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Quantification of carnitine esters by high-performance liquid chromatography

Effect of feeding medium-chain triglycerides on the plasma carnitine ester profile

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ABSTRACT

A high-performance liquid chromatographic (HPLC) technique was developed using commercially available derivatization reagents and commonly used reversed-phase HPLC column chemistry to analyze plasma samples for their carnitine ester content. The method proved to be sufficiently sensitive to determine changes in the carnitine ester profile in plasma resulting from metabolic disorders or metabolic insults. The method was tested using plasma samples obtained from pigs fed medium-chain triglycerides (MCT) of different chain lengths (four to seven carbons). The MCT feeding was associated with transient increases in plasma carnitine and carnitine esters, and feeding odd-chain MCT (tri-C_5 or tri-C_7) led to elevated levels of propionylcarnitine in plasma.

INTRODUCTION

The role of L-carnitine (β-hydroxy-γ-trimethylaminobutyric acid) in the transport of long-chain fatty acids across the inner mitochondrial membrane has long been acknowledged [1]. More recently, carnitine has been shown to have a similar role in the export of the products of β-oxidation from the peroxisomes [2]. It has also been proposed [3] that carnitine has a role in releasing mitochondrial coenzyme A (CoA) from acyl-CoA when the free CoA supply becomes limiting due to activation and subsequent accumulation of metabolites in the mitochondrion. Carnitine might act to buffer the mitochondrial acyl-CoA/CoA ratio by translocating excess acyl groups into the cytosol.

Carnitine is considered to be a conditionally essential nutrient, especially for the neonate. This nutritional aspect of carnitine has been widely accepted when long-chain fatty acids ($\geqslant C_{14}$) provide the majority of the energy for the neonate [4]. Recent evidence, however, has suggested that carnitine supplementation might also be important in neonates fed medium-chain triglycerides (MCT, containing C_6 to C_{12} fatty acids) [3,5].

These functions of carnitine make it an important compound in lipid metabolism, and studying the composition of the carnitine ester pool can add valuable information to our understanding of metabolism. Indeed, metabolism of carnitine has been studied extensively with regard to its

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role in infant nutrition [6], ketogenesis [7,8], aciduria [9], and metabolic disorders involving oxidation of fatty acids, such as acyl-CoA dehydrogenase deficiencies [10]. The carnitine profile in tissues and plasma can function as an indicator of steps limiting the oxidation of fatty acids. Accumulated acyl-CoA esters are converted to carnitine esters, which can be excreted into the cytosol and can enter the plasma pool. Plasma carnitine esters are incompletely reabsorbed in the kidney, resulting in the excretion of these esters in urine [11]. Determination of the composition of the urinary or plasma carnitine esters can therefore provide information about the steps in oxidation which may be limiting.

Currently available methods to analyze the different carnitine esters are, however, limited. A commonly used assay is the radioisotopic exchange assay [12]. While this method is adequate for crude separations of free, acid-soluble (short- and medium-chain) and acid-insoluble (long-chain) acylcarnitines, it is incapable of distinguishing specific carnitine esters that are of interest in metabolic studies.

Carnitine esters have been analyzed using radioisotopic exchange, followed by high-performance liquid chromatographic (HPLC) separation and quantification using a β-flow monitor [13]. Unfortunately, carnitine acetyltransferase (EC 2.3.1.7), used to exchange carnitine of acylcarnitine esters with radiolabelled carnitine, is not sufficiently active toward long-chain acylcarnitines to obtain quantitative yields, nor do medium-chain acylcarnitines and branched-chain acylcarnitines yield quantitative information without inclusion of appropriate standards. Another confounding issue is the presence of an acylcarnitine hydrolase associated with commercial preparations of carnitine acetyltransferase, which results in the breakdown of acylcarnitines [14]. Although this method is extremely sensitive, these complications make it of limited applicability.

Other methods to quantify acylcarnitines have been reported which use HPLC separation followed by UV detection of derivatized acylcarnitines [15,16]. The derivatization procedure employs phenacyl reagents which react with the carboxyl group of carnitine, creating a UV-absorbing chromophore. Although these methods do not have the limitation imposed by the limited chain-length specificity of commercial acylcarnitine acetyltransferase, the use of complex derivatization reagents, ternary gradients or atypical columns prompted us to develop a simplified method.

