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Quantification of Carnitine and Acylcarnitines in Biological Matrices by HPLC Electrospray Ionization–Mass Spectrometry

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BACKGROUND: Analysis of carnitine and acylcarnitines by tandem mass spectrometry (MS/MS) has limitations. First, preparation of butyl esters partially hydrolyzes acylcarnitines. Second, isobaric nonacylcarnitine compounds yield false-positive results in acylcarnitine tests. Third, acylcarnitine constitutional isomers cannot be distinguished.

METHODS: Carnitine and acylcarnitines were isolated by ion-exchange solid-phase extraction, derivatized with pentafluorophenacyl trifluoromethanesulfonate, separated by HPLC, and detected with an ion trap mass spectrometer. Carnitine was quantified with d_3-carnitine as the internal standard. Acylcarnitines were quantified with 42 synthesized calibrators. The internal standards used were d_6-acetyl-, d_3-propionyl-, undecanoyl-, undecanedioyl-, and heptadecanoylcarnitine.

RESULTS: Example recoveries [mean (SD)] were 69.4% (3.9%) for total carnitine, 83.1% (5.9%) for free carnitine, 102.2% (9.8%) for acetylcarnitine, and 107.2% (8.9%) for palmitoylcarnitine. Example imprecision results [mean (SD)] within runs (n = 6) and between runs (n = 18) were, respectively: total carnitine, 58.0 (0.9) and 57.4 (1.7) μmol/L; free carnitine, 44.6 (1.5) and 44.3 (1.2) μmol/L; acetylcarnitine, 7.74 (0.51) and 7.85 (0.69) μmol/L; and palmitoylcarnitine, 0.12 (0.01) and 0.11 (0.02) μmol/L. Standard-addition slopes and linear regression coefficients were 1.00 and 0.9998, respectively, for total carnitine added to plasma, 0.99 and 0.9997 for free carnitine added to plasma, 1.04 and 0.9972 for octanoylcarnitine added to skeletal muscle, and 1.05 and 0.9913 for palmitoylcarnitine added to skeletal muscle. Reference intervals for plasma, urine, and skeletal muscle are provided.

CONCLUSIONS: This method for analysis of carnitine and acylcarnitines overcomes the observed limitations of MS/MS methods.

Today, the usual method for analyzing carnitine and acylcarnitines uses butyl ester formation and tandem mass spectrometry (MS/MS) *(1–4)*. Because of its simplicity and rapidity, this method has been applied with great success to high-throughput newborn-screening programs *(5–8)*; however, limitations to this method have been identified. First, the preparation of acylcarnitine butyl esters partially hydrolyzes acylcarnitines. This hydrolysis produces artificially increased free carnitine values *(9)*, and the basic procedure has been modified in response to this problem *(10–12)*. Second, MS/MS is not sufficiently selective in some cases. False-positive test results due to the presence of isobaric contaminants have been reported *(13, 14)*. Third, acylcarnitine constitutional isomers cannot be distinguished. This lack of specificity can lead to false-positive results due to contributions from other acylcarnitine species *(15, 16)*.

We have developed HPLC methods for quantifying carnitine and acylcarnitines *(17–20)*. These assays possess several unique features: (*a*) sample isolation via weak cation exchange with silica gel; (*b*) derivatization with highly reactive reagents under mild reaction conditions *(21, 22)* that do not hydrolyze acylcarnitines, (*c*) HPLC for selective elution, and (*d*) detection by ultraviolet or fluorescence spectrophotometry. We reasoned that linking electrospray ionization–mass spectrometry (ESI-MS)[4] detection to our HPLC method would permit highly specific detection of carnitine and

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[4] Nonstandard abbreviations: ESI-MS, electrospray ionization–mass spectrometry; MS/MS, tandem or recursive mass spectrometry; RIC, reconstructed ion chromatogram.

acylcarnitines in biological specimens, and we have presented a detailed report on the conceptual basis for this strategy and the empirical characterization of its components *(23)*. We report on the application of that procedure to the quantification of carnitine and acylcarnitines in biological samples.

Materials and Methods

CHEMICALS

Compounds labeled with stable isotopes were purchased from Cambridge Isotope Laboratories. More than 40 acylcarnitines were synthesized in our laboratory *(24–26)*. We used Bovine Albumin Fraction V Solution (75 g/L in PBS; Invitrogen) as a proxy for biological samples. Solid-phase extraction columns (50-mg silica Bond Elut) were purchased from Varian. Other chemicals were obtained and used as previously described *(23)*.

EQUIPMENT

The HPLC-ESI-MS system consisted of an HP 1100 Series HPLC instrument (quaternary pump and degasser, column compartment, and autosampler) from Agilent Technologies and an LCQ Deca mass spectrometer from Thermo Finnigan. The HPLC column was 100 mm × 4.6 mm (inner diameter) and contained Hypersil MOS-1 C_8, 3 μm (Thermo Hypersil-Keystone). Four chromatographic eluents were used (relative volumes): eluent A, acetonitrile/water (80/20); eluent B, acetonitrile/water (20/80); eluent C, acetonitrile/water/acetic acid/triethylamine (20/80/0.5/0.5); and eluent D, acetonitrile/water/acetic acid/triethylamine (90/10/0.5/0.5) *(23)*.

BIOLOGICAL SAMPLES

The procedures followed were reviewed by the University Hospitals Case Medical Center Institutional Review Board. Human skeletal muscle, plasma, urine, blood, and blood spot samples were obtained from the Center for Inherited Disorders of Energy Metabolism (CIDEM, Cleveland, OH). Tissues (10–40 mg) were placed in 1 mL of ice-cold deionized water and homogenized for 30 s with an Omni 2000 tissue homogenizer (Omni International). Plasma was collected into tubes containing EDTA. Whole blood was collected by finger lance. Blood spots were 3/16-in disks punched from blood spotted to Schleicher & Schuell Grade 903 filter paper and dried; each disk was assumed to contain 7.6 μL of blood *(4)*. Urine samples were routinely diluted with 4 volumes of water.

CARNITINE AND ACYLCARNITINE SAMPLE ANALYSIS

Carnitine solutions were calibrated *(27)*, and stock solutions were prepared. Acylcarnitine solutions were calibrated with the calibrated carnitine solutions. The phosphate-buffered BSA solution (which is free of carnitine and acylcarnitines) replaced the biological sample in calibrators and QC samples. Calibrators and QC samples were then analyzed identically to biological samples.

