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Analysis of Carnitine Biosynthesis Metabolites in Urine by HPLC–Electrospray Tandem Mass Spectrometry

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Background: **We developed a method to determine the urinary concentrations of metabolites in the synthetic pathway for carnitine from N^6-trimethyllysine and applied this method to determine their excretion in control individuals. In addition, we investigated whether newborns are capable of carnitine synthesis from deuterium-labeled N^6-trimethyllysine.**

Methods: **Urine samples were first derivatized with methyl chloroformate. Subsequently, the analytes were separated by ion-pair, reversed-phase HPLC and detected online by electrospray tandem mass spectrometry. Stable-isotope-labeled reference compounds were used as internal standards.**

Results: **The method quantified all carnitine biosynthesis metabolites except 4-*N*-trimethylaminobutyraldehyde. Detection limits were 0.05–0.1 μmol/L. The interassay imprecision (CV) for urine samples with added compounds was 6–12%. The intraassay imprecision (CV) was 1–5% (3–10 μmol/L). Recoveries were 94–106% at 10–20 μmol/L and 98–103% at 100–200 μmol/L. The mean (SD) excretions of N^6-trimethyllysine and 3-hydroxy-N^6-trimethyllysine were 2.8 (0.8) and 0.45 (0.15) mmol/mol creatinine, respectively. γ-Butyrobetaine and carnitine excretions were more variable with values of 0.27 (0.21) and 15 (12) mmol/mol creatinine, respectively. After oral administration of deuterium-labeled N^6-trimethyllysine, all urines of newborns contained deuterium-labeled N^6-trimethyllysine, 3-hydroxy-N^6-trimethyllysine, γ-butyrobetaine, and carnitine.**

Conclusions: **HPLC in combination with electrospray ionization tandem mass spectrometry allows rapid determination of urinary carnitine biosynthesis metabolites. Newborns can synthesize carnitine from exogenous N^6-trimethyllysine, albeit at a low rate.**

Carnitine (3-hydroxy-4-trimethylaminobutyrate) is a vital compound that plays an indispensable role in the transport of activated fatty acids across the inner mitochondrial membrane into the matrix, where β-oxidation takes place *(1, 2)*. Furthermore, carnitine is involved in the transfer of the products of peroxisomal β-oxidation, including acetyl-CoA, to the mitochondria for oxidation to CO_2 and H_2O in the Krebs cycle *(3, 4)*. Apart from the dietary intake of carnitine, most eukaryotes are able to synthesize this compound from N^6-trimethyllysine (TML)[6] *(5–7)*. This TML is generated within lysosomes by the hydrolysis of proteins containing lysines that are trimethylated at their ε-amino group by a protein-dependent methyltransferase. The synthesis of carnitine from TML is shown in Fig. 1. TML is first hydroxylated at the 3 position by TML hydroxylase, after which the resulting 3-hydroxy-N^6-trimethyllysine (HTML) is cleaved by HTML aldolase into 4-*N*-trimethylaminobutyraldehyde

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[6] Nonstandard abbreviations: TML, N^6-trimethyllysine; HTML, 3-hydroxy-N^6-trimethyllysine; TMABA, 4-*N*-trimethylaminobutyraldehyde; γ-BB, γ-butyrobetaine; MS, mass spectrometry; and IS, internal standard.

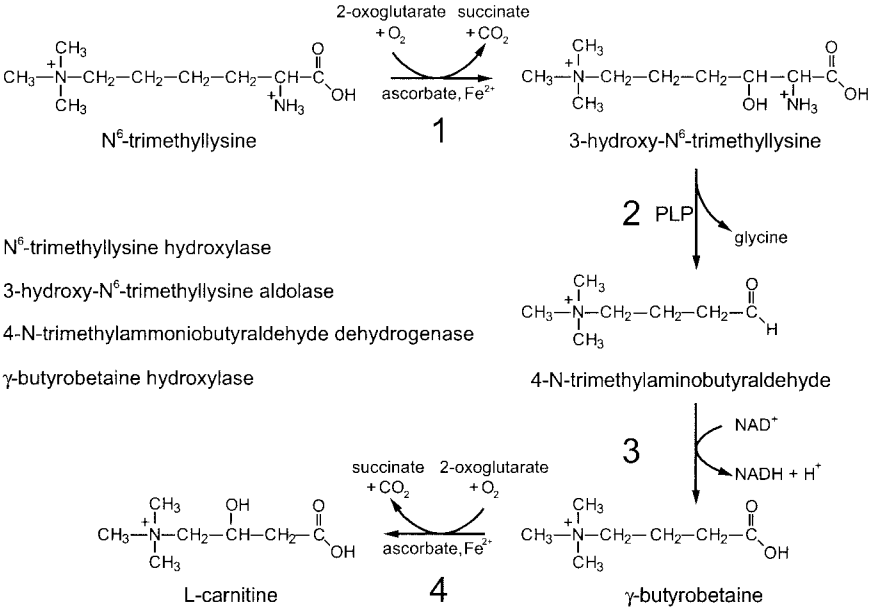

Fig. 1. Biosynthesis of carnitine from TML.
PLP, pyridoxal phosphate.

