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Journal of Chromatography B, 731 (1999) 89–95

JOURNAL OF CHROMATOGRAPHY B

# Measurement of carnitine precursors, ε-trimethyllysine and γ-butyrobetaine in human serum by tandem mass spectrometry

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**Abstract**

Methods using tandem mass spectrometry for measurement of ε-trimethyllysine and γ-butyrobetaine in human serum are described. Precursor ion scan analysis of a methylated sample was applied for γ-butyrobetaine measurement. However, for ε-trimethyllysine measurement, homoarginine interfered with the methylated sample during precursor ion scan analysis. To overcome this interference, the sample was propylated and acetylated prior to precursor ion scan analysis. The obtained values resembled those obtained by enzymatic or HPLC measurement. Using tandem mass spectrometry, all members of the carnitine family, free carnitine, acylcarnitines, γ-butyrobetaine, ε-trimethyllysine can be analyzed in 0.1 ml of serum. Thus, the proposed method appears to be suitable for clinical application, especially in the pediatric field. 

*Keywords:* Carnitine precursors; ε-Trimethyllysine; γ-Butyrobetaine

## 1. Introduction

Carnitine is a known transporter of long chain fatty acyl CoA for fatty acid oxidation. It also plays a role in detoxification of abnormal accumulation of acyl CoAs in mitochondria in metabolic disorders such as organic acidemia and fatty acid oxidation disorders, leading to secondary carnitine deficiency [1,2]. Thus, a better understanding of the regulation system of carnitine synthesis in primary and secondary carnitine deficiency is required. ε-Trimethyllysine (TML) and γ-butyrobetaine (BB) are precursors of carnitine biosynthesis in humans. Several methods have been proposed to measure TML [3,4] and BB [5]. However, their accuracy, sensitivity and ease of application are not adequate for clinical use. We developed a simple and accurate method for analysis of TML and BB using tandem mass spectrometry (MS–MS) in combination with liquid secondary ion mass spectrometry (LSIMS).

## 2. Experimental

### 2.1. Materials

ε-Trimethyllysine, γ-butyrobetaine hydrochloride and octyl sodium sulfate were purchased from Sigma (St. Louis, MO, USA) and ε-trimethyllysine and γ-butyrobetaine hydrochloride were recrystallized before use. L-homoarginine hydrochloride and acetylacetone were obtained from Nacalai tesque (Kyoto, Japan). Deuterium labeled internal standards, $(CD_3)_3$-*N*-trimethyllysine ($d_9$-TML) and $(CD_3)_3$-*N*-

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0378-4347/99/$ – see front matter 
PII: S0378-4347(99)00112-7

butyrobetaine ($d_9$-BB) were synthesized according to the method of Ingalls [6]. The isotope enrichment was checked by mass spectrometry and $^1$H-NMR.

### 2.2. Equipment

A triple stage quadrupole mass spectrometer was used, a QUATTRO with Masslynx data system (Micromass, Manchester, UK). The QUATTRO used a cesium LSIMS ion gun. Argon gas was used for collision induced dissociation (CID). The CID energy was set at 8–12 eV.

### 2.3. Sample preparation

#### 2.3.1. Butyrobetaine analysis

A 0.02-ml sample of serum and $d_9$-BB (internal standard) was deproteinized with 1 ml methanol and analyzed according to the protocol for carnitine analysis by MS–MS [7]. The supernatant was dried under a nitrogen stream. Methylation was carried out by 0.05 ml of 5% methanol/HCl at 65°C for 15 min. After drying under a nitrogen stream, the sample was dissolved in 0.05 ml of 50% methanol in glycerol with 1% w/v octyl sodium sulfate matrix (OSS matrix).

### 2.4. Trimethyllysine analysis

#### 2.4.1. Methylation method

TML was methylated by 5% methanol/HCl at 100°C for 30 min. For serum TML analysis, 0.05 ml of serum containing $d_9$-TML (internal standard) was treated in the same manner as that for BB analysis except for the methylation step.

### 2.5. Dimethylpyrimidyl derivatization method

Homoarginine (HA) was treated with acetylacetone according to Mori [8] for conversion into a dimethylpyrimidyl derivative, and then dried under a nitrogen stream. The dried sample was methylated with 0.1 ml of 5% methanol/HCl at 100°C for 30 min and dried again. The sample was dissolved in 0.05 ml OSS matrix. For serum TML analysis, a 0.1-ml sample of serum and $d_9$-TML was deproteinized with 1 ml methanol and the supernatant was dried under a nitrogen stream. The dried sample was treated in the same manner as HA.