ANALYSIS BATCHES

We prepared samples in batches that contained different sample types (urine, plasma, skeletal muscle, and so forth), and each batch was completed in 3 days. Our average batch size was 24 samples, with the largest batch having 48 samples.

TOTAL AND FREE CARNITINE

We added 50 μL of sample (tissue homogenate, plasma, urine, or other specimen) or 2 blood-spot disks to a microcentrifuge tube. We then added 50 μL of internal-standard solution (25 μmol/L of d_3-carnitine) and 1 mL of acetonitrile/methanol solution (3 volumes/1 volume). Samples were vortex-mixed for 2 s (blood spots were placed on an orbital shaker for 30 min) and centrifuged (5 min at 13 200*g*). For measurement of free carnitine, we applied a 400-μL aliquot to a solid-phase extraction column. For total carnitine, we combined a 400-μL aliquot with 100 μL of 1 mol/L of KOH in methanol and placed the solution in a water bath at 50 °C for 60 min. After acylcarnitine hydrolysis, we transferred the sample to a microcentrifuge tube, added 50 μL of 1.5 mol/L phosphoric acid and 750 μL of acetonitrile/methanol solution (relative volumes, 3/1), centrifuged the tube for 5 min at 13 200*g*, and decanted the supernatant into a solid-phase extraction column.

ACYLCARNITINES

We added 10 μL of sample (tissue homogenate, plasma, urine, or other specimen) or 1 blood-spot disk to a microcentrifuge tube. We then added 10 μL of internal-standard solution (50 μmol/L of d_6-acetyl-, 5.0 μmol/L of d_3-propionyl-, and 2.5 μmol/L each of undecanoyl-, undecanedioyl-, and heptadecanoylcarnitine) and 1 mL of acetonitrile/methanol (relative volumes, 3/1). We vortex-mixed the samples for 2 s (blood spots were placed on an orbital shaker for 30 min), centrifuged the samples, and decanted the supernatants into solid-phase extraction columns.

SOLID-PHASE EXTRACTION

Silica gel solid-phase extraction columns were prepared by washing with 0.5 mL of methanol (gravity flow was used throughout). Samples were applied to the columns, and the columns were washed with 0.5 mL methanol. We eluted carnitine and acylcarnitines with 1.0 mL of water/methanol/acetic acid so-

lution (relative volumes, 10/9/1) and collected the effluents. We evaporated the effluents to dryness, reconstituted the samples in 200 μL of the 3/1 acetonitrile/methanol solution described above, transferred them to HPLC autosampler vials, and then evaporated the vials to dryness.

DERIVATIZATION

Each sample received *N*,*N*-diisopropylethylamine solution (10 μL in 10 mL acetonitrile) followed by pentafluorophenacyl trifluoromethanesulfonate solution (0.1 mol/L in acetonitrile). Samples received 50 μL of each reagent for total and free carnitine assays and 10 μL each for acylcarnitine assays. Samples were injected directly into the HPLC column (2 μL for total and free carnitine measurements and 5 μL for acylcarnitine measurements).

HPLC

Acylcarnitine pentafluorophenacyl esters were separated by sequential ion-exchange/reversed-phase chromatography on a single non–end-capped C_8 column *(23)*. Elution began with 100% eluent A at a flow rate of 1.75 mL/min. At 2 min after injection, eluent A was replaced with 100% eluent B. At 4 min after injection, we instituted a gradient from 100% eluent C to 80% eluent C and 20% eluent D over a 3-min period. We then reduced the eluent flow rate to 0.50 mL/min and started a gradient to 70% eluent C and 30% eluent D over a 15-min period. We followed this step with a gradient to 50% eluent C and 50% eluent D over a 20-min period and then finished with a final gradient to 100% eluent D over 20 min. The system then maintained 100% eluent D for 8 min. At 70 min after injection, we increased the eluent flow rate to 1.75 mL/min and returned the system to 100% eluent A for an additional 5 min (total run time, 75 min per injection). We used an abbreviated version of this eluent system for analysis of total and free carnitine (total run time, 13.5 min per injection). Flow was diverted from the ion source to waste during the washing and equilibration phases (i.e., when the flow rate was 1.75 mL/min).

ESI-MS

Carnitine and d_3-carnitine pentafluorophenacyl esters (370.1 *m/z* and 373.1 *m/z*, respectively) were detected with 2 alternating selected reaction-monitoring scans and fragmented by the application of a relative collision energy of 30%. For acylcarnitines, we observed that the product ion spectra of the acylcarnitine pentafluorophenacyl esters have analogous patterns: generation of a compound-specific ion (molecular ion -59 *m/z*)$^+$ and a common ion at 293 *m/z*. We performed full-scan mass spectrometry (with 30% source collision energy) and full-scan MS/MS sampling routines. We used full-scan mass spectrometry for qualitative and semiquantitative analyses and full-scan MS/MS for quantification.

QUANTIFICATION

For total and free carnitine, chromatographic peaks were integrated from the total ion current response and fit to linear equations. For acylcarnitines, we summed product ions [i.e., (molecular ion -59 *m/z*)$^+$ and 293 *m/z*] to produce reconstructed ion chromatogram (RIC) peak signals, which were fit to quadratic equations.

DETECTION AND SEMIQUANTIFICATION OF ADDITIONAL ACYLCARNITINES

The collection of full-scan mass spectrometry spectra in acylcarnitine analysis qualitatively reveals unusual acylcarnitines and permits their semiquantification. For each biological sample, a qualitative acylcarnitine chromatogram was produced from the full-scan mass spectrometry data. We measured newly observed acylcarnitines by evaluating the RICs of their molecular ions (compared with the peak areas of one of the internal standards) and calculated their concentrations semiquantitatively by assuming that the response factors for the compound and the internal standard were identical.