(TMABA) and glycine *(6, 8)*. TMABA is subsequently oxidized by TMABA dehydrogenase to form 4-*N*-trimethylaminobutyrate (γ-butyrobetaine; γ-BB) *(9)*. In the last step, γ-BB is hydroxylated at the 3 position by a second hydroxylase, γ-BB hydroxylase, yielding L-carnitine *(5, 7, 10)*. γ-BB hydroxylase is differentially expressed; its activity has been detected in only human kidney, liver, and brain *(11)*. In our laboratory, we have identified the cDNAs that encode TMABA dehydrogenase *(12)*, γ-BB hydroxylase *(13, 14)*, and recently, TML hydroxylase *(15)*.

Measurement of the concentrations of the metabolites in this pathway in body fluids is necessary to further investigate the carnitine biosynthetic pathway and its regulation. Although several methods have been developed to measure one or more of the carnitine biosynthesis intermediates in urine and/or plasma, these procedures are labor-intensive, and sample preparation differs for each compound *(16–23)*. We developed a new method that enables fast and easy determination of urinary concentrations of the metabolites of carnitine biosynthesis and applied the method to the study of de novo carnitine biosynthesis in newborns. The method incorporates derivatization with methyl chloroformate, separation of the analytes by ion-pair HPLC, and detection by electrospray ionization tandem mass spectrometry (MS).

Previous experiments with full-term infants showed a moderate increase in urinary carnitine excretion after TML loading, which suggested that infants are able to synthesize carnitine from exogenous TML *(24)*. Using fast atom bombardment MS, Melegh et al. *(25)* could not detect isotope-labeled carnitine in the urine of premature infants after 1 day of oral N^6-[*Me*-$^2H_9$]TML loading. To further investigate carnitine biosynthesis in newborns, we extended the latter study and performed a 5-day loading test with deuterium-labeled TML on seven newborns. We used our newly developed method to analyze the urines before and after loading to investigate whether newborns have the capacity to use exogenous TML as a precursor for carnitine synthesis.

Materials and Methods

CHEMICALS

Analytical grade methanol and methyl chloroformate were obtained from Merck. Heptafluorobutyric acid was from Pierce Chemical Company. All other reagents were of analytical grade. Deionized water was passed through a MilliQ Labo system (Millipore). HTML was synthesized from TMABA and glycine as described previously *(6)*.

The internal standards (ISs) used were N^6-[*Me*-$^2H_9$]TML, N^6-[*Me*-$^2H_6$]TML, 4-*N*-[*Me*-$^2H_3$]-γ-BB, and 4-*N*-[*Me*-$^2H_3$] carnitine. 4-*N*-[*Me*-$^2H_3$]Carnitine was obtained from Cambridge Isotope Laboratories. Other ISs were synthesized as described below.

SYNTHESIS OF N^6-[*Me*-$^2H_9$]TML AND N^6-[*Me*-$^2H_6$]TML

We synthesized N^6-[*Me*-$^2H_9$]TML from L-lysine HCl (Sigma) by the following method, using [*Me*-$^2H_6$]dimethyl sulfate (Isotec) as methyl donor. After 2 g of L-lysine was dissolved in 20 mL of distilled water, 2 g of alkaline $CuCO_3$ was added, and the mixture was boiled for 10 min. [For the preparation of alkaline $CuCO_3$, see Melegh et al. *(25)*.] The reaction mixture was cooled to room temperature and filtered over Whatman 3 MM paper. The clear filtrate was mixed with 4 mL of deuterium-labeled dimethyl sulfate at 20 °C, after which 13 mL of 100 g/L NaOH solution was added dropwise. The solution containing the deuterium-labeled TML was applied to a 4-mL Dowex 50WX8 column, and after the column was washed with 10 volumes of distilled water, the bound N^6-[*Me*-$^2H_9$]TML was eluted with 120 mL of 2 mol/L NH_4OH.

The effluent was concentrated and lyophilized. Chemical purity, determined by thin-layer chromatography and nuclear magnetic resonance, was >98%. The isotopic purity, determined by tandem MS, was >99%.

N^6-[*Me*-$^2H_6$]TML was synthesized by the same procedure used for the synthesis of N^6-[*Me*-$^2H_9$]TML except that N^6-methyl-L-lysine HCl was used as the precursor rather than L-lysine.