### 2.6. Acetylation method

TML was propylated by 0.2 ml of n-propanol and 0.01 ml of HCl at 100°C for 30 min. The sample was then dried under a nitrogen stream and the dried sample was acetylated by 0.1 ml of acetate anhydrate and 0.4 ml of pyridine at 80°C for 30 min. The acetylated sample was dried under a nitrogen gas steam. A half milliliter of ether was poured into the test tube with the dried sample, and then decanted. The test tube was then dried again under a nitrogen gas stream and dissolved in 0.025 ml of OSS matrix. For serum TML analysis, a 0.05-ml sample of serum and $d_9$-TML was deproteinized with 1 ml methanol and the supernatant was dried under a nitrogen stream. The dried sample was treated in the same manner as TML.

## 3. Results

### 3.1. Butyrobetaine analysis

Mass spectrometry of BB and $d_9$-BB methyl esters revealed peaks corresponding to the $[M+1]^+$ ion (pseudo-molecular ion) at Da/e 160 and Da/e 169, respectively. Daughter spectra produced by CID of Da/e 160 for BB methyl ester and Da/e 169 for $d_9$-BB methyl ester are showed in Fig. 1. A common peak at Da/e 101 was observed. Consequently, Da/e 101 was chosen for precursor ion scan analysis. The calibration curve obtained by Da/e 101 precursor ion scan for BB was linear over wide range ($r=0.998$). The coefficient variation (C.V.) in the serum was 12.9%. Recovery following addition of 2.81 nmol ml$^{-1}$ of BB to the serum was 109.5%. The serum level of BB in six adults was $1.78\pm0.23$ nmol ml$^{-1}$.

### 3.2. Trimethyllysine analysis

#### 3.2.1. Methylation method

LSIMS mass spectrometry of TML and $d_9$-TML methyl esters revealed pseudo-molecular ions at Da/e 203 and Da/e 212, respectively. Subsequently,

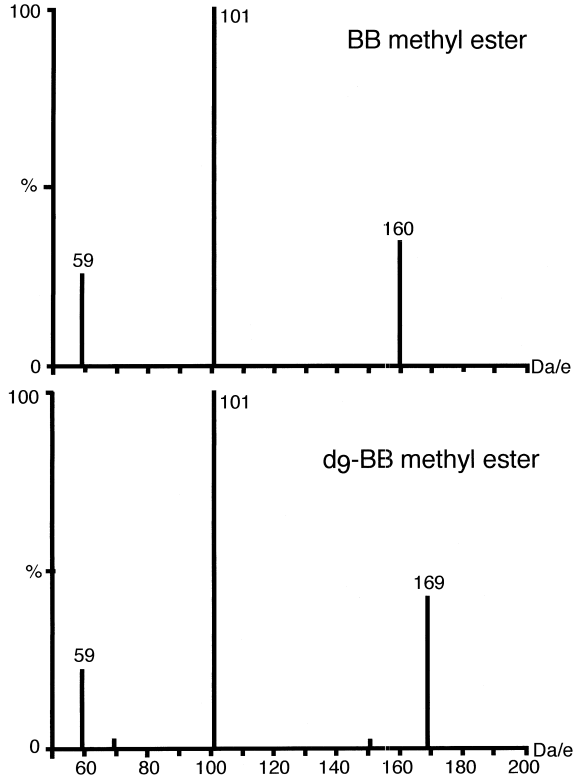


Fig. 1. Daughter ion scan of BB methyl ester (Da/e 160) and $d_9$-BB methyl ester (Da/e 169). Da/e 101 was common daughter ion for both BB and $d_9$-BB methyl esters.