Results and Discussion

The basic goal of this procedure is to augment the high-throughput screening analysis of acylcarnitines by addressing (*a*) the unintended hydrolysis of acylcarnitines, (*b*) false positives, and (*c*) isomer detection. In addition, quantification is carried out with multiple-point calibration curves. First, derivatization under mild conditions with pentafluorophenacyl trifluoromethanesulfonate does not hydrolyze acylcarnitines. Second, false positives can be reduced greatly by increasing the selectivity. We accomplished this objective with more selective sample-isolation and HPLC-separation procedures. The described cation-exchange solid-phase extraction process isolates both carnitine and acylcarnitines with high recoveries yet excludes potential interferences [e.g., this procedure removes palmitic acid *(23)*]. Other investigators who also have used cation-exchange solid-phase extraction have reported high recoveries of acylcarnitines from urine and plasma *(28)*. The use of HPLC separation in acylcarnitine analysis before detection has been shown to distinguish false positives from true positives *(29)*, and our sequential ion-exchange/reversed-phase HPLC method adds even more selectivity *(23)*. We synthesized more than 40 acylcarnitines [e.g., *cis*-3,4-methyleneheptanoylcarnitine *(26)*] to be used as calibrators or

internal standards and generated 42 individual multiple-point calibration curves (Figs. 1 and 2) for MS/MS quantification of these acylcarnitines. Fig. 1A displays both a representative free carnitine chromatogram for the 5.0-μmol/L point on the calibration curve and a calibration curve. The injection volume contained 10 pmol carnitine pentafluorophenacyl ester and 50 pmol d_3-carnitine pentafluorophenacyl ester. The mass spectrometer monitored the transitions 370.1 *m/z*→311.0 *m/z* and 373.1 *m/z*→311.0 *m/z*, respectively. Fig. 1B displays a representative palmitoylcarnitine chromatogram for the 0.10-μmol/L point on the calibration curve. The injection volume contained 500 fmol palmitoylcarnitine pentafluorophenacyl ester and 25 pmol heptadecanoylcarnitine pentafluorophenacyl ester. The mass spectrometer monitored the transitions 608.3 *m/z*→product ion spectrum 165–650 *m/z* and 622.3 *m/z*→product ion spectrum 170–650 *m/z*, respectively. Also shown in Fig. 1B are the MS/MS full-scan spectra for palmitoylcarnitine and heptadecanoylcarnitine pentafluorophenacyl esters, along with the calibration curve that incorporated this point. Fig. 2 shows a representative acylcarnitine high QC injection (75.0 μmol/L acetylcarnitine, 15.0 μmol/L propionylcarnitine, and 1.50 μmol/L of each of the other acylcarnitines) including internal standards (50 μmol/L d_6-acetylcarnitine, 5.0 μmol/L d_3-propionylcarnitine, and 2.5 μmol/L each of undecanoyl-, undecanedioyl-, and heptadecanoylcarnitine). The chemical structures beneath the chromatogram illustrate a fragmentation of acylcarnitine pentafluorophenacyl esters consistent with the observed spectra. All of the acylcarnitine pentafluorophenacyl esters examined have similar product ion spectra that generate ions (molecular ion -59 $m/z)^+$ and 293 *m/z*. The ion at 293 *m/z* is common to all acylcarnitine pentafluorophenacyl esters.

VALIDATION STUDIES

Extraction efficiency. We assessed recoveries from the sample-isolation procedure by comparing results obtained with calibrators carried through the entire procedure with results obtained for identical calibrators that were simply evaporated. We added internal-standard solutions just before derivatization, evaluated chromatographic peak area ratios for compounds and internal standards, and calculated recoveries. Example results [mean (SD), n = 6] are as follows: total carnitine, 69.4% (3.9%); free carnitine, 83.1% (5.9%); acetylcarnitine, 102.2% (9.8%); palmitoylcarnitine, 107.2% (8.9%). The recovery of dicarboxylic acylcarnitines was not as high as for the other acylcarnitines [e.g., glutaroylcarnitine, 58.6% (7.7%)] because of the second carboxylate functional group on these molecules. To accommodate this lower recovery, we use a dicarboxylic acylcarnitine (undecanedioylcarnitine) as the internal standard for these compounds.

Standard-addition studies. Fig. 3 summarizes the results of standard-addition studies with carnitine and representative acylcarnitines. Increasing amounts of carnitine or an acylcarnitine added to biological samples display a linear relationship with the detected carnitine or acylcarnitine, with all the slopes and linear regression coefficient values very close to 1. Fig. 3B shows that adding increasing amounts of acetylcarnitine to a plasma sample does not change the free carnitine concentration. On the other hand, the amount of acetylcarnitine detected is directly proportional to the added acetylcarnitine. This result proves that acetylcarnitine is not hydrolyzed to carnitine with this method. We added increasing concentrations of the 42 acylcarnitines to aliquots of samples of nonpathologic human plasma, urine, and homogenized skeletal muscle and assayed for acylcarnitines. The results of the analyses for octanoylcarnitine and palmitoylcarnitine are shown in Fig. 3, C and D.

Imprecision assessment with biological samples. We assessed the imprecision of the method via multiple analyses of identical aliquots of pooled samples of nonpathologic human plasma. Example within-batch results (n = 6) and between-batch results (n = 18) [mean (SD)] are respectively as follows: total carnitine, 58.0 (0.9) and 57.4 (1.7) μmol/L; free carnitine, 44.6 (1.5) and 44.3 (1.2) μmol/L; acetylcarnitine, 7.74 (0.51) and 7.85 (0.69) μmol/L; and palmitoylcarnitine, 0.12 (0.01) and 0.11 (0.02) μmol/L.

QC tolerance limits. We analyzed 3 QC samples (low, medium, and high concentrations) as a part of each analysis batch. The values were 5.00, 50.0, and 400 μmol/L for free carnitine and total carnitine; 10.0, 35.0, and 75.0 μmol/L for acetylcarnitine; and 1.00, 7.00, and 15.0 μmol/L for propionylcarnitine. For all other acylcarnitines, the values were 0.20, 0.70, and 1.50 μmol/L. We evaluated the percent differences from expected values. The tolerance limits for free carnitine, total carnitine, and each of the 42 acylcarnitines for the low QC concentration were within ±20% of expected values. The tolerance limits for free carnitine, total carnitine, and each of the 42 acylcarnitines for the medium and high QC concentrations were within ±15% of expected values. Typically, within-batch analyses met >90% of the 132 tolerance-limits tests.

