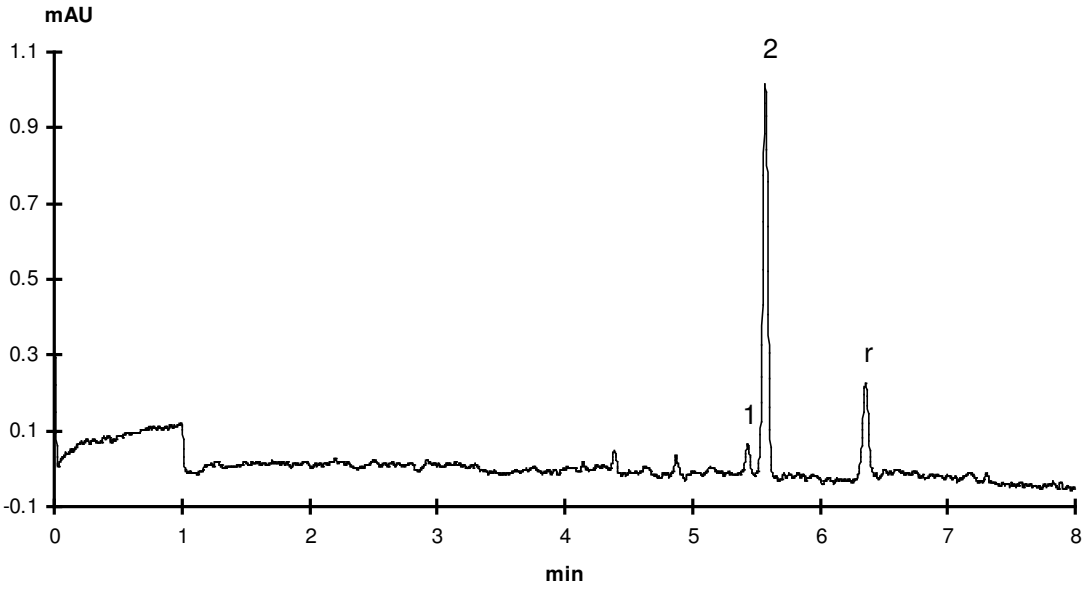


Figure 4: Electropherogram of hydrolyzed carnitine and short-chain acylcarnitine standards. The concentrations are 100 µmol/L for carnitine/acylcarnitines and 50 µmol/L for the internal standard. The final preparation was diluted 10 times before injection. Conditions and peaks as in Figure 3.

2.4.3 Method characterization

The optimized method was characterized for carnitine and acetylcarnitine standards.

The linearity was tested over a concentration range of 5 to 100 µmol/L for both carnitine and acetylcarnitine. Regression lines were calculated with the corrected area/migration time versus concentration. As shown in the Table 1, a good linearity ($r > 0.99$) was obtained for both compounds.

Table 1: Regression data for carnitine and acetylcarnitine standard solutions[a]

	y = ax + b		Correlation
	a	b	r
Carnitine	1.03 ± 0.05	-0.002 ± 0.024	0.999 ± 0.001
Acetylcarnitine	1.10 ± 0.06	0.014 ± 0.026	0.999 ± 0.001

[a] The concentration range was 5-100 µmol/L. n = 5 individual determination for every curve.

The limit of detection, defined as a signal-to-noise ratio of 3, was established by injecting serial dilutions of the corresponding 100 µmol/L standard solutions. For both carnitine and acetyl-carnitine the limit of detection was 3 µmol/L.

The inter-day reproducibility performed over 5 days (complete workup of four samples at four different concentrations) is shown in Table 2. For both compounds the relative standard deviation (RSD) is below 7% for the higher concentrations (25-100 µmol/L) but increases to approximately 20% with the lowest concentration (5 µmol/L). The intra-day reproducibility, also shown in Table 2, was performed by analyzing three individual samples on the same day. The results obtained are similar to the ones obtained for the inter-day assays.

Table 2: Reproducibility of the quantification of carnitine and acetylcarnitine standard solutions[a]

	Concentration (µmol/L)	Corrected Area/Migration time		
		Average	RSD (%)	n
Inter-day				
Carnitine	100	2.092	5.4	5
	50	0.968	2.6	5
	25	0.530	6.6	4
	5	0.110	21.0	4
Acetylcarnitine	100	2.211	5.0	5
	50	1.137	6.5	5
	25	0.577	5.9	5
	5	0.117	16.1	3
Intra-day				
Carnitine	100	2.216	1.5	3
	25	0.635	4.6	3
Acetylcarnitine	100	2.514	6.4	3
	25	0.722	10.2	3

[a] Individual samples of standard solutions were worked up and analyzed on the same or on different days as indicated in the Table.

2.4.4 Application to urine samples

The next step was to analyze urine spiked with a mixture of the same acylcarnitines (Figure 5). The separation of the acylcarnitines added proved to be as efficient as for the standard solutions in Figure 3. Peak attribution was performed by spiking with individual acylcarnitines and by hydrolyzing the spiked urine samples (not shown). An additional peak can be observed in urine which elutes just before the internal standard, is not hydrolyzed and does not interfere with the analytes of interest.

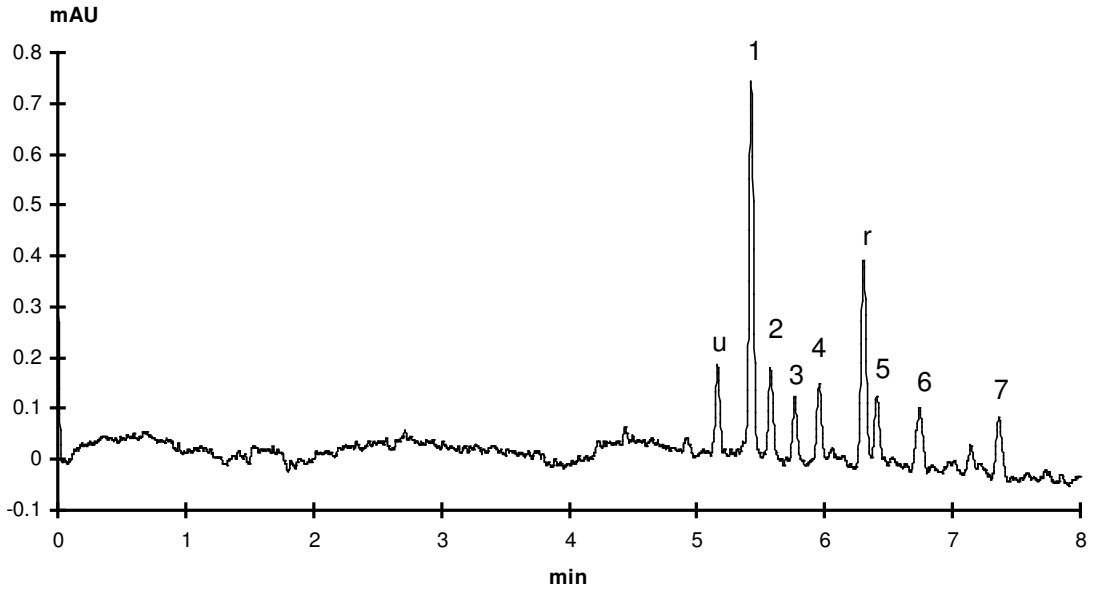


Figure 5: Electropherogram of human urine spiked with 100 µmol/L of each acylcarnitine and 50 µmol/L of internal standard. Conditions and peaks are as in Figure 3. u : represents a peak which is observed with urine samples but not with standard solutions.

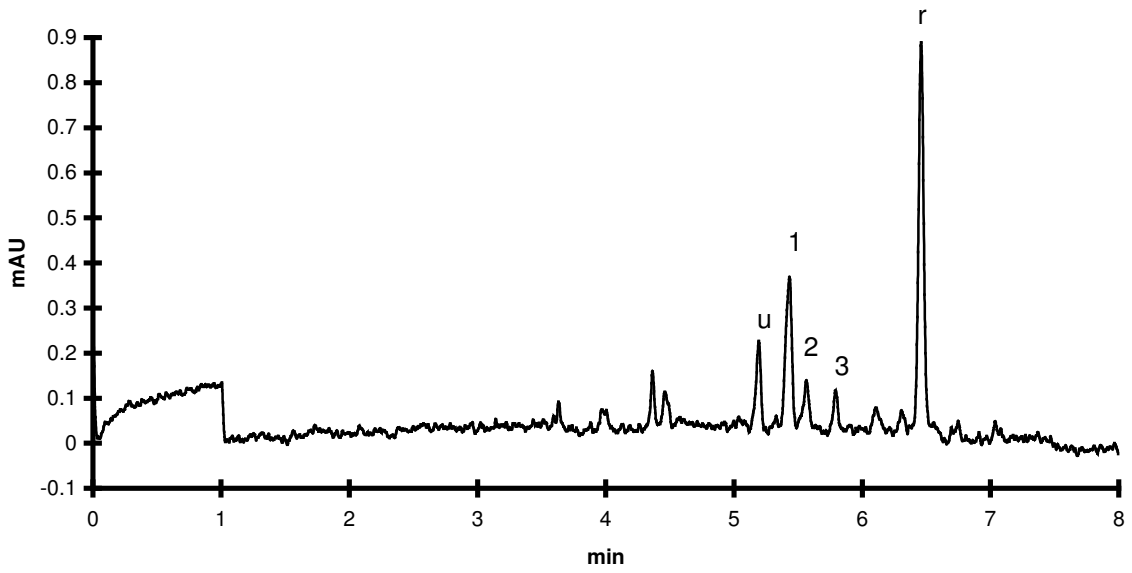


Figure 6: Electropherogram of human urine spiked with internal standard (50 µmol/L). The final preparation was diluted five times before injection. Conditions and peaks are as in Figure 3.

Free and total carnitine were quantified in five urines of healthy persons (Figure 6). The urines were appropriately diluted so that their content in free and total carnitine were in the calibration range. The results were compared with those of the radioenzymatic method and are presented in Table 3. The correlation between the two methods was in an acceptable range (r=0.998 for free and 0.959 for total carnitine). On the other hand, the slopes were different from one (0.77 for free carnitine and 1.55 for total carnitine), indicating that in urine, the free carnitine concentration tends to be over- and the total carnitine concentration underestimated by the current CE method.

Additional urine samples contained variable amounts of propionylcarnitine but no other acylcarnitines (results not shown). Unfortunately, we did not have access to urine samples of patients with organic acidurias.

Table 3: Quantification of free and total carnitine in samples of human urines[a]

Urine sample	Free carnitine (µmol/L)		Total carnitine (µmol/L)	
	CE	REA	CE	REA
1	8.8	10.0	30.4	22.8
2	27.9	40.0	119	112
3	7.6	10.5	14.6	16.5
4	11.0	7.1	137	143
5	113	144	379	233

[a] Free and total carnitine was determined by capillary electrophoresis (CE) and by the radioenzymatic assay (REA) in five human urines.

2.5 CONCLUSION

A new CE method was developed to analyze carnitine and acylcarnitines in standard aqueous solutions and in human urine samples. Seven compounds (carnitine, different acylcarnitines and internal standard) could be separated and identified in less than 8 minutes. In comparison to similar HPLC methods, analysis of carnitine/acylcarnitines by CE is faster and consumes less solvents, rendering CE less expensive. While the current method is usable for separation of carnitine and acylcarnitines, the quantification of carnitine and acylcarnitines should still be improved.

Acknowledgements

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CHAPTER

3

DETERMINATION OF CARNITINE AND ACYLCARNITINES IN URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY – ELECTROSPRAY IONIZATION ION TRAP TANDEM MASS SPECTROMETRY

Laurence Vernez[1], Gérard Hopfgartner[2], Markus Wenk[1], Stephan Krähenbühl[1]*

[1]Division of Clinical Pharmacology & Toxicology, University Hospital of Basel, Switzerland.

[2] Laboratory of Pharmaceutical Analytical Chemistry, University of Geneva, Switzerland.

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3.1 SUMMARY

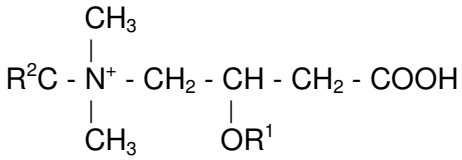
A high-performance liquid chromatography – mass spectrometry method has been developed for the simultaneous determination of native carnitine and eight acylcarnitines in urine. The procedure uses a solid phase extraction on a cation exchange column and the separation is performed without derivatization within 17 min on a reversed-phase C_8 column in the presence of a volatile ion-pairing reagent. The detector was an ion trap mass spectrometer and quantification was carried out in the MS/MS mode. Validation was done for aqueous standards at ranges between 0.75 and 200 µmol/L, depending on the compound. Carnitine was quantified in urine and comparison with a radioenzymatic assay gave a satisfactory correlation (R^2 = 0.981). The assay could be successfully applied to the diagnostic of pathological acylcarnitines profile of metabolic disorders in urines of patients suffering from different organic acidurias.

3.2 INTRODUCTION

Carnitine is an endogenous compound present in most mammalian tissues, reaching particularly high concentrations in skeletal muscle and heart (Bremer, 1983; Friolet et al., 1994). The most important role of carnitine is the transport of fatty acids into the mitochondrial matrix, where they are metabolized via β-oxidation to produce energy (Fritz, 1955; Bremer, 1983). For this purpose carnitine can be esterified on the hydroxyl group in position 3 (see R^1 in Figure 1), generating various acylcarnitines with different chain lengths. A second function of carnitine is the maintenance of the cellular pool of free (unesterified) coenzyme A by the transfer of acyl-groups from acyl-CoAs to carnitine (Brass and Hoppel, 1980; Bremer, 1983). While free carnitine and acetylcarnitine represent the major constituents of the carnitine pool in normal subjects (Bremer, 1983; Friolet et al., 1994), analysis of the different acylcarnitines can provide important information about inherited or acquired metabolic disorders such as organic acidurias (Minkler and Hoppel, 1993b).

Carnitine determination has been performed with numerous methods. A radioenzymatic assay (Hoppel, 1991) allows the specific determination of L-carnitine, whereas high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been used to get enantiomeric separation of D- and L- carnitine (De Witt et al., 1994; Hirota et al., 1994; Vogt and Kiessig, 1996; Mardones et al., 1999a) and to obtain the acylcarnitine profile (Minkler and Hoppel, 1993a; b; Kamimori et al., 1994; Longo et al., 1996; Vernez et al., 2000). The main drawback of these methods is the need of a derivatization step to introduce

a lacking chromophoric group on carnitine and its esters to gain UV or fluorescent absorption. Different chromophores have been used, reacting either on the hydroxyl (De Witt et al., 1994; Vogt and Kiessig, 1996; Mardones et al., 1999a) or on the carboxyl groups (Minkler and Hoppel, 1993a; b; Hirota et al., 1994; Kamimori et al., 1994; Longo et al., 1996; Vernez et al., 2000) of carnitine. Concerning capillary electrophoresis, another approach is to use indirect UV detection (Mardones et al., 1999b). The acylcarnitine profile can also be obtained with gas chromatography (GC) (Kumps et al., 1994).



R^2	R^1	Name	Transitions (m/z)
H_3	H	Carnitine	162.2 → 103.0 + 60.1
D_3	H	Carnitine-d_3	165.2 → 103.0 + 63.1
H_3	$COCH_3$	Acetylcarnitine	204.2 → 144.9 + 85.1
H_3	$COCH_2CH_3$	Propionylcarnitine	218.2 → 158.8 + 85.1
H_3	$COC(CH_3)_3$	Isovalerylcarnitine	246.2 → 186.9 + 85.1
H_3	$CO(CH_2)_4CH_3$	Hexanoylcarnitine	260.2 → 200.9 + 85.1
H_3	$CO(CH_2)_6CH_3$	Octanoylcarnitine	288.2 → 229.0 + 85.1
D_3	$CO(CH_2)_6CH_3$	Octanoylcarnitine-d_3	291.2 → 229.0 + 85.1
H_3	$CO(CH_2)_{12}CH_3$	Myristoylcarnitine	372.3 → 313.0 + 211.0
H_3	$CO(CH_2)_{14}CH_3$	Palmitoylcarnitine	400.4 → 341.1 + 239.1
H_3	$CO(CH_2)_{16}CH_3$	Stearoylcarnitine	428.4 → 369.1 + 267.2

Figure 1: Chemical structures of carnitine and the acylcarnitines analyzed and MS/MS transitions used for quantification.