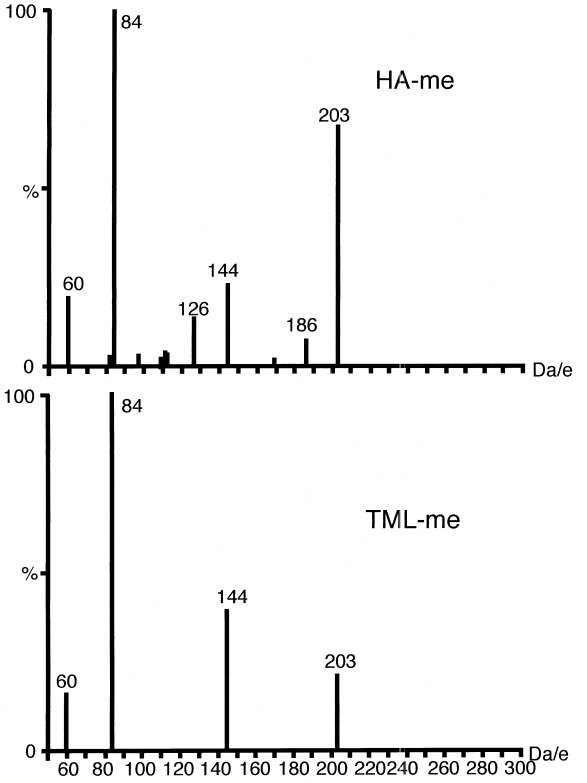


Fig. 2. Daughter ion scan of HA methyl ester (HA-me) and TML methyl ester (TML-me). HA-me and TML-me had same pseudo-molecular ion at Da/e 203 and common daughter ion at Da/e 84.

daughter ion scan analysis of both esters revealed a common ion at Da/e 84. The calibration curve obtained by Da/e 84 precursor ion scan analysis was linear ($r=0.999$). The C.V. in the serum was 9.1%. Recovery following addition of 9.0 nmol $ml^{-1}$ of TML to the serum was 96.0%. The serum level of TML in six adults was $4.43\pm0.66$ nmol $ml^{-1}$, which is almost ten times that reported using HPLC [4]. This suggested the presence of interference of a substance with a similar molecular weight and chemical character.

### *3.3. Dimethylpyrimidyl derivatization method*

HA which has the same molecular weight and is a strong cation as TML was identified as the source of interference in TML measurement using MS–MS. HA methyl ester had the same mass unit in LSIMS and same daughter ion scan pattern (Fig. 2). HA has a guanidino group which can be modified by acetylacetone (dimethylpyrimidyl derivatization), following which the mass unit of HA methyl ester shifts to Da/e 267. Fig. 3 shows mass change by dimethylpyrimidyl derivatization in serum analysis using Da/e 84 precursor ion scan. After dimethylpyrimidyl derivatization, the peak height ratio of Da/e 203 (TML methyl ester) against to Da/e 212 ($d_9$-TML methyl ester) was reduced to approximately 30%, and peak at Da/e 267 elevated, reflecting the presence of the HA dimethylpyrimidyl derivative.

### *3.4. Acetylation method*

The number and position of amino groups differ between TML and HA. TML propyl ester and HA propyl ester were acetylated by acetate anhydrate and pyridine at different temperatures. At room tempera-

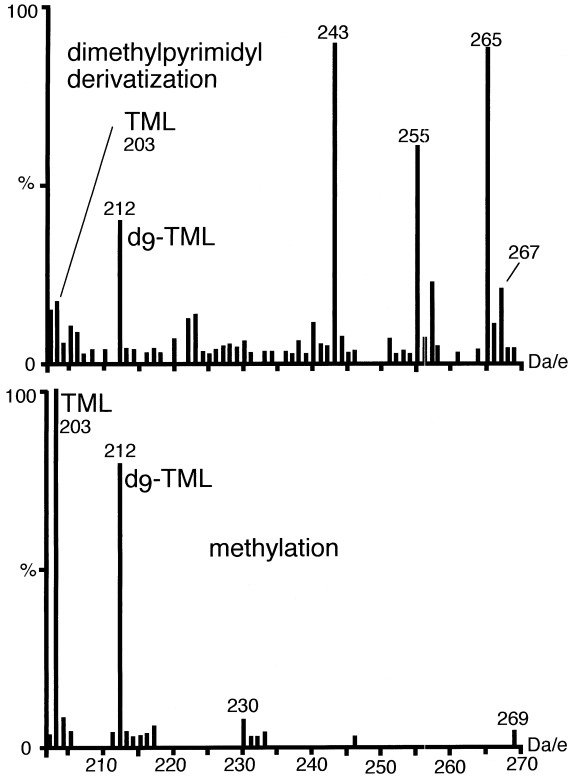


Fig. 3. Comparison of methylation method and dimethylpyrimidyl derivatization method in serum TML analysis. $d_9$-TML was added to serum as a internal standard. Following dimethylpyrimidyl derivatization, the peak height ratio of TML methyl ester (Da/e 203) against to $d_9$-TML methyl ester (Da/e 212) decreased to approximately 30%. The peak at Da/e 267 reflected the presence of the HA.

