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Quantification of carnitine and specific acylcarnitines by high-performance liquid chromatography: application to normal human urine and urine from patients with methylmalonic aciduria, isovaleric acidemia or medium-chain acyl-CoA dehydrogenase deficiency

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ABSTRACT

This paper describes the development of a high-performance liquid chromatographic method for the quantitation of free carnitine, total carnitine, acetylcarnitine, propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine and octanoylcarnitine in human urine. Carnitine and acylcarnitines were isolated from 10 or 25 μl of urine using 0.5-ml columns of silica gel, derivatized with 4′-bromophenacyl trifluoromethanesulfonate and separated by high-performance liquid chromatography. Using 4-(N,N-dimethyl-N-ethylammonio)-3-hydroxybutanoate ("e-carnitine") as the internal standard, standard curves (10–300 nmol/ml) were generated. Carnitine and acylcarnitines were quantified (when they were present) in normal human urine and the urine of patients diagnosed with one of three different disorders of organic acid metabolism: methylmalonic aciduria, isovaleric acidemia, and medium-chain acyl-CoA dehydrogenase deficiency.

INTRODUCTION

The occurrence and distribution of free carnitine (4-N-trimethylammonio-3-hydroxybutanoate) and specific acylcarnitines (carnitinyl esters) in urine can be characteristic of disorders of organic acid metabolism [1]. Knowledge of urinary free carnitine and specific acylcarnitine concentrations greatly assists the diagnosis and management of these diseases.

One approach to carnitine/acylcarnitine analysis is based on a two-step procedure. First, free carnitine is determined. Then the procedure is repeated, except that the acylcarnitines are first hydrolyzed to form carnitine. The total carnitine present is then quantified and the acylcarnitine concentration is calculated: total carnitine − free carnitine = acylcarnitine. Although radioenzymatic procedures [2–6] are the most common of the methods that utilize this approach, there also are procedures that use either fast atom bombardment ionization coupled with mass spectroscopy (FAB–MS) [7] or pre-column chemical derivatization followed by high-performance liquid chromatography (HPLC) [8]. However, these methods are sometimes inadequate because the

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identity of the individual acylcarnitines contained in the total carnitine value is unknown.

A second approach is based on analytical techniques that determine the individual acylcarnitines. Among the reported procedures are those which use radioisotope exchange HPLC [9,10], FAB–MS [11], on-column derivatization gas chromatography followed by mass spectroscopy (GC–MS) [12], pre-column acylcarnitine deamination GC–MS [13] and pre-column chemical derivatization HPLC [14,15].

A published procedure for urinary free and total carnitine quantification by HPLC [8] demonstrated that values determined by pre-column chemical derivatization HPLC were not different from results obtained using a radioenzymatic procedure. Now that procedure has been expanded and improved to permit quantification of specific acylcarnitines that are characteristic of three organic acid metabolism diseases: methylmalonic aciduria, isovaleric acidemia and medium-chain acyl-CoA dehydrogenase (MCAD) deficiency. Therefore, what follows is a simple, rapid, accurate and precise pre-column chemical derivatization HPLC method for the simultaneous quantification of free carnitine, acetyl-, propionyl-, isovaleryl-, hexanoyl- and octanoylcarnitine along with the follow-up determination of total carnitine in human urine.

EXPERIMENTAL

Equipment

The HPLC system consisted of a 1050 Series quaternary pump, autosampler and multiple-wavelength detector (operated at 260 nm) purchased from Hewlett-Packard (Avondale, PA, USA). The HPLC column was a 100 mm × 4.6 mm I.D. column packed with Hypersil (MOS-1) C_8 (3 μm particle size) manufactured by Alltech Assoc., (Deerfield, IL, USA). A Hewlett-Packard Vectra QS/20 386DX PC running under Windows 3.1 (Microsoft, Redmond, WA, USA) and Hewlett-Packard 3365 ChemStation were used for collection of chromatographic data. Disposable polypropylene chromatography columns were purchased from Evergreen Scientific (Los Angeles, CA, USA). These columns were slurry packed with 0.5 ml of silica gel using a repeater pipettor. Evaporation of sample tubes was accomplished with an Evapo-O-Rac (Cole-Parmer, Chicago, IL, USA) evaporation manifold attached to the house-compressed air outlet.

Materials

B&J UV-grade acetonitrile was purchased from Baxter Scientific (Obetz, OH, USA). HPLC-grade acetonitrile, methanol, acetic acid, triethylamine and potassium phosphate monobasic were purchased from Fisher Scientific (Cleveland, OH, USA). N,N-Diisopropylethylamine and phosphoric acid were purchased from Aldrich (Milwaukee, WI, USA). Silica gel 60 (230–400 mesh) was purchased from Curtin Matheson Scientific (Broadview Heights, OH, USA). *l*-Carnitine was a generous gift from Sigma-Tau (Rome, Italy). [^{14}C-methyl]Carnitine was synthesized as described [16]. The internal standard, 4-(N,N-dimethyl-N-ethylammonio)-3-hydroxybutanoate ("e-carnitine"), and 4′-bromophenacyl trifluoromethanesulfonate were prepared as described [8,17]. Acetylcarnitine, propionylcarnitine, isovalerylcarnitine, hexanoylcarnitine and octanoylcarnitine were synthesized from the appropriate acid (Aldrich) and carnitine [18]. Betaine was purchased from Aldrich while trimethyllysine (6-N,N,N-trimethyllysine) and butyrobetaine (4-N,N,N-trimethylammonio butanoate) were synthesized as before [19,20].

Urine acylcarnitine (including free carnitine) sample preparation

Urine (25 μl) or diluted urine (10 μl urine plus 20 μl water) samples were placed in microcentrifuge tubes. To each of these tubes was added 25 μl of the e-carnitine internal standard (100 nmol/ml), 50 μl of 1 *M* potassium phosphate monobasic and 1 ml of acetonitrile–methanol (3:1, v/v).

Urine total carnitine sample preparation

Urine (25 μl) or diluted urine (10 μl urine plus 20 μl water) samples were placed in microcentrifuge tubes. Each tube received 10 μl of a 1 *M* KOH solution. The tubes were vortex-mixed and

allowed to stand at room temperature for 10 min. To each of these tubes were then added 25 μl of the e-carnitine internal standard (100 nmol/ml), 50 μl of 1 *M* potassium phosphate monobasic and 1 ml of acetonitrile–methanol (3:1, v/v).

Sample isolation

The microcentrifuge tubes were capped, vortex-mixed for 2 s, and centrifuged for 5 min at 13 600 *g*. The supernatants were decanted into 0.5-ml silica gel columns. The columns were then washed with 2.0 ml of methanol followed by 1.0 ml of 1% acetic acid in methanol (these washes were discarded). Carnitine, acylcarnitines and e-carnitine (internal standard) were eluted into tubes with 4.0 ml of 1% acetic acid in methanol. The tubes were then placed under the evaporation manifold, immersed in a 35°C water bath, and the contents of the tubes were evaporated to dryness.

Derivatization with 4′-bromophenacyl trifluoromethanesulfonate

To the dried residue in each tube were added 250 μl of acetonitrile–methanol (3:1, v/v). The tubes were vortex-mixed (2 s) and the contents decanted into microcentrifuge tubes. These tubes were centrifuged (5 min at 13 600 *g*) and the resulting supernatants transferred into 300-μl autosampler vial micro inserts. The contents of these inserts were evaporated to dryness using the evaporation manifold. Each insert then received 10 μl of a diisopropylethylamine solution (25 μl in 10 ml methanol) and 20 μl of a 4′-bromophenacyl trifluoromethanesulfonate solution (0.035 g in 1 ml acetonitrile; 0.1 *M*). The tubes were crimp-capped, vortex-mixed (2 s) and allowed to stand for 10 min; then 6 μl of the reaction mixture were ready to be injected directly into the HPLC system.