Mass spectrometry approaches have been introduced first using fast atom bombardment tandem mass spectrometry (FAB-MS/MS) (Millington et al., 1989) and then electrospray tandem mass spectrometry (ESI-MS/MS). ESI-MS/MS is frequently used for screening purposes in newborn blood spots (Chace et al., 1997; Clayton et al., 1998; Inoue et al., 1999; Zytkovicz et al., 2001), where acylcarnitines are usually determined after formation of butyl (Chace et al., 1997; Clayton et al., 1998; Zytkovicz et al., 2001) or methyl (Inoue et al., 1999) esters. This technique allows the determination of amino acids within the same experiment. Quantification is performed with help of a triple stage quadrupole using either multiple reaction monitoring or precursor ion scans of the common acylcarnitines product m/z 85. Mass spectrometry was also used as a detection method after prior separation of carnitine

and acylcarnitines by HPLC (Tallarico et al., 1998; Zhu et al., 2000; Vaz et al., 2002), CE (Heinig and Henion, 1999; Deng et al., 2001) or GC (Huang et al., 1991; Costa et al., 1997). Very recently, a method for the analysis by HPLC-MS/MS of biosynthetic carnitine precursors has been published (Vaz et al., 2002). The method uses derivatization with methyl chloroformate for the extraction and an ion pair reagent for the HPLC separation. So far, quantification of carnitine and acylcarnitines was performed with quadrupole mass spectrometers, single or triple, except in one study in which an ion trap method was used (Zhu et al., 2000).

Since separation and quantification of carnitine and acylcarnitines in urine has currently not been described for ion trap systems, we decided to develop such a method. We describe a novel reversed-phase HPLC separation of un-derivatized carnitine and eight biological relevant acylcarnitines using an electrospray ion trap mass spectrometer detector. We will discuss the application of this system to urine samples of volunteers as well as of patients suffering from organic acidurias and describe the problems we were facing for quantification with an ion trap system.

3.3 EXPERIMENTAL

3.3.1 Chemicals

L-carnitine was from Fluka (Buchs, Switzerland), and acetylcarnitine, hexanoylcarnitine, octanoylcarnitine, myristoylcarnitine, palmitoylcarnitine and stearoylcarnitine were from Sigma (St. Louis, MO, USA). Propionylcarnitine and isovalerylcarnitine were a gift from Sigma-Tau (Zofingen, Switzerland). The deuterated internal standards [$^2H_3$]carnitine (carnitine-d_3) and [$^2H_3$]octanoylcarnitine (octanoylcarnitine-d_3) were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Methanol and water were of HPLC grade from Merck. Other reagents were of analytical grade and purchased either from Fluka or from Merck.

3.3.2 Preparation of internal standards and standard solutions

Internal standard stock solutions of 1 mmol/L carnitine-d_3 and 1 mmol/L octanoylcarnitine-d_3 were prepared in methanol. A mixed internal standard spiking solution was prepared by diluting these stock solutions to have respective concentrations of 100 and 20 µmol/L.

Standard stock solutions of carnitine and acylcarnitines for calibration were prepared in methanol in concentrations between 5 and 20 mmol/L. A mix standard stock solution was prepared by diluting the respective stock solutions to obtain a solution containing all acylcarnitines of concentrations ranging from 80 to 800 µmol/L. This solution was further diluted in methanol to obtain the calibration standard solutions. The carnitine concentration was standardized using a spectrophotometric method (Marquis and Fritz, 1964). Carnitine contamination due to spontaneous hydrolysis was estimated for each acylcarnitine by injecting the individual standard stock solution and measuring the respective peaks area. The carnitine content was not found to be relevant in any of them.

3.3.3 Urine sample collection

Urine samples were obtained from the chemical laboratory of the University Hospital of Basel. One of these urines was used as a quality control sample and to establish calibration curves. Patient urines were obtained from the Children Hospital in Basel.

3.3.4 Urine sample preparation

Urine pretreatment included dilution, acidification and solid phase extraction. Urine (200 µL) was mixed with 50 µL internal standard spiking solution and 50 µL 1 mol/L HCl. For spiked urine samples, 50 µL of the appropriate standard stock solution was also added. The volume was adjusted to 1 mL with water. The solid-phase extraction was performed as described by Kamimori et al. (Kamimori et al., 1994), after some modifications, using disposable cation-exchange columns (SCX 100 mg, Varian, Harbor City, CA, USA) and a Vac-Elut system (Analytichem, Harbor City, CA, USA). After preconditioning with 1 mL methanol, 1 mL water and 1 mL of 10 mmol/L aqueous HCl, samples (1 mL) were loaded, washed with 3 mL of 10 mmol/L aqueous HCl and eluted with 2 mL of 75 mmol/L pyridine in methanol-water (1:1, v/v). The eluate was collected in silanised glass tubes an evaporated to dryness at 40 ^{0}C using a TurboVap evaporator (Zymark, Hopkinton, MA, USA). The residue was dissolved in 1 mL methanol and reevaporated. The residue was reconstituted in 200 µL methanol-water (1:1, v/v) containing both 10 mmol/L heptafluorobutyric acid and 10 mmol/L ammonium acetate.

3.3.5 HPLC-MS/MS analysis

The HPLC separation was performed on a HP 1100 system (Agilent Technologies, Palo Alto, CA, USA) equipped with a heated column compartment. The analytical column was a reversed-phase LUNA, C_8, 150 x 2.0 mm I.D. (5 µm particles) (Phenomenex, Torrance, CA, USA), equipped with a corresponding pre-column MOS, C_8, 4.0 x 2.0 mm (Phenomenex). The column was maintained at 30^0C. A gradient elution with two mobile phases was used: 10 mmol/L heptafluorobutyric acid and 10 mmol/L ammonium acetate in water (A) or in methanol (B). The gradient started with 20% B, went up to 90% B between 0.1 min and 4 min and was followed by a plateau at 90% B for 14 minutes. The column was then reequilibrated for 10 minutes with 20% B. The flow was set at 200 µL/min and the injected sample volume was 2 µL.

Mass spectrometry detection was performed on a Finnigan LCQ^{DECA} ion trap instrument (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization source that was run in the positive ion mode (4.0 kV). Sheath gas (N_2) pressure was set at 70 arbitrary units. The temperature of the heated capillary was kept at 300 ^{0}C. Quantitative analysis of carnitine and acylcarnitines was monitored in MS-MS mode. For this purpose each of the nine peaks chromatographed was associated with a segment. During the analysis time of each segment, one precursor ion was stored in the trap and further fragmented with a collision energy that allowed a sufficient fragmentation of the selected ion. In the case of carnitine and octanoylcarnitine, as their respective deuterated derivative used as internal standards are co-eluting, two ions were attributed to their respective segment. The relative collision energy was set up at 35% for each compound, the isolation width fixed at 1.8 and the product ions were scanned. Automatic gain control was employed using one microscan and a maximum injection time of 200 ms. Carnitine-d_3 was used as internal standard at a concentration of 20 µmol/L for carnitine, acetylcarnitine and propionylcarnitine, whereas octanoylcarnitine-d_3 was used as internal standard at a concentration of 5 µmol/L for all other acylcarnitines. Areas obtained for the two most intense product ions (a common and a specific one) were corrected with the respective areas of the internal standard and were used for quantification. The observed transitions are given in Figure 1. The HPLC and MS system were computer controlled using Xcalibur 1.2 software (Finnigan). The same software was also used for all quantitative calculations. Linear regressions were calculated with a weighting factor of 1/X.

3.3.6 Method validation

Linearity of the developed HPLC-MS/MS method was investigated for carnitine and five short- and medium-chain acylcarnitines on aqueous standards. Calibrations (six points) were prepared in concentration ranges of 5-200 µmol/L for carnitine, 2.5-100 µmol/L for acetylcarnitine and 0.75-30 µmol/L for propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine and octanoylcarnitine. Precision and accuracy were determined for carnitine, acetyl-, propionyl-, isovaleryl-, hexanoyl- and octanoylcarnitine by running aqueous quality controls at three different concentrations covering the calibration range, on the same (intra-day) and on different days (inter- day variability). Urine quality controls were run simultaneously at two different concentrations, namely a blank (unspiked) urine and the same urine after spiking with carnitine, acetyl-, propionyl-, isovaleryl-, hexanoyl- and octanoylcarnitine.

3.3.7 Urine

Blank and spiked urines were extracted as described above. Carnitine quantitation results of quality control urine obtained with an aqueous standard calibration were compared with the one obtained with a calibration done with spiked urine. Free carnitine was determined in 15 different urine samples and the results were compared with radioenzymatic assay. The radioenzymatic assay used has been described by Hoppel (Hoppel, 1991).

3.4 RESULTS AND DISCUSSION

Carnitine and acylcarnitines belong to a family of compounds with a large span of polarities, from the polar carnitine to the quite apolar long-chain acylcarnitines, such as for instance stearoylcarnitine. From these characteristics one can easily presume that their simultaneous extraction from a biological matrix and their chromatographic separation will be a rather delicate task. For instance, it is not possible to use the common C_8 or C_{18} reversed-phase material, because carnitine and short-chain acylcarnitines are not properly chromatographied under these conditions. So far, the use of reversed-phase HPLC was used after a derivatization step that does not only introduce a chromophore group for UV or fluorescence detection, but also decreases the polarity of carnitine, enabling thereby its retention on such material. To develop our method we used the zwitterionic feature of these analytes for their

extraction and exploited the formation of ion-pair for their separation on reversed phase material.

3.4.1 Optimization of solid-phase extraction

In acidic conditions the carboxylic group of analytes (see Figure 1) is protonated, resulting in the presence of permanently positively charged quaternary amines. Under these conditions, the choice of a cation exchange column for the extraction is particularly well suited. According to the literature, ammonia (Kumps et al., 1994) and pyridine (Kamimori et al., 1994) were evaluated to displace carnitine and its esters from the column. The entire extraction process was investigated using [^{14}C]-carnitine and [^{14}C]-palmitoylcarnitine, a hydrophilic and a lipophilic representant of the analyte family. As we were facing hydrolysis of acylcarnitines during the extraction process when using ammonia, what was absolutely not desired, we changed to pyridine. The minimal effective concentration able to displace in a satisfactory way both analytes was found to be at 75 mmol/L pyridine in a water-methanol (1:1, v/v) mixture. Recovery for [^{14}C]-carnitine was 75 and 73% for [^{14}C]-palmitoylcarnitine. Silanised glass tubes were used to collect the eluate in order to prevent interactions of positively charged carnitines with the hydroxyl groups of glass. It was impossible to resolubilize the dried eluate using the starting HPLC mobile phase, since with 20% methanol the long-chain acylcarnitines could not be brought into solution. We, therefore, used a water-methanol (1:1, v/v) mixture, containing 10 mmol/L heptafluorobutyric acid and 10 mmol/L ammonium acetate.

3.4.2 Analysis of carnitine and acylcarnitines by HPLC-MS/MS

For the HPLC separation we investigated different volatile ion-pairing agents, in order to mask the positive charges on the nitrogen atom and to increase the interactions of the polar compounds with reversed-phase material. While the addition of trifluoroacetic acid was not successful (carnitine was still not retained by the C_8 column), heptafluorobutyric acid at an optimal concentration of 10 mmol/L showed enough ion-pairing interactions in order to get retention of carnitine and acetylcarnitine. The pH of the eluant was fixed to 3.2 with 10 mmol/L ammonium acetate, as a more acidic mobile phase was not required to increase the number of positive charge and in order to protect the column material. With this composition of the mobile phase and using a gradient, from 20 to 90% organic solvent, to increase the elution strength, we could elute stearoylcarnitine within 17 min. A typical chromatogram

obtained under the developed conditions, recorded in MS mode, shows a good baseline separation of carnitine and eight acylcarnitines (see Figure 2). With higher concentrations of heptafluorobutyric acid, as described in the literature for the determination of acetylcholine and related quaternary amines such as carnitine and acetylcarnitine (Zhu et al., 2000) or, most recently, for the determination of carnitine and carnitine precursors (Vaz et al., 2002), the retention times of long-chain acylcarnitines increases without any further benefit for the retention of carnitine and acetylcarnitine. As samples had to be solubilized in a water-methanol (1:1, v/v) mixture, their elution strengths were higher than the one of the mobile phase and we were facing peak broadening, especially for carnitine and acetylcarnitine. By reducing the injection volume from 10 to 2 µL, the resulting peak sharpening compensated for the water-methanol (1:1, v/v) mixture, and we did not lose to much sensitivity, since the number of scans through the peaks was still sufficient.

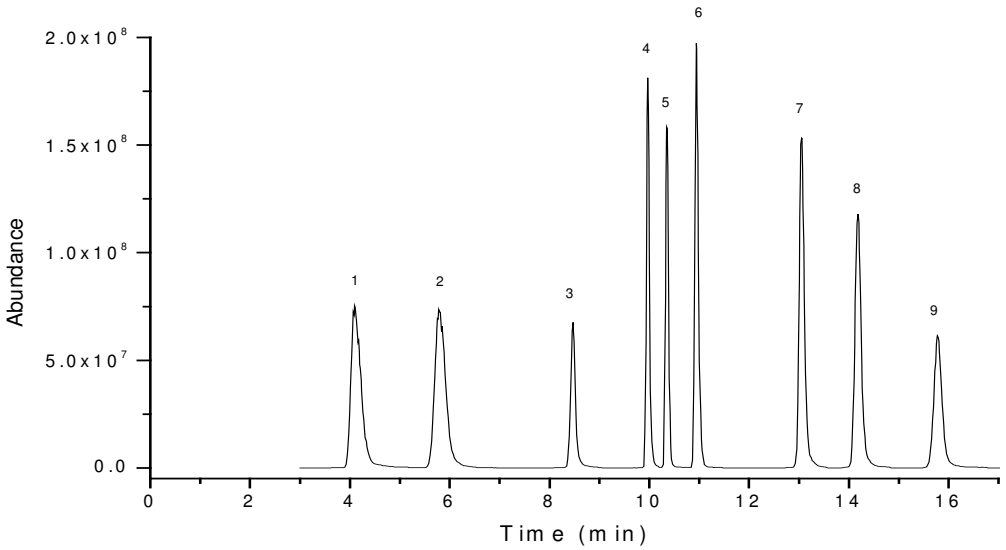


Figure 2: Total ion current trace of a mixed standard containing: 1, carnitine (80 µmol/L; 160 pmol injected); 2, acetylcarnitine (40 µmol/L; 80 pmol injected); 3, propionylcarnitine (12 µmol/L; 24 pmol injected); 4, isovalerylcarnitine (12 µmol/L; 24 pmol injected); 5, hexanoylcarnitine (12 µmol/L; 24 pmol injected); 6, octanoylcarnitine (12 µmol/L; 24 pmol injected); 7, myristoylcarnitine (8 µmol/L; 16 pmol injected); 8, palmitoylcarnitine (8 µmol/L; 16 pmol injected); 9, stearoylcarnitine (8 µmol/L; 16 pmol injected).

The electrospray ionization process shows a predominant protonated molecular ion of $[M + H]^+$ for all analyzed compounds (data not shown). The formation of these positively charged molecular ions may also be supported by the presence of heptafluorobutyric acid in the mobile phase. These peaks were selected as precursor ions to undergo collision induced dissociation in the ion trap and produce the MS/MS data. The fragmentation pattern of these ions follows a similar process, namely loss of the trimethylamine moiety (see Figure 3).

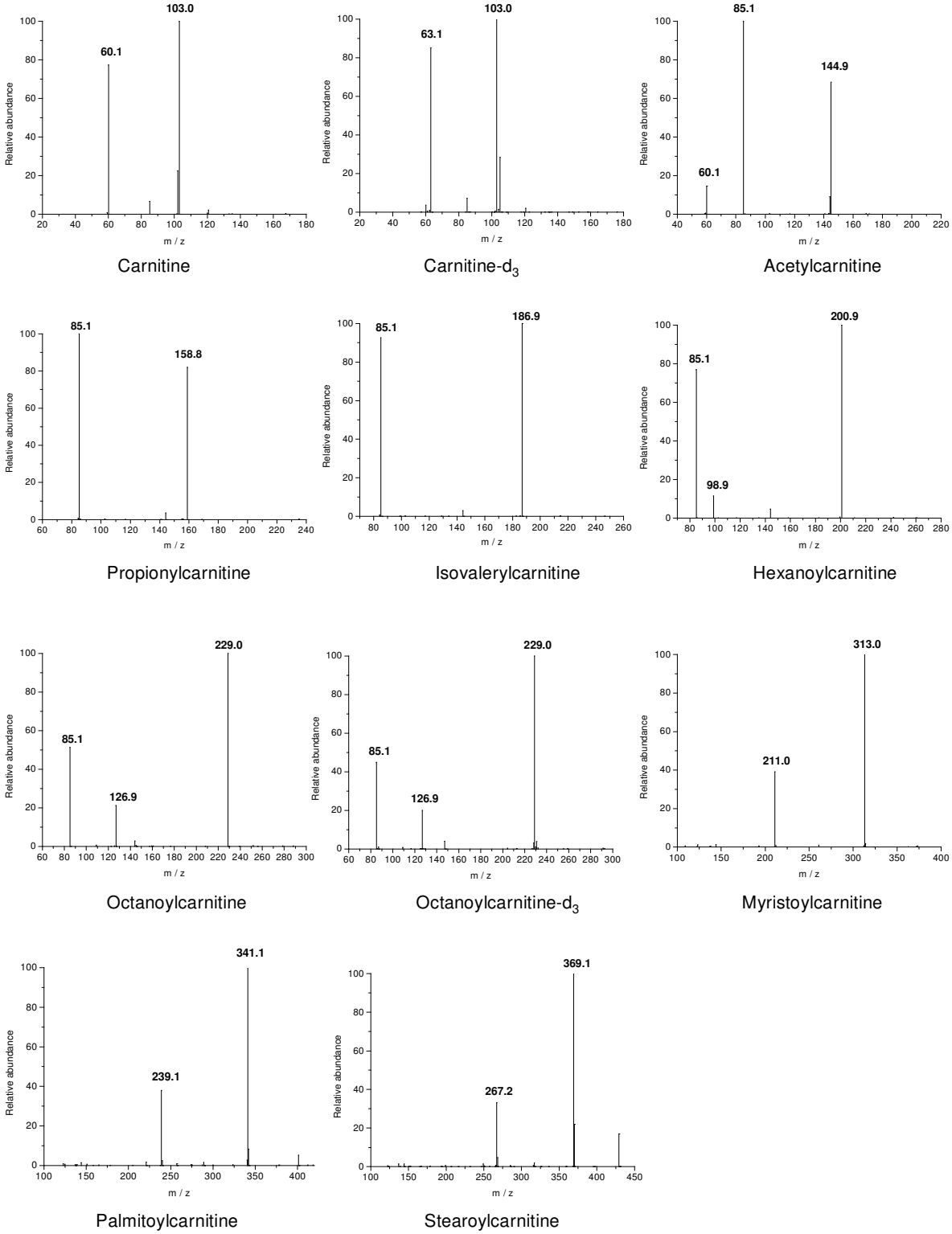


Figure 3: Product ion spectra (MS/MS) of analyzed compounds with relative collision energy of 35% and an isolation width of 1.8.