SYNTHESIS OF 4-*N*-[*Me*-$^2H_3$]-γ-BB

4-*N*-[*Me*-$^2H_3$]-γ-BB was synthesized from 4-*N*-dimethylaminobutyric acid HCl as follows, with [*Me*-$^2H_3$]methyl iodide (both from Sigma) as the methyl donor. 4-*N*-Dimethylaminobutyric acid (1 g; 6 mmol) was dissolved in 1 mL of distilled water and mixed with 3.16 g (10 mmol) of $Ba(OH)_2 \cdot 8\ H_2O$ dissolved in 20 mL of distilled water; 200 mL methanol was then added. After the addition of 600 μL (9 mmol) of [*Me*-$^2H_3$]methyl iodide, the mixture was stirred overnight at room temperature. The solution was concentrated under slightly reduced pressure to obtain a volume of ~20 mL, after which 20 mL of distilled water was added. The addition of 3–4 mL of 2 mol/L H_2SO_4 precipitated the barium ions as $BaSO_4$, which was removed by centrifugation. The supernatant was made alkaline by the addition of ~7 mL of 2 mol/L NaOH, and the mixture was kept at 65 °C for 4 h. The solvent was evaporated under reduced pressure, and the residue was redissolved in 1 mol/L HCl and extracted with 150 mL of CCl_4. The aqueous phase was concentrated to 1–2 mL in a rotary evaporator. 4-*N*-[Me-$^2H_3$]-γ-BB was purified by Dowex ion-exchange chromatography essentially as described above for deuterium-labeled TML.

N^6-[*Me*-$^2H_9$]TML LOADING TEST

Seven orally fed male infants were studied. The mean (SD) gestational age was 38.5 (0.87) weeks (range, 38–41 weeks). Postnatal age at the beginning of the study was 8 (range, 5–11) days, and body weight was 3360 (100) g (range, 3100–4300 g). The infants were fed 70 g of formula (Aptamil) seven times daily. The study period lasted 5 consecutive days, with 24-h urines collected on the day before administration of N^6-[*Me*-$^2H_9$]TML (day 0) and on the last day (day 5) of the study. After the preloading control day, the newborns received 0.5 mmol of N^6-[*Me*-$^2H_9$]TML daily in their formula during the 5-day study period.

The study design was approved by the local Ethics Committee, and informed consent was obtained from the parents. N^6-[*Me*-$^2H_6$]TML was used as an IS for TML and N^6-[*Me*-$^2H_9$]TML for analysis of the urine samples of the N^6-[*Me*-$^2H_9$]TML loading test.

CARNITINE BIOSYNTHESIS INTERMEDIATES IN CONTROL INDIVIDUALS

The 24-h urines used for determination of the carnitine biosynthesis intermediates in controls were obtained from 40 healthy individuals (23 males and 17 females) with a mean age of 21 years (SD, 17; range, 2–66 years).

SAMPLE PREPARATION

Urine samples were stored at 4 °C if analyzed within 1 week; otherwise, they were stored at −20 °C until analysis. Urinary creatinine concentrations were determined by the conventional alkaline creatinine-picrate method *(26)*. Urine samples were centrifuged at 10 000*g* for 5 min to remove debris, and a portion of the cleared urine was diluted to a creatinine concentration of 1 mmol/L. Urines with creatinine concentrations <1 mmol/L were used undiluted. One hundred microliters of each urine sample was used for the derivatization with methyl chloroformate. Methyl chloroformate reacts with primary, secondary, and tertiary amino groups, but not with quaternary amino groups. TML and HTML both contain a primary amino group, which reacts with methyl chloroformate, forming 2-*N*-methylformyl-N^6-TML and 2-*N*-methylformyl-3-hydroxy-N^6-TML, respectively. Carnitine and γ-BB do not react with methyl chloroformate. Interfering compounds (especially amino acids) readily react with methyl chloroformate and can be separated from the compounds of interest by acidic extraction with ethyl acetate. The carnitine biosynthesis intermediates, however, remain in the aqueous phase by virtue of their constitutively positive quaternary amino group.

We added 25 μL of the IS mixture (25 μmol/L N^6-[*Me*-$^2H_9$]TML or N^6-[*Me*-$^2H_6$]TML, 4-*N*-[Me-$^2H_3$]-γ-BB, and 4-*N*-[*Me*-$^2H_3$]carnitine each) and 20 μL of a 1:3 (1 part NH_4OH plus 3 parts sodium phosphate buffer) mixture of 1.5 mol/L NH_4OH and 0.5 mol/L sodium phosphate buffer, pH 7.2, to each urine sample. Derivatization was started by the addition of 20 μL of methyl chloroformate and the mixture was vortex-mixed. After a 5-min incubation at room temperature, the reaction was terminated by the addition of 40 μL of 40 mL/L heptafluorobutyric acid. The reaction mixture was extracted with 1 mL of ethyl acetate, and 5 μL of the aqueous phase was injected into the HPLC-tandem MS. When stored at 4 °C, the reaction mixture remained stable for several weeks.

