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Effect of sports activity on carnitine metabolism Measurement of free carnitine, γ-butyrobetaine and acylcarnitines by tandem mass spectrometry

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Abstract

The effects of sports activity on carnitine metabolism were studied using mass spectrometry. Serum levels of free carnitine, acylcarnitines (acetylcarnitine, propionylcarnitine, C4-, C5- and C8-acylcarnitine) and γ-butyrobetaine, a carnitine precursor, were determined by tandem mass spectrometry in liquid secondary ion mass ionization mode. The coefficients of variation at three different concentrations were 2.8~7.9% for γ-butyrobetaine, and 1.2~6.7% for free carnitine. The recoveries added to serum were 109.1% for γ-butyrobetaine, 89.3% for free carnitine. Sports activity caused increased serum levels of γ-butyrobetaine, acetylcarnitine, C4- and C8-acylcarnitines and decreased serum levels of free carnitine. This method requires a small amount of sample volume (20 μl of serum) and short total instrumental time for the analysis (1 h for preparation, 2 min per sample for mass spectrometric analysis). Therefore, this method can be applied to study carnitine metabolism under various conditions that affect fatty acid oxidation.

Keywords: Acylcarnitine; γ-Butyrobetaine; Carnitine

1. Introduction

Carnitine is known to play an important role in fatty acid oxidation [1]. Many studies concerning carnitine metabolism in sports activity have been reported [2]. However, no studies have described individual acylcarnitine level or γ-butyrobetaine level. Carnitine has been measured by the radioenzymatic method [3], and γ-butyrobetaine has been measured by the radioenzymatic method combined with high-performance liquid chromatography (HPLC) [4]. To analyze acylcarnitines, several methods [fast atom bombardment mass spectrometry (FAB-MS) [5], gas chromatography (GC)–MS [6], HPLC [7], electrospray ionization (ESI)-MS [8]] have been reported. We developed a simple method to simultaneously analyze free carnitine, γ-butyrobetaine and acylcarnitines (acetylcarnitine, propionylcarnitne, C4-, C5- and C8-acylcarnitine) using tandem mass spectrometry and applied this method to study metabolism during sports activity.

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2. Experimental

2.1. Sports activity

Blood samples were obtained from 10 healthy male college students who belonged to the volleyball club before and after 150 min of regular exercise. Before the initiation of exercise, the subjects fasted for at least 3 h. Body composition was accessed by bioelectrical impedance analysis. Serum levels of free fatty acid, 3-hydroxybutyrate and lactate were determined by enzymatic methods using an automatic analyzer. Characteristics of the subjects are summarized in Table 1.

2.2. Analytical methods

2.2.1. Chemicals

DL-$^2H_9$-carnitine and $^2H_9$-γ-butyrobetaine were synthesized according to the method of Ingalls [9]. The products were used as internal standards without further purification. The chemical purity checked by MS and ^{1}H-nuclear magnetic resonance (NMR) was 98% in free carnitine and 41% in γ-butyrobetaine, respectively, and their isotopic purity was greater than 99%. $^2H_3$-Acetylcarnitine, $^2H_3$-propionylcarnitine, $^2H_7$-*n*-butyrylcarnitine, $^2H_9$-isovalerylcarnitine and $^2H_3$-octanoylcarnitine were obtained from Dr. Ten Brink at the Free University Hospital of Amsterdam [10]. Five percent hydrogen chloride in anhydrous methanol was purchased from Wakenyaku (Osaka, Japan). Carnitine hydrochloride, γ-butyrobetaine hydrochloride and octylsalfate, sodium salt were purchased from Sigma (St. Louis, MO, USA). Iodomethane-d_3 was purchased from Aldrich (Milwaukee, WI, USA). Other chemicals used were of analytical grade, and purchased from Nacalai Tesque (Kyoto, Japan).

2.2.2. Sample preparation

Due to the presence of a small amount of hydrolysed free carnitine in internal standards of acylcarnitines, two sets of samples, one for free carnitine and γ-butyrobetaine and another for acylcarnitines, were prepared separately. For the analysis of free carnitine and γ-butyrobetaine, 20 μl of serum was mixed with 1 ml of methanol containing 500 pmol of DL-$^2H_9$-carnitine and 50 pmol of $^2H_9$-γ-butyrobetaine. To analyze acylcarnitines, 20 μl of serum was mixed with 1 ml of methanol containing 100 pmol of $^2H_3$-acetylcarnitine, 50 pmol of $^2H_3$-propionylcarnitine, $^2H_7$-*n*-butyrylcarnitine, $^2H_9$-isovalerylcarnitine and $^2H_3$-octanoylcarnitine. The mixture was centrifuged for 3 min in an Eppendorf microcentrifuge. The supernatant was transferred to microtube and methanol was evaporated under a stream of nitrogen. The residue was methylated by the addition of 50 μl of 5% hydrogen chloride in methanol and heated at 65°C for 15 min. Methanol was evaporated under a stream of nitrogen and residue was reconstituted with 50 μl of 50% glycerol in methanol containing 1% of octylsulfate, sodium salt. An aliquot (usually 1 to 2 μl) of sample was used for the analysis by MS.