While the corresponding m/z 60 ion is seen for carnitine and acetylcarnitine, only the complementary ions $[M+H - N(CH_3)_3]^+$, that are compound-specific, are seen for all the other acylcarnitines. In addition for short- and medium-chain acylcarnitines, a common fragment at m/z 85 is present, corresponding to a rearrangement of the butyric acid chain ($^+CH_2$-CH=CH-COOH), as suggested in the literature (Tallarico et al., 1998; Heinig and Henion, 1999). This common fragment ion is the most intense for the short-chain acylcarnitines, whereas the specific fragment ion $[M+H - N(CH_3)_3]^+$ is the most intense for the medium-chain acylcarnitines. With a chain length larger than six carbons, the fragmentation process produces the specific ion (due to the loss of trimethylamine), and a less intense ion corresponding to the fatty acid moiety itself (R^1 in Figure 1).

3.4.3 Method validation

Quantification was carried out in MS/MS mode using two product ions, a common and a specific one, as in the fragmentation patterns these two ions were of relative intensities between 40 to 90%. This way we could gain selectivity. Although carnitine exhibits a lower electrospray sensitivity than the acylcarnitines, the resulting increase in the limit of quantification does not disturb its determination in biological matrix, since the urine concentrations of carnitine are usually higher than that of acylcarnitines. We did not perform any quantification experiments on long-chain acylcarnitines because they are usually not present in urine (Hoppel, 1991).

The linearity of the method was evaluated for carnitine and five short- and medium-chain acylcarnitines on aqueous standards. Ranges were 5-200 µmol/L for carnitine, 2.5-100 µmol/L for acetylcarnitine and 0.75-30 µmol/L for propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine and octanoylcarnitine. Limits of quantification (LOQs) were determined as the lowest concentration with a relative deviation of replicate runs of less than 20%. They correspond to 5 µmol/L for carnitine, 2.5 µmol/L for acetylcarnitine and 0.75 µmol/L for propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine and octanoylcarnitine, respectively, and were chosen as the lowest concentration of the respective calibration standards. For quantification, areas of the 2 most intense product ions were used after division by the respective areas of the selected internal standard. The linearity of the method was validated by recalculating the aqueous calibration standard concentrations (n=6) with the obtained linear regression. Results for carnitine are given in Table 1. With precisions smaller than 8% and accuracies of 99.2 ± 9.3% we found these results within acceptable limits. Precision and accuracy data for intra- and inter-day assays on aqueous quality controls were performed at

three different concentrations, covering the concentration range of the calibration standards used. For intra-day analysis, the precision was between 1.2 and 8.0% for carnitine and acylcarnitines, whereas the values for inter-day precision were between 2.7 and 14.6%. Results for carnitine are presented in Table 2.

Table 1: Accuracy and precision for the determination of carnitine in calibration standards

Added concentration (µmol/L)	Found concentration (µmol/L)	SD	Precision (%)	Accuracy (%)
5	5.8	0.4	7.2	116.5
10	9.8	0.6	5.7	98.0
20	17.8	1.3	7.1	89.2
50	46.9	3.5	7.4	93.7
100	100.4	4.5	4.5	99.6
200	204.2	3.5	1.7	97.9

n = 6 determinations

Table 2: Intra- and inter-day variability of free carnitine in aqueous standard and in urine

	Inter-day assay (n=12)			Intra-day assay (n=7)		
	Low	Medium	High	Low	Medium	High
Standard quality control						
Added concentration (µmol/L)	5	80	150	15	80	150
Found concentration (µmol/L)	13.6	78.3	150.9	14.7	86.0	150.3
Precision (%)	5.3	8.1	10.9	1.2	2.7	2.7
Accuracy (%)	91.0	97.8	100.6	98.0	107.5	100.2
	Inter-day assay (n=4)			Intra-day assay (n=8)		
	Unspiked	Spiked		Unspiked	Spiked	
Urine quality control						
Added concentration (µmol/L)	0	50		0	60	
Found concentration (µmol/L)	63.7	111.5		63.8	127.6	
Precision (%)	6.0	4.0		1.8	6.1	
Accuracy (%)	nd	95.7		nd	106.3	

nd: not determined.

3.4.4 Application to urine

A chromatogram obtained for a urine sample is shown in Figure 4A. We see a typical urine profile with predominant peaks of carnitine (peak 1) and acetylcarnitine (peak 2). Depending on the urine concentration, propionylcarnitine (peak 3) and isovalerylcarnitine (peak 4) can also occasionally been seen. In contrast to standards where the method could easily be validated with acceptable intra- and inter-day precisions and accuracies for all compounds, we were facing more difficulties using urine, where only the quantification of carnitine was validated.

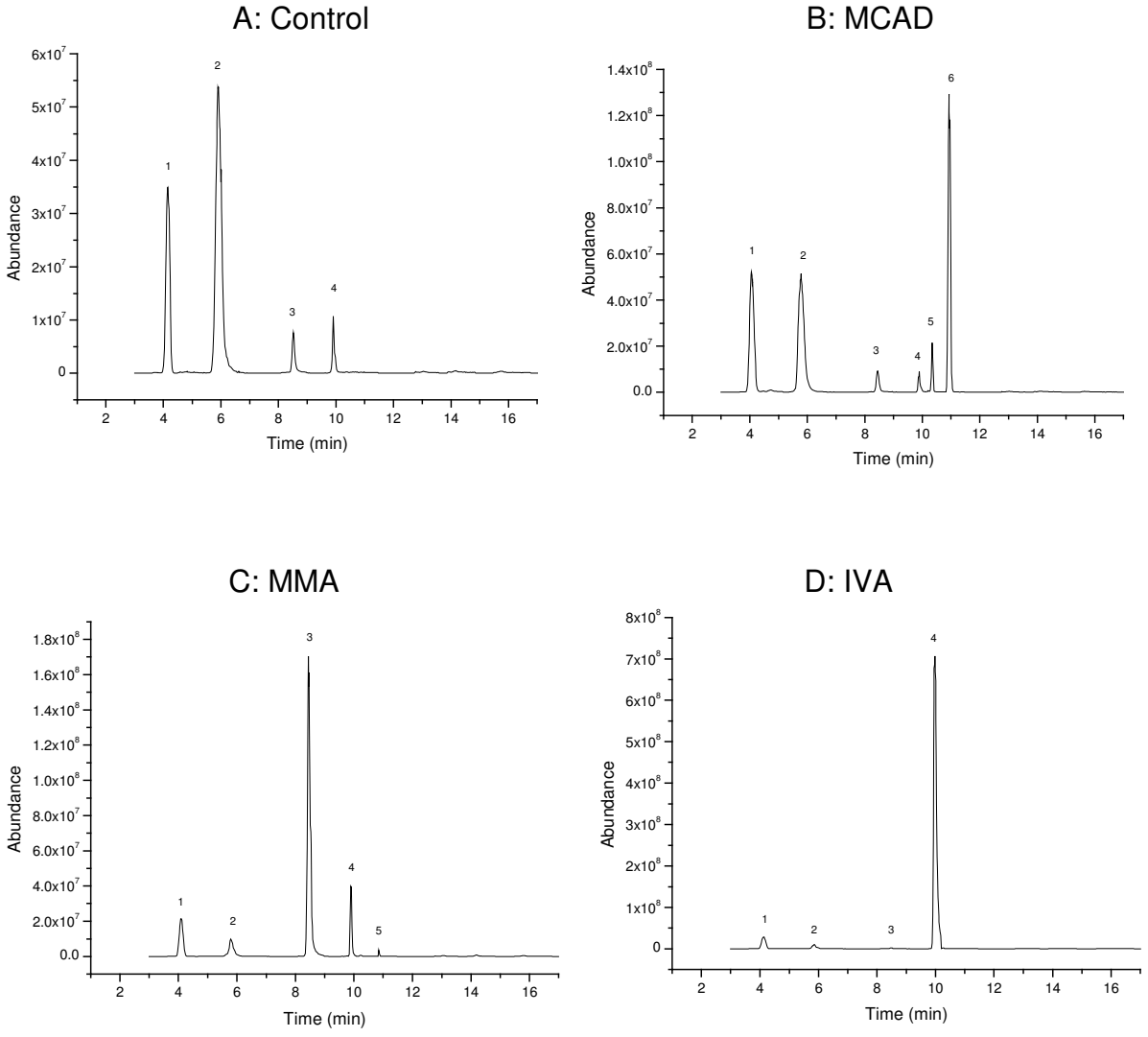


Figure 4: Total ion current trace. A: Control urine. B: Medium chain acyl-CoA dehydrogenase deficiency (MCAD). C: Methylmalonic aciduria (MMA). D: Isovaleric academia (IVA). The samples were extracted and analyzed as described in the Materials and methods section. Peaks are: 1, carnitine; 2, acetylcarnitine; 3, propionylcarnitine; 4,isovalerylcarnitine; 5, hexanoylcarnitine; 6, octanoylcarnitine.

As already mentioned, carnitine and acylcarnitines are endogenous compounds, rendering it impossible to get carnitine-free urine. The presence of endogenous carnitine and acylcarnitines makes it difficult to use urine as a matrix for calibration standards. To facilitate the determination of carnitine in urine, we therefore investigated the possibility of using a calibration curve performed with aqueous calibration standards instead of spiked urine. As compared to calibration standards in urine, aqueous calibration standards yielded similar results, with deviations of 5.6 or 3.0% for non-spiked or spiked urine, respectively (n = 8 determinations for each condition). These results indicated that urine could be substituted with water to prepare the calibration standards. Further experiments showed also that the calibration curves of standards undergoing extraction and HPLC-MS/MS were not different to curves of standards undergoing HPLC-MS/MS only (results not shown), as the use of an internal standard compensates for any extraction loss. Intra- and inter-day variabilities for unspiked and spiked urine quality controls are shown in Table 2 and are, with precisions smaller than 6.1%, as good as for standard quality controls.

The quantification of free carnitine in 15 urines was finally compared with the standard radioenzymatic method (Figure 5) and a linear relationship is observed (R^2 = 0.981). However, the concentrations found with the HPLC-MS/MS method were approximately 10% lower than the ones obtained with the radioenzymatic assay and there was a positive intercept on the x-axis. Taking into consideration the quantification of carnitine in calibration standards (Table 1) and the recovery of carnitine added to urine (Table 2), the problem appears to be more likely due to the radioenzymatic assays than to the HPLC-MS/MS assay. For acylcarnitines, the intra-day precisions were below 15% except for octanoylcarnitine, which had a variability of 40%. As for carnitine, the inter-day variabilities for acylcarnitines were higher than intra-day, with variabilities in the range of 20% for most of them. The use of only two internal standards for quantification of all analytes is probably associated with an increased variability, as observed also by others (Vaz et al., 2002). Better results might be observed with a deuterated derivative for each analyzed acylcarnitine, since the suppression effects would be the same for each compound and its internal standard, as they will enter the trap under the same conditions of ion trap gating and HPLC mobile phase composition.

Figure 4B-D displays urine HPLC chromatograms from patients suffering from different metabolic disorders. Figure 4B is from a patient with medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. As expected with this metabolic disorder (Chace et al., 1997), the peaks for octanoylcarnitine and hexanoylcarnitine are increased (peak 6 and 5, respectively) and the ratio octanoylcarnitine/acetylcarnitine is higher than 0.1. Figure 4C depicts a chromatogram of a patient suffering from methylmalonic acidemia (MMA) where the

propionylcarnitine peak (peak 3) is more than 8 fold increased when compared with a control urine (Zytkovicz et al., 2001). Figure 4D shows a urine of a patient with isovaleric acidemia (IVA). As expected, the isovalerylcarnitine peak (peak 4) is increased.

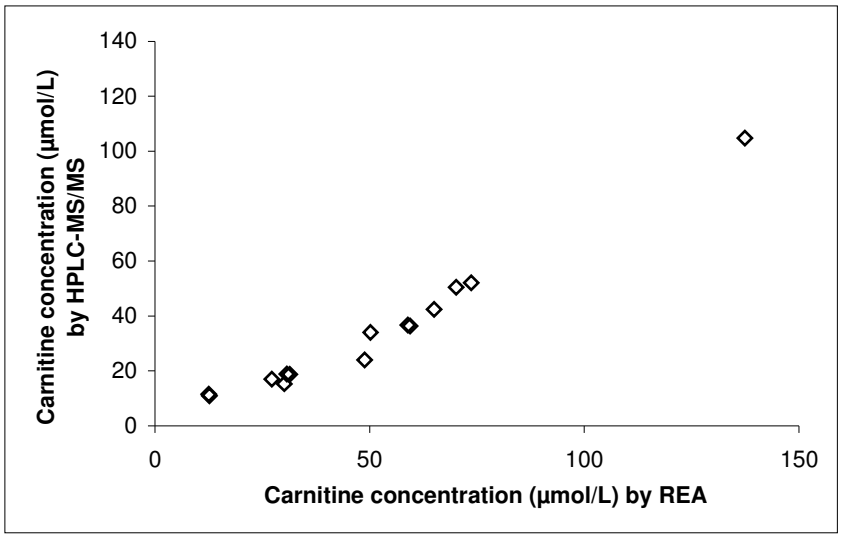


Figure 5: Correlation between the quantification of free carnitine in urine, using a radioenzymatic method (REA) and HPLC-MS/MS. The relationship is characterized by the following equation $y = 0.909\ x - 7.32$ ($R^2 = 0.9806$).

3.5 CONCLUSION

In this paper we presented a reversed-phase HPLC separation of un-derivatized carnitine and eight acylcarnitines with ion trap mass spectrometry detection. The method is based on the detection of common and specific daughter ions produced by collision-induced dissociation of the molecular cation. This method could be applied successfully to the identification and quantification of carnitine and acylcarnitines. The application to patient urines proved that the developed HPLC-MS/MS method is capable to identify metabolic disorders and can therefore be used for diagnostic purposes in a non-invasive way. The application of this method to plasma and tissue samples and the extension towards quantification of long-chain acylcarnitines is currently investigated.

Acknowledgements

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CHAPTER

4

DETERMINATION OF CARNITINE AND ACYLCARNITINES IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY – ELECTROSPRAY IONIZATION ION TRAP TANDEM MASS SPECTROMETRY

Laurence Vernez, Markus Wenk, Stephan Krähenbühl

Division of Clinical Pharmacology & Toxicology and Department of Research,
University Hospital of Basel, Switzerland.

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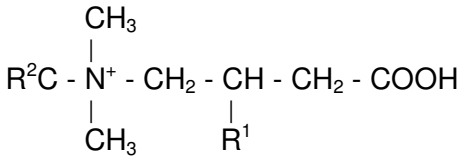
4.1 SUMMARY

A high-performance liquid chromatography-mass spectrometry method was developed for the determination of carnitine, its biosynthetic precursor butyrobetaine and eight acylcarnitines in plasma. The procedure includes a solid-phase extraction for carnitine, short- and medium-chain acylcarnitines and a liquid-liquid extraction for protein-bound long-chain acylcarnitines, followed by separation on a reversed-phase column in the presence of a volatile ion-pairing reagent. Detection was carried out using an ion-trap mass spectometer run in the MS/MS mode. The choice of the matrix for calibrators used for quantification of these endogenous compounds was also investigated. Validation was performed for standard quality controls diluted with 4% bovine serum albumin solution and for spiked plasma quality control samples at concentrations between 0.5 and 80 µmol/L, depending on the compound. Intra- and inter-day precisions for the determination of carnitine were below 3.4% and accuracies between 95.2 and 109.0%. Application of the method for the diagnosis of pathological acylcarnitine profiles of metabolic disorders in a patient suffering from methylmalonic aciduria is presented. The method allows quantification of carnitine, butyrobetaine, acetylcarnitine and propionylcarnitine and semi quantitative analysis of medium- and long-chain acylcarnitines. In comparison to other methods, no derivatization step is needed.