The objective of this paper was therefore to describe a method to analyze carnitine esters using commonly available equipment and chemicals. This method was subsequently tested using plasma samples obtained from pigs fed MCT to determine specifically whether plasma carnitine esters are affected by feeding MCT.

EXPERIMENTAL

Chemicals

Biosafe II scintillation cocktail was obtained from Research Products International (Mount Prospect, IL, USA). [^{14}C]Carnitine was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Methanol (HPLC grade) was purchased from Baxter Healthcare (Muskegon, MI, USA). Phenacyl-8 was purchased from Pierce (Rockford, IL, USA). Acetonitrile (HPLC grade) was from Fisher (Fair Lawn, NJ, USA), and ninhydrin was obtained from Beckman (Palo Alto, CA, USA). MCT were synthesized by Karlshamns Lipid Specialties (Columbus, OH, USA). All other chemicals and the Kieselgel 60 were supplied by Sigma (St. Louis, MO, USA). Vacutainers were obtained from Becton Dickenson (Rutherford, NJ, USA).

Equipment

A Beckman System Gold HPLC apparatus was used (Beckman, Palo Alto, CA, USA). The system consisted of two 110B pumps, a Model 406 analog interface and a Model 166 variable-wavelength UV detector. The system was equipped with a Model 507 autoinjector with a 50-μl injection loop. The column used was an Ultrasphere ODS-IP column (5 μm particle size, 25 cm $\times$ 0.46 cm I.D., Beckman) with a cartridge system guard column (Absorbosphere HS C_{18}, 5

μm particle size, Alltech, Deerfield, IL, USA). To accommodate three eluents, pump A was equipped with a three-way switching valve (General Valve, Fairfield, NJ, USA), connected with a 12-V power supply (Radio Shack, Tandy, Fort Worth, TX, USA), controlled by a relay in the 406 module (using a second 3-V power supply and a relay). The valve was positioned so as to switch solvents at the inlet line of the pump. From the same pump A, the pulse dampener (6 ml void volume) was removed to reduce the volume between the pump and the injector (final volume was approximately 4 ml).

To verify retention times of phenacylcarnitine esters and to determine the recovery of [^{14}C]carnitine, a Model 265 β-flow monitor was used (Radiomatics, Tampa, FL, USA). Fractions containing radioactivity were collected using a Model 204 fraction collector (Gilson, Middleton, WI, USA) and quantified using a Model LS6000IC liquid scintillation counter (Beckman).

Isolation of carnitine esters from plasma samples

Carnitine esters (including free carnitine and γ-butyrobetaine) were isolated from plasma using a methanol extraction procedure modified after Minkler *et al.* [15]. The isolation and derivatization procedure is summarized in Fig. 1. This procedure can be used to isolate carnitine esters from liver as well (unpublished data).

The internal standard (hexanoylcarnitine or [^{14}C]carnitine) was added to a known amount of plasma (250 μl). The plasma was extracted with five volumes of 100% methanol and centrifuged at 30 000 *g* in a 2-ml microcentrifuge tube (non-siliconized). The supernatant was transferred into another vial, and 50 μl of ninhydrin were added. This mixture was evaporated to dryness using a Model AS160 Speed-Vac (Savant, Farmingdale, NY, USA) allowing the ninhydrin to react with amino acids contained in the methanol extract. The methanol-soluble products were reconstituted in 1 ml of 100% methanol and recentrifuged if cloudy. The methanol fraction (supernatant) was then loaded onto a 1-ml silica gel column (Kieselgel 60) contained in a pasteur pipet, and washed three times with a volume of methanol equal to the column volume. Carnitine esters were subsequently eluted using 1.25 ml of a mixture of methanol–water–acetic acid (MWA, 50:45:5, v/v/v) into a glass vial. The MWA fraction was then evaporated to dryness.

The efficiency of the isolation procedure was assessed by adding a known amount of [^{14}C]carnitine to the plasma samples before methanol extraction. In subsequent steps of the isolation procedure 'waste' was collected, and radioactivity in this waste was determined.