High-performance liquid chromatography

Four chromatographic eluents were used: eluent A contained 800 ml of acetonitrile and 200 ml of water, eluent B contained 200 ml of acetonitrile and 800 ml of water, eluent C contained 5 ml of triethylamine, 4 ml of phosphoric acid, 200 ml of acetonitrile and 800 ml of water, and eluent D contained 2.5 ml of triethylamine, 2 ml of phosphoric acid, 900 ml of acetonitrile and 100 ml of water. Initially, 100% eluent A was pumped at a flow-rate of 1.75 ml/min. At 0.20 min after sample injection, eluent A was replaced by 100% eluent B. At 1.00 min after sample injection, eluent B was replaced by 100% eluent C and there began a linear gradient over 10.00 min of 100% eluent C to 100% eluent D. At 11.01 min the eluent was switched to 100% eluent A. At 12.00 min the chromatographic run was finished and the next injection could then be performed within 2 min.

Quantification

Quantification of total carnitine, free carnitine, acetyl-, propionyl-, isovaleryl-, hexanoyl- and octanoylcarnitine in human urine specimens was accomplished by interpolation from generated standard curves. First, a standardized solution of carnitine was formulated: an approximately 1 μmol/ml solution of carnitine was prepared and its carnitine concentration was determined using the spectrophotometric method of Marquis and Fritz [21]. Dilutions of this solution were prepared and free carnitine and total carnitine standard curves were then generated. Individual acylcarnitine solutions (approximately 1 μmol/ml of each acylcarnitine) were prepared and standardized by determining the free and total carnitine in these solutions. The acylcarnitine concentrations were then calculated: total carnitine concentration − free carnitine concentration = acylcarnitine concentration. Dilutions of these standardized solutions were prepared and simultaneous standard curves for acetyl-, propionyl-, isovaleryl-, hexanoyl- and octanoylcarnitine were generated by combining these solutions, evaporating to dryness with compressed air, reconstituting in 25 μl of water, and then treating each mixture as if it were an acylcarnitine urine specimen. Using this approach, the standard curves of all the acylcarnitines were generated simultaneously. Although the free carnitine contamination in the acylcarnitine solutions required that the free carnitine and the individual acylcarnitine

standard curves be generated separately, the free carnitine and acylcarnitines were simultaneously quantified within the acylcarnitine urine preparation.

Recovery studies

The recovery of carnitine from the sample isolation was determined by subjecting replicate samples from three human urine specimens (25 μl) to the above described procedures for acylcarnitines and total carnitine isolation. However, in place of the 25-μl aliquot of e-carnitine (internal standard) normally used, there was substituted 25 μl of a solution containing [^{14}C-methyl]carnitine. The radioactivity was determined in both the acylcarnitine (including free carnitine) and total carnitine sample preparations (1) after the extraction with acetonitrile–methanol, (2) after the silica gel chromatography and (3) after reconstitution in acetonitrile–methanol and centrifugation.

In a second experiment, the recoveries of free carnitine, acetyl-, propionyl-, isovaleryl-, hexanoyl- and octanoylcarnitine from the entire acylcarnitine sample procedure (along with the recovery of total carnitine from the total carnitine sample procedure) were determined by external standardization. First, standard solutions of free carnitine, acetyl-, propionyl-, isovaleryl-, hexanoyl-, octanoylcarnitine or total carnitine (25 μl; 100 nmol/ml) were prepared as if they were urine samples, except that the 25-μl aliquot of e-carnitine was replaced with water. Just prior to derivatization, 25 μl of e-carnitine (100 nmol/ml) was added to each sample as an external standard. These samples were derivatized, chromatographed, and the peak-height ratios of carnitine or acylcarnitine *versus* e-carnitine external standard were calculated. These ratios were compared to the peak-height ratios of standards and e-carnitine which were simply evaporated, derivatized, and chromatographed. The recovery of e-carnitine from the acylcarnitine and total carnitine procedures were determined exactly the same way, but carnitine was used as the external standard for e-carnitine.

Extent of derivatization study

Four replicate samples of an isovaleric acidemia human urine specimen (25 μl) were prepared; two samples underwent the described acylcarnitine (including free carnitine) procedure while the other two replicates underwent total carnitine isolation. In place of the 25-μl aliquot of e-carnitine (internal standard) normally used, 25 μl of a solution containing 34 000 dpm of [^{14}C-methyl]carnitine solution was used. One of the two acylcarnitine and total carnitine samples were then derivatized and injected into the HPLC column. The other acylcarnitine and total carnitine samples were treated the same, except that the 4′-bromophenacyl trifluoromethanesulfonate–acetonitrile solution was replaced with acetonitrile only. Fractions (0.5 min) of the HPLC effluent from these four injections were collected and the radioactivity contained in each was determined.

Reproducibility and reliability studies

Three human urine specimens, one each from a patient diagnosed with methylmalonic aciduria, isovaleric acidemia or MCAD deficiency, were selected. For each specimen, six 10-μl replicates were analyzed for both acylcarnitines (including free carnitine) and total carnitine on three different days to calculate the sample-to-sample and day-to-day reproducibility, respectively. In a second experiment, various amounts of carnitine, acetyl-, propionyl-, isovaleryl-, hexanoyl- or octanoylcarnitine were added to microcentrifuge tubes. Following evaporation, each of these tubes received 25 μl of a normal human urine specimen. These tubes were vortex-mixed and analyzed for acylcarnitines (including free carnitine) and total carnitine.

RESULTS

The pre-column chemical derivatization HPLC method described uses the same sample isolation procedure as described previously [8]. In the course of the development of the method, the recovery of carnitine from the sample isolation procedure was examined. [^{14}C-methyl]Carnitine

TABLE I

STEP-BY-STEP RECOVERY OF CARNITINE FROM THE SAMPLE ISOLATION PROCEDURE USING THREE DIFFERENT URINES WITH ADDED [^{14}C]CARNITINE

Sample preparation step	Recovery (mean ± S.D., n = 3) (%)	
	Free carnitine	Total carnitine
Radioactivity isolated following acetonitrile–methanol (3:1) extraction	93 ± 4	92 ± 2
Radioactivity isolated following small-column silica gel chromatography	77 ± 3	78 ± 3
Radioactivity isolated following the acetonitrile–methanol (3:1) reconstitution centrifugation step	67 ± 3	66 ± 6

was added to urine (Table I). There was an apparent loss of between 7 and 16% of the added radioactive carnitine with each sample isolation step. The recoveries of standard solutions of carnitine, e-carnitine and acylcarnitines through the entire procedures using HPLC peak-height ratios of these compounds *versus* an external standard were also determined (Table II). As shown, the recoveries of the acylcarnitines examined were similar to but different from carnitine. The extent of derivatization of carnitine was determined by adding [^{14}C-methyl]carnitine to human urine from a patient with isovaleric acidemia. Fig. 1 displays the superimposition of the four different HPLC chromatograms generated from this study. The derivatized carnitine radioactivity eluted between 4.0 and 4.5 min, thus co-chromatographing with carnitine 4′-bromophenacyl ester. Underivatized carnitine eluted between 1.5 and 2.5 min. In the derivatized specimens, there was a small amount of radioactivity eluting between 2.0 and 2.5 min, which accounted for 5 and

TABLE II

RECOVERY OF CARNITINE, E-CARNITINE AND ACYLCARNITINES THROUGH THE ENTIRE ACYLCARNITINE (INCLUDING FREE CARNITINE) AND TOTAL CARNITINE PROCEDURES

Calculations were performed based on external standardization and HPLC chromatographic peak heights).

Carnitine	Standard solutions subjected to derivatization and injection		Standard solutions subjected to sample isolation, derivatization and injection		Recovery (%)
	Average peak-height ratio[a]	Error[b] (%)	Average peak-height ratio[a]	Error[b] (%)	
Free carnitine	0.92	1	0.45	1	50
Total carnitine	0.92	1	0.50	2	54
Free e-carnitine	1.09	1	0.66	3	60
Total e-carnitine	1.09	1	0.63	0	58
Acetylcarnitine	1.14	1	0.72	2	63
Propionylcarnitine	1.18	0	0.80	2	68
Isovalerylcarnitine	1.19	2	0.86	3	72
Hexanoylcarnitine	1.18	0	0.79	3	67
Octanoylcarnitine	1.13	0	0.66	6	58

[a] Average of two determinations of the (peak height of compound)/(peak height of external standard).

[b] [(Larger value − average)/average] · 100%.