HPLC-TANDEM MS

The HPLC system consisted of a HP 1100 series binary gradient pump, a vacuum degasser, and a column temperature controller (Hewlett Packard) and was connected to a Gilson 231 XL autosampler with a 402 single dilutor (Gilson). The autosampler maintained the temperature of the samples at 4 °C. The Aqua analytical column (250 × 2.0 mm; particle size, 5 μm; Phenomenex) was protected by a guard column (SecurityGuard C_{18} ODS; 4 × 2.0 mm; Phenomenex). Column temperature was maintained at 15 °C. The mobile phases were as follows: eluant A, 11 mL/L heptafluorobutyric acid; eluant B, 900 mL/L methanol. The elution gradient was as follows (flow rate, 0.3 mL/min): 0–3 min, 95% A; 3–6 min, 95% A to 50% A; 6–8 min, 50% A to 30% A; 8–9 min, 30% A to 0% A; 9–10 min,

0% A; 10–16 min, equilibration with 95% A. All gradient steps were linear, and the total analysis time, including equilibration, was 16 min. An accurate splitter (LC-packings) was used between the HPLC column and the mass spectrometer (split ratio of 1:20), which introduced the eluate at a flow rate of 15 μL/min into the mass spectrometer. An electrically operated valve was used so that only the eluate from 5.5 to 16 min was introduced into the mass spectrometer (preventing early-eluting salts from contaminating the mass spectrometer). A Quattro II tandem mass spectrometer (Micromass) was used in the positive electrospray ionization mode. Nitrogen was used as both the nebulizing and the drying gas. The collision gas was argon, and the cell pressure was 0.25 Pa. The source temperature was set at 80 °C, and the capillary voltage was maintained at 3.5 kV. The detector was used in tandem MS mode with multiple-reaction monitoring to detect the transition of a specific precursor ion to a fragment for each analyte. The transitions, cone voltages, and collision energies established for each compound are listed in Table 1.

VALIDATION

The linear ranges and detection limits for each compound were established by injection of calibration mixtures containing different concentrations. The stable-isotope-labeled compound of each analyte was used as IS except for HTML, for which N^6-[*Me*-$^2H_9$]TML or N^6-[*Me*-$^2H_6$]TML was used as IS. Analyte concentrations were determined based on the slope and intercept of the calibration curve, which were obtained from a linear least-squares regression for the analyte/IS peak-area ratio vs the concentration of the calibrator.

The intraassay (within-day) imprecision (CV) of the method was established by measuring, 10 times, a blank urine and the same urine, diluted to 1 mmol/L creatinine, enriched with synthetic compounds at low (3–10 μmol/L), medium (30–100 μmol/L), and high (300–1000 μmol/L) concentrations.

The interassay (between-day) CV was established by measuring blank urines and urines enriched with synthetic compounds (30–100 μmol/L) during 5 separate weeks. The recovery of the method was established by measuring 13 different urines before and after enrichment with known concentrations of synthetic compounds (10–20 and 100–200 μmol/L). The synthetic compounds were added after dilution of the urine to 1 mmol/L creatinine. The IS mixture was added to compensate for losses in the preparation of samples and to compensate for losses in sensitivity because of quenching of the signal by coeluting components.

Results

ASSAY DEVELOPMENT AND VALIDATION

To measure the carnitine biosynthesis intermediates in urine, samples were first derivatized with methyl chloroformate. This enabled the separation of interfering compounds, mainly amino acids, from the compounds of interest. Because all amino acids contain a primary amino group, they react readily with methyl chloroformate. The resulting amino acid derivatives, which are neutral at pH <2, can be extracted with ethyl acetate from the aqueous phase. The derivatives of the carnitine biosynthesis intermediates, however, remain in the aqueous phase by virtue of their constitutively positive quaternary amino group. The removal of the contaminating amino acids and other ethyl acetate-soluble compounds increased the sensitivity of the analysis dramatically.

A representative urine analysis is shown in Fig. 2. Although specific transitions were used for each analyte, a HPLC step was needed to separate interfering compounds with the same transition from the compounds of interest in the urine sample. This was particularly important for γ-BB, which showed two major interfering compounds with the same transition at different elution times (see Fig. 2). Another important benefit of the HPLC step was that salts present in urine eluted in the void volume of the column, thereby preventing contamination of the mass spectrometer.

The calibration curves for each compound were linear up to at least 250 μmol/L (r^2 >0.995). Above this concentration, deviation from linearity was observed for most

Table 1. Transitions, cone voltages, and collison energies of each compound in positive electrospray ionization mode.