Derivatization procedure

Acylcarnitines were derivatized using commercially available phenacyl bromide which reacts with the carboxyl group of carnitine forming a phenacyl ester [17]. The dried MWA fraction was reconstituted in (in the order specified) 20 μl of water, 0.1 ml of phenacyl-8 (0.1 *M* phenacyl bromide and 0.005 *M* crown ether in acetonitrile) and 0.9 ml of acetonitrile. This mixture was either heated at 80°C under vigorous stirring or sonicated at 60°C in an ultrasonic water bath for 90 min (Model 3200, Branson, Danbury, CT, USA). Similar derivatization efficiencies of [^{14}C]carnitine were obtained from each procedure.

The reaction mixture was evaporated to dryness, and the phenacylcarnitine esters were reconstituted in 500 μl of a mixture of acetonitrile–water (25:75, v/v). Up to 50 μl of this mixture were injected into the HPLC system.

The efficiency of derivatization was determined based on recovery of [^{14}C]carnitine and measured as the percentage of radioactivity associated with the UV peak corresponding to phenacylcarnitine, compared to total radioactivity per injection. Underivatized carnitine does not coelute with derivatized carnitine.

To allow quantification of phenacylcarnitine esters, [^{14}C]carnitine and [^{14}C]hexanoylcarnitine of known specific activity were derivatized and chromatographed. The peak area and the radioactivity associated with the corresponding peaks were determined, and the peak area per nmol of phenacylcarnitine ester was calculated (analogous to a molar absorptivity).

To verify that the isolation and derivatization

procedure yielded similar results for free carnitine and medium-chain acylcarnitines, the recovery of exogenous [^{14}C]carnitine and hexanoylcarnitine from plasma samples was determined.

Chromatography

A constant flow-rate of 1.6 ml/min was utilized, which resulted in a head pressure ranging from 115 to 175 bar over the course of the gradient employed. Three eluents were used with the following composition: (I) acetonitrile–water (70:30, v/v); (II) 100% water; (III) acetonitrile–TEAP (80% acetonitrile, 20% water, 0.8% triethylamine, 0.64% phosphoric acid).

All eluents were passed through a 0.2-μm filter (Anodisk 47, Alltech) and degassed using an Alltech filter apparatus. The water used was distilled and deionized (Barnsted 4-module E-pure system, Barnsted, Dubuque, IA, USA).

The gradients are presented graphically in Fig. 2. The acetonitrile–water eluent (100% I) was used to equilibrate the column and was maintained until 1 min after an injection was made (dependent on the void volume of the system between switch valve and column; for our system this was approximately 4 ml). Then, 1 min after injection, the switch valve was activated on pump A, causing a flow of 100% water (eluent II). The acetonitrile remaining in the system was sufficient to elute all excess derivatization agent. In our experience, extending eluent I for longer duration caused free carnitine and acetylcarnitine phenacyl esters to co-elute in the void volume.

Eluent II was subsequently maintained at 100% for 5 min. A shorter time shifted the free carnitine and acetylcarnitine derivatives to a shorter retention time, and resulted in very poor peak shapes. Times of 2 min, however, resulted in good peak shapes for carnitine and acetylcarnitine derivatives, but not for the medium-chain ester derivatives.

At 6 min after injection, a gradient was initiated, delivered through pump B, with the acetonitrile–TEAP eluent (eluent III) initially at 15%. This was increased linearly over a 25-min time period to 100% (replacing eluent II). Then, 36 min after the injection, acetonitrile–TEAP (eluent III via pump B) was switched to 100% eluent I (via pump A). The switch valve was typically deactivated 25 min after injection such that heat produced by the switch valve was not a problem. The system was re-equilibrated for 19 min; injections could thus be made at 55-min intervals.

This method was optimized for short- and medium-chain carnitine esters (C_2–C_{10}) and tested up to palmitoylcarnitine. Preliminary analyses indicated that long-chain carnitine esters were not present at detectable levels in samples used for this and similar studies. Extending the 100% acetonitrile–TEAP period elutes longer-chain esters.

Phenacylcarnitine esters have a maximum UV absorption at 245 nm (based on spectral analysis obtained with a scanning Gilford Response II spectrophotometer, Ciba Corning Diagnostics, Oberlin, OH, USA). Therefore, the detector was programmed to monitor absorbance at 245 nm. Acetonitrile and triethylamine absorbed at 245 nm, and this resulted in a gradual rise in baseline with the progression of the gradient. This baseline shift could be reduced by using a higher wavelength, *e.g.* 254 nm, but this resulted in a 20% decrease in sensitivity.