Table 1
Characteristics of subjects

Subjects	Age (years)	Height (cm)	Weight (kg)	BMI	Fat (%)	LBM (kg)
1	19	181.0	74.0	22.6	17.2	61.3
2	19	178.0	66.0	21.6	13.9	56.8
3	19	185.0	74.0	21.6	16.0	62.2
4	21	181.0	87.0	26.6	23.2	66.8
5	20	178.0	67.0	21.1	18.4	54.7
6	19	173.0	64.0	21.4	13.2	55.6
7	19	188.0	76.0	21.5	16.3	63.6
8	19	175.5	70.0	22.3	17.2	58.0
9	21	177.0	65.0	20.7	13.7	56.1
10	19	178.4	73.0	22.9	11.3	64.8
Mean	19.5	179.5	71.6	22.2	16.0	60.0
SD	0.85	4.44	6.88	1.68	3.34	4.29

2.2.3. Mass spectrometry

A triple stage quadrupole mass spectrometer, QUATTRO (Micromass, Manchester, UK) with an ion gun of cesium iodide as a secondary ion mass ionization mode was used. Argon was used as collision gas, and collision energy was set at 12 eV. As previously demonstrated [11], predominant daughter ions of both labeled and unlabeled standards were m/z 117 for free carnitine, m/z 101 for γ-butyrobetaine, and m/z 99 for acylcarnitines. An aliquot (usually 1 to 2 μl) of sample was placed on the probe, and precursor ion scans of m/z 117, m/z 101 and m/z 99 were obtained to analyze free carnitine, γ-butyrobetaine and acylcarnitines, respectively. About 100 scans were accumulated for each compound in the multi channel analysis mode, and the peak height of protonated molecular ions of unlabeled and labeled compounds was used for quantification after smoothing, background subtraction and centering.

2.2.4. Statistical analysis

Statistical analysis was carried out by software, StatView version 4.02 (Abacus Concepts, Berkeley, CA, USA). Differences between the values before and after exercise were analyzed by a paired *t*-test.

3. Results

3.1. Reliability of analytical methods

Standard curves were obtained in the concentration range of interest, 0.1 to 4 nmol/ml for γ-butyrobetaine and 5 to 200 nmol/ml for free carnitine. The peak height ratios of unlabeled to labeled were calculated and evaluated by linear regression analysis. All calibration curves showed a linear relationship between the concentration and peak height, while correlation coefficients were 1.000 for free carnitine, and 0.999 for γ-butyrobetaine. Detection limits were determined in plasma samples and varied from 0.01 nmol/ml for γ-butyrobetaine to 0.2 nmol/ml for free carnitine. Fig. 1 shows raw spectra of methyl esters of free carnitine and γ-butyrobetaine without smoothing and background subtraction. Recoveries were determined as follows: 1.10 nmol/ml of γ-butyrobetaine and 50 nmol/ml of free carnitine were added to serum, and the increment observed was shown as a percent of theoretical values. Recovery for γ-butyrobetaine was 109.1%, and that for free carnitine was 89.3%. Extraction recoveries were determined by stable isotope labeled analogs. The labeled internal standards were added to an aliquot of the serum, and none to another aliquot of the serum. After extraction of both samples, the same quantities of labeled internal standards were added to the extract of the second aliquot. The extracts were methylated and analyzed to determine the isotopic abundance ratios for each pair of labeled and unlabeled compound. The recoveries of γ-butyrobetaine and free carnitine from serum were 77.3% and 92.7%, respectively. Coefficients of variations (CVs) at three different concentrations (low, medium and high) were determined as shown in Table 2. CVs for γ-butyrobetaine were 2.8% (low), 7.9% (medium) and 3.8% (high). CVs for free carnitine were 1.2% (low), 6.7% (medium) and 1.4% (high). CVs of the same sample on five different days were 5.1% for γ-butyrobetaine, and 6.6% for free carnitine, respectively.

3.2. Effects of sports activity

The effects of sports activity are summarized in Table 3. Serum levels of free fatty acids and 3-hydroxybutyrate showed marked increases due to sports activity. As shown in Fig. 2, the serum level of acetylcarnitine, C4- and C8-acylcarnitine increased by two-fold. The serum level of free carinitine decreased slightly ($P<0.05$). On the contrary, the serum level of γ-butyrobetaine increased slightly after exercise ($P<0.01$).