Compound	Mass, Da	Parent ion, *m/z*	Daughter, *m/z*	Cone voltage, V	Collision energy, eV
2-*N*-Methylformyl-TML	247	247	142	35	25
2-*N*-Methylformyl-[$^2H_9$]TML	256	256	142	35	25
2-*N*-Methylformyl-[$^2H_6$]TML	253	253	142	35	25
2*N*-Methylformyl-HTML	263	263	158	20	20
2-*N*-Methylformyl-[$^2H_9$]TML	272	272	158	20	20
γ-BB	146	146	87	25	15
$^2H_3$-γBB	149	149	87	25	15
$^2H_9$-γBB	155	155	87	25	15
Carnitine	162	162	103	35	16
[$^2H_3$]Carnitine	165	165	103	35	16
[$^2H_9$]Carnitine	171	171	103	35	16

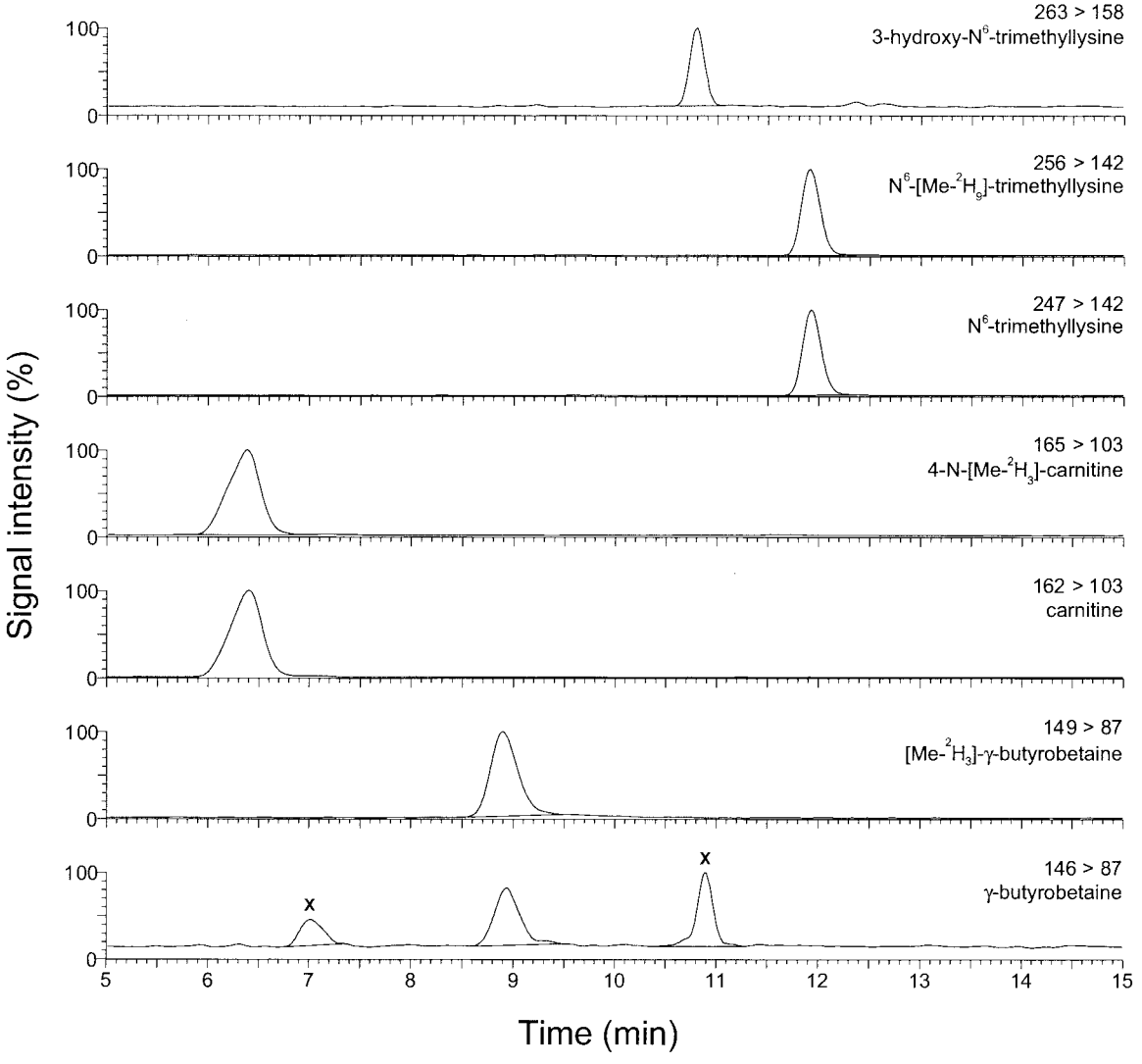

Fig. 2. HPLC-tandem MS traces of a representative urine analysis.
Peaks X in the γ-BB trace (*bottom*) are interfering compounds with the same transition.

compounds. When the concentrations of one or more metabolites were above the linear part of the calibration curve (in practice, only in carnitine-treated patients), the samples were diluted to be within the linear range of the curve. The detection limit was established and defined as the lowest signal with a signal-to-noise ratio of 3. The detection limits were as follows: carnitine, 0.05 μmol/L; γ-BB, 0.05 μmol/L; HTML, 0.1 μmol/L; and TML, 0.05 μmol/L.

The intraassay (within-day) imprecision, interassay (between-day) imprecision, and recovery data are summarized in Tables 2, 3, and 4, respectively.