Elution profile of carnitine esters

Retention times of different carnitine esters were determined in order to identify peaks in chromatograms obtained from plasma samples. Carnitine esters were either purchased or synthesized according to the following procedure. CoA esters (0.5 m*M*) were suspended in a 100 m*M* Tris [tris(hydroxymethyl)aminomethane] buffer (pH 8.0) containing 1 m*M* L-carnitine (plus [^{14}C]carnitine). To this mixture was added carnitine acetyltransferase in order to convert the CoA esters to carnitine esters.

The elution profile of γ-butyrobetaine (deoxycarnitine) and trimethyllysine was also evaluated. Both components are precursors for the *de novo* synthesis of carnitine and have been detected in plasma [18]. The addition of ninhydrin during the purification of carnitine esters destroys trimethyllysine; therefore, ninhydrin should be omitted for the quantification of total carnitine precursors.

Effect of feeding MCT of varying chain-length on the carnitine ester profiles in newborn pigs

To determine the effect of feeding MCT on the plasma carnitine profile, plasma samples were obtained from sixteen MCT-fed pigs. The pigs were removed from the sow 24 h after birth (four pigs per litter), and were fasted for an additional 4 h while housed in pens maintained at 32°C. MCT oils consisting of different chain-length (saturated) fatty acids (four, five, six or seven carbons, indicated as tri-C_4, tri-C_5, tri-C_6, or tri-C_7, respectively) were subsequently fed via gastric intubation in doses of 6 ml so that all four pigs per litter received MCT oil containing a different chain-length fatty acid. Blood samples were obtained via jugular venipuncture using heparinized vacutainers at 0, 1, 2, 4, and 8 h after oral gavage. Plasma was obtained following centrifugation at 4°C and was stored at −20°C until analyzed. The samples used were obtained in the course of another study [19].

The data were analyzed by the analysis of variance procedure using SAS [20] according to a split–splot design, blocked by litter [21]. Results are given as means ± standard deviation (S.D.).

RESULTS

A graphic summary of the isolation procedure as well as the losses of [^{14}C]carnitine internal standard in the subsequent steps is given in Fig. 1. [^{14}C]Carnitine was lost in the methanol precipitate (10.9 ± 0.3% of initial ^{14}C present, n = 6), in the waste effluents from the silicagel column (2.7 ± 4.5%), and in non-specified losses (1%; transfer pipettes, remnants in vials). The overall percentage of carnitine recovered in the phenacylcarnitine peak was 79.9 ± 6.7%.

The efficiency of derivatization was 94.2 ± 4.1% (n = 6). The recovery of [^{14}C]carnitine and hexanoyl-carnitine (UV) correlated with an r^2 of 0.95, and the absorption area per nmol of carnitine (7.42) and hexanoyl-carnitine (7.70) were not statistically different ($p > 0.1$). The minimal detection limit was 20 pmol, and the coefficient of variation was 8% (data not shown).

The complete elution profile of all tested esters and γ-butyrobetaine is given in Fig. 2. This elution profile is actually a composite of two chromatograms since the reaction conditions used to synthesize the acylcarnitines caused a broad car-