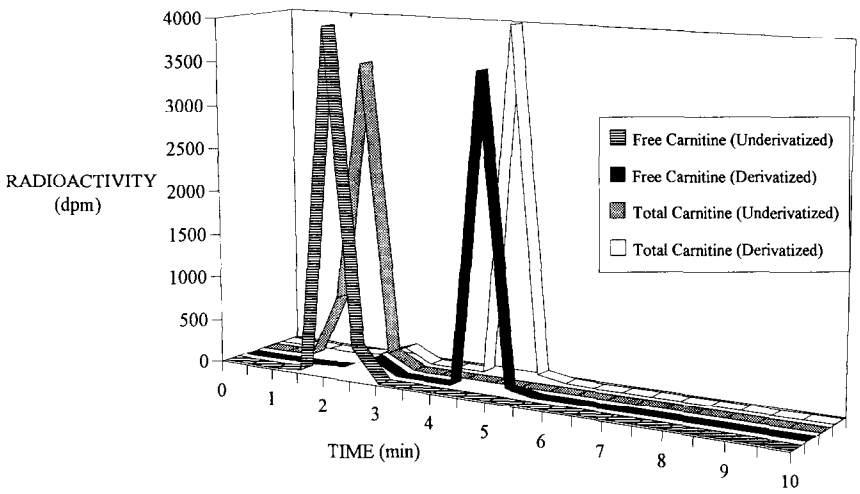

Fig. 1. Extent of derivatization experiment. To four aliquots of a urine specimen from a patient with isovaleric acidemia were added radioactive carnitine. Two of these aliquots were prepared by the acylcarnitine (including free carnitine) procedure and two were prepared by the total carnitine procedure. One of each of these acylcarnitine and total carnitine pairs was derivatized with 4′-bromophenacyl trifluoromethanesulfonate, while the other pair was simply injected. The post-column eluent following each injection was collected as 0.5-min fractions and the radioactivity was determined. The data presented in this graph reflects the superimposition of these four different HPLC chromatograms, with each chromatographic result represented by a different pattered ribbon. The radioactivity peak in the free derivatized and total derivatized samples (black and white ribbons, respectively) co-chromatographed with carnitine 4′-bromophenacyl ester and contained 95%+ of the radioactivity injected.

4% of the radioactivity injected for acylcarnitine and total carnitine procedures, respectively. With 95%+ of the radioactivity in this urine specimen appearing in the carnitine 4′-bromophenacyl ester peak, the reaction of carnitine with 4′-bromophenacyl trifluoromethanesulfonate is essentially quantitative (the same quantitative results were reported using 4′-bromophenacyl trifluoromethanesulfonate with the biosynthetic precursor of carnitine, butyrobetaine [20]). The amount of radioactivity appearing in the [^{14}C]carnitine 4′-bromophenacyl ester peaks (when corrected for injection volume) was 56 and 62% of the original radioactivity committed to the acylcarnitine and total carnitine procedures, respectively. Comparing this to the recovery of free carnitine and total carnitine presented in Table II (50 and 54% recovered, respectively), it was noted that these values are in close agreement.

The described procedure had the ability to resolve not only carnitine and the various acylcarnitines from each other, but it could also distinguish these compounds from other similar quaternary carboxylates. Fig. 2 shows a chromatogram of a mixture containing 2 nmol of carnitine and 2.5 nmol each of e-carnitine, acetyl-, propionyl-, isovaleryl-, hexanoyl-, octanoylcarnitine, betaine, butyrobetaine and trimethyllysine. The compounds in this solution were isolated, derivatized and chromatographed using the sample preparation scheme for acylcarnitines. There is no peak (peak 10) for trimethyllysine. This was because under these sample isolation conditions, trimethyllysine was not recovered. With full-scale detection at 0.10 a.u.f.s., these peaks were more than 500 times greater than the detection sensitivity limit [20]. The free carnitine peak (peak 1) in the chromatogram resulted from added carnitine and was not the result of acylcarnitine hydrolysis.

Standard curves for acetyl-, propionyl-, isovaleryl-, hexanoyl- and octanoylcarnitine (using 25 μl of solutions containing 10, 25, 50, 100 and 300 nmol/ml of each compound) were generated together. Separate free carnitine and the total car-

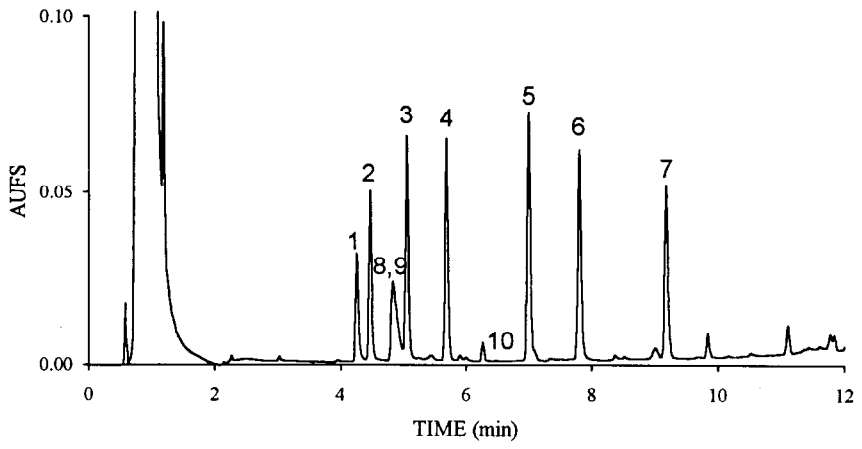

Fig. 2. Chromatograms of standard solutions of carnitine, acylcarnitines and related compounds; 2.5 nmol of these compounds (or 2 nmol in the case of carnitine) were combined, evaporated, reconstituted in 25 μl of water and then prepared as if they were a urine specimen. Peaks: 1 = carnitine; 2 = e-carnitine; 3 = acetylcarnitine; 4 = propionylcarnitine; 5 = isovalerylcarnitine; 6 = hexanoylcarnitine; 7 = octanoylcarnitine; 8 = betaine; 9 = butyrobetaine; 10 = trimethyllysine.

nitine standard curves were also generated using 25 μl of solutions containing 10, 25, 50, 100 and 300 nmol/ml and 50, 100, 300, 500 and 800 nmol/ml carnitine, respectively. The HP3365 software determined the chromatographic peak heights of the standard chromatograms and (using the internal standard as its basis of comparison) fit a linear equation to the chromatographic peak-height data *versus* carnitine or acylcarnitine concentration. The resulting linear regression coefficients (r^2) were greater than 0.99. Despite this, concentration values at both the low and high

TABLE III

SUMMARY OF THE SAMPLE-TO-SAMPLE AND DAY-TO-DAY REPRODUCIBILITY STUDY PERFORMED WITH URINES FROM PATIENTS DIAGNOSED WITH METHYLMALONIC ACIDURIA, ISOVALERIC ACIDEMIA AND MCAD DEFICIENCY

Carnitine	Concentration (mean ± S.D.) (nmol/l)					
	Methylmalonic aciduria		Isovaleric acidemia		MCAD deficiency	
	Sample-to-sample (n = 6)	Day-to-day (n = 17)	Sample-to-sample (n = 5)	Day-to-day (n = 15)	Sample-to-sample (n = 6)	Day-to-day (n = 16)
Free carnitine	161 ± 4	166 ± 14	599 ± 30	599 ± 26	124 ± 4	118 ± 12
Acetylcarnitine	52 ± 2	53 ± 3	86 ± 4	84 ± 6	36 ± 1	37 ± 3
Propionylcarnitine	304 ± 17	309 ± 29	N.D.[a]	N.D.	N.D.	N.D.
Isovalerylcarnitine	N.D.	N.D.	271 ± 8	256 ± 25	N.D.	N.D.
Hexanoylcarnitine	N.D.	N.D.	N.D.	N.D.	21 ± 1	23 ± 6
Octanoylcarnitine	N.D.	N.D.	N.D.	N.D.	411 ± 14	420 ± 60
Total carnitine	675 ± 36	680 ± 26	1041 ± 28	1029 ± 83	757 ± 25	728 ± 34

[a] N.D. = not detected.