Calibration curve tolerance limits. All points on the calibration curves for total and free carnitine (13 calibration points for each curve) and the 42 acylcarnitine calibrators (7 calibration points for each compound, excluding the zero point) were back-calculated, and

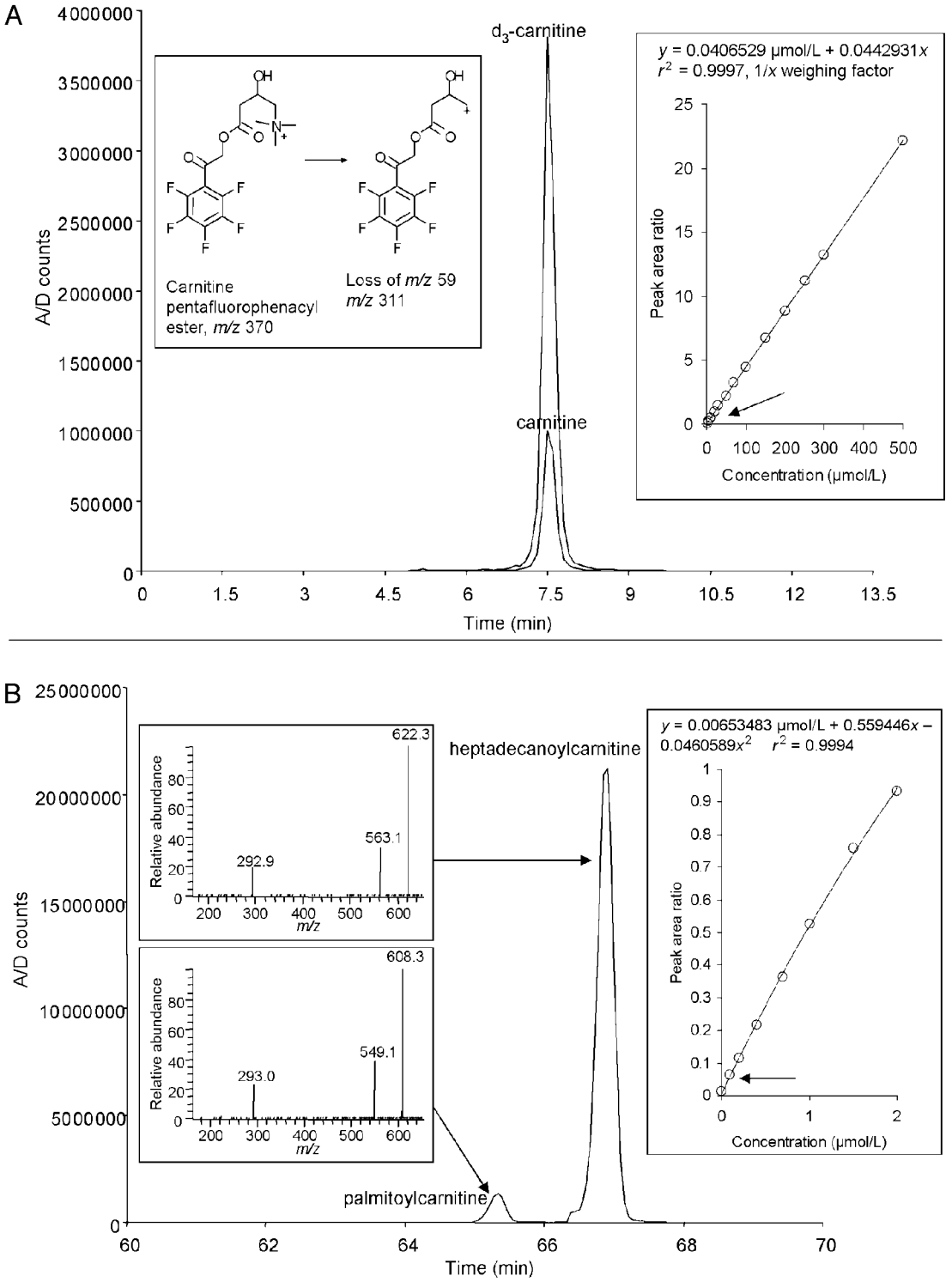

Fig. 1. Calibration curve chromatograms.

Free carnitine (A) and palmitoylcarnitine (B). The arrow in the calibration curves indicates the concentration corresponding to the chromatograms. A/D, analog-to-digital.

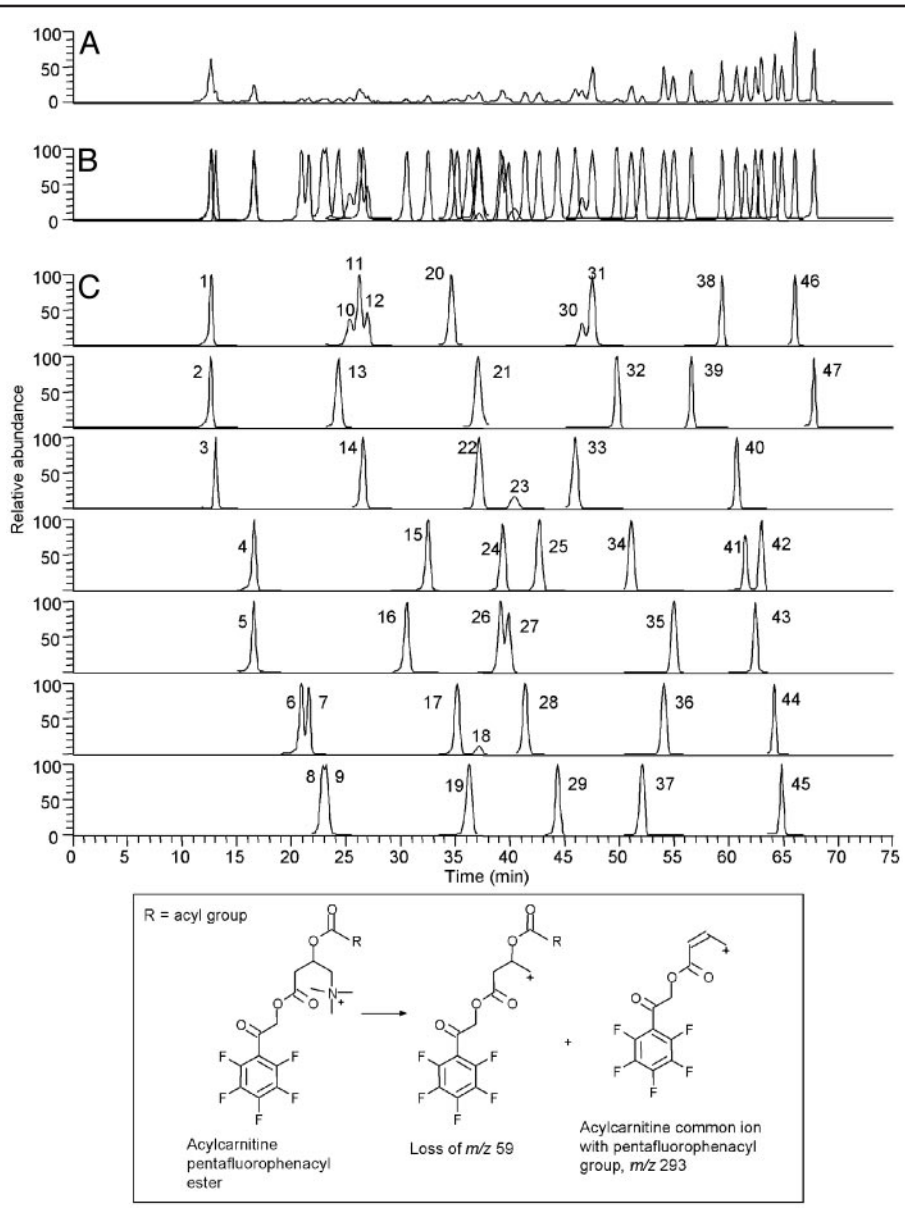

Fig. 2. Acylcarnitine QC chromatograms.