4.2 INTRODUCTION

Carnitine (3-hydroxy-4-N-trimethylaminobutyrate) is a small, ubiquitous molecule in mammalian tissues, mainly located in skeletal muscle and heart (Bremer, 1983; Friolet et al., 1994). Carnitine has a critical role in energy metabolism, as it is an essential factor for transport of fatty acids into the mitochondrial matrix, where they are metabolized via β-oxidation (Bremer, 1983). Fatty acids bind carnitine by the formation of an ester on the hydroxyl group in position 3 (see R^1 in Figure 1), generating various acylcarnitines with different chain lengths. Carnitine is also involved in the maintenance of the cellular pool of unesterified coenzyme A (CoA), by the transfer of acyl-groups from acyl-CoAs to carnitine (Brass and Hoppel, 1980). In normal subjects, carnitine and acetylcarnitine represent the major constituents of the body fluid and tissue carnitine pools (Bremer, 1983; Friolet et al., 1994). Under pathological conditions such as acquired or inherited metabolic disorders, other acylcarnitines can accumulate (Minkler and Hoppel, 1993b). For diagnostic purposes, profiling of the acylcarnitines in body fluids is therefore beneficial.

Several methods have been developed for the determination of carnitine and acylcarnitines. Beside the radioenzymatic method (Hoppel, 1991), high-performance liquid chromatography (HPLC) (Minkler and Hoppel, 1993a; De Witt et al., 1994; Kamimori et al., 1994; Longo et al., 1996) and capillary electrophoresis (CE) (Vogt and Kiessig, 1996; Mardones et al., 1999a; Vernez et al., 2000) have been used for enantiomeric separation of D- and L-carnitine and to obtain acylcarnitine profiles. Due to the lack of a chromophoric group, detection is performed by UV or fluorescence, after derivatization with different chromophores.



R^2	R^1	Name	Transitions (m/z)
H_3	OH	Carnitine	162.2 → 103.0 + 60.1
D_3	OH	Carnitine-d_3	165.2 → 103.0 + 63.1
H_3	H	Butyrobetaine	146.2 → 87.0 + 60.1
H_3	$OCOCH_3$	Acetylcarnitine	204.2 → 144.9 + 85.1
H_3	$OCOCH_2CH_3$	Propionylcarnitine	218.2 → 158.8 + 85.1
H_3	$OCOC(CH_3)_3$	Isovalerylcarnitine	246.2 → 186.9 + 85.1
H_3	$OCO(CH_2)_4CH_3$	Hexanoylcarnitine	260.2 → 200.9 + 85.1
H_3	$OCO(CH_2)_6CH_3$	Octanoylcarnitine	288.2 → 229.0 + 85.1
D_3	$OCO(CH_2)_6CH_3$	Octanoylcarnitine-d_3	291.2 → 229.0 + 85.1
H_3	$OCO(CH_2)_{12}CH_3$	Myristoylcarnitine	372.3 → 313.0 + 211.0
H_3	$OCO(CH_2)_{14}CH_3$	Palmitoylcarnitine	400.4 → 341.1 + 239.1
D_3	$OCO(CH_2)_{14}CH_3$	Palmitoylcarnitine-d_3	400.4 → 341.1 + 239.1
H_3	$OCO(CH_2)_{16}CH_3$	Stearoylcarnitine	428.4 → 369.1 + 267.2

Figure 1: Chemical structures of carnitine, butyrobetaine and the acylcarnitines analyzed and MS/MS transitions used for quantification.

In order to improve detection and identification of acylcarnitines, methods using mass spectrometry were developed. Fast atom bombardment tandem mass spectrometry (FAB-MS/MS) was used first for the determination of carnitine and acylcarnitines as methyl esters (Millington et al., 1989), followed by electrospray tandem mass spectrometry (ESI-MS/MS) of their methyl or butyl esters (Chace et al., 1997; Inoue et al., 1999; Zytkovicz et al., 2001). Although this technique became the method of choice for the determination of carnitine and acylcarnitines in the field of newborn screening (Hardy et al., 2001; Carpenter and Wiley, 2002), the results should be interpreted with caution, since the derivatization with butanol

might be only partial and hydrolysis of acylcarnitines could occur (Johnson, 1999; Ho et al., 2003).

In the present paper, we describe the adaptation and validation of our previously developed HPLC-MS/MS method in urine (Vernez et al., 2003) for the determination of carnitine, butyrobetaine and acylcarnitines in plasma.

4.3 EXPERIMENTAL

4.3.1 Chemicals

L-carnitine was obtained from Fluka (Buchs, Switzerland), butyrobetaine (4-trimethylaminobutyrate), acetylcarnitine, hexanoylcarnitine, octanoylcarnitine, myristoylcarnitine, palmitoylcarnitine, stearoylcarnitine and bovine serum albumin were from Sigma (St. Louis, MO, USA). Propionylcarnitine and isovalerylcarnitine were a gift from Sigma-Tau (Zofingen, Switzerland). The deuterated internal standards carnitine-d_3, octanoylcarnitine-d_3 and palmitoylcarnitine-d_3 were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Methanol, ethyl acetate and water were of HPLC grade from Merck (Darmstadt, Germany). Other reagents were of analytical grade and purchased either from Fluka or from Merck.

4.3.2 Instrumentation

The HPLC separation system consisted of a HP 1100 series (Agilent Technologies, Palo Alto, CA, USA) including a heated column compartment. The analytical column was a reversed-phase LUNA C_8, 150 X 2.0 mm, 3 µm (Phenomenex, Torrance, CA, USA), equipped with the corresponding pre-column. Mass spectrometry detection was performed on a Finnigan LCQ^{DECA} ion trap instrument (Finnigan MAT, San Jose, CA, USA) equipped with an electrospray ionization source. The HPLC and MS systems were run using the Xcalibur 1.2 software (Finnigan).

4.3.3 Standard and internal standard solutions

Standard stock solutions were prepared by dissolving the respective compound in water to obtain concentrations ranging from 5 to 20 mmol/L. These stock solutions were further

diluted with water to get three solutions: a 320 µmol/L carnitine solution, a mixed solution containing butyrobetaine, acetylcarnitine, propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine and octanoylcarnitine at individual concentrations of 80 µmol/L and a solution containing 500 µmol/L of myristoylcarnitine, palmitoylcarnitine and stearoylcarnitine. After appropriate dilution with water, 3 sets of standard spiking solutions, ranging from 40 to 320 µmol/L for carnitine, from 2 to 80 µmol/L for butyrobetaine and short- and medium-chain acylcarnitines, and from 0.25 to 10 µmol/L for the long-chain acylcarnitines were obtained. The carnitine concentration was standardized using a spectrophotometric method (Marquis and Fritz, 1964). The contamination with carnitine of the solutions containing acylcarnitines (due to spontaneous hydrolysis) was estimated by injecting the individual standard stock solution and measuring the respective peak areas. However, the carnitine content was not relevant in any of the solutions.

Internal standard stock solutions containing either 5 mmol/L carnitine-d_3, 1 mmol/L octanoylcarnitine-d_3, or 1 mmol/L palmitoylcarnitine-d_3 were prepared in methanol. A mixed internal standard-spiking solution was prepared by diluting the carnitine-d_3 and octanoylcarnitine-d_3 stock solutions with water to yield concentrations of 80 and 20 µmol/L, respectively. A palmitoylcarnitine-d_3 spiking solution of 1 µmol/L was prepared by dilution of the stock solution with water.

4.3.4 Plasma sample collection

Plasma samples were obtained either from the chemical laboratory or from the blood collection center of the University Hospital, Basel. Plasma calibrators and plasma quality samples were prepared from 3 different plasma pools. Patient serum was obtained from the University Children's Hospital, Basel.

4.3.5 Sample preparation

Carnitine, butyrobetaine and short- and medium-chain acylcarnitines were extracted from plasma by solid phase extraction after a protein precipitation step. Two hundred µL plasma or bovine serum albumin 4% in water (for calibrators and standard quality controls) were mixed with 50 µL internal standard spiking solution, 50 µL of each standard spiking solution (calibrators and standard quality controls) or 100 µL water (unspiked plasma samples) and acidified with 70 µL HCl (1 mol/L). Proteins were precipitated with 1 mL acetonitrile-methanol

(3:1, v/v). After vigorous mixing and centrifugation, 1200 µL of the supernatant was applied on a disposable cation-exchange column, according to a previously published protocol (Vernez et al., 2003). The only alteration to the published procedure was the modified washing step, which was done with 20 mmol/L HCl -methanol (1:1, v/v). An aliquot of 2 µL of the reconstituted sample was injected into the HPLC-MS/MS system.

Myristoyl-, palmitoyl- and stearoyl-carnitine were extracted using a separate liquid-liquid extraction procedure. To 200 µL plasma or bovine serum albumin 4% in water (for calibrators and standard quality controls), 50 µL internal standard spiking solution and 50 µL standard spiking solution (calibrators and standard quality controls) or water (unspiked plasma samples) were added. Extraction was performed by adding 1 mL ethyl-acetate and shaking for 5 minutes. The organic layer was removed and the aqueous residues were extracted a second time with 1 mL ethyl-acetate. The combined organic layers were evaporated under nitrogen at 30°C. The evaporated residues were reconstituted in 50 µL methanol-water (1:1, v/v), containing 10 mmol/L heptafluorobutyric acid and 10 mmol/L ammonium acetate. The HPLC-MS/MS analysis was performed on 10 µL of the reconstituted samples.

4.3.6 Chromatographic conditions

The HPLC-MS/MS system was run as previously described (Vernez et al., 2003). Briefly, the separation was carried out using a binary gradient with a volatile ion-pair reagent, which consisted of 10 mmol/L heptafluorobutyric acid and 10 mmol/L ammonium acetate in water (A) or in methanol (B) at a flow rate of 200 µL/min. The gradient started at 20% B, went up to 90% B within 4 min, and then stayed at a plateau at 90% B for another 14 min. For detection, analytes were ionized by positive ion (4.0 kV) electrospray and the selected ions were monitored in the MS/MS mode. Each compound was associated with a segment, except for butyrobetaine, which was in the same segment as carnitine. As the internal standards are stable isotopes from analyzed compounds, they were in the same segment as their respective analyte.

Carnitine-d_3 was used as internal standard at a concentration of 20 µmol/L for the determination of carnitine, butyrobetaine, acetylcarnitine and propionylcarnitine. Octanoylcarnitine-d_3 was used as internal standard at a concentration of 5 µmol/L for the determination of all medium-chain acylcarnitines. Palmitoylcarnitine-d_3 served as the internal standard for the long-chain acylcarnitines at a concentration of 1 µmol/L. Areas obtained for the 2 most intense product ions (a common and a specific one) were corrected with the

respective areas of the internal standard and were used for quantification. The observed transitions are given in Figure 1. The Xcalibur 1.2 software was used for quantification of the analytes. Linear regressions of the calibration curves were calculated with a weighting factor of 1/X.

4.3.7 Validation procedure

The linearity of the method was investigated using the sample preparation procedure described above for carnitine, five short- and medium-chain acylcarnitines, and the long-chain palmitoylcarnitine. Calibration curves (six points) were prepared in concentration ranges of 10-80 µmol/L for carnitine, 0.5-20 µmol/L for butyrobetaine, acetylcarnitine, propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine and octanoylcarnitine and 0.25-10 µmol/L for palmitoylcarnitine. Precision and accuracy were determined by running standard quality controls at 3 different concentrations covering the calibration range (2 concentrations for palmitoylcarnitine), on the same (intra-day) and on different days (inter-day variability). Pooled plasma quality controls were run simultaneously at 3 different concentrations (in double), namely a blank (unspiked) plasma and the same plasma after spiking with 2 different concentrations. To investigate the method under clinical conditions a plasma sample of a patient suffering from methylmalonic aciduria (MMA) was analyzed. This disease is caused by defects of the cobalamine-dependent enzyme methylmalonyl-CoA mutase.

4.4 RESULTS AND DISCUSSION

4.4.1 Extraction procedure

In a previous paper (Vernez et al., 2003) we discussed in detail the difficulties facing the extraction of the compounds of the carnitine family. The difficulties originate from the zwitterionic nature of carnitine and from the different length of the acyl-group regarding the acylcarnitines, leading to a wide range of polarities. After introducing a protein precipitation step, the solid phase extraction developed for urine samples could be successfully applied to plasma samples for the determination of carnitine, butyrobetaine and short- and medium-chain acylcarnitines. However, a different protocol had to be developed for the extraction of long-chain acylcarnitines in plasma, as they are bound to plasma proteins (Marzo et al., 1991). We therefore investigated liquid-liquid extraction (Heinig and Henion, 1999) to extract these compounds efficiently. Several solvents were tested and the entire extraction process

was investigated using ^{14}C-palmitoylcarnitine dissolved in 200 µL plasma. Hexane, ether, chloroform and dichloromethane gave extraction recoveries below 10%, whereas 2 mL ethyl acetate gave a recovery of 40%, which could not be improved further by other solvents. Since ethyl-acetate turned out to be to lipophilic to extract medium- and short-chain acylcarnitines, the solid phase extraction could be performed also on the liquid-liquid extraction residue, resulting in the same efficiency as for non-extracted plasma (data not shown). Nevertheless, under routine conditions, we preferred to use a different aliquot to extract carnitine and short- and medium- chain acylcarnitines, because these two extraction procedures were usually not performed on the same day.

4.4.2 HPLC-MS/MS separation

Carnitine, the carnitine precursor butyrobetaine and 8 acylcarnitines could be separated in less than 16 minutes (see Figure 2). As for the extraction procedure, the separation of these compounds in plasma could be performed using the same conditions as for urine (Vernez et al., 2003). In comparison to the method developed for urine, the plasma method was worked out also for the determination of butyrobetaine and long-chain acylcarnitines. In comparison to carnitine, butyrobetaine has no hydroxyl group in position 3 (see R^1 in Figure 1). We therefore expected its elution close to carnitine, but with a higher retention time, as it is less hydrophilic than carnitine and should be retained better on the reversed-phase column. Figure 2 shows that butyrobetaine is indeed eluted after carnitine, but the two compounds are not baseline separated, which is not disturbing when using mass spectrometry for detection. The mass spectrometer was set so that butyrobetaine and carnitine were in the same analysis segment. The presence of 3 compounds in the same segment (carnitine, butyrobetaine and deuterated carnitine) did still allow a sufficient number of scans through each peak to obtain sufficient sensitivity and precision.

4.4.3 Matrix used for calibrators

The presence of endogenous carnitine and acylcarnitines in biological fluids renders it difficult to use plasma as a matrix for calibrators, as it is difficult to get carnitine-free plasma. To investigate, whether water could be used as a calibration matrix instead of plasma, we compared the results using a calibration performed with standards in water with a calibration performed with spiked plasma. Values resulting from the calibration in water were 10 to 20% higher than those obtained with plasma. The use of plasma prepared differently (citrate

instead of heparin), or obtained from different patients, or the use of bovine serum albumin 4% in water, to better mimic the plasma matrix, resulted in a similar calibration as obtained with water. A plasma pool was therefore dialyzed using a 6-8000 Da cut off membrane (Longo et al., 1996), and used as a calibration matrix. The results were identical to those in the presence of 4% bovine serum albumin. Matrix effect and ion suppression were further evaluated using the post-column infusion technique (Bonfiglio et al., 1999). Carnitine and the different acylcarnitines were continuously infused, one by one, and dialyzed plasma samples were injected into the system. No suppression area could be observed for any compound when dialyzed plasma was injected. The experiment could not be performed using non-dialyzed plasma, since the endogenous carnitine and acylcarnitines in plasma interfered with the infused compounds.

Taking into consideration all the experiments mentioned above, we decided that the use of a calibration in the presence of 4% bovine serum albumin (see sample preparation in the Method section) was the best alternative to the use of plasma as a calibration matrix. This is in accordance to Hoppel (Hoppel, 1991) who also adds a bovine serum albumin solution to the aqueous calibrators for the radioenzymatic determination of carnitine. The obtained accuracies for carnitine in plasma controls, which were between 95.2 and 109.0% (see section below), supported us in our choice. These values are in the same range as the values obtained with plasma as a matrix for calibration (95.4 to 100.4%).