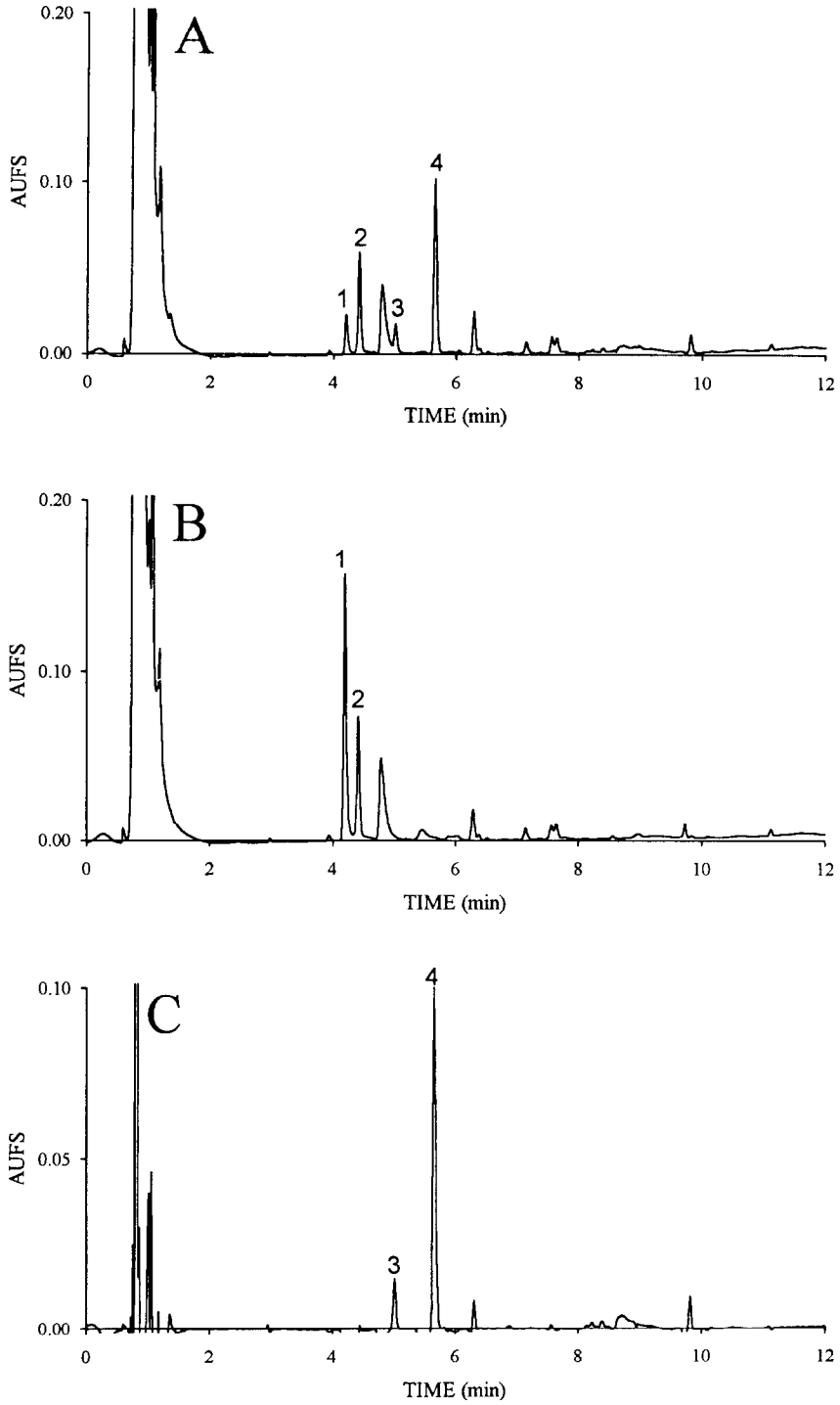

Fig. 3. Chromatograms of 10 μl of urine obtained from a two-year-old female patient with methylmalonic aciduria who was receiving 100 mg/kg carnitine per day as treatment. The identities of peaks 1–4 are as in Fig. 2. (A) Acylcarnitines (including free carnitine); (B) total carnitine; (C) the result of chromatogram A subtracted from chromatogram B. Determined concentrations: free carnitine = 169 nmol/ml; acetylcarnitine = 56 nmol/ml; propionylcarnitine = 311 nmol/ml; total carnitine = 690 nmol/ml.

ends of the standard curve ranges were better represented using standard curves calculated with second-order equations rather than with linear equations. This was due to the disproportional broadening of the peaks (particularly hexanoyl- and octanoylcarnitine) as their concentrations increased. Therefore, standard curves were calculated using second-order equations and these standard curves were used in the quantification of sample concentrations.

The precision of the assay was examined by a three day sample-to-sample and day-to-day reproducibility study (Table III). In each case, replicate 10-μl aliquots of urine from patients with methylmalonic aciduria, isovaleric acidemia or MCAD deficiency were prepared and quantified. As a monitor of the consistency of the assay, (pippetting precision, sample isolation, derivatization reproducibility, etc.) it was noted that over the course of the three-day study, the variability ($\pm$ standard deviation) of the internal standard peak heights in the urine samples were $\pm$ 15% for acylcarnitine and $\pm$ 11% for total carnitine determinations. The variability of the internal standard retention times was less than $\pm$0.4%. The small variability of these quantities implied the entire procedure functioned in a reproducible fashion. This reproducibility is also reflected in the small sample-to-sample and day-to-day variabilities reported in Table III.

Fig. 3 displays three chromatograms derived from the urine of a patient with methylmalonic aciduria receiving carnitine therapy: (A) acylcarnitines (including free carnitine), (B) total carnitine and (C) the result of the subtraction of these two chromatograms from each other. Likewise, Fig. 4 displays chromatograms from a patient with isovaleric acidemia receiving carnitine therapy, Fig. 5 displays chromatograms from a patient with MCAD deficiency not receiving carnitine therapy and Fig. 6 displays chromatograms prepared from replicate 25-μl aliquots of a normal human urine not receiving carnitine.

Method validation studies were performed. First, we wanted to determine if the assay's response to the addition of known amounts of carnitine or acylcarnitines to a urine specimen was linear. This was necessary in order to verify that the carnitine and acylcarnitine standard curves would in fact reflect the amount of detected material within a urine sample. Our second concern was with the stability of the acylcarnitines during the sample isolation procedure. Some conditions, (for instance those conditions which were used to determine total carnitine) can result in the decomposition of acylcarnitines, resulting in free carnitine. It was therefore necessary to prove that the amount of free carnitine was not artificially increased due to the experimental procedure. These two questions were examined by adding increasing amounts of carnitine or acylcarnitines to a human urine (Fig. 7; in performing this study, it was taken into account that the synthesized acylcarnitine standards were contaminated with between 7 and 21% free carnitine; therefore, the free and total carnitine concentration values were corrected by subtracting the determined carnitine contamination values from the free and total carnitine concentration values). The concentration of free carnitine and total carnitine *versus* carnitine added to the urine specimen generated linear responses with slopes of 1 for both free carnitine and total carnitine (see Fig. 7A). The y-intercepts were 128 and 252 nmol/ml (due to endogenous free carnitine and total carnitine, respectively). The calculated acylcarnitine concentration (total carnitine $-$ free carnitine) resulted in a line with a zero slope, indicating that the empirically derived acylcarnitine concentration did not change with the addition of carnitine. Next, the assay's response to the addition of acetyl-, propionyl-, isovaleryl-, hexanoyl- or octanoylcarnitine was examined (Fig. 7B–F). The total carnitine concentrations *versus* added acylcarnitine resulted in linear equations with slopes of approximately 1 and the same y-intercept as with Fig. 7A, while the free carnitine concentrations did not change (zero slopes). This indicated that the acylcarnitines were not hydrolyzed during the course of the assay, since any hydrolysis would have increased the free carnitine concentration. The individual acylcarnitine concentrations *versus* added acylcarnitine generated linear responses with slopes of approximately 1, indi-