CARNITINE BIOSYNTHESIS INTERMEDIATES IN CONTROLS

To study the excretion of the carnitine biosynthesis intermediates in urine of healthy controls, the concentrations of the metabolites were determined in 24-h urines from 40 healthy individuals 2–66 years of age. The urinary concentrations of the four metabolites measured with our method are shown in Table 5 and were in agreement with previously published data.

CARNITINE BIOSYNTHESIS IN NEWBORNS

To determine whether full-term newborns can utilize exogenous TML for carnitine biosynthesis, we performed a loading test with deuterium-labeled TML in seven healthy newborns. Figs. 3 and 4 show the urinary concentrations of endogenous and $^2H_9$-labeled TML, HTML, γ-BB, and carnitine before and after [$^2H_9$]TML administration. Both before and after loading, endogenous concentrations of TML and HTML appeared to be proportional to creatinine (Fig. 3). After [$^2H_9$]TML administration, the mean concentrations of endogenous TML and HTML increased moderately but significantly ($P < 0.001$). The concentration of endogenous γ-BB before [$^2H_9$]TML administration varied greatly, ranging from 0.7 to 9.7 mmol/mol creatinine. After

Table 2. Intraassay imprecision.

	Blank[a]		Low[b]		Medium[b]		High[b]	
Compound	mmol/mol creatinine[c]	CV, %	mmol/mol creatinine[c]	CV, %	mmol/mol creatinine[c]	CV, %	mmol/mol creatinine[c]	CV, %
TML	4.30	1	4.69	1	8.9	2	51	1
HTML	0.59	4	0.98	5	4.9	2	45	2
γ-BB	0.23	5	0.89	3	7.9	1	80	2
Carnitine	10.7	1	12.0	1	21.7	3	116	3

[a] Urine without supplementation.

[b] Urine supplemented (before dilution to 1 mmol/L creatinine) with low (3–10 μmol/L), medium (30–100 μmol/L), or high (300–1000 μmol/L) concentrations of the four measured compounds. The urine used had a creatinine value of 8.7 after addition of the supplement.

[c] Mean concentration (n = 10).

loading, the γ-BB excretion increased considerably, ranging from 1.5 to 6.7 times the original concentration (Fig. 3). Carnitine concentrations showed a similar behavior, ranging from 2.5 to 48 mmol/mol creatinine before [$^2H_9$]TML administration and increasing 1.5- to 12-fold after [$^2H_9$]TML administration (Fig. 3). The urine samples obtained after [$^2H_9$]TML administration (Fig. 4) all contained [$^2H_9$]TML, [$^2H_9$]HTML, [$^2H_9$]-γ-BB, and [$^2H_9$]carnitine, demonstrating that newborns are capable of synthesizing carnitine. [$^2H_9$]TMABA was below the detection limit in these urines.

For all seven full-term newborns, the products of one or more enzymatic steps of the carnitine biosynthetic pathway accounted for 23% (SD, 4%) of the total amount of excreted $^2H_9$-labeled compounds, and 10% (SD, 3%) of the free carnitine in urine was $^2H_9$-labeled carnitine (range, 5–16%). The concentrations of endogenous and $^2H_9$-labeled carnitine measured in the urine samples (results not shown) were in agreement with the results obtained with the established procedure for the analysis of acylcarnitines by tandem MS *(27, 28)*. Acylcarnitine analysis also revealed the presence of acetyl-[$^2H_9$]carnitine, propionyl-[$^2H_9$]carnitine, and butyryl-]$^2H_9$]carnitine in most of the samples after [$^2H_9$]TML loading.

Discussion

VALIDATION

We have developed an easy and fast HPLC-tandem MS method to analyze four of the five metabolites of carnitine biosynthesis in urine. Urine samples were first derivatized with methyl chloroformate and then extracted with ethyl acetate. This extraction removed interfering compounds, whereas the carnitine biosynthesis metabolites remained in the aqueous phase. Subsequently, the analytes were separated on a reversed-phase HPLC column equilibrated with an ion-pairing agent, heptafluorobutyric acid, and detected by tandem MS. This is the first published method that allows simultaneous analysis of four of the five metabolites of the carnitine biosynthetic pathway, using the same sample preparation procedure and detection method.

The presence of interfering compounds with the same transitions as the analytes makes separation by HPLC mandatory and underlines the importance of the use of ISs, preferably stable-isotope-labeled analogs. The lack of an IS for HTML most likely caused the relatively large intra- and interassay imprecision and lower recovery for this compound. To overcome this, we intend to synthesize [$^2H_9$]HTML from [$^2H_9$]TML enzymatically with rat kidney TML hydroxylase, which has recently been purified in our laboratory *(15)*, to obtain better results for this compound.

The only compound that could not be detected was TMABA. Although we optimized the detection of this compound with a calibrator (detection limit, 0.1 μmol/L), TMABA could not be detected in urine. Considering the reactivity of aldehydes, this compound might either be oxidized chemically to γ-BB or enzymatically by the

Table 3. Interassay imprecision.