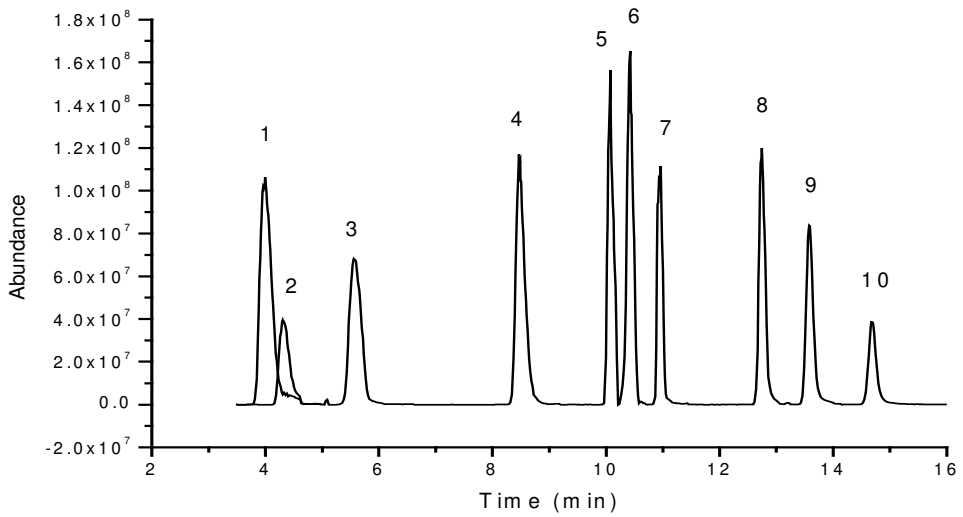


Figure 2: Total ion current trace of a mixed standard containing (in pmol on column): 1, carnitine (80); 2, butyrobetaine (40); 3, acetylcarnitine (40); 4, propionylcarnitine (40); 5, isovalerylcarnitine (40); 6, hexanoylcarnitine (40); 7, octanoylcarnitine (40); 8, myristoylcarnitine (24); 9, palmitoylcarnitine (24); 10, stearoylcarnitine (24).

4.4.4 Quantification in standard and plasma quality controls

Quantification was carried out in the MS-MS mode using two product ions (Vernez et al., 2003). The linearity of the method including extraction was evaluated between 10 and 80 µmol/L for carnitine, between 0.5 and 20 µmol/L for butyrobetaine, acetylcarnitine, propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine and octanoylcarnitine, and between 0.25 and 10 µmol/L for palmitoylcarnitine.

The recalculated calibrator concentrations with the obtained linear regression (n = 6) were used to validate the linearity. The results for carnitine show accuracies of 100.0 ± 0.4% and precisions smaller than 2.4% (Table 1). Butyrobetaine and short- and medium-chain acylcarnitines had precisions between 0.9 and 10.3% and accuracies between 80 and 126%, except for octanoylcarnitine with accuracies between 70 and 150%.

Table 1: Validation of the linearity of the method for carnitine in calibration standards by recalculation of the calibrator concentrations

Added concentration (µmol/L)	Found concentration (µmol/L)	SD	Precision (%)	Accuracy (%)
10	10.0	0.2	1.9	100.1
30	30.1	0.7	2.4	100.3
40	39.9	0.3	0.8	99.7
50	49.7	1.2	2.3	99.4
60	60.2	0.4	0.7	100.4
80	80.1	1.0	1.2	100.1

n = 6 determinations

Precision and accuracy data for intra- and inter-day variabilities on standard quality controls were obtained at three different concentrations, covering the calibration ranges used for carnitine, butyrobetaine, short- and medium-chain acylcarnitines (Table 2) and at two different concentrations for palmitoylcarnitine. For intra-day analysis, the precisions were between 1.4 and 10.1% (18.6% for palmitoylcarnitine), whereas the values for inter-day precisions varied between 1.0 and 11.0% (22.5% for palmitoylcarnitine). Accuracies were between 85.5% (78 and 68% for the low concentration of propionylcarnitine and octanoylcarnitine, respectively) and 104.8% for intra-day analysis, and between 80.4% (77 and 68% for the low concentration of propionylcarnitine and octanoylcarnitine, respectively) and 108.4% (119% for palmitoylcarnitine) for inter-day assays.

Table 2: Intra- and inter-day variabilities of standards quality controls for free carnitine and short- and medium-chain acylcarnitines

		C[1]	AC	BB	PC	IVC	HC	OC
Concentration ranges (µmol/L)		10-80	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20
Concentration (µmol/L)								
QC[2] low		15	1.5	1.5	1.5	1.5	1.5	1.5
QC medium		35	8.0	8.0	8.0	8.0	8.0	8.0
QC high		55	15	15	15	15	15	15
Inter-day (n=5)								
QC low	Precision (%)	4.0	4.0	2.7	2.4	6.3	11.0	3.5
	Accuracy (%)	95.9	89.7	103.3	77.3	85.4	80.4	67.6
QC medium	Precision (%)	1.0	4.2	2.0	3.5	2.7	3.8	3.4
	Accuracy (%)	102.1	84.7	92.2	81.6	101.6	108.4	95.7
QC high	Precision (%)	1.9	5.4	2.5	4.0	2.7	6.8	1.9
	Accuracy (%)	100.1	92.4	92.4	95.4	96.4	102.3	98.4
Intra-day (n=5)								
QC low	Precision (%)	2.6	3.6	1.4	2.8	3.6	2.5	5.7
	Accuracy (%)	97.1	85.5	104.8	77.8	87.1	94.1	68.3
QC medium	Precision (%)	1.8	5.3	2.9	1.6	2.5	2.8	1.6
	Accuracy (%)	102.2	92.2	93.4	78.4	98.3	99.8	96.3
QC high	Precision (%)	1.8	3.1	1.5	10.1	5.6	3.3	1.8
	Accuracy (%)	102.0	99.1	95.0	102.9	95.1	94.5	100.6

[1] C = carnitine, AC = acetylcarnitine, BB = butyrobetaine, PC = propionylcarnitine, IVC = isovalerylcarnitine, HC = hexanoylcarnitine, OC = octanoylcarnitine
[2] QC = quality control

Table 3: Intra- and inter-day variabilities of plasma quality controls for free carnitine and short- and medium-chain acylcarnitines

		C[1]	AC	BB	PC	IVC	HC	OC
Concentration ranges (µmol/L)		10-80	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20	0.5-20
Concentration (µmol/L)								
QC[2] unspiked		0	0	0	0	0	0	0
QC low		15	1.5	1.5	1.5	1.5	1.5	1.5
QC medium		35	8.0	8.0	8.0	8.0	8.0	8.0
Inter-day (n=10)								
QC unspiked	Precision (%)	3.4	13.4	11.9	3.2	nd[3]	nd	nd
	Accuracy (%)	nd	nd	nd	nd	nd	nd	nd
QC low	Precision (%)	3.0	4.6	6.9	7.3	13.7	20.5	9.7[4]
	Accuracy (%)	97.6	124.6	119.3	70.5	100.0	60.5	58.2[4]
QC medium	Precision (%)	2.8	8.8	7.3	8.2	7.8	25.3	32.6
	Accuracy (%)	109.0	107.6	105.6	96.8	129.9	88.3	73.8
Intra-day (n=10)								
QC unspiked	Precision (%)	2.9	4.0	8.1	nd	nd	nd	nd
	Accuracy (%)	nd	nd	nd	nd	nd	nd	nd
QC low	Precision (%)	2.2	9.5	6.5	7.4	2.8	3.6	1.0
	Accuracy (%)	95.2	77.0	93.9	114.9	114.8	97.6	60.0
QC medium	Precision (%)	2.2	7.9	6.0	4.6	7.9	5.6	3.5
	Accuracy (%)	98.3	109.2	106.2	101.3	121.3	116.3	102.1

[1] C = carnitine, AC = acetylcarnitine, BB = butyrobetaine, PC = propionylcarnitine, IVC = isovalerylcarnitine, HC = hexanoylcarnitine, OC = octanoylcarnitine
[2] QC = quality control
[3] nd: not determined
[4] n = 6

Precision and accuracy data for intra- and inter-day assays on plasma quality controls were obtained at three different concentrations, a blank plasma (unspiked) and 2 different concentrations covering the respective calibration ranges (Table 3). Mean inter-day concentrations measured in blank plasma were 34.3 µmol/L for carnitine, 5.35 µmol/L for acetylcarnitine, 0.76 µmol/L for butyrobetaine, and 0.65 µmol/L for propionylcarnitine, whereas isovalerylcarnitine, hexanoylcarnitine, and octanoylcarnitine were below the quantification limits. Precisions were between 1.0 and 9.5% for intra-day analysis of carnitine, short- and medium-chain acylcarnitines, and between 2.8 and 13.7% (except 25 and 33% for hexanoylcarnitine and octanoylcarnitine, respectively) for inter-day assays. For intra-day analysis, the accuracies were between 77.0% (60% for octanoylcarnitine) and 116.3% (121% for isovalerylcarnitine). The values for inter-day precisions were between 70.3% (61 and 58% for hexanoylcarnitine and octanoylcarnitine, respectively) and 129.9%. The long-chain acylcarnitines (myristoylcarnitine, palmitoylcarnitine and stearoylcarnitine) were determined only qualitatively (see Figure 3), but not quantified.

All the most relevant compounds, namely carnitine, butyrobetaine, acetylcarnitine and propionylcarnitine exhibit acceptable precisions and accuracies for intra- and inter-day assays in standards and in plasma quality controls. Medium chain acylcarnitines present more imprecision, especially at low concentrations in plasma quality controls. This could be explained by the fact that medium chain acylcarnitines are partially bound to plasma protein (Marzo et al., 1991) and that their extraction could have been affected by the protein precipitation step. Since the method will be used primarily for the diagnosis of patients with metabolic disorders, semi-quantitative determination of medium- and long-chain acylcarnitines is sufficient (see below).

4.4.5 Application to a serum sample from a patient with a metabolic disorder

Figure 3 represents on the panel A a chromatogram obtained from a plasma of a healthy person. This shows the typical carnitine profile with predominant peaks of carnitine (peak 1) and acetylcarnitine (peak 3). Butyrobetaine (peak 2) and other acylcarnitines can also be detected. The panel B of Figure 3 depicts a chromatogram of a serum from a patient suffering from methylmalonic academia (MMA). As expected, the propionylcarnitine level (peak 4) is clearly increased in this plasma.

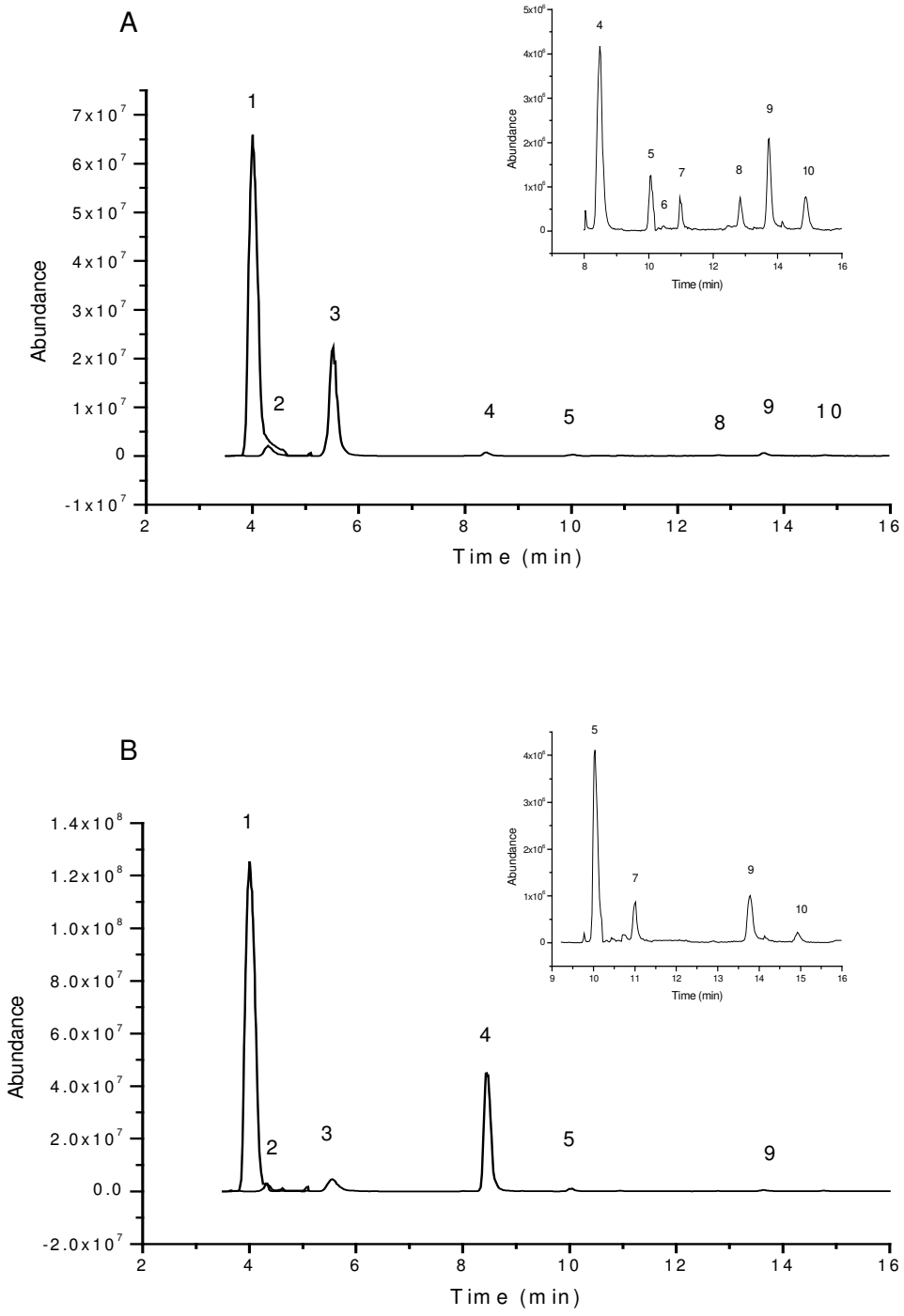


Figure 3: Total ion current trace of a control plasma (A) and serum of a patient with methylmalonic aciduria (B). The samples were extracted and analyzed as described in the Experimental section. The traces obtained for carnitine and for short- and medium-chain acylcarnitines were combined with the ones obtained for long-chain acylcarnitines (2 different traces resulting from 2 different injections): 1, carnitine; 2, butyrobetaine; 3, acetylcarnitine; 4, propionylcarnitine; 5, isovalerylcarnitine; 8, myristoylcarnitine; 9, palmitoylcarnitine; 10, stearoylcarnitine. The inserts show an expended view of final parts of the respective chromatograms. As expected, the patient has an increased peak for propionylcarnitine.

4.5 CONCLUSION

In the present paper we present the application of a reversed-phase high-performance liquid chromatography with MS/MS detection for the determination of carnitine, its biosynthetic precursor butyrobetaine, and eight short-, medium- and long-chain acylcarnitines. The method could successfully be validated for standards and plasma quality controls, using a bovine serum albumin 4% in water as a calibration matrix. A metabolic disorder with a pathological acylcarnitine profile could be successfully diagnosed.

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CHAPTER 5

EFFECT OF L-CARNITINE SUPPLEMENTATION ON THE KINETICS OF CARNITINE, ACYLCARNITINES AND BUTYROBETAINE IN PATIENTS WITH LONG-TERM HEMODIALYSIS

Laurence Vernez[1], Michael Dickenmann[2], Markus Wenk[1] and Stephan Krähenbühl[1]

[1]Division of Clinical Pharmacology & Toxicology and Department of Research,
and [2]Division of Nephrology & Transplantation Medicine,
University Hospital, Basel, Switzerland

5.1 SUMMARY

Background: The current study was performed to investigate the kinetics of carnitine, individual acylcarnitines and butyrobetaine in patients on hemodialysis.
Methods: Seven stable long-term hemodialysis patients were studied under basal conditions (no carnitine supplementation) and 3 weeks after i.v. supplementation with L-carnitine (10 or 20 mg/kg body weight) after each hemodialysis session. The kinetic studies included serial determinations of carnitine and metabolites just before, during or between hemodialysis sessions. Analysis was performed by HPLC-MS/MS.
Results: Before hemodialysis, the plasma concentrations were (µmol/L) 15.1 ± 1.4 for carnitine, 5.9 ± 1.7 for acetylcarnitine, 0.66 ± 0.13 for propionylcarnitine and 0.96 ± 0.25 for butyrobetaine (basal conditions), and 147 ± 68 for carnitine, 73 ± 35 for acetylcarnitine, 6.3 ± 3.3 for propionylcarnitine and 2.6 ± 0.8 for butyrobetaine (carnitine 20 mg/kg). During hemodialysis, the plasma concentrations dropped by approximately 80% for all compounds determined, with extraction coefficients ranging from 0.81 to 0.87. In patients supplemented with 20 mg/kg carnitine, the amount of carnitine removed by hemodialysis equaled 56% of the administered dose, consisting of 3.08 mmol carnitine, 1.47 mmol acetylcarnitine and 0.070 mmol propionylcarnitine. Between the hemodialysis sessions, carnitine, acylcarnitines and butyrobetaine reached steady state concentrations within 1 day both under basal conditions and after supplementation.
Conclusions: Patients on hemodialysis have reduced carnitine, acylcarnitine and butyrobetaine plasma levels, which can be normalized or increased by supplementing carnitine. Propionylcarnitine is an important constituent of the acylcarnitine pool, which can be removed by hemodialysis. Removal of potentially toxic acyl-groups may represent a mechanism for the beneficial effect of carnitine in these patients.