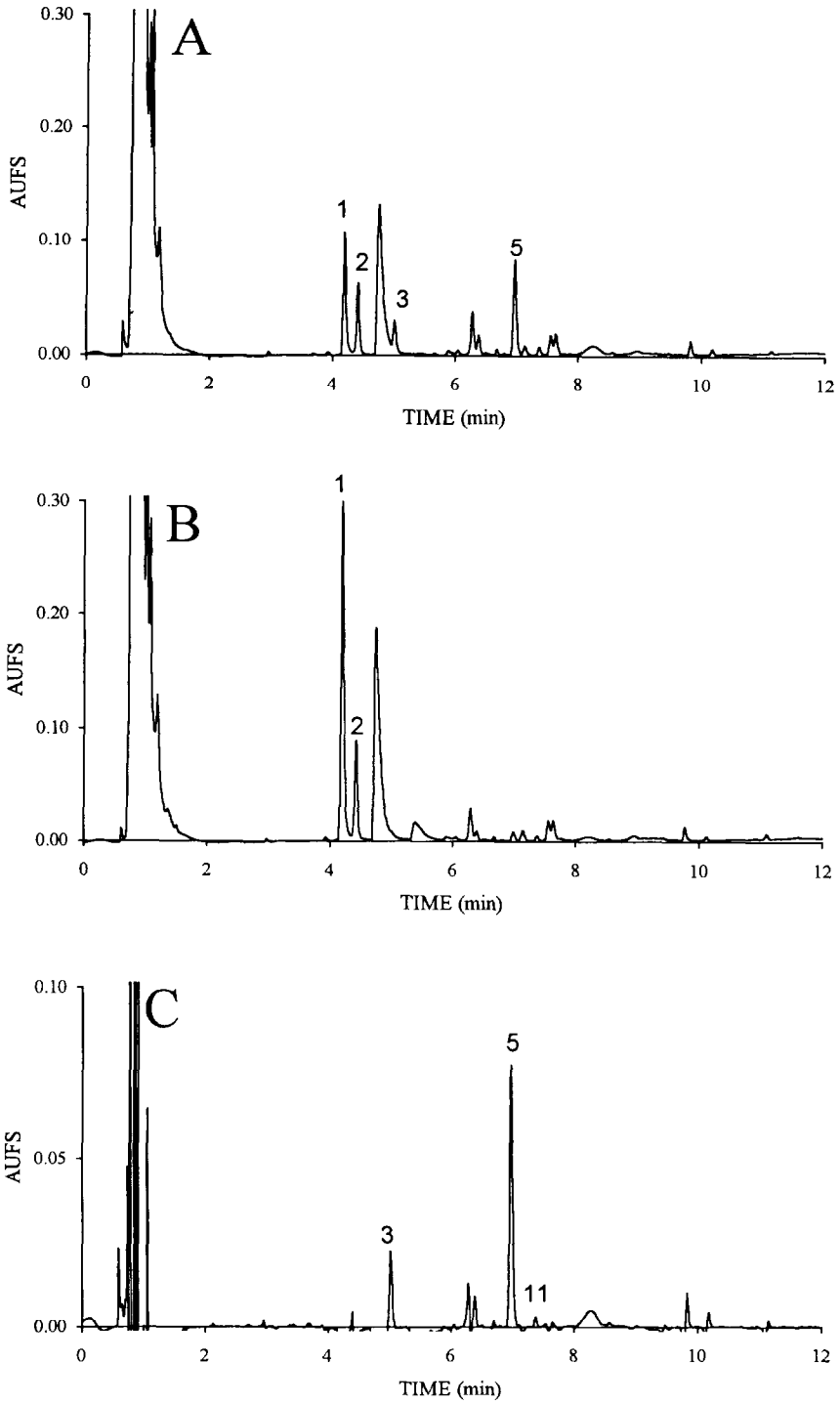

Fig. 4. Chromatograms of 10 μl of urine obtained from a seventeen-month-old female patient with isovaleric acidemia who was receiving 150 mg/kg carnitine per day as treatment. Peak identities 1–3 and 5 are as in Fig. 2; peak 11 is a possible unidentified acylcarnitine. (A) Acylcarnitines (including free carnitine); (B) total carnitine; (C) the result of chromatogram A subtracted from chromatogram B. Determined concentrations: free carnitine = 594 nmol/ml; acetylcarnitine = 90 nmol/ml; isovalerylcarnitine = 244 nmol/ml; total carnitine = 1027 nmol/ml.

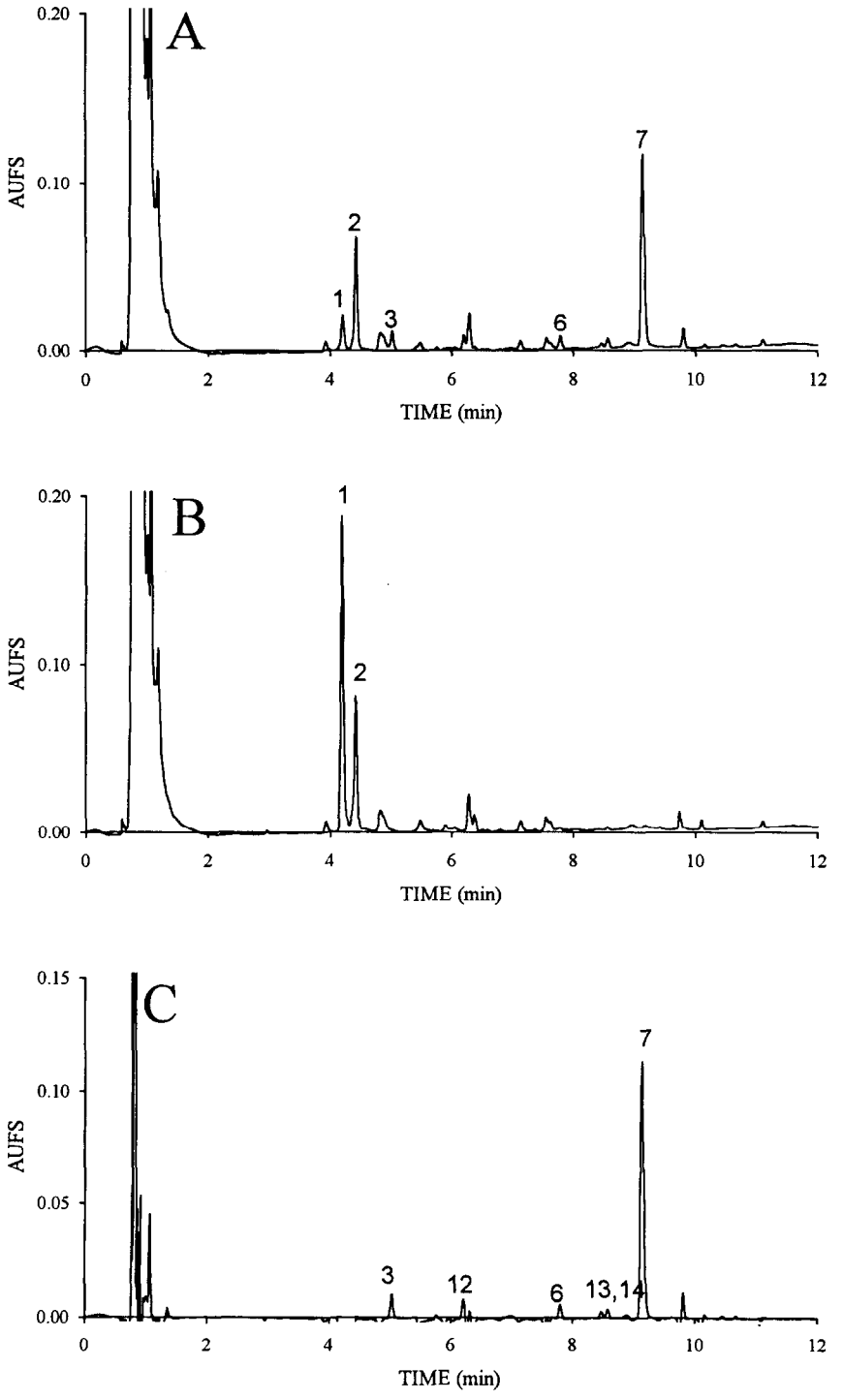

Fig. 5. Chromatograms of 10 μl of urine obtained from a fourteen-month-old female who was acutely ill (but not receiving carnitine therapy) with MCAD deficiency. The identities of peaks 1–3, 6 and 7 are as in Fig. 2; peaks 12–14 are possible unidentified acylcarnitines. (A) Acylcarnitines (including free carnitine); (B) total carnitine; (C) the result of chromatogram A subtracted from chromatogram B. Determined concentrations: free carnitine = 123 nmol/ml; acetylcarnitine = 37 nmol/ml; hexanoylcarnitine = 23 nmol/ml; octanoylcarnitine = 397 nmol/ml; total carnitine = 735 nmol/ml.