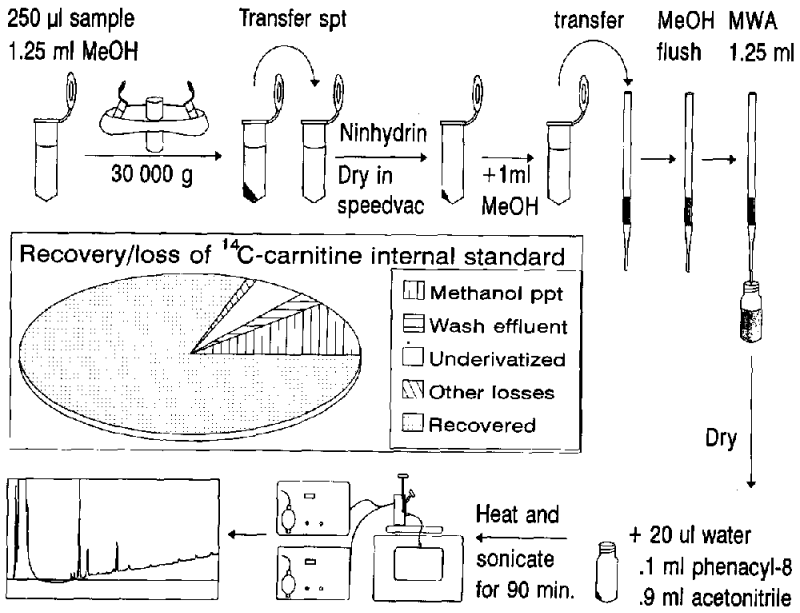

Fig. 1. Graphic summary of the isolation procedure for acylcarnitines from plasma samples and the loss of [^{14}C]carnitine in the subsequent steps of the isolation procedure (ppt = precipitate, spt = supernatant, MeOH = methanol, MWA = methanol–water–acetic acid).

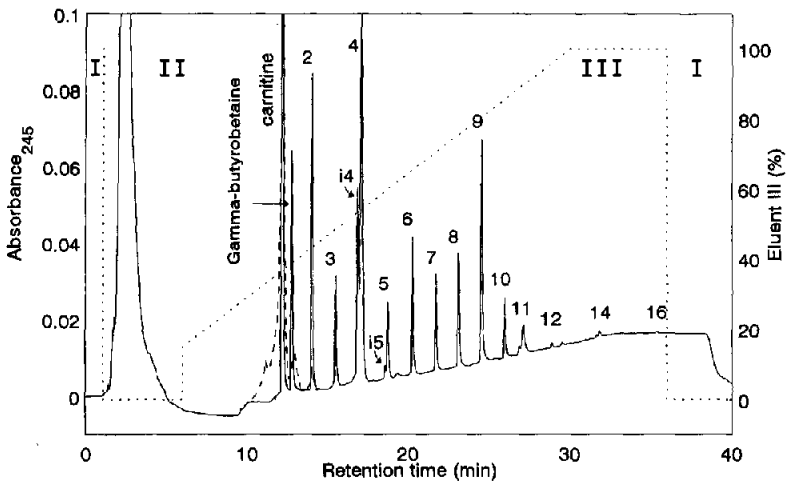

Fig. 2. Elution profile of phenacyl esters of carnitine, γ-butyrobetaine, and various carnitine esters, identified by numbers representing the chain-length of the acylgroup (i4 = isobutyrylcarnitine; i5 = isovalerylcarnitine). The Roman numbers and dotted lines represent the eluents used to obtain the chromatogram (see text for details).

nitine peak, overlapping γ-butyrobetaine (see dashed line). To illustrate the separation of carnitine esters, another chromatogram (10–15 min) with carnitine, γ-butyrobetaine, and acetylcarnitine is overlaid.

Two chromatograms, obtained from plasma sample analysis (equivalent to approximately 20 μl of plasma) for carnitine esters are given in Fig. 3. Both chromatograms show the presence of free carnitine, the carnitine precursor γ-butyrobetaine, acetylcarnitine, and C_4-carnitine. In the chromatogram obtained from a pig fed tri-C_7 (Fig. 3B), propionylcarnitine and possibly valerylcarnitine were present.

The acyl chain-length of the MCT fed to the piglets significantly affected the concentration of free carnitine as well as the concentration of propionylcarnitine. Propionyl-carnitine was undetectable in animals fed even-chain MCT (tri-C_4 and tri-C_6), as well as at time 0 in all treatments.