5.2 INTRODUCTION

Carnitine (L-3-hydroxy-4-N-trimethylaminobutyrate) is an endogenous compound present in most mammalian tissues, which is ingested by the diet and produced endogenously by biosynthesis. Carnitine is an essential factor for transport of long-chain fatty acids into the mitochondrial matrix, where they are metabolized via β-oxidation (Fritz, 1955; Bremer, 1983). By reacting with activated fatty acids, carnitine can generate various acylcarnitines with different chain lengths. By accepting acyl-groups from acyl-CoAs, carnitine is also involved in the regulation of the cellular pool of free coenzyme A (CoASH), thus acting as a buffer

(Brass and Hoppel, 1980; Bieber et al., 1982). This buffer effect may be important for the detoxification and elimination of potentially toxic acyl-groups, originating from exposure to xenobiotics or from blockage of metabolic pathways. In contrast to acyl-CoAs, the corresponding acylcarnitines can be excreted in the urine (Bremer, 1983; Roe et al., 1983).

In healthy subjects, carnitine and acetylcarnitine represent the major constituents of the body fluid and tissue carnitine pools (Bremer, 1983; Friolet et al., 1994). Skeletal muscle contains more than 95% of the total carnitine body stores, and the tissue concentrations are considerably higher than the concentration in plasma (Friolet et al., 1994), necessitating active transporter into tissues. Kidneys play a crucial role in carnitine homeostasis, since they reabsorb more than 90% of the filtered carnitine (Krahenbuhl and Reichen, 1997), so that the plasma levels of free carnitine are maintained at 37±8 µmol/L (Hoppel, 1991).

Since carnitine is a small molecule (molecular weight = 161.2), and not protein-bound, it is efficiently removed from blood through the dialyzer membrane (Evans, 2003). Patients with end-stage renal disease undergoing long-term hemodialysis have therefore reduced carnitine plasma (Bellinghieri et al., 1983; Rodriguez-Segade et al., 1986a; Wanner et al., 1990; Hiatt et al., 1992; Evans et al., 2000) and muscle levels (Bellinghieri et al., 1983; Hiatt et al., 1992), and may suffer from complications similar to those observed in patients with carnitine deficiency, e.g. skeletal muscle weakness and pain, intradialytic cramps and hypotensive episodes or impaired exercise performance (Bellinghieri et al., 2003; Evans, 2003). Relative to the decreased carnitine, acylcarnitines are increased, so that the acylcarnitine to total carnitine ratio (free carnitine plus acylcarnitines) is higher than in healthy subjects (Rodriguez-Segade et al., 1986a; Wanner et al., 1990; Hiatt et al., 1992; Evans et al., 2000). Supplementation with carnitine, either orally or intravenously, at the end of hemodialysis has been shown to increase the carnitine plasma concentration (Bellinghieri et al., 1983; Wanner et al., 1990; Siami et al., 1991; Evans et al., 2000) and carnitine skeletal muscle concentration (Bellinghieri et al., 1983; Siami et al., 1991). Despite the fact that carnitine supplementation is clearly associated with an increase in carnitine plasma and tissue levels, carnitine administration has been shown to be associated with clinical improvement of muscular symptoms only in some (Bellinghieri et al., 1983; Ahmad et al., 1990; Sakurauchi et al., 1998) but not in all studies or patients (Hurot et al., 2002). In several studies, the metabolism of carnitine in patients on long-term hemodialysis has been investigated. Most of them were restricted to the observation of free and acylcarnitines (Leschke et al., 1983; Rumpf et al., 1983; Panzetta et al., 1985), but in some studies, the acylcarnitines were differentiated (Jackson and Lee, 1996; Evans et al., 2000). In one recent study, the plasma concentration of carnitine, acetylcarnitine and total carnitine was investigated in patients on

hemodialysis before and after intravenous supplementation of different doses of carnitine (Evans et al., 2000).

In the current study, we investigated the composition of the plasma carnitine, acylcarnitine and butyrobetaine pools in patients on long-term hemodialysis under baseline conditions and during intravenous supplementation with two different doses of carnitine. The kinetics of carnitine, individual acylcarnitines and butyrobetaine were evaluated during and between hemodialysis sessions. The analytical method utilized was HPLC-MS/MS (Vernez et al., 2003), which allows the quantification of all analytes mentioned above. We were particularly interested to answer the question which acylcarnitines other than acetylcarnitine accumulate in the plasma of patients on long-term hemodialysis and how the plasma butyrobetaine pool is influenced by the administration of carnitine.

5.3 EXPERIMENTAL

The study was approved by the Ethics Committee of the Cantons of Basel and patients provided written informed consent before the start of the study.

5.3.1 Study population

Patients aged between 20 and 80 years were recruited at the dialysis center of the University Hospital of Basel. Enrolment requirements included treatment with hemodialysis three times a week for more than 6 months and the absence of acute illnesses. Patients already supplemented with carnitine, either orally or intravenously, were also excluded from the study.
Seven patients, 3 women and 4 men, with a mean age of 59 years (see details in Table 1) were included. They underwent 3 hemodialysis sessions per week with a duration of 240 minutes and a dialysate flow of 0.5 L/min. A HF 80 dialysis membrane (Fresenius, Bad Homburg, Germany), the most commonly used membrane in our dialysis center, was utilized for all patients.

Table 1: Characterization of the patients that completing the study. Patients 1, 5 and 7 were recruited for intra- and inter-dialysis kinetics (see Table 2)

Patient	Sex	Age (year)	Urine production per day (ml)	Target weight (kg)	Dialysis blood flow rate (L/min)	Carnitine dose (mmol) for 10 mg/kg
1	female	46	0	59.0	0.30	3.66[3]
2	male	75	200	83.5	nr[2]	5.17
3	male	38	50	66.0	nr	4.08
4	female	60	0	76.0	nr	4.69
5	female	59	0	72.5[1]	0.30	4.39
6	male	57	650	75.5	nr	4.69
7	male	76	800	74.5	0.30	4.65

5.3.2 Study design

The study was performed over 7 weeks (21 hemodialysis sessions) and included three different observation periods. During the first week (hemodialysis sessions 1, 2 and 3), patients were studied under baseline conditions (no carnitine supplementation). During the three next weeks (hemodialysis sessions 4-12), all patients were supplemented after each hemodialysis session with 10 mg/kg body weight carnitine i.v. (low carnitine supplementation). During the last three weeks (hemodialysis sessions 13-21), carnitine supplementation was increased to 20 mg/kg body weight carnitine i.v. (high carnitine supplementation). Carnitine (Carnitene sigma-tau®, Sigma-Tau Pharma AG, Zofingen, Switzerland) was administered by slow injection using the venous line installed for hemodialysis, just before it was removed at the end of the hemodialysis session. After the administration of carnitine, the line was flushed with saline. Blood samples were collected in 2 mL heparinized tubes which were centrifuged and the plasma was removed and stored in polystyrene tubes at -20^{0}C until analysis.

5.3.3 Steady state carnitine and acylcarnitines profiles between hemodialysis sessions

To establish the carnitine and acylcarnitine profiles over the entire study period, blood collections were performed in all patients twice under baseline conditions (before hemodialysis sessions 1 and 3), thrice under low carnitine supplementation (before hemodialysis sessions 6, 9 and 12) and thrice under high carnitine supplementation (before hemodialysis sessions 15, 18 and 21). Thus, the first blood samples collections under the

respective supplementation conditions were performed after 3 administrations of the respective carnitine dosage. All samples were withdrawn immediately before the respective hemodialysis session was started.

5.3.4 Carnitine and acylcarnitines kinetics during hemodialysis sessions (intra-dialysis)

Intra-dialysis kinetics of carnitine was studied in 3 patients (patients 1, 5 and 7) under baseline conditions (session 3), after 3 weeks of low carnitine supplementation (session 12) and after 3 weeks of high carnitine supplementation (session 21). Blood samples were collected before hemodialysis was started, during and at the end of hemodialysis. During hemodialysis, arterial blood (blood entering in the dialysis system), venous blood (blood leaving the dialysis system) and 10 mL dialysate were collected at 10, 30, 60, 120 and 180 minutes after beginning hemodialysis. The arterial blood was obtained just before the venous one, and the dialysate was collected just after the blood samples had been obtained.

5.3.5 Carnitine and acylcarnitines kinetics between two hemodialysis sessions (inter-dialysis)

Inter-dialysis kinetics of carnitine were studied on 3 patients (patients 1, 5 and 7) under baseline conditions (between sessions 2 and 3) and at the end of the study (between sessions 20 and 21, after 3 weeks of treatment with 20 mg/kg carnitine). Blood samples were collected before the start of hemodialysis, immediately after hemodialysis had been completed (for session 20, just before carnitine was administered) and 5, 10, 15, 20, 30, 45 minutes and 1, 2, 4, 6, 20 and 44 hours after carnitine had been administered. At the end of hemodialysis, the venous line was kept open during 6 hours for the collection of blood. The 20 hours blood sample was performed by venopuncture, and the 44 hours collection time point corresponded to the beginning of the next hemodialysis session. Patients received a vegetarian meal between the blood collections at 1 and 2 hours.

5.3.6 Analysis of carnitine and acylcarnitines

Carnitine, the different acylcarnitines and butyrobetaine present in plasma and dialysate samples were analyzed by high-performance liquid chromatography - tandem mass spectrometry (HPLC-MS/MS) according to a method published previously for urine and

adapted for plasma (Vernez et al., 2003). Briefly, samples were subjected to a solid phase extraction on a cation-exchange column and separation was performed using a reversed-phase column in the presence of a volatile ion-pairing reagent. Detection was carried out using an ion-trap mass spectrometer run in the MS/MS mode. The method linearity was between 10-80 µmol/L for carnitine and 0.5-20 µmol/L for acetylcarnitine, propionylcarnitine, isovalerylcarnitine and butyrobetaine. Inter-day and intra-day plasma quality controls had precisions ranging from 2.2 to 13.7% and accuracies between 70.5 and 129.9%, for both carnitine and acylcarnitines.

Plasma samples having high carnitine and acetylcarnitine levels were diluted up to 40 fold with a 4% bovine serum albumin solution in water before analysis in order to reach the calibration range. Modified calibration ranges for carnitine (between 5-60 µmol/L) were used for the analysis of samples containing a low carnitine concentration.

The acylcarnitine concentration used to calculate total carnitine (sum of free and acylcarnitine) and the acylcarnitine to total carnitine ratios was obtained by summing the concentration of each individual acylcarnitine.

5.3.7 Statistics and pharmacokinetic analysis

Data are presented as mean ± SEM unless stated otherwise. Kinetics of carnitine, acylcarnitine and butyrobetaine in the inter-dialysis period were analyzed under baseline conditions (between hemodialysis sessions 2 and 3) and after 3 weeks of supplementation with 20 mg/kg body weight carnitine (between hemodialysis sessions 20 and 21). The area under the curve from time zero to 44 hours ($AUC_{0\text{-}44}$) was determined with the linear trapezoidal rule using TopFit software (Tanswell and Koup, 1993). The baseline corrected AUC was calculated by subtracting the AUC obtained under baseline conditions from the corresponding AUC after carnitine substitution.

The intra-dialysis period was evaluated under baseline conditions (hemodialysis session 3), after 3 weeks treatment with 10 mg/kg body weight carnitine (hemodialysis session 12) and after 3 weeks supplementation with 20 mg/kg body weight carnitine (hemodialysis session 21). The extraction coefficient (E) was calculated as follows:

$$E = \frac{C_{pre} - C_{post}}{C_{pre}} \qquad (1)$$

where C_{pre} is the concentration of the analyte in arterial blood (entering the dialysis system) at the start of hemodialysis and C_{post} the concentration in the arterial blood at the end of hemodialysis.

Alternatively, E was calculated as:

$$E' = \frac{Cart - Cven}{Cart} \qquad (2)$$

where Cart is the arterial and Cven the venous concentration at a given time point. Since the values for E and the average of E' (average of several determinations during hemodialysis) were not different, E is given in Table 2 and was used for the following calculations.
The clearance by hemodialysis was calculated as:

$$Cl_h = Q \times E \qquad (3)$$

Where Q is the hemodialysis blood flow (Table 1) and E the extraction coefficient (equation 1).
The amount of carnitine and acylcarnitines eliminated by hemodialysis (A_h) was calculated as follows:

$$A_h = AUC_{art;\ 0\text{-}240} \times Cl_h \qquad (4)$$

with $AUC_{art;\ 0\text{-}240}$ being the area under the curve in arterial blood from time zero until completion of hemodialysis after 240 minutes.

5.4 RESULTS

Using a sensitive HPLC-MS/MS method, we investigated the intra- and inter-dialysis kinetics of carnitine in patients with chronic renal failure treated by hemodialysis under baseline conditions and during treatment with different amounts of intravenous carnitine. Under baseline conditions, all patients had lower free carnitine plasma concentrations as compared to patients without renal disease (Hoppel, 1991; Krahenbuhl and Reichen, 1997), ranging from 12.8 to 16.4 µmol/L. The acylcarnitine to carnitine ratio was 0.42 ± 0.09 (range 0.33 and 0.59), a value which is higher than observed in healthy persons (Hoppel, 1991; Krahenbuhl and Reichen, 1997). Acetylcarnitine was the prominent short-chain acylcarnitine, but propionylcarnitine was also detectable in 4 patients and isovalerylcarnitine in one patient. The carnitine precursor butyrobetaine could be found in the plasma of all patients at a concentration of 0.96 ± 0.25 µmol/L (range 0.66 – 1.30 µmol/L).

5.4.1 Carnitine and acylcarnitines profiles

The evolution of the carnitine and acylcarnitine pools over the entire study is depicted in Figure 1. After 1 week of supplementation with 10 mg/kg carnitine (low dosage) at the end of

each hemodialysis session, the carnitine pre-dialysis concentration increased in all patients between 130 to 440 % as compared to baseline (mean plasma concentration 40.4 ± 16.1 µmol/L, range 21.1 – 70.8). The acetylcarnitine concentration increased from 5.9 ± 1.7 to 18.0 ± 9.1 µmol/L, propionylcarnitine from 0.66 ± 0.13 to 1.20 ± 0.63 µmol/L and butyrobetaine from 0.96 ± 0.25 to 1.78 ± 0.72 µmol/L. Propionylcarnitine was detectable in the plasma of 2 patients and isovalerylcarnitine in 1 patient. After 3 weeks of low dosage carnitine supplementation, the carnitine, acetylcarnitine and propionylcarnitine plasma levels had further increased in most patients, while the isovalerylcarnitine and butyrobetaine levels remained constant. After one week of carnitine supplementation at low dosage, the acylcarnitine to carnitine ration had reached a value of 0.46 ± 0.11, which dropped to 0.41 ± 0.07 at the end of the low carnitine supplementation period.