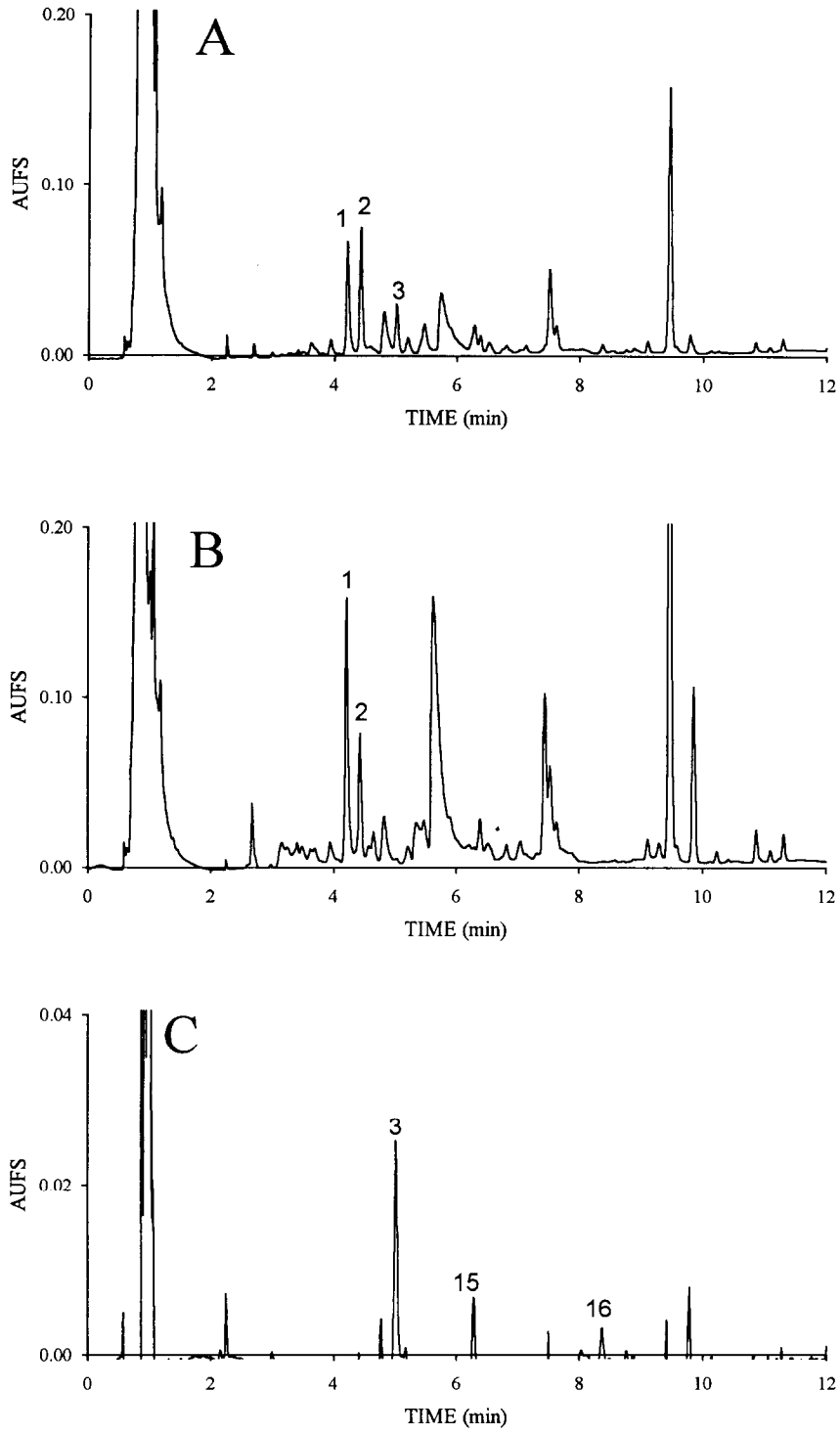

Fig. 6. Chromatograms of 25 μl of urine obtained from a normal 35-year-old adult male. The identities of peaks 1–3 are as in Fig. 2; peaks 15 and 16 are possible unidentified acylcarnitines. (A) Acylcarnitines (including free carnitine); (B) total carnitine; (C) the result of chromatogram A subtracted from chromatogram B. Determined concentrations: free carnitine = 128 nmol/ml; acetylcarnitine = 32 nmol/ml; total carnitine = 250 nmol/ml.

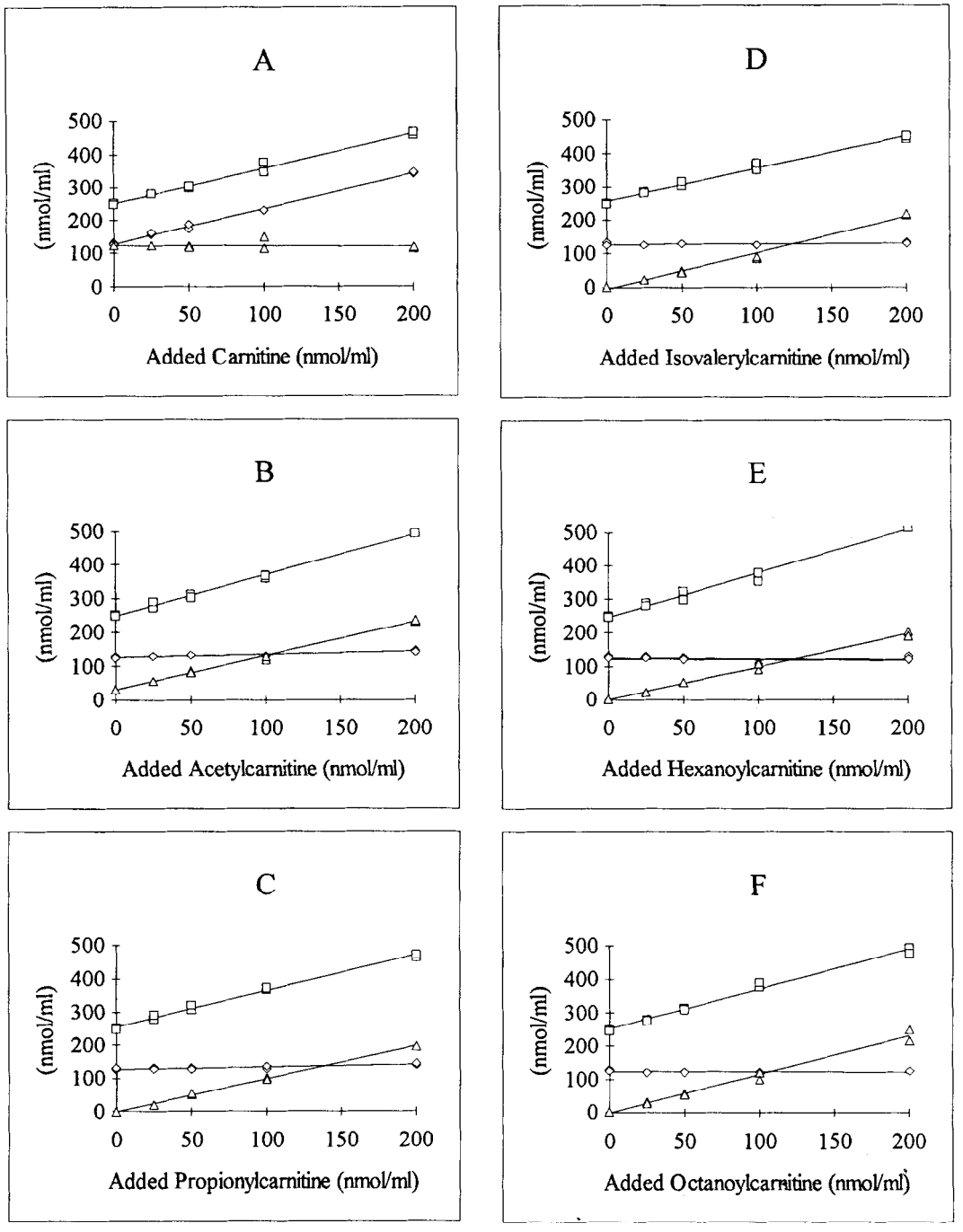

Fig. 7. Graphs displaying the results of adding various amounts of carnitine or a specific acylcarnitine to a normal human urine. Total carnitine values are represented by squares, free carnitine values are represented by diamonds, and acylcarnitine values are represented by triangles. Added compounds: (A) Carnitine; (B) acetylcarnitine; (C) propionylcarnitine; (D) isovalerylcarnitine; (E) hexanoylcarnitine; (F) octanoylcarnitine.