	Unenriched urine[a]		Enriched urine[a,b]	
Compound	mmol/mol creatinine[c]	CV, %	mmol/mol creatinine[c]	CV, %
TML	4.33	13	8.1	8
HTML	1.28	18	7.9	12
γ-BB	0.25	8	9.0	6
Carnitine	11.7	9	22.8	7

[a] The results were obtained by measuring the same urines over 5 different weeks.

[b] Enriched with 30–100 μmol/L each compound.

[c] Mean (n = 5).

Table 4. Recovery.

	Endogenous concentration,[a]	Recovery at 10–20 μmol/L[b]		Recovery at 100–200 μmol/L[b]	
Compound	mmol/mol creatinine	Mean, %	SD, %	Mean, %	SD, %
TML	3.1 (1.03–4.89)	97	4	98	2
HTML	0.55 (0.13–1.13)	106	20	103	18
γ-BB	0.49 (0.08–1.26)	100	2	101	1
Carnitine	26 (0.87–73)	94	14	98	3

[a] Mean (range) endogenous concentrations of compounds in urines (n = 13) used for the enrichment.

[b] Urine supplemented (after dilution to 1 mmol/L creatinine) with low (10–20 μmol/L) or medium (100–200 μmol/L) concentrations of four measured compounds (n = 13).

Table 5. Urinary concentrations of carnitine biosynthesis metabolites in controls.

Compound	Mean (SD),[a] mmol/mol creatinine	n	Ref.
TML	2.8 (0.8) [1.3–4.7]	40[b]	This study
	3.4	3	*(17)*
	4.3	25	*(33)*
	5.3 (1.7) [3.8–10.4]	13	*(18)*
	4.7 (0.9) [3.5–6.2]	7	*(19)*
	6.2 (0.3)	16	*(32)*
HTML	0.45 (0.15) [0.13–0.91]	40	This study
γ-BB	0.27 (0.21) [0.05–0.79]	40	This study
Carnitine	15 (12) [1.6–48]	40	This study
	16 (11)	28	*(34)*
	18 (12)	5	*(24)*

[a] Where available, range is given in brackets.

[b] Study group includes 23 males and 17 females; mean age, 21 years (range, 2–66 years) *(17)*.

action of the enzyme TMABA dehydrogenase, which is highly active in human kidney *(29)*.

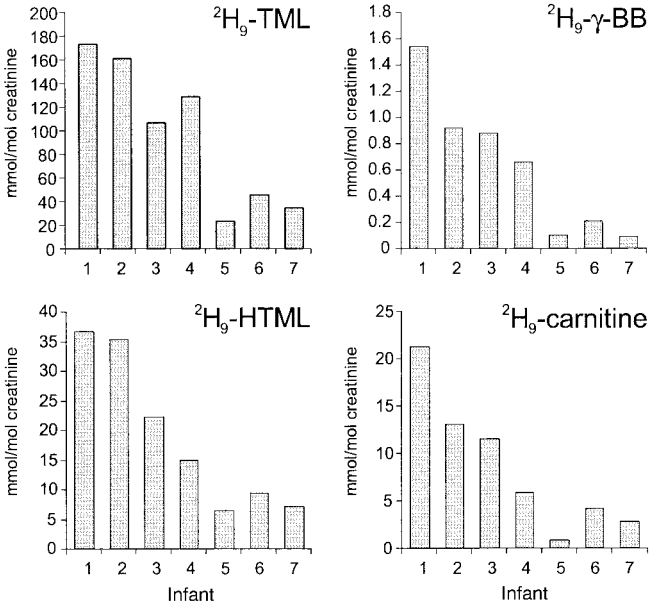

Fig. 4. $^2H_9$-labeled carnitine biosynthesis metabolites (except TMABA) in seven newborns after loading with [$^2H_9$]TML.

CONTROLS

The analysis of the carnitine biosynthesis intermediates in 40 control individuals (Table 5) showed that the excretion of TML is proportional to that of creatinine. This is in agreement with previous results obtained from clearance experiments, which showed that TML is not reabsorbed by the kidney *(17)*. The presence of HTML in urine has never been reported. The excretion of HTML shows a profile similar to that of TML and is proportional to creatinine excretion, which would suggest that HTML, like TML, is not reabsorbed by the kidney. The urinary concentrations of γ-BB were low (0.3 mmol/mol creatinine) compared with the concentrations reported in plasma [4.8 μmol/L *(23)* and 1.8 μmol/L *(16, 30)*]. This could be explained by the high activity of γ-BB hydroxylase in human kidney, which converts most of the γ-BB into carnitine. Additionally, γ-BB is efficiently reabsorbed