(A), Acylcarnitine pentafluorophenacyl ester common ion (293 *m/z*) RIC, generated from full-scan mass spectrometric sampling. (B), Superimposed RICs [(molecular ion −59 *m/z*)$^+$ and 293 *m/z*] generated from full-scan MS/MS scans for the different acylcarnitine pentafluorophenacyl esters. (C), Separated RICs generated from full-scan MS/MS scans [(molecular ion −59 *m/z*)$^+$ and 293 *m/z*] for the different acylcarnitine pentafluorophenacyl esters. Peak identities are as follows: 1, acetyl-; 2, d_6-acetyl-; 3, 3-hydroxy-isovaleryl-; 4, propionyl-; 5, d_3-propionyl-; 6, isobutyryl-; 7, butyryl-; 8, tigloyl-; 9, 3-methyl-crotonyl-; 10, 2-methyl-butyryl-; 11, isovaleryl-; 12, valeryl-; 13, benzoyl-; 14, phenylacetyl-; 15, hexanoyl-; 16, phenylpropionyl-; 17, succinyl-; 18, methylmalonyl-; 19, 4-methyl-hexanoyl-; 20, 4-phenyl-butyryl-; 21, *cis*-3,4-methylene-heptanoyl- [Yang et al. *(26)*]; 22, glutaroyl-; 23, ethylmalonyl-; 24, valproyl-; 25, octanoyl-; 26, adipoyl-; 27, 3-methyl-glutaroyl-; 28, 5-decynoyl-; 29, suberoyl-; 30, *cis*-3,4-methylene-nonanoyl-; 31, *cis*-4-decenoyl-; 32, sebacoyl-; 33, 4-methyl-octanoyl-; 34, decanoyl-; 35, *trans*-2-dodecenoyl-; 36, undecanoyl-; 37, undecanedioyl-; 38, *trans*-2-tetradecenoyl-; 39, lauroyl-; 40, myristoyl-; 41, palmitoleoyl-; 42, *trans*-2-hexadecenoyl-; 43, linoleoyl-; 44, palmitoyl-; 45, oleoyl-; 46, heptadecanoyl-; 47, stearoylcarnitine pentafluorophenacyl ester.

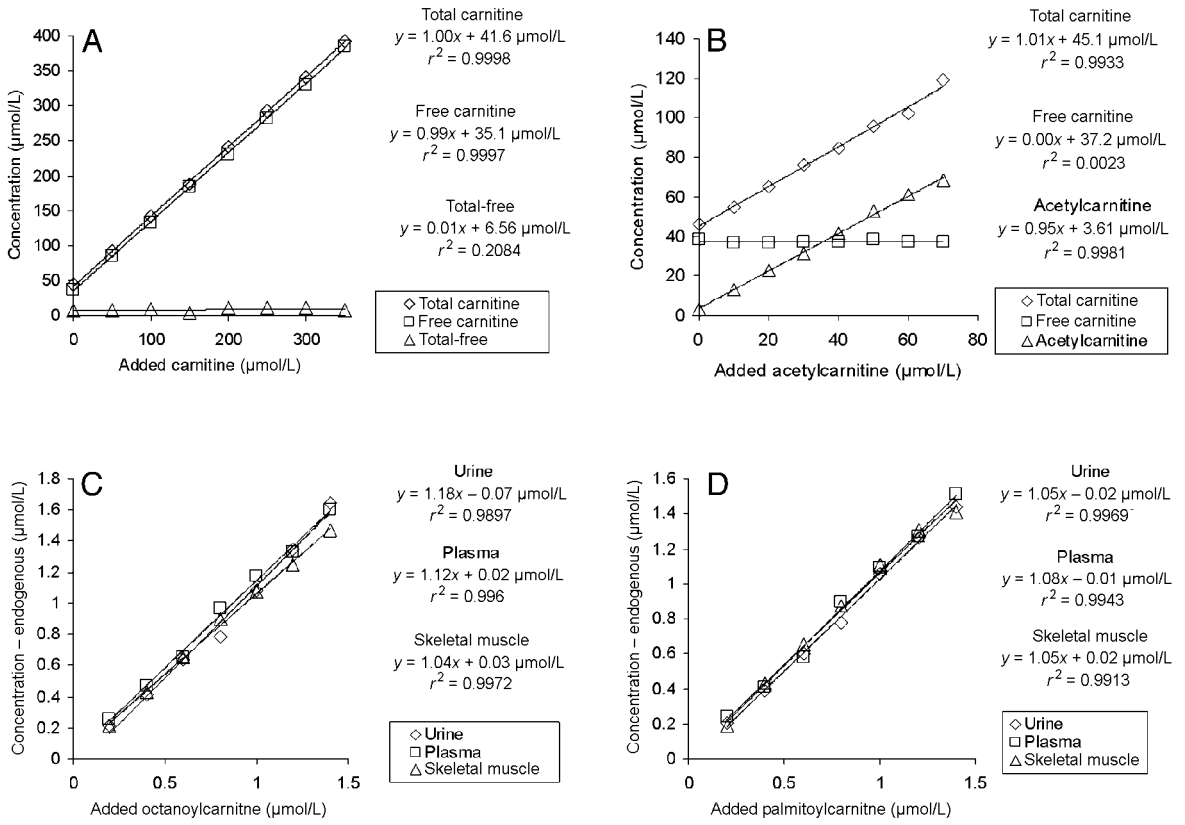

Fig. 3. Standard-addition study.