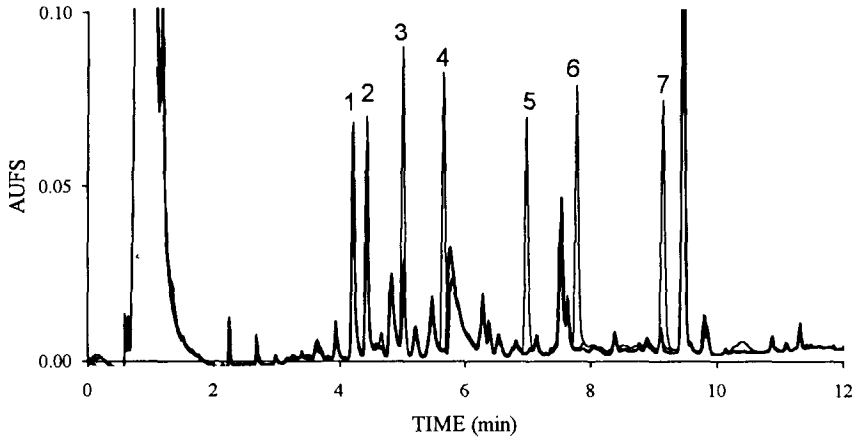

Fig. 8. Five superimposed chromatograms of a normal human urine with 100 nmol/ml of added acetyl-, propionyl-, isovaleryl-, hexanoyl- or octanoylcarnitine.

cating that the increase in acylcarnitine concentration was in proportion to the amount of acylcarnitine added. The only endogenous acylcarnitine identified in this urine was acetylcarnitine (32 nmol/ml). Because of this, the added acetylcarnitine experiment (Fig. 7B) yielded a non-zero y-intercept. All the other individual acylcarnitine concentration *versus* added acylcarnitine linear response plots had y-intercepts of approximately zero (Figs. 7C–F).

Fig. 8 is the superimposition of five chromatograms from a normal human urine to which was added 100 nmol/ml acetyl-, propionyl-, isovaleryl-, hexanoyl- or octanoylcarnitine (acetylcarnitine = 129 nmol/ml, propionylcarnitine = 101 nmol/ml, isovalerylcarnitine = 101 nmol/ml, hexanoylcarnitine = 107 nmol/ml and octanoylcarnitine = 99 nmol/ml). This figure clearly shows the endogenous acetylcarnitine (peak 3). The sum of the added acetylcarnitine (100 nmol/ml) and the determined endogenous acetylcarnitine (32 nmol/ml) is very close to the determined concentration of the acetylcarnitine in this urine (129 nmol/ml *versus* 132 nmol/ml). Together, Figs. 7 and 8 offer clear evidence that the assay can accurately quantify carnitine and acylcarnitines added to urine. This, of course, very strongly implies that endogenous compounds will likewise be correctly quantified. Again, as an indication of the consistency of the assay (including pippetting precision and sample isolation and derivatization reproducibility), the internal standard peak-height and retention variability were monitored. It was noted that over the course of the study, the acylcarnitine determinations had an internal standard peak-height variability ($\pm$ standard deviation) of $\pm 14\%$ and the total carnitine urine determinations had an internal standard peak-height variability of $\pm 6\%$. The variability of the internal standard retention times was less than $\pm 0.2\%$.

Balance studies, which determine the percentage of the acylcarnitines not accounted for by the individual acylcarnitine procedure, are contained in Table IV. All the patients with methylmalonic aciduria and isovaleric acidemia were receiving carnitine. Two of the MCAD deficiency patients shown were not receiving carnitine therapy and the five normal urines were from persons not receiving carnitine therapy. The percentage acylcarnitines found was calculcated as follows: (sum of the concentrations of the identified acylcarnitines)/(total carnitine concentration − free carnitine concentration). This value indicated that there were large amounts of unaccounted for acylcarnitines in most of the samples examined.

TABLE IV

BALANCE STUDY

Sample	Methylmalonic aciduria				
	Total carnitine (nmol/ml)	Free carnitine (nmol/ml)	Acetyl-carnitine (nmol/ml)	Propionyl-carnitine (nmol/ml)	Percentage acylcarnitine found
1	680	166	53	309	70
2	1786	607	175	573	63
3	794	306	62	178	49
4	1349	605	164	414	78
5	1509	595	165	533	76
	Isovaleric acidemia				
	Total carnitine (nmol/ml)	Free carnitine (nmol/ml)	Acetyl-carnitine (nmol/ml)	Isovaleryl-carnitine (nmol/ml)	Percentage acylcarnitine found
1	1029	599	84	256	79
2	3162	928	702	1360	92
3	2241	281	69	1281	69
	MCAD deficiency				
	Total carnitine (nmol/ml)	Free carnitine (nmol/ml)	Acetyl-carnitine (nmol/ml)	Hexanoyl-carnitine (nmol/ml)	Octanoyl-acylcarnitine (nmol/ml)	Percentage acylcarnitine found
1[a]	728	118	36	23	420	79
2	2886	1897	572	N.D.	169	75
3[a]	52	10	6	N.D.	11	40
	Normal				
	Total carnitine (nmol/ml)	Free carnitine (nmol/ml)	Acetyl-carnitine (nmol/ml)	Percentage acylcarnitine found	
1[a]	250	128	32	26	
2[a]	137	89	22	46	
3[a]	216	81	28	21	
4[a]	431	227	29	14	
5[a]	420	290	47	36	

[a] Samples from persons not receiving carnitine therapy.

DISCUSSION

HPLC system

The HPLC system was developed for rapid sample turn around and resolution of derivatized carnitine and acylcarnitines from other UV-absorbing compounds. The HPLC column used for this procedure contained packing material which was not end-capped. The free silanols on the chromatographic material permitted the on-line

extraction of the cationic carnitine, acylcarnitines and internal standard 4′-bromophenacyl esters from the large reagent peak using eluent A (80% acetonitrile, no triethylamine–phosphoric acid; despite the high acetonitrile content, carnitine and acylcarnitines were retained while excess reagent was washed from the column). Switching to eluent B at 0.20 min lowered the acetonitrile concentration to 20% acetonitrile. This was necessary to prepare the column for the inclusion of triethylamine–phosphoric acid into the mobile phase at 1.00 min (eluent C). Carnitine and acylcarnitine 4′-bromophenacyl esters were then eluted by disrupting their ionic retention with a competing cation (acidified triethylamine) in the presence of an acetonitrile gradient (eluents C and D).

The HPLC eluents were formulated from two different brands of acetonitrile. The B&J UV-grade acetonitrile had slightly more background absorbance at 260 nm than the HPLC-grade acetonitrile from Fisher Scientific and this difference was used to the method's advantage. The B&J acetonitrile was used in the eluent that contained 20% acetonitrile (eluent C) and the Fisher Scientific acetonitrile was used in the eluent that contained 80% acetonitrile (eluent D). The result was a flat baseline (despite the acetonitrile gradient) between 1.00 and 11.00 min.