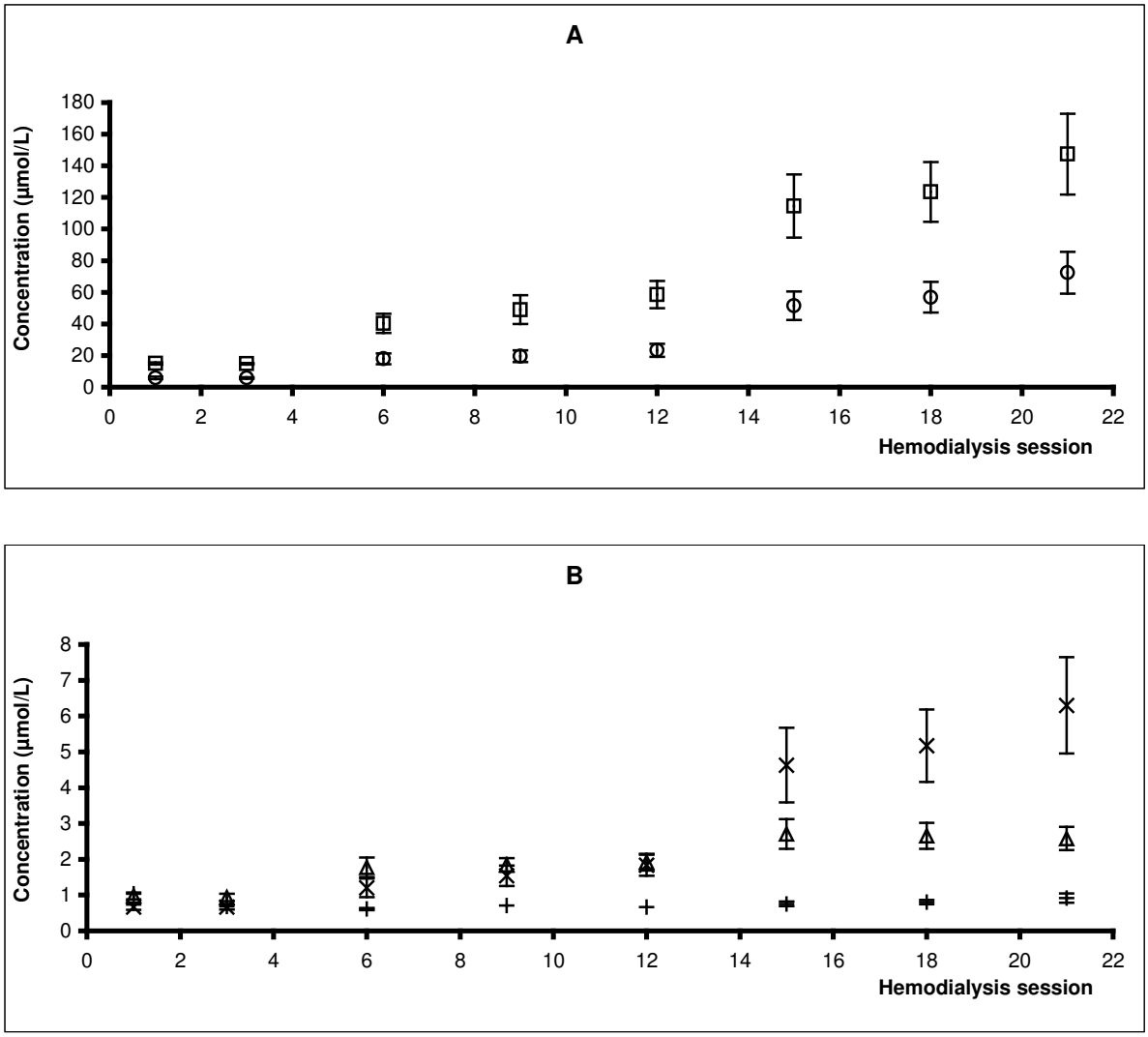

Figure 1: Pre-dialysis plasma concentrations of carnitine, acylcarnitines and butyrobetaine. Values are expressed as mean ± standard error, n = 7 patients. Analysis was performed by HPLC-MS/MS as described in Methods. A: carnitine ( ) and acetylcarnitine (o). B: propionylcarnitine (x), isovalerylcarnitine (+) and butyrobetaine (Δ).

After one week of supplementation with 20 mg/kg carnitine (high dosage) at the end of each hemodialysis, the carnitine plasma concentration showed a further increase by a factor of 1.94 ± 0.51 (range 1.28 to 2.62), reaching values between 46 and 186 µmol/L. Similar to carnitine, acetylcarnitine increased by a factor of 2.17 ± 0.49 (range 1.30 to 2.74), propionylcarnitine by a factor of 2.75 ± 0.96 (range 1.56 to 3.98), and butyrobetaine by a factor of 1.39 ± 0.23 (1.09 to 1.68). Isovalerylcarnitine appeared in the plasma of 2 more patients. At the end of the study, after 3 weeks of high dose carnitine supplementation, 6 patients showed further increases in their carnitine levels, reaching concentrations between 78-229 µmol/L. In contrast, patient number 4 showed a constant carnitine plasma concentration at 48 µmol/L over the entire 3 weeks of high dose carnitine supplementation. Further increases were also observed for acetylcarnitine, propionylcarnitine and isovalerylcarnitine, reaching plasma concentrations of 72.5 ± 35.0 µmol/L, 6.30 ± 3.30 µmol/L and 0.92 ± 0.26 µmol/L, respectively. The mean plasma concentration of butyrobetaine reached 2.58 ± 0.85 µmol/L at the end of the high supplementation period. After one week of carnitine supplementation at high dosage, the acylcarnitine to carnitine ratio had reached a value of 0.48 ± 0.13, which increased to 0.52 ± 0.10 at the end of the high carnitine supplementation period.

5.4.2 Intra-dialysis kinetics

The plasma concentration time curves for carnitine, acylcarnitines and butyrobetaine are given in Figure 2. As known from previous studies (Evans et al., 2000), carnitine and acetylcarnitine can be removed efficiently by hemodialysis. As could be expected, this is also the case for propionylcarnitine and butyrobetaine (see Figures 2B and 2C). For carnitine, acylcarnitines and butyrobetaine, new steady state plasma concentrations are reached after 2 to 3 hours of hemodialysis. This steady state concentration is 50 to 80% lower for all analytes than the respective pre-dialysis plasma concentrations, indicating an efficient removal by hemodialysis.

The kinetic analysis of the plasma concentration-time curves during hemodialysis allowed the calculation of the extraction (E) by hemodialysis, hemodialytic clearance (Cl_h) and the amount of analytes removed by hemodialysis (A_h) (see Table 2). These calculations show that supplementation with carnitine is associated with a dose-dependent removal of acyl-groups by hemodialysis.

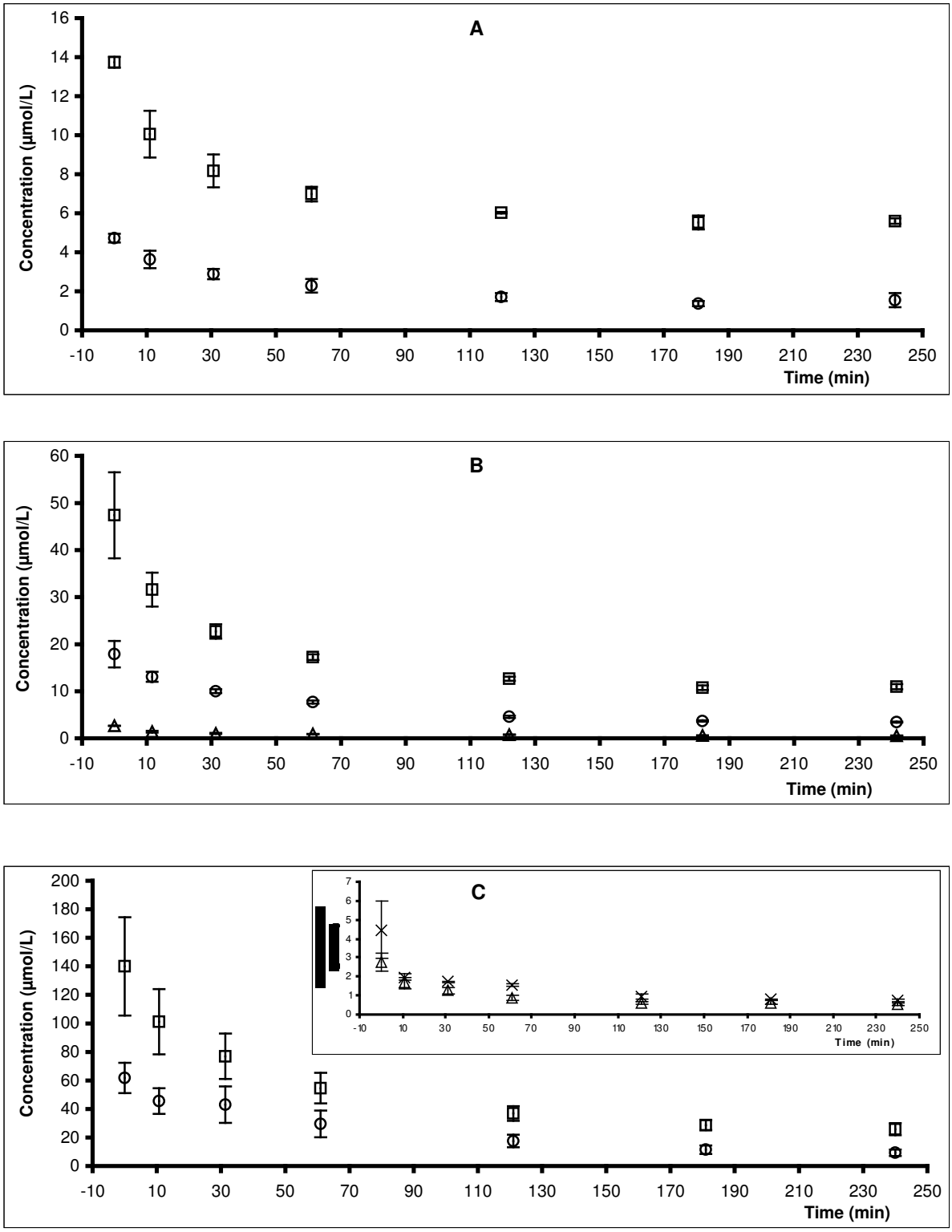

Figure 2: Kinetics of carnitine, acylcarnitines and butyrobetaine during hemodialysis (intra-dialysis). Values are expressed as mean ± standard error, n = 3 patients. Analysis was performed by HPLC-MS/MS as described in Methods. A: Baseline conditions (hemodialysis session 3), B: During supplementation with 10 mg/kg body weight carnitine (hemodialysis session 12), C: During supplementation with 20 mg/kg body weight carnitine (hemodialysis session 21). Symbols are: carnitine ( ), acetylcarnitine (o), propionylcarnitine (x) and butyrobetaine (Δ).

Table 2: Kinetics for carnitine, acylcarnitines and butyrobetaine during and between hemodialysis sessions. Values are expressed as mean ± standard error, n = 3 patients. Kinetic calculations were performed as described in Methods.

	Carnitine	Acetylcarnitine	Propionylcarnitine	Butyrobetaine
Intra-dialysis				
No substitution				
$AUC_{art;\ 0\text{-}240min}$ (µmol/L x h)	24.9 ± 2.60	7.65 ± 1.06	nd[2]	2.12[3]
CL_h (L/h)	10.5 ± 0.15	12.4 ± 1.21	nd	10.7[3]
E	0.585 ± 0.008	0.689 ± 0.067	nd	0.594[3]
A_h[1] (mmol)	0.264 ± 0.031	0.098 ± 0.008	nd	0.023[3]
10 mg/kg carnitine				
$AUC_{art;\ 0\text{-}240min}$ (µmol/L x h)	64.25 ± 3.31	25.14 ± 0.76	2.86[3]	3.45 ± 0.15[4]
CL_h (L/h)	13.52 ± 0.83	14.32 ± 0.67	11.03[3]	14.07 ± 0.46
E	0.751 ± 0.046	0.795 ± 0.037	0.613[3]	0.782 ± 0.025[4]
A_h[1] (mmol)	0.920 ± 0.091	0.387 ± 0.002	0.047[3]	0.049 ± 0.004[4]
20 mg/kg carnitine				
$AUC_{art;\ 0\text{-}240min}$ (µmol/L x h)	189.8 ± 33.7	90.79 ± 23.82	4.92 ± 0.21	3.37 ± 0.42
CL_h (L/h)	14.50 ± 0.41	15.32 ± 0.31	14.00 ± 1.51	14.42 ± 0.27
E	0.806 ± 0.023	0.851 ± 0.017	0.778 ± 0.084	0.801 ± 0.015
A_h[1] (mmol)	3.077 ± 0.282	1.473 ± 0.270	0.070 ± 0.010	0.052 ± 0.008
Inter-dialysis				
$AUC_{0\text{-}44h}$ (µmol/L x h) – no substitution	537.8 ± 49.8	201.9 ± 28.9	nd	45.0 ± 10.4
$AUC_{0\text{-}44h}$ (µmol/L x h) – 20 mg/kg carnitine	9228 ± 1475	2691 ± 569	240.8 ± 52.6	107.6 ± 26.5
Baseline-corrected $AUC_{0\text{-}44h}$ (µmol/L x h)	8691 ± 1494	2489 ± 558	240.8 ± 52.6	62.6 ± 16.6

[1]: Amount excreted in urine of patient 7 is included
[2]: nd: could not be determined
[3]: n=1
[4]: n=2

5.4.3 Inter-dialysis kinetics

At the end of hemodialysis, the plasma levels of all analytes were reduced by 50-80% as compared to pre-dialysis values (see Figure 2). When carnitine was not supplemented, carnitine, acetylcarnitine and butyrobetaine plasma concentrations started to increase already 5 minutes after the end of hemodialysis (Figure 3A). This increase was more marked during the first 6 hours after hemodialysis, and steady-state concentrations were reached after 8 to 24 hours.

In patients supplemented with 20 mg/kg carnitine, the plasma carnitine concentration reached 1340 ± 550 µmol/L 5 minutes after intravenous administration of carnitine (see Figure 3B). After having reached the peak concentration, the plasma carnitine concentration decreased with a half-life of approximately 2 hours, reaching steady state concentrations 8 to

24 hours after injection. After intravenous administration of carnitine, the plasma concentrations of acetylcarnitine, propionylcarnitine and butyrobetaine increased rapidly, reaching steady state concentration after 8 hours (see Figure 3B). In patient number 1, isovalerylcarnitine was also detected and showed a similar kinetic behavior as the other acylcarnitines.

The AUCs for the inter-dialysis period obtained without carnitine supplementation and after 3 weeks of supplementation with 20 mg/kg are given in Table 2. In comparison to baseline, the AUCs increased by a factor of 17.7 ± 7.0 for carnitine, 13.5 ± 5.4 for acetylcarnitne and 2.4 ± 0.3 for butyrobetaine during supplementation with carnitine.

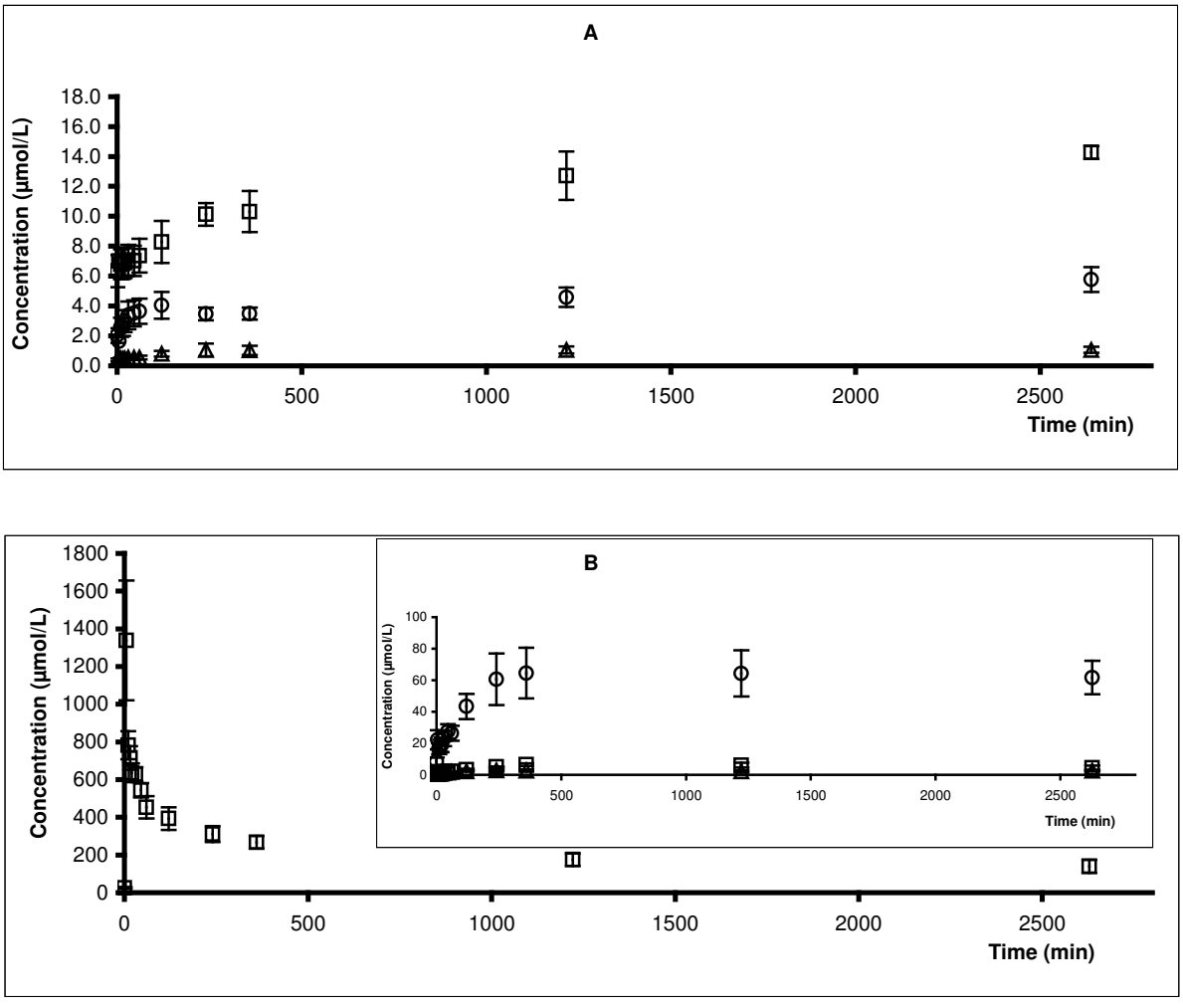

Figure 3: Kinetics of carnitine and acylcarnitines between two hemodialysis sessions (inter-dialysis). Values are expressed as mean ± standard error, n = 3 patients. Analysis was performed by HPLC-MS/MS as described in Methods. A: Baseline conditions between hemodialysis sessions 2 and 3 (no carnitine supplementation), B: Supplementation with 20 mg/kg body weight carnitine between hemodialysis sessions 20 and 21. Symbols are: carnitine ( ); acetylcarnitine (o), propionylcarnitine (x) and butyrobetaine (Δ).