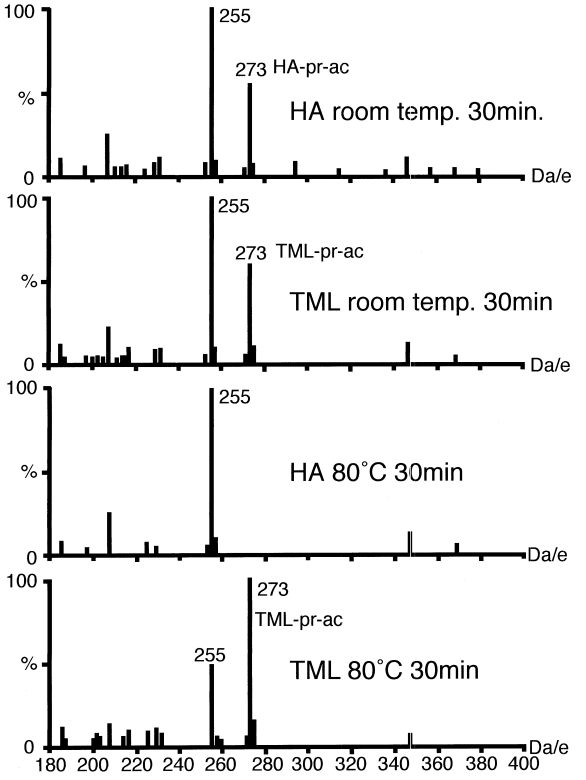


Fig. 4. Temperature dependency of acetylation of HA propyl ester and TML propyl ester. Da/e 273 represented either HA propyl-acetyl ester (HA-pr-ac) or TML propyl-acetyl ester (TML-pr-ac) as indicated. At room temperature, both HA-pr-ac and TML-pr-ac can be seen, but at 80°C, HA-pr-ac disappears.

ture, both TML and HA propyl esters were derivatized to propyl-acetyl ester, whereas at 80°C, the TML propyl ester was acetylated but the HA propyl ester was not (Fig. 4). TML propyl-acetyl ester (Da/e 273) and $d_9$-TML propyl-acetyl ester (Da/e 282) showed common peaks at Da/e 84 and Da/e 126 in the daughter ion scan (Fig. 5). When serum was analyzed without purification after acetylation step, the peak height ratio of Da/e 273 against to Da/e 282 (internal standard) by Da/e 126 precursor ion scan was approximately 60%, but that by Da/e 84 precursor ion scan was approximately 30% in the same sample (Fig. 6). However, this discrepancy disappeared when the acetylated sample was washed with ether (Fig. 7). The calibration curves obtained by both Da/e 84 and Da/e 126 precursor ion scan with ether wash for authentic TML propyl-acetyl ester were similar and quite linear over a wide range ($r=0.999$ for both). A strong correlation was found between serum levels of TML by precursor ion scan of Da/e 84 and Da/e 126 with ether wash ($r=0.970$, slope=0.951, range 0.15–1.8 in ratio of peak height of Da/e 273 to Da/e 282, $n=9$). The Da/e 84 precursor ion scan was chosen for serum analysis, because the sensitivity of Da/e 84 precursor ion scan was higher than Da/e 126 precursor ion scan. The C.V. of serum analysis was 5.2%. Recovery following addition of 1.06 nmol $ml^{-1}$ of TML to the serum was 91.9%. The addition of 37 nmol $ml^{-1}$ of HA to the serum induced a 5% increase in the TML value. The average serum level of TML was $0.88\pm0.28$ nmol $ml^{-1}$ ($n=9$, Table 1).