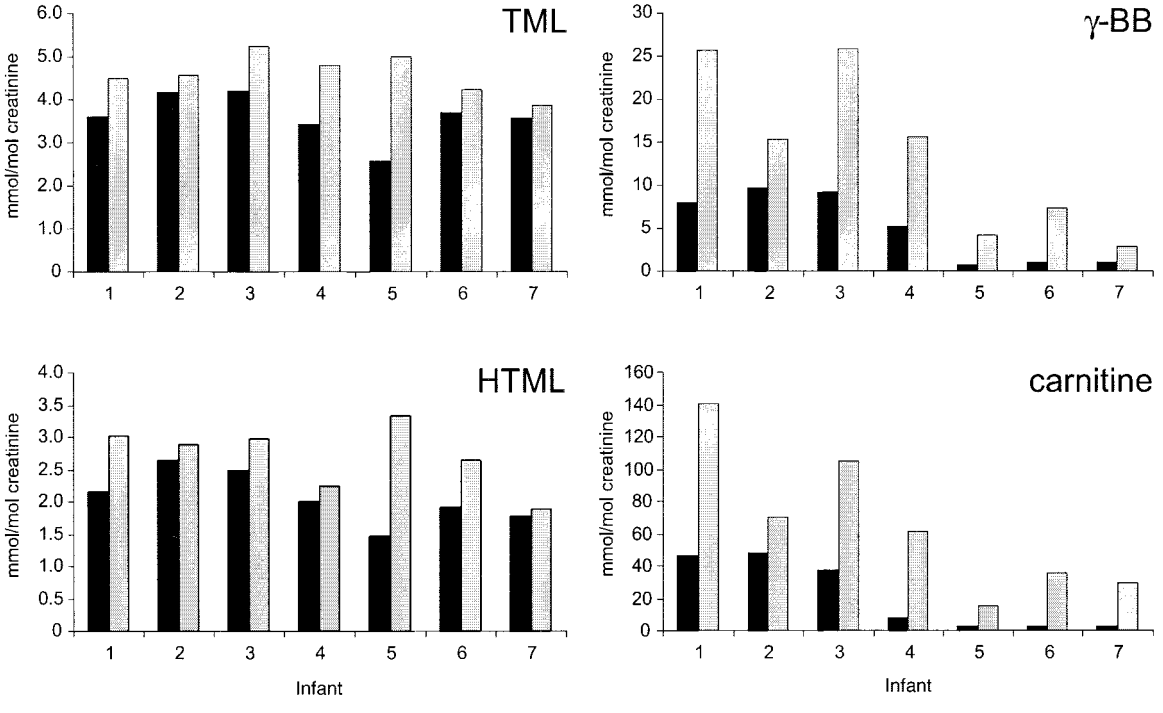

Fig. 3. Urinary concentrations of endogenous carnitine biosynthesis metabolites (except TMABA) in seven newborns before (■) and after (▨) loading with [$^2H_9$]TML.

by the renal tubules by the action of organic cation transporter 2, which further lowers the urinary excretion of γ-BB.

CARNITINE BIOSYNTHESIS IN NEWBORNS

In a recent study, no labeled carnitine could be detected by fast atom bombardment MS in the urine of premature infants after 1 day of oral [$^2H_9$]TML loading *(25)*. As an extension of this study, we performed a similar experiment with seven full-term newborns, who received [$^2H_9$]TML for 5 days, and analyzed the urines with our newly developed assay. After loading, all the metabolites of carnitine biosynthesis could be detected in the urine in the $^2H_9$-labeled form, except for [$^2H_9$]TMABA. In addition, [$^2H_9$]carnitine was also incorporated into acylcarnitines. These results show that human infants are able to convert exogenous TML into downstream carnitine precursors, carnitine, and carnitine esters at a low rate. Most of the TML (>75%), however, was excreted unchanged in the urine, which confirms that human infants do not use exogenous TML efficiently as a precursor for carnitine biosynthesis. Although TML loading studies make an important contribution to our understanding of carnitine biosynthesis, it should be noted that in these experiments, the intracellular metabolism of TML is bypassed. Tissues such as heart and muscle typically synthesize TML but do not readily absorb it from the circulation. It is believed that TML produced intracellularly is converted to butyrobetaine in the tissue of origin, after which butyrobetaine is excreted into the circulation and converted to carnitine in tissues that contain γ-BB hydroxylase. Unlike TML, γ-BB is absorbed readily by the liver and converted to carnitine. These processes could be important for carnitine biosynthesis.

After [$^2H_9$]TML loading, the concentrations of endogenous γ-BB and carnitine were considerably higher than before [$^2H_9$]TML administration. This phenomenon was also observed by Melegh and coworkers *(25, 31)*. In previous studies in which unlabeled precursors were used *(24, 32)*, urinary carnitine excretion was used to calculate the rate of carnitine biosynthesis, assuming that this carnitine was a result of actual biosynthesis. Especially in the case of TML-loading studies, these results should be reevaluated and additional experiments performed with stable-isotope-labeled carnitine precursors.

Our future plans include developing a similar assay for plasma, whole blood, and cells/tissues. Although defects in carnitine biosynthesis have not been reported to date, the ability to measure the intermediates of carnitine biosynthesis may facilitate the identification of patients suffering from such a defect.

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