5.5 DISCUSSION

Our study demonstrates that patients on long-term hemodialysis have reduced plasma carnitine concentrations before and after hemodialysis, that carnitine, acylcarnitines and butyrobetaine are removed by hemodialysis and that the plasma carnitine and butyrobetaine levels can be increased to physiological or even supraphysiological levels by supplementation of carnitine following each hemodialysis session.

Under baseline conditions, all patients investigated were carnitine-deficient with a mean plasma free carnitine concentration of 15.0 ± 1.4 µmol/L, which is well below the normal range of 37±8 µmol/L (Hoppel, 1991). The acylcarnitine to free carnitine ratio was 0.42 ± 0.09, indicating a redistribution of the plasma carnitine pool towards acylcarnitines (Hoppel, 1991; Krahenbuhl and Reichen, 1997). Acylcarnitines were mainly represented by acetylcarnitine, as reported in other studies (Bellinghieri et al., 1983; Jackson and Lee, 1996; Evans et al., 2000). Interestingly, propionylcarnitine could be detected in 4 patients with plasma concentrations ranging between 0.53 and 0.82 µmol/L. Propionylcarnitine has also been detected in plasma of healthy persons, but at lower concentrations as compared to the patients in our study (Minkler and Hoppel, 1993a; Inoue et al., 1999). In addition, still under baseline conditions, isovalerylcarnitine was present in a single patient at a level of 0.86 µmol/L, also a higher concentration than the 0.11 ± 0.06 µmol/L reported in healthy sportive subjects (Inoue et al., 1999). Since acylcarnitines are formed from the respective acyl-CoAs (Brass and Hoppel, 1980; Bieber et al., 1982; Bremer, 1983), it can be assumed that the tissue concentrations of propionyl-CoA and isovaleryl-CoA are increased at least in some patients on long-term hemodialysis. Depending on the metabolic situation, the plasma carnitine profile can reflect both the carnitine pool of the liver (Sandor et al., 1990) or of skeletal muscle (Friolet et al., 1994). In the case of propionate, the plasma carnitine profile primarily reflects the liver carnitine and coenzyme A pools, because propionate degradation is located in liver mitochondria (Frenkel and Kitchens, 1975). It is well known that propionate and other short- and medium chain fatty acids can impair mitochondrial function, in particular mitochondrial β-oxidation (Brass et al., 1986; Brass and Beyerinck, 1988). This inhibition may explain increased plasma concentrations of free fatty acids in patients on long-term hemodialysis (Maeda et al., 1989), and suggests that removal of acyl-groups may be potentially beneficial for this group of patients. Since the administration of exogenous carnitine is not only associated with an increase in free carnitine, but also in the respective acylcarnitine fractions, and since acylcarnitines are removed efficiently by hemodialysis, carnitine supplementation is associated with an increased removal of acyl-groups by

hemodialysis (see Table 2). In support of our hypothesis suggesting a beneficial effect of carnitine on mitochondrial metabolism, carnitine supplementation has been associated with a drop in the plasma free fatty acid concentration in patients on long-term hemodialysis (Maeda et al., 1989) and with an increase in cardiac fatty acid metabolism (Sakurabayashi et al., 1999). In addition, beneficial results on hepatic mitochondrial function have also been reported in patients suffering from methylmalonic aciduria who were treated with high doses of carnitine (Roe et al., 1983). The administration of carnitine to patients on long-term hemodialysis may therefore act in two ways, namely by increasing the low carnitine levels in skeletal muscle (Bellinghieri et al., 1983; Spagnoli et al., 1990; Siami et al., 1991) and by eliminating potentially toxic acyl-groups mainly from liver.

Butyrobetaine, the direct carnitine biosynthesis precursor (Vaz and Wanders, 2002), was present in all patients at concentrations between 0.66 and 1.30 µmol/L under baseline conditions. These values are below reported mean butyrobetaine plasma concentrations in healthy persons of 1.80 µmol/L determined by tandem mass spectrometry (Inoue et al., 1999) or 4.66 µmol/L determined by an enzymatic assay (Sandor et al., 1988). Similar to carnitine, butyrobetaine is stored mainly in skeletal muscle (Krahenbuhl et al., 2000; Vaz and Wanders, 2002). In order to be converted to carnitine, butyrobetaine has to be transported from skeletal muscle into the blood and then into liver, kidney or testes, where butyrobetaine hydroxylase is expressed and carnitine can be synthesized (Vaz and Wanders, 2002). Since butyrobetaine can be removed by hemodialysis (see Table 2), patients on long-term hemodialysis have lower butyrobetaine plasma concentrations than healthy subjects. Considering that butyrobetaine is taken up actively by tissues and that the K_m value of this transport is in the range of 5 µmol/L (Berardi et al., 1998), it can be assumed that transport into tissues shows an almost linear relationship with the plasma concentrations, suggesting that patients on hemodialysis have reduced tissue butyrobetaine concentrations. Impaired carnitine biosynthesis may therefore contribute to the low carnitine plasma and tissue concentrations (Bellinghieri et al., 1983; Rodriguez-Segade et al., 1986a; Wanner et al., 1990; Hiatt et al., 1992; Evans et al., 2000) in patients on long-term hemodialysis. In support of this hypothesis, patients on long-term hemodialysis have lower plasma and tissue concentrations of carnitine as compared to healthy subjects despite hemodialysis removes less carnitine than renal excretion in healthy subjects (Leschke et al., 1983; Rumpf et al., 1983). Our study supports the hypothesis that carnitine biosynthesis is reduced in patients on long-term hemodialysis and offers a possible mechanism for this assumption.

Interestingly, supplementation of carnitine in patients on long-term hemodialysis is not only associated with an increase in plasma carnitine and acylcarnitines, but also in butyrobetaine

(see Figure 1). How carnitine enters skeletal muscle is so far not completely clear, but it has to be an active transport, since the tissue concentrations are much higher than the plasma carnitine concentration (Bremer, 1983; Friolet et al., 1994). One possible transporter is OCTN2, a sodium-dependent carnitine transporter with a high expression in the kidney (Tamai et al., 1998). In humans, mRNA expression of OCTN2 is also high (Tamai et al., 1998), whereas rats have a low expression of OCTN2 in skeletal muscle (Spaniol et al., 2001). In the rat, a potential-driven transport of carnitine into skeletal muscle plasma membrane vesicles has been described with different substrate specificities than OCTN2, suggesting additional carnitine transporters (Berardi et al., 2000). The observed increase in the plasma butyrobetaine concentration during carnitine supplementation (see Figure 1) suggests the possibility that uptake of carnitine into and release of butyrobetaine from skeletal muscle are related with each other, possibly by an exchange mechanism similar to the carnitine-acylcarnitine translocase in the inner mitochondrial membrane (Indiveri et al., 1997). In support of the existence of such an exchange mechanism, the administration of butyrobetaine to rats was associated with a decrease in skeletal muscle carnitine and increases in the carnitine plasma concentration and renal excretion (Sartorelli et al., 1989).

In conclusion, our study shows that carnitine, short-chain acylcarnitines and butyrobetaine are removed efficiently by hemodialysis and that supplementation with exogenous carnitine increases the plasma concentrations of carnitine itself, but also of acylcarnitines and butyrobetaine in patients on long-term hemodialysis. Removal of potentially toxic acyl-groups as acylcarnitines by hemodialysis may explain some of the beneficial effects associated with the administration of carnitine in this group of patients. The increase in the plasma butyrobetaine concentration after carnitine supplementation may be explained by an exchange between carnitine and butyrobetaine across the skeletal muscle plasma membrane.

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CHAPTER 6

CONCLUSION

Using capillary electrophoresis and high-performance liquid chromatography, we developed new analytical methods for the determination of endogenous carnitine, acylcarnitines and butyrobetaine in biological fluids.

Despite the fact that carnitine is a good candidate for capillary electrophoresis analysis, this technique had not been widely exploited to analyze carnitine and its esters when we started to set up our method. Most of the available assays focused on the determination of the D- and L-isomers of carnitine. Some of them enabled nonetheless the separation of carnitine and acylcarnitines, but required up to 25 minutes to separate the medium-chain acylcarnitines. Our developed capillary electrophoresis assay enables the separation of carnitine and six short- and medium-chain acylcarnitines in standard solutions and in urine samples in a short analysis time, namely within 8 minutes. When setting up the assay, we did not include the analysis of long-chain acylcarnitines because they are usually not present in urine. However, further investigations, for instance increasing the SDS concentration to widen the detection windows and increasing the acetonitrile percentage in the running buffer to facilitate their solubility, would probably enable the extension of this assay towards long-chain acylcarnitines. The detection limits of 3 µmol/L achieved for carnitine and acetylcarnitine are in the same range as the detection limits obtained by most HPLC and CE methods for the determination of these compounds and were sufficient for the quantification of carnitine in urine samples. Relative standard deviations of corrected area/migration time for intra- and inter-day assays in standard solutions were below 10% and reached 21% at the quantification limits.

Analysis of endogenous compounds renders it impossible to obtain a biological matrix that is free of the substance to quantitate. Nevertheless, most published methods for the determination of carnitine and acylcarnitines use pooled plasma or urine samples, to which increasing amounts of analytes are added, as a calibration matrix (Chace et al., 1997; Vreken et al., 1999; Stevens et al., 2000). Because the samples to analyze may have less carnitine and acylcarnitines than the pooled matrix samples used for the calibration, and thus be out of the calibration ranges, we considered the use of water as a matrix to establish our quantitative data. The quantification of carnitine in urine using water or spiked urine as the calibration matrix yielded similar results (see chapter 3). Because of the presence of plasma proteins, the use of water as a calibration matrix appears to be less obvious when analyzing plasma samples. A solution of 4% bovine serum albumin in water as the calibration matrix

turned out to be suitable for the quantification of endogenous carnitine, acylcarnitines and butyrobetaine in plasma samples (see chapter 5).

Mass spectrometry has been proven in the last years to be a powerful tool for the determination of carnitine and acylcarnitines, especially for the screening of metabolic disorders in newborns. ESI-MS/MS analysis, combined with a simple methanol extraction of dried blood spots samples and the absence of a chromatographic separation is used in screening programs. Nonetheless, because of possible matrix interferences, particularly when working with complex matrix such as urine, the use of previous adapted extraction and separation is recommended to get reliable results.

The HPLC-MS/MS method that we have developed here enables the determination of carnitine and acylcarnitines, including long-chain acylcarnitines, in aqueous standards, urine and plasma samples in a short analysis time of 17 minutes. The separation of these compounds, which have a large span of polarities, on a reversed-phase column was achieved by the addition of an ion-pairing reagent into the mobile phases and has the advantage that pre-column derivatization is not necessary. More interestingly, butyrobetaine, the direct carnitine biosynthesis precursor, can be determined during the same analysis.

The assay exhibits sufficient sensitivity, precision and accuracy to quantify carnitine, short-chain acylcarnitines and butyrobetaine in urine and plasma samples. The precision for these determinations (below 10%) is similar to those obtained with other MS/MS assays.
Medium-chain acylcarnitines could not be determined with enough sensitivity to enable their determination in plasma and urine samples, but could be quantified in aqueous standard solutions, in spiked urine and spiked plasma samples with precision similar to those obtained for short-chain acylcarnitines. As presented in chapter 3, this assay allows the identification, in a non-invasive way, of medium-chain acylcarnitines in urine samples from patients suffering from MCAD deficiency, thus enabling the diagnosis of metabolic disorders.
In order to apply the developed assay for the quantitative determination of long-chain acylcarnitines in plasma, further investigations would be necessary, principally to improve the recovery of 40% we achieved with liquid-liquid extraction. Unfortunately we did not have access to plasma samples from patients having increased long-chain acylcarnitines to assess the ability of our plasma assay for the qualitative identification of pathological profile. But, as long-chain acylcarnitines could be determined in spiked plasma samples, we can reasonably presume, that such qualitative determinations are possible.

The qualitative detection of metabolic disorders using the developed HPLC-MS/MS method was shown to be feasible. Nonetheless, an access to more samples from patients with abnormal carnitine metabolism should be provided and a comparative study with the other methods used for screening should be performed before our assay can be used in a routine manner for the quantitative detection of metabolic disorders.

Our quantitative results were produced using an ion trap mass spectrometer in selected-reaction monitoring mode. As it is known that triple quadrupole mass spectrometers display improved sensitivity in selected-reaction monitoring mode, it would be interesting to compare the detection limits which could be achieved when using such a mass spectrometer coupled with our HPLC separation. This could be a way of increasing sensitivity towards the determination of medium- and long-chain acylcarnitines in plasma samples.

As described in chapter 5, the developed HPLC-MS/MS method could successfully be used for the quantitative determination of carnitine, short-chain acylcarnitines and butyrobetaine profiles in the plasma of patients under hemodialysis. The effects of the dialysis process on the plasma concentrations of these compounds could be evaluated. Additionally, the distribution of the plasma acylcarnitine pools in these patients and the outcome of carnitine supplementation on carnitine, acylcarnitines and butyrobetaine plasma levels could be assessed.

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Curriculum vitae

Contact l.vernez@hotmail.com

EDUCATION

1999 - 2004	**PhD thesis** at the Division of Clinical Pharmacology & Toxicology, University Hospital of Basel, University of Basel, under the supervision of Prof. S. Krähenbühl) Thesis topic: *Analysis of carnitine and acylcarnitines in biological fluids and application to a clinical study*
1990 - 1996	**Study of pharmacy**, University of Geneva
1985 - 1990	**High school**, Thônex, Geneva Maturity type C (option science)

PROFESSIONAL EXPERIENCES

1998	Six months practical training, Laboratory for Pharmaceutical Analysis and Pharmacokinetic, Department of clinical research, University of Bern Topic: *Application and establishment of analytical methods (HPLC-DAD, GC-FID, GC-MS) for determination of narcotics (opioids and cannabinoids) in biological fluids*
1997 - 1998	One year PhD position, Central Clinical Chemistry Laboratory, University Hospital of Geneva Topic: Establishment of capillary electrophoresis method for the separation of proteins; application of mono- and bidimensional electrophoresis and molecular methods for proteins analysis
1996	Three months ERASMUS exchange, Faculty of Science, Design and Construction, Nescot's College, Epsom, UK Topic: *Establishment and evaluation of an ELISA system for detection of paroxetine in biological fluids*
1993 - 1996	Substitute for pharmacists in several pharmacies, Geneva
1992 - 1993	Practical year in a pharmacy during the third year study, Geneva

PUBLICATIONS

Vernez, L., W. Thormann and S. Krähenbühl. **2000**. Analysis of carnitine and acylcarnitines in urine by capillary electrophoresis. J Chromatogr A 895 (1-2): 309-16

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CONGRESSES PARTICIPATION AND POSTER PRESENTATION

15th IFCC-FESCC European Congress of Clinical Chemistry and Laboratory Medicine and 22nd National Congress of the Spanish Society of Clinical Biochemistry and Molecular Pathology, June 1-5 2003, Barcelona, Spain (EUROMEDLAB 2003)
poster presentation

26th International Symposium on high-performance liquid phase separations and related techniques, June 2-7 2002, Montreal, Canada (HPLC 2002) **poster presentation**

11th International Symposium on Pharmaceutical and Biomedical Analysis, May 14-18 2000, Basel, Switzerland (PBA 2000)

13th International Symposium on high-performance Capillary Electropjhoresis & Related Microscale Techniques, February 20-24 2000, Saarbrücken, Germany (HPCE 2000), with one day short course on CE/MS **poster presentation**

LANGUAGES

French	mother tongue
German	fluent in written and speaking (5 years in Swiss Germany)
English	fluent in written and speaking (Certificate in Advanced English)

INFORMATIC SKILLS

Word, Excel, Powerpoint, Origin, Endnote, TopFit, Internet, Xcalibur (mass spectrometer software), different softwares for driving HPLC (Agilent-HP 1100) and CE (Biorad-BioFocus 300) apparatus