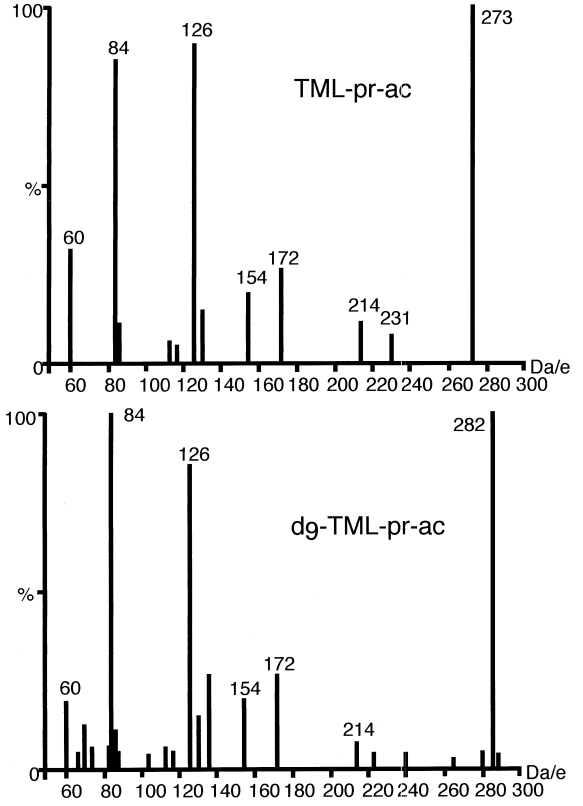


Fig. 5. Daughter ion scan of TML propyl-acetyl ester (TML-pr-ac) and $d_9$-TML propyl-acetyl ester ($d_9$-TML-pr-ac). Da/e 84 and Da/e 126 were common daughter ions which was able to use for precursor ion scan analysis.

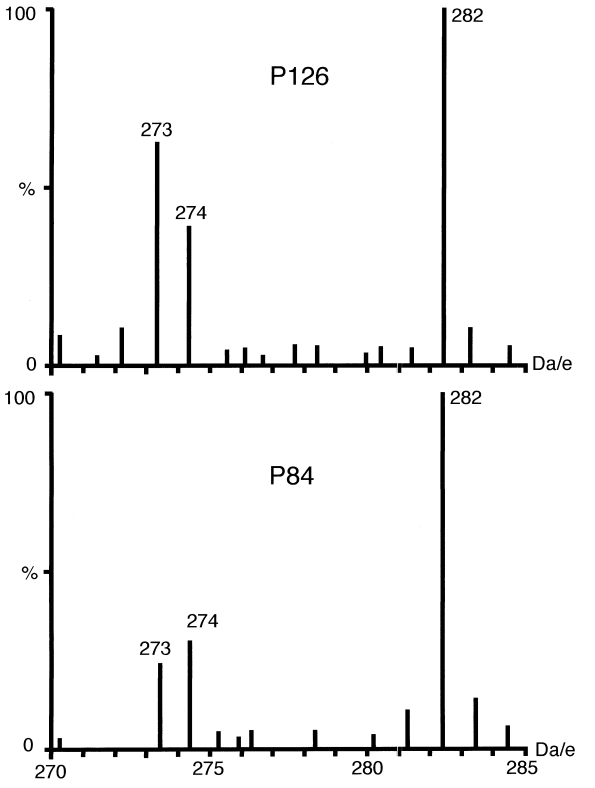


Fig. 6. Serum measurement of TML by propyl-acetyl ester without ether wash. The peak height ratio of Da/e 273 (TML propyl-acetyl ester) against Da/e 282 ($d_9$-TML propyl-acetyl ester) by Da/e 126 precursor ion scan (P126) was approximately twice than by Da/e 84 precursor ion scan (P84).

## 4. Discussion

MS–MS has been applied to carnitine analysis including acylcarnitines which has been analyzed by Da/e 99 precursor ion scan for methyl ester and Da/e 85 precursor ion scan for butyl ester and free carnitine which has been analyzed by Da/e 103 precursor ion scan for butyl ester and Da/e 117 precursor ion scan for methyl ester [2,7,9,10]. This analysis can be performed using only 0.02 ml of serum, requires no chromatographical purification and can be performed in only 1–2 min.

In the case of a small molecule such as carnitine, it has been thought that precursor ion scan analysis based on CID is sufficient for clinical sample analysis without requiring an additional separation step.

MS–MS analysis showed the similar BB level in serum as that determined by enzymatic assay [5]. However, the analysis of TML was complicated by interference. Dimethylpyrimidyl derivatization and acetylation of the serum revealed that the interference was caused mainly by the presence of HA. Dimethylpyrimidyl derivatization had poor sensitivity and the derivatization step was time consuming. TML propyl-acetyl ester was able to be analyzed by both precursor ion scan of Da/e 84 and Da/e 126. The analysis of authentic TML and $d_9$-TML by acetylation showed good sensitivity and high $S/N$ ratio with both Da/e 84 and Da/e 126 precursor ion scan. But serum TML analysis needed purification step after acetylation, because its poor $S/N$ ratio and the discrepancy between Da/e 84 and Da/e 126 precursor ion scan analysis. HA itself seemed not to