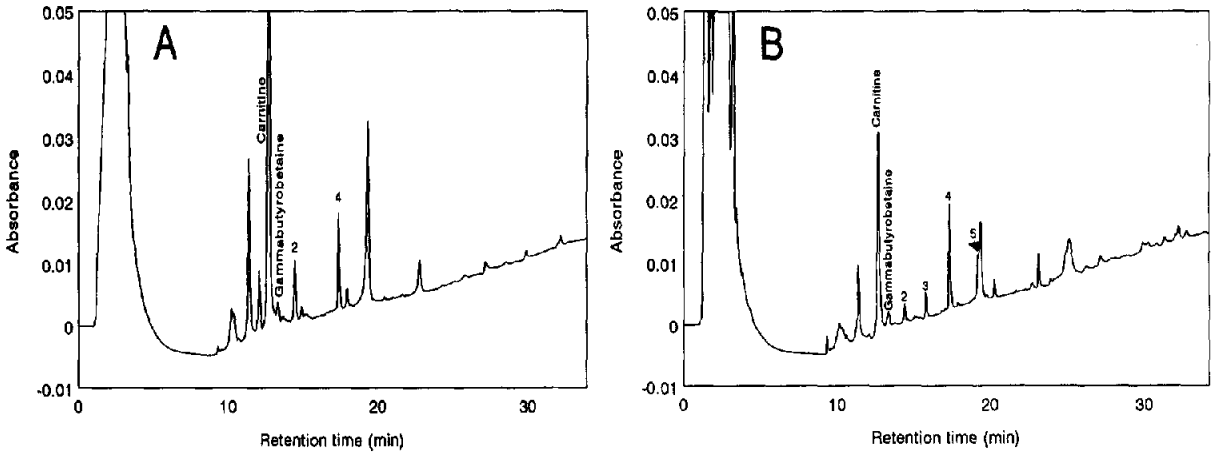

Fig. 3. Samples of carnitine ester plasma profiles obtained from pigs 4 h after feeding 6 ml of medium-chain triglycerides containing (saturated) fatty acyl chains of six (A) or seven (B) carbons.

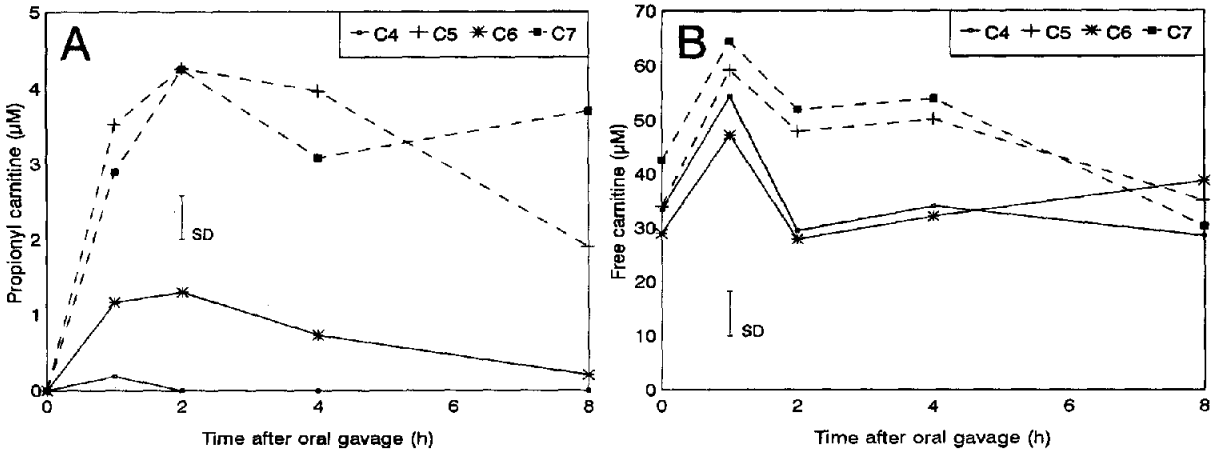

Fig. 4. Effect of feeding MCT of varying chain-lengths (tri-C_4 through tri-C_7) on the concentrations of propionylcarnitine (A) and free carnitine (B) in plasma of newborn pigs. Propionylcarnitine: time effect $p < 0.01$ and chain-length effect $p < 0.05$; free carnitine: time effect $p < 0.01$ and chain-length effect $p < 0.06$; $n = 3$ per mean. SD = standard deviation.

In animals fed odd-chain MCT (tri-C_5 or tri-C_7), propionylcarnitine increased with increasing oxidation rate of the odd-chain fatty acids [22] and declined in a similar fashion as the oxidation rate declined (see Fig. 4A).