(A), We added increasing concentrations of carnitine to aliquots of a nonpathologic human plasma and analyzed the aliquots for total and free carnitine. (B), Increasing concentrations of acetylcarnitine were added to aliquots of nonpathologic human plasma, and the aliquots were analyzed for total and free carnitine and acetylcarnitine. Increasing concentrations of the 42 acylcarnitines were added to aliquots of nonpathologic human plasma, urine, and homogenized skeletal muscle samples, and the samples were analyzed for acylcarnitines. (C), Added octanoylcarnitine. (D), Added palmitoylcarnitine.

these values were compared with expected values. The tolerance limit for the percent difference was within ±20% of expected values for the lowest concentration and within ±15% of expected values for all the other concentrations. Typically, within-batch analyses met >90% of the 320 tolerance-limits tests.

APPLICATION TO PATIENT SAMPLES

"Balance" studies. We calculated the total acylcarnitine concentration (Sum) by adding the concentration values for the individual acylcarnitines and calculated the difference between total and free carnitine concentrations (Total-free). The Sum/Total-free ratio will have a value of 1 if all acylcarnitines have been accounted for. Table 1 presents the concentrations and results of "balance" studies obtained with this procedure for representative patient samples. The patients with methylmalonic aciduria, glutaric acidemia type I, and isovaleric acidemia were receiving carnitine as a part of their treatment. The whole-blood sample and the blood spot were from a single healthy volunteer and were collected at the same time. The close agreement in concentrations demonstrates the precision of this procedure, despite the error expected for the sampling procedure of collecting blood onto filter paper *(30)*.

Fig. 4A shows the separation and detection of valproylcarnitine and octanoylcarnitine in urine from a patient receiving valproate therapy. The top chromatogram is the mass spectrometry qualitative chromatogram. The middle chromatogram is the MS/MS quantitative chromatogram for valproyl- and octanoylcarnitine in the patient's urine. The bottom chromatogram is a calibrator analyzed during the same batch. These RICs were constructed from full MS/MS

Table 1. Measured concentrations for selected samples, including the results of balance studies.

Plasma: patient with methylmalonic aciduria, μmol/L		**Urine: patient receiving valproate therapy, μmol/g creatinine**	
Total carnitine	477.1	Total carnitine	138.8
Free carnitine	216.4	Free carnitine	17.8
Acetylcarnitine	61.3	Acetylcarnitine	8.81
Propionylcarnitine	136.4	Propionylcarnitine	1.93
Butyrylcarnitine	2.75	Isobutyrylcarnitine	1.20
Methylmalonylcarnitine	1.14	Valproylcarnitine	1.61
Other acylcarnitines	4.24	Octanoylcarnitine	0.22
Total-free[a]	260.7	*cis*-3,4-Methylene-heptanoylcarnitine	3.72
Sum	205.8	Succinylcarnitine	1.62
Sum/Total-free ratio	0.79	Glutaroylcarnitine	12.37
Plasma: patient with glutaric acidemia type I, μmol/L		Adipoylcarnitine	8.51
Total carnitine	75.6	Sebacoylcarnitine	13.21
Free carnitine	47.8	Suberoylcarnitine	2.56
Acetylcarnitine	18.1	Other acylcarnitines	5.45
Glutaroylcarnitine	7.0	Total-free	121.0
Other acylcarnitines	3.0	Sum	48.65
Total-free	27.8	Sum/Total free ratio	0.40
Sum	28.1	**Skeletal muscle: increased long chains, nmol/g ww**	
Sum/Total-free ratio	1.01	Total carnitine	2110
Urine: patient with isovaleric acidemia, μmol/g creatinine		Free carnitine	918
Total carnitine	857.3	Acetylcarnitine	855
Free carnitine	132.7	Myristoylcarnitine	9
Acetylcarnitine	258.7	Palmitoleoylcarnitine	12
Isovalerylcarnitine	379.2	Linoleoylcarnitine	25
cis-3,4-Methylene-heptanoylcarnitine	70.6	Palmitoylcarnitine	56
Other acylcarnitines	8.9	Oleoylcarnitine	81
Total-free	724.6	Stearoylcarnitine	20
Sum	717.4	Other acylcarnitines	39
Sum/Total-free ratio	0.99	Total-free	1192
Blood spot: healthy adult, μmol/L		Sum	1097
Total carnitine	69.4	Sum/Total-free ratio	0.92
Free carnitine	46.8	**Whole blood: healthy adult, μmol/L**	
Acetylcarnitine	9.32	Total carnitine	71.3
Propionylcarnitine	1.71	Free carnitine	44.8
cis-3,4-Methylene-heptanoylcarnitine	0.08	Acetylcarnitine	14.2
Linoleoylcarnitine	0.87	Propionylcarnitine	1.84
Oleoylcarnitine	1.28	*cis*-3,4-Methylene-heptanoylcarnitine	0.13
Palmitoylcarnitine	0.92	Linoleoylcarnitine	0.91
Stearoylcarnitine	0.81	Oleoylcarnitine	1.64
Other acylcarnitines	1.40	Palmitoylcarnitine	0.87
Total-free	22.6	Stearoylcarnitine	0.81
Sum	16.4	Other acylcarnitines	2.60
Sum/Total-free ratio	0.72	Total-free	26.5
		Sum	22.2
		Sum/Total-free ratio	0.84

[a] Total-free, the difference between the total carnitine concentration and the free carnitine concentration; Sum, sum of the measured acylcarnitine concentrations; ww, wet weight.