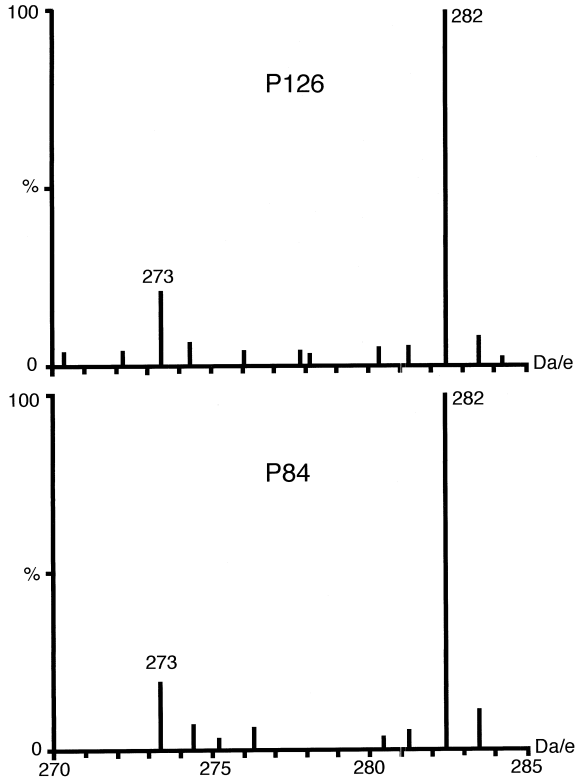


Fig. 7. Serum measurement of TML by propyl-acetyl ester with ether wash. The peak height ratio of Da/e 273 (TML propyl-acetyl ester) against Da/e 282 ($d_9$-TML propyl-acetyl ester) by Da/e 126 precursor ion scan (P126) was as same as by Da/e 84 precursor ion scan (P84).

have any relation to this discrepancy. We tested many purification procedures and found that ether wash after acetylation increased $S/N$ ratio, eliminated the discrepancy and gave equivalent values of TML levels in serum obtained by both precursor ion scan of Da/e 84 and Da/e 126. The compounds removed by ether wash were not identified, and not removed by hexane or ethyl acetate. The serum HA levels were 0.5–2.8 nmol $ml^{-1}$ in normal children, 1.98±0.634 in normal men, 1.51±0.609 in normal women, and reached 20–40 nmol $ml^{-1}$ in hyperarginenemia [11,12]. The addition of 37 nmol $ml^{-1}$ of HA to the serum caused only a 5% increase in the TML value. Thus, the proposed MS–MS method appeared to have negated the interference and yielded the similar values as HPLC method [4] in the serum TML analysis.

Table 1
BB and TML measurement in human blood by MS–MS compared to enzyme and HPLC analysis[a]

| | BB | TML |
|---|---|---|
| | nmol $ml^{-1}$ | nmol $ml^{-1}$ |
| Enzyme assay[b] | 4.66±0.8 ($n$=5) | |
| HPLC assay[c] | | 0.556±0.173 ($n$=6) |
| LISMSMS[d] | 1.78±0.23 ($n$=6) | 0.88±0.28 ($n$=9) |

[a] Values listed represent means±SD.
[b] In serum, A. Sandor et al. [5].
[c] In plasma L.J. Lehman et al. [4].
[d] In serum, methylation for BB, propyl-acetyl derivatization with ether wash for TML (Proposed method).

Enzymatic analysis of BB required the use of a special enzyme [5], limiting its widespread application. Furthermore, this enzymatic analysis requires 1 ml of plasma, as does HPLC analysis of TML [4]. In contrast, the proposed MS–MS requires only 0.05 ml or less. The major components of carnitine biosynthesis, free carnitine, acylcarnitines, BB and TML can be measured by MS–MS. Analysis of these components by MS–MS requires only 0.1 ml of serum. Thus, the proposed MS–MS method appears suitable for clinical application especially in pediatric field because of its requirement of small sample volume.

## Acknowledgements

We appreciate Dr. S. Sawada for obtaining the data of $^1$H-NMR. We thank Ms. A. Suzuki for her secretarial assistance in preparation of this manuscript. This study was supported in part by a Grant-in-Aid for Scientific Research 08307008 and 09670804, Japan Ministry of Education, Science and Culture.

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