Plasma free carnitine concentrations varied considerably during the sampling period. A significant chain-length effect ($p < 0.06$) as well as a significant time effect was observed ($p < 0.05$). Odd-chain MCT invoked a higher plasma free carnitine concentration compared to even-chain MCT. A significant increase in plasma carnitine concentration was observed 1 h after gastric intubation, while no increase was observed 2 h after gastric intubation (plasma fatty acid concentrations peaked between 1 and 2 h [19]).

No significant effects were observed in γ-butyrobetaine and acetylcarnitine esters ($p > 0.1$). Acetylcarnitine varied considerably, and tended to be higher when tri-C_6 and tri-C_7 were fed, but due to the high variation, no significant effects were observed (Fig. 5A).

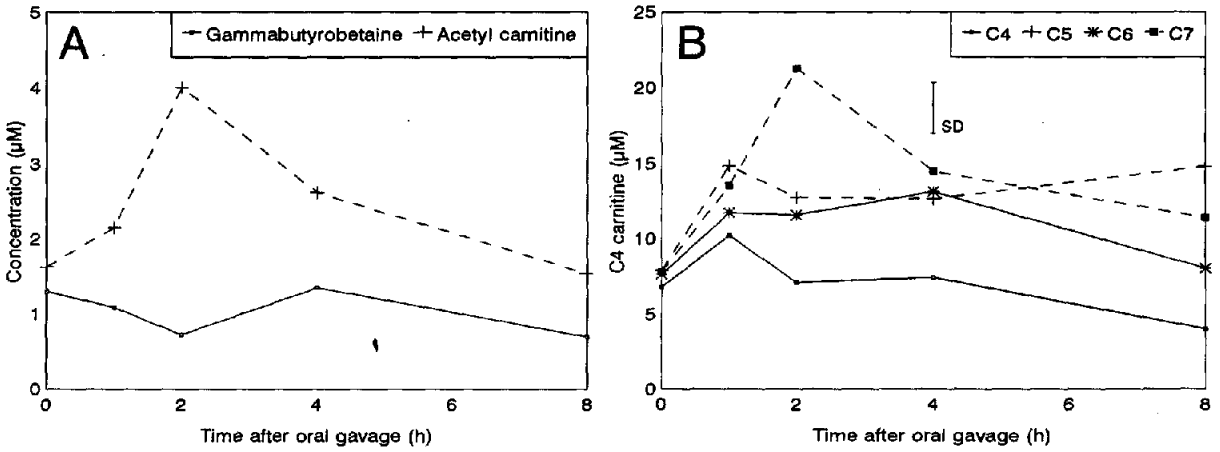

Fig. 5. Effect of feeding MCT of varying chain-length on (A) the concentrations of γ-butyrobetaine and acetylcarnitine averaged over treatments ($n = 12$ per mean) and (B) C_4-carnitine ($n = 3$ per mean) in plasma of newborn pigs. SD = standard deviation.

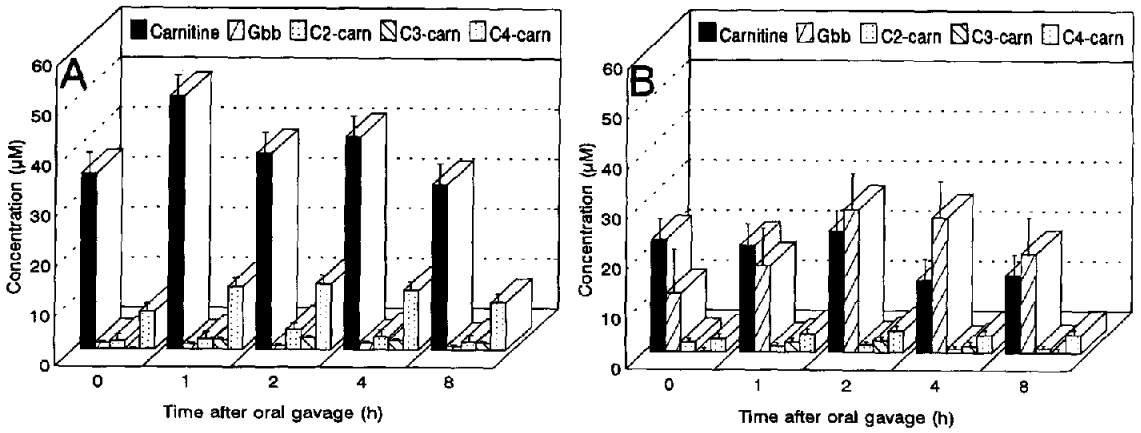

Fig. 6. Effect of feeding MCT on the carnitine profile over time: (A) averaged over twelve pigs (three litters) with normal γ-butyrobetaine (Gbb) levels or (B) averaged over four pigs (one litter) with high γ-butyrobetaine levels (C2-carn = acetylcarnitine; C3-carn = propionylcarnitine; C4-carn = butyrylcarnitine). Data are given as means + standard deviation.