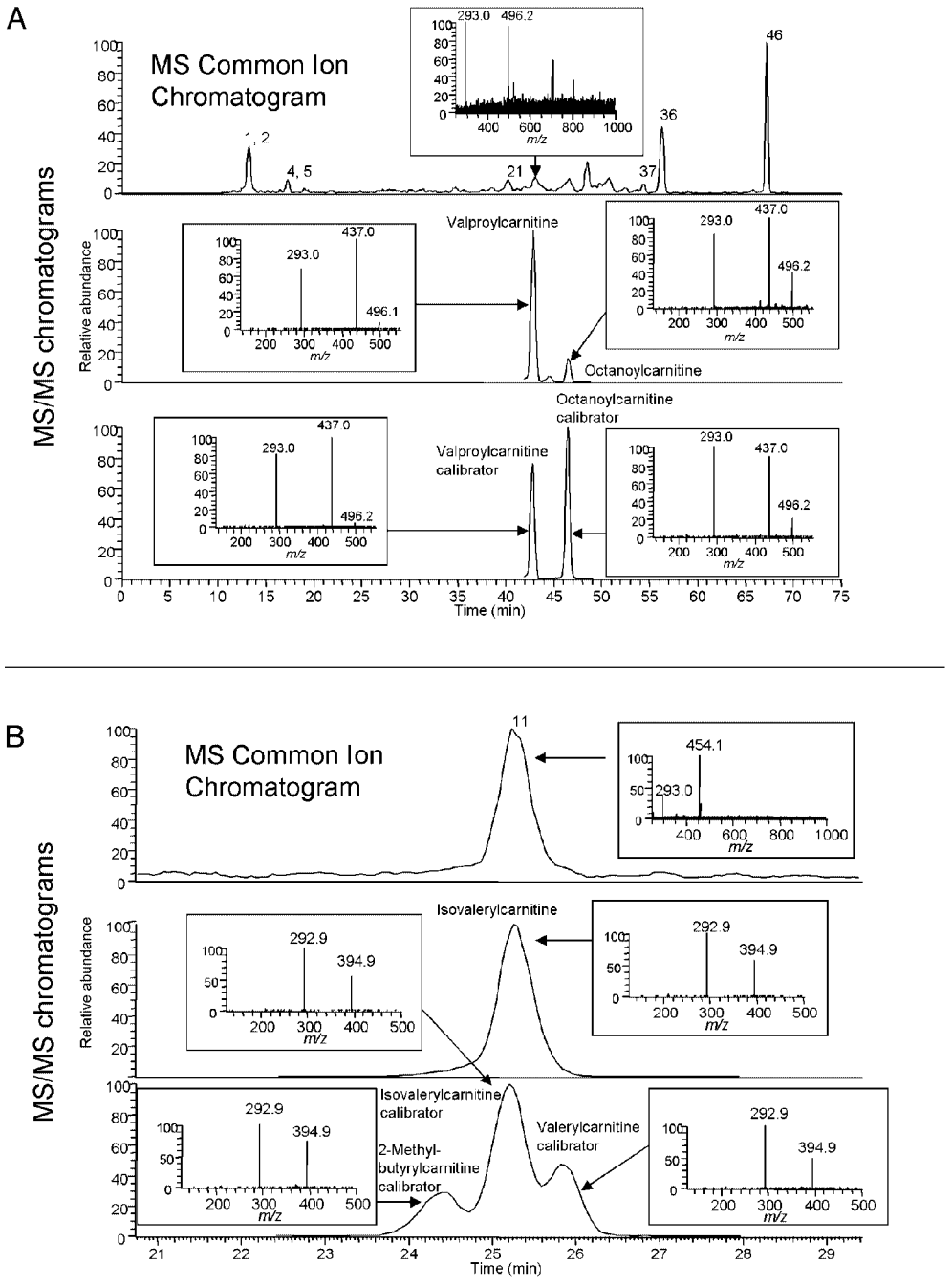

Fig. 4. Chromatograms of patient samples.

(A), Patient receiving valproate therapy. The top chromatogram is the mass spectrometry (MS) qualitative chromatogram. Peak labels are the same as in Fig. 2. The inset image is the mass spectrum for valproylcarnitine pentafluorophenacyl ester (496.2 *m/z*). The middle chromatogram is the MS/MS quantitative chromatogram for valproyl- and octanoylcarnitine pentafluorophenacyl esters. The bottom chromatogram is from a calibration curve point analyzed during the same batch. The inset spectra are the product ion mass spectra for valproyl- and octanoylcarnitine pentafluorophenacyl esters. (B), Separation and detection of isovalerylcarnitine in urine from a patient with isovaleric acidemia. The top chromatogram is the MS qualitative chromatogram. The middle chromatogram is the MS/MS quantitative chromatogram for isovalerylcarnitine pentafluorophenacyl ester. The bottom chromatogram is from a calibration curve point analyzed during the same batch.