Brief summary of the limitations of other acylcarnitine methods

(1) Radioisotope exchange HPLC [9] assumes that all acylcarnitines are substrates for the acylcarnitine transferase enzyme(s) used, and therefore engage in radioisotope exchange. However, it is now known that some acylcarnitines undergo incomplete radioisotope exchange, whereas other acylcarnitines do not exchange at all [22]. Worse yet, it has been reported that some unusual acylcarnitines inhibit carnitine acyltransferases [23], putting into question any values derived from this process. Radioisotope exchange HPLC procedures do not quantify free carnitine or total carnitine, and therefore separate procedures must be employed to make these determinations. Unknown acylcarnitines must be identified using separate procedures.

(2) FAB–MS [11] can identify acylcarnitine fragments, but cannot differentiate among structural isomers without additional fragmentation (FAB-MS–MS) or separation techniques (LC–FAB-MS). Quantification of acylcarnitines by FAB-MS requires the inclusion of a stable labeled isotope internal standard for each of the compounds to be quantified [24]. Those acylcarnitines for which their respective internal standard is not included are not precisely quantifiable. Therefore, only qualitative information is provided for those compounds.

(3) On-column derivatization GC–MS [12] provides MS data on acylcarnitine fragments, but does not quantify free carnitine or total carnitine. Therefore, as with radioisotope exchange HPLC, separate procedures for these determinations are required. The on-line derivatization used is not selective for acylcarnitines, resulting in chromatographic peaks that may interfere with acylcarnitines. In addition, this type of on-column GC derivatization produces erratic yields of derivatization.

(4) Pre-column acylcarnitine deamination GC–MS [13] does not quantify free carnitine or total carnitine. The derivatization is not selective for acylcarnitines. Although good qualitative MS information can be obtained, the accurate quantification of acylcarnitines by this approach has yet to be demonstrated.

Limitations and advantages to pre-column chemical derivatization HPLC

Pre-column chemical derivatization HPLC is not without shortcomings. Like on-column derivatization GC–MS and pre-column acylcarnitine deamination GC–MS, the derivatization used is not selective for acylcarnitines. Furthermore, the procedure does not entail direct interface with a mass spectrometer, making identification of unknown acylcarnitines a separate issue. However, pre-column chemical derivatization HPLC has advantages which make it appropriate in many situations. (1) Pre-column chemical derivatization HPLC does not suffer from the substrate selectivity and inhibition limitations of radioisotope exchange HPLC. Unlike radioisotope

exchange HPLC, pre-column chemical derivatization HPLC does not use radioactivity (and the associated problems of disposal, toxic chemical scintillates, licensing procedures and paper work). (2) Unlike FAB-MS, quantification of multiple compounds using pre-column chemical derivatization HPLC does not require synthesis of individual internal standards for each compound to be quantified. It is possible to simply generate multiple standard curves using a single internal standard. Because chromatography is used, structural isomers can generally be distinguished [15]. In addition, pre-column chemical derivatization HPLC requires considerably less complex and expensive analytical equipment. (3) Unlike radioisotope exchange HPLC, on-column derivatization GC–MS or pre-column acylcarnitine deamination GC–MS, pre-column chemical derivatization HPLC can determine free carnitine and individual acylcarnitines simultaneously. Pre-column chemical derivatization HPLC also can determine total carnitine, making it possible to calculate the balance among total carnitine, free carnitine and acylcarnitines (see Table IV). The ability to determine both free carnitine and total carnitine is required in order to standardize individual acylcarnitine solutions. This is necessary since all acylcarnitine standards contain some free carnitine which is not totally removed by current purification procedures. (4) As with on-column derivatization GC–MS and pre-column acylcarnitine deamination GC–MS, the derivatizations used with pre-column chemical derivatization HPLC procedures are not carnitine or acylcarnitine specific. Therefore, non-carnitine endogenous compounds appear in the chromatogram. However, unlike on-column derivatization GC–MS or pre-column acylcarnitine deamination GC–MS, the pre-column derivatization procedures used in pre-column chemical derivatization HPLC can be performed under extremely mild conditions that are not destructive to carnitine or acylcarnitines. Pre-column chemical derivatization HPLC reactions are reproducible, widely used, well behaved and well characterized [25].

Value of balance studies

The concept that the relative amounts and identities of individual acylcarnitines can indicate the presence of accumulated unusual organic acids (which are characteristic of unusual and sometimes life-threatening conditions) is well known. However, what is frequently ignored is that these concentration values can be misleading if not coupled with free carnitine and total carnitine concentration values. Specifically, it is highly recommended that the "percentage acylcarnitines found" (see Table IV) or some other measure be calculated to determine the concentration of the acylcarnitines which were not accounted for by the acylcarnitine procedure. This is because the undetermined acylcarnitines may be a substantial fraction of the acylcarnitine pool and this knowledge may be crucial to avoid misinterpreting the metabolic condition. As Table IV indicates, there are substantial amounts of acylcarnitines present in normal and patient urine samples that are not among the specific acylcarnitines evaluated. The data presented are not inconsistent with published results. Using a fluorescence method for the determination of acetylcarnitine, this laboratory showed that acetylcarnitine accounted for only 26% of the acylcarnitines in the urine of normal-weight subjects on an isocaloric low-carnitine diet [26]. Using radioisotope exchange HPLC, Schmidt-Sommerfeld *et al.* [22] reported that acetylcarnitine accounted for less than 25% of the acylcarnitines in normal human urines. Using FAB-MS–MS, Millington *et al.* [27] showed that in a normal human urine, acetylcarnitine is not the primary acylcarnitine.

The above described HPLC procedure and FAB-MS are the only methods that report the determination of free carnitine, individual acylcarnitines and total carnitine. However, this HPLC procedure has the advantage of providing a visual comparison of the acylcarnitine chromatogram with the total carnitine chromatogram. This qualitative comparison can be helpful, since chromatographic peaks that appear in the free and acylcarnitine chromatogram but not in the total carnitine chromatogram may represent additional acylcarnitine species. For exam-

ple, the subtraction of a total carnitine chromatogram from its respective acylcarnitine chromatogram (displayed in Figs. 3C, 4C, 5C and 6C) yielded a figure which clearly showed the presence of the acylcarnitines which were identified, along with some possible acylcarnitines which were not identified. From the results of the balance studies shown (Table IV), it appears that a substantial proportion of the acylcarnitines are not accounted for among the acylcarnitines quantified in many of the urine specimens examined. However, Fig. 5C shows three peaks (peaks 12–14) that are not present in the corresponding total carnitine chromatogram. It is therefore possible that these peaks represent the "missing" acylcarnitines in this chromatogram. The normal urine chromatogram displayed in Fig. 6 has only one identified acylcarnitine (acetylcarnitine at 32 nmol/ml) even though there were 90 nmol/ml of unaccounted acylcarnitines (Table IV). Fig. 6C shows some possible unidentified acylcarnitine peaks (peaks 15–16).

CONCLUSION

The advantages of this pre-column chemical derivatization HPLC method over other procedures were several, among them: (1) The sample clean-up of carnitine and acylcarnitines from human urine was performed using an inexpensive, easy and rapid two-step procedure. (2) The derivatization of isolated carnitine and acylcarnitines to their 4′-bromophenacyl ester derivatives was quantitative and accomplished in less than 10 min at room temperature. (3) The HPLC run time for the separation and detection of 4′-bromophenacyl esters of carnitine and acylcarnitines was brief (12 min per sample). This chromatographic run time is twice as fast as on-column derivatization GC–MS [12] or pre-column acylcarnitine deamination GC–MS [13] and five times faster than the radioisotope exchange HPLC method [9]. (4) The UV detection of acylcarnitine 4′-bromophenacyl esters was possible over a large sample range (10–300 nmol/ml) with high sensitivity. On the basis of initial sample volume required, this procedure is five times more sensitive than FAB–MS [11], twenty times more sensitive than radioisotope exchange HPLC [9] and fifty times more sensitive than on-column derivatization GC–MS [12] and pre-column acylcarnitine deamination GC–MS [13]. (5) With the internal standard used, quantification of free carnitine, acylcarnitines and total carnitine was easy and straightforward. (6) The reliability and reproducibility studies demonstrate that the procedure was both precise and accurate. With these features, we believe that this pre-column chemical derivatization HPLC procedure is ideally suited to clinical laboratories and we proposed that this procedure be considered as the preferred method for the evaluation of urinary carnitine and acylcarnitines in patients with metabolic disorders.

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