The C_4-carnitine peak, presumptively identified as butyrylcarnitine, followed a pattern which mirrored the pattern of free carnitine (Fig. 5B); however, no significant effects were observed ($p > 0.1$).

Other peaks occurred in the chromatograms, from which the retention time of one peak corresponded with the retention time of C_5-carnitine (from pigs fed tri-C_7). Due to sporadic co-elution of a reagent peak, later identified as originating from the use of dated ninhydrin reagent, the C_5-carnitine peak could not be quantified consistently (Fig. 3B). Other peaks were not identified since their retention time did not correspond to the retention time of a saturated, straight-chain acylcarnitine.

With the exception of one litter of pigs, γ-butyrobetaine was low throughout the experiment. These animals were not included in the final analysis of the data. Pigs from this litter exhibited extremely high concentrations of carnitine precursor (γ-butyrobetaine) and relatively low levels of free carnitine. No elevation in free carnitine was observed 1 h after oral gavage, while a rise was observed in γ-butyrobetaine. Fig. 6 shows data averaged over treatments from this litter contrasted to data averaged over treatments from the other three litters.

DISCUSSION

The method presented provides a powerful tool to analyze carnitine esters. Because it employs standard reversed-phase column chemistry and commercially available reagents, this method is easy to implement in laboratories using an HPLC system. The observation that odd-chain MCT can give rise to propionylcarnitine in plasma (Fig. 4A) and (abnormally) elevated γ-butyrobetaine (Fig. 6) in one litter of pigs illustrates the importance of a method which can distinguish between different carnitine esters in plasma samples and, therefore, detect potential abnormalities in the profile. Several metabolic disorders can be characterized via analysis of the carnitines profile as observed in plasma. [10,23].

The isolation procedure of plasma carnitine esters proved to be effective for both free and medium-chain acylcarnitines. Steric hindrance of the carboxyl group of (medium-chain) acylcarnitine by the fatty-acid chain, which could possibly prevent efficient derivatization, as mentioned by Minkler *et al.* [24] was not observed to be a problem using phenacyl bromide as a derivatization agent.

The data obtained from pigs fed MCT indicate that MCT feeding does affect the concentration of free carnitine in plasma (Fig. 4B). The concen-

tration of free carnitine increased 1 h after feeding, but dropped to the 0-h level at 2 h. The increase in plasma carnitine at 1 h after feeding MCT suggests that the pig has the ability to mobilize carnitine from tissues such as the intestines, as suggested by Li *et al.* [25]. The biological significance of this possible mobilization is unknown, but raises the question of whether plasma carnitine is a correct parameter for assessing the carnitine status of a subject under certain nutrional and/or disease states.

The drop in plasma carnitine between 1 and 2 h after feeding MCT might be caused by an impaired reabsorption of free carnitine by the kidneys due to the high levels of plasma fatty acids, as suggested by Penn *et al.* [26] and Stadler and Rebouche [27]. Further experiments are needed to determine the exact cause.

Odd-chain fatty acids (upon β-oxidation) can yield propionate (as well as valerate). Propionylcarnitine was present in the plasma of pigs fed tri-C_5 or tri-C_7, suggesting that the production of propionyl-CoA exceeds the oxidation in certain tissues (Fig. 4A).

The anecdotal observation that one litter of pigs had high concentrations of the carnitine precursor γ-butyrobetaine and relative low levels of free carnitine suggests that these pigs had a genetic deficiency, impairing either the uptake of γ-butyrobetaine by the liver or the synthesis of carnitine from γ-butyrobetaine in the liver (Fig. 6). A similar condition was previously observed in an infant by Dr. S. C. Winter (personal communication). Detailed information on this case is, however, lacking as well.

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