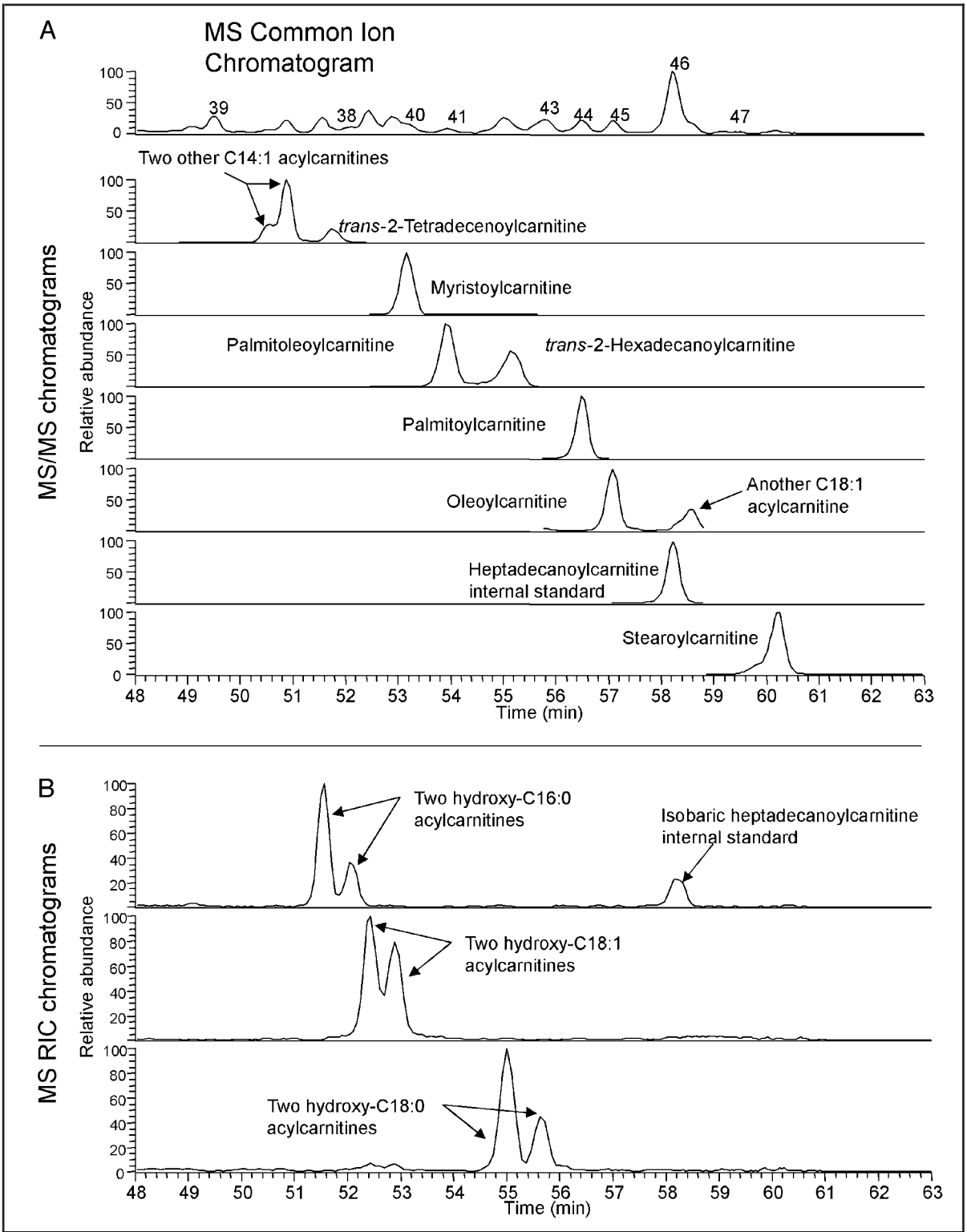

Fig. 5. Patient with deficiency in long-chain 3-hydroxy-acyl-CoA dehydrogenase.

(A), The top chromatogram is the mass spectrometry (MS) qualitative chromatogram, and the remaining chromatograms are the MS/MS quantitative chromatograms. (B), MS semiquantitative chromatograms.

scans for C8 acylcarnitine pentafluorophenacyl esters (496.2 *m/z*→product ion spectrum 135–550 *m/z*). The inset spectra are the product ion mass spectra for valproyl- and octanoylcarnitine pentafluorophenacyl esters. We observed that the product ion mass spectra for valproyl- and octanoylcarnitine pentafluorophenacyl esters have identical features and are essentially indistinguishable. Therefore, although we used MS/MS to detect these acylcarnitines, the identification of these acylcarnitines rests with the chromatographic system rather than the mass spectrometer. The results of the balance study for this urine sample are shown in Table 1. Our examination of the mass spectrometry common ion chromatogram revealed several chromatographic peaks that are not among those for which we have reference compounds. These peaks include those for 2 isomeric forms of C9 acylcarnitine and 2 isomeric forms of C9:1 acylcarnitine. The semiquantified concentrations obtained for these compounds are as follows: C9a, 2.36 μmol/g creatinine; C9b, 0.55 μmol/g; C9:1a, 0.61 μmol/g; and C9:1b, 1.93 μmol/g. Inclusion of these values in the calculation increases the Sum/Total-free ratio from 0.40 to 0.45; however, the difference between the implicit acylcarnitine content (Total-free) and what can be accounted for (Sum) is still substantial. This difference does not occur in all urine samples (e.g., the Sum/Total-free ratio for the patient with isovaleric acidemia is 0.99; Table 1), but many urine samples contain acylcarnitines in addition to those for which we can account.

Fig. 4B shows the separation and detection of isovalerylcarnitine in urine from a patient with isovaleric acidemia. The top chromatogram is the mass spectrometry qualitative chromatogram. The middle chromatogram is the MS/MS quantitative chromatogram for isovalerylcarnitine pentafluorophenacyl ester. The bottom chromatogram is from a calibrator analyzed during the same batch, showing 2-methylbutyrylcarnitine, isovalerylcarnitine, and valerylcarnitine pentafluorophenacyl esters. The product ion mass spectra are essentially indistinguishable. Therefore, although the detection of these acylcarnitines is by MS/MS, the identification of these acylcarnitines rests with the chromatographic system rather than the mass spectrometer. Table 1 presents the quantitative results and the results of the balance study for this sample.

Fig. 5 shows the separation and detection of long-chain acylcarnitines in plasma from a patient with deficiency of long-chain 3-hydroxy-acyl-CoA dehydrogenase. In Fig. 5A, the top chromatogram is the mass spectrometry qualitative chromatogram, and the remaining chromatograms are the MS/MS quantitative chromatograms. These RICs [(molecular ion -59 *m/z*)$^+$ and 293 *m/z*] were generated from full-scan MS/MS spectra of the molecular ions for *trans*-2-tetradecenoyl-, myristoyl-, palmitoleoyl-, *trans*-2-hexadecenoyl-, linoleoyl-, palmitoyl-, oleoyl-, heptadecanoyl-, and stearoylcarnitine pentafluorophenacyl esters. We observed isomeric forms of C14:1 acylcarnitines in addition to *trans*-2-tetradecenoylcarnitine and observed another C18:1 acylcarnitine in addition to oleoylcarnitine. Fig. 5B shows the mass spectrometry semiquantitative chromatograms, which reveal 2 isomeric forms each of hydroxy-C16:0, hydroxy-C18:1, and hydroxy-C18:0 acylcarnitines that are present in this plasma sample. Measured values for this sample were as follows: total carnitine, 77.8 μmol/L; free carnitine, 52.9 μmol/L; acetylcarnitine, 8.83 μmol/L; propionylcarnitine, 0.48 μmol/L; hexanoylcarnitine, 0.13 μmol/L; octanoylcarnitine, 0.13 μmol/L; decanoylcarnitine, 0.24 μmol/L; lauroylcarnitine, 0.81 μmol/L; *trans*-2-dodecenoylcarnitine, 0.15 μmol/L; myristoylcarnitine, 0.30 μmol/L; *trans*-2-tetradecenoylcarnitine, 0.18 μmol/L; palmitoleoylcarnitine, 0.43 μmol/L; palmitoylcarnitine, 0.31 μmol/L; oleoylcarnitine, 0.44 μmol/L; stearoylcarnitine, 0.11 μmol/L; and other acylcarnitines, 1.28 μmol/L. We quantified the additional isomeric forms of C14:1 acylcarnitine with the *trans*-2-tetradecenoylcarnitine calibration curve (C14:1a, 0.53 μmol/L; C14:1b, 0.14 μmol/L) and quantified the other isomeric form of C18:1 acylcarnitine with the oleoylcarnitine calibration curve (C18:1a, 0.33 μmol/L). We also obtained semiquantitative values for the other observed acylcarnitines: hydroxy-C16:0a, 0.40 μmol/L; hydroxy-C16:0b, 0.18 μmol/L; hydroxy-C18:1a, 0.72 μmol/L; hydroxy-C18:1b, 0.59 μmol/L; hydroxy-C18:0a, 0.53 μmol/L; hydroxy-C18:0b, 0.23 μmol/L. The results of the balance study were as follows: Total-free, 24.9 μmol/L; Sum, 17.5 μmol/L; Sum/Total-free ratio, 0.70. See the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue9 for the reference intervals for plasma, urine, and skeletal muscle generated with this procedure.

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