4. Discussion

Liquid secondary ion mass spectrometry (LSIMS) is an important technique for analyzing nonvolatile organic compounds such as peptides, nucleotides and quarternary amines such as carnitines and γ-butyrobetaine. Tandem mass spectrometry combined with stable isotope dilution analysis is widely used to analyze various compounds. In collision activated dissociation (CAD), the ion in question is chosen as the parent ion and directed into a collision cell,

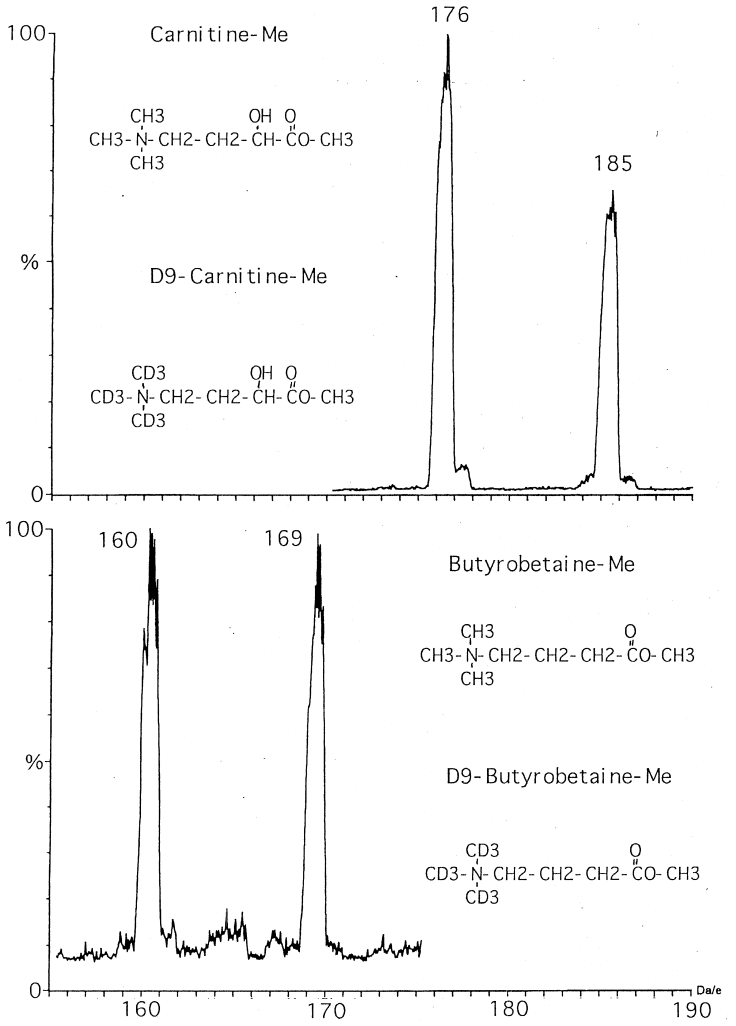

Fig. 1. Typical raw spectra of precursor ion scans of methyl esters of carnitine (upper panel) and γ-butyrobetaine (lower panel) in a serum sample. Background levels are low for both compounds.

where it collides with a collision gas to acquire energy, which leads to its decomposition into daughter ions. The CAD process has been used to characterize fragmentation pathways and to obtain structurally characteristic fragmentation patterns that can be used to identify selected analytes in complex mixtures. Therefore, the precursor ion scan acts not only in sample analysis but also as a sample purification step, so the time consuming preparation step can be reduced. Stable isotope dilution analysis provides accurate quantification [12]. A stable isotope labeled analogue of the analyte serves as an ideal standard, that has nearly the same chemical and physical characteristics as the analyte. In this way, the analyte and the internal standard undergo the same losses during sample preparation including chemical reactions and physical processes. It requires small amounts of sample volume and has superior specificity and sensitivity than previous methods. A previous method required 1 ml of plasma for γ-butyrobetaine determination [4], however this method requires only 20 μl. The total instrumental time to

Table 2
Precision of within- and between-day assay

			Free carnitine (nmol/ml)	γ-Butyrobetaine (nmol/ml)
Within-day assay	Low[a]	Mean±SD	18.32±0.22	0.37±0.01
	(*n*=10)	CV (%)	1.2	2.8
	Medium[b]	Mean±SD	55.62±3.62	2.04±0.16
	(*n*=10)	CV (%)	6.7	7.9
	High[c]	Mean±SD	100.26±1.44	3.24±0.12
	(*n*=9)	CV (%)	1.4	3.8
Between-day assay	Medium[b]	Mean±SD	54.00±3.55	2.06±0.10
	(*n*=5)	CV (%)	6.6	5.1

[a] Low sample was made by diluting normal serum 1.
[b] Medium sample was normal serum 2 without dilution.
[c] High sample was normal serum 2 supplemented with carnitine and γ-butyrobetaine corresponding 50 nmol/ml and 1.10 nmol/ml, respectively.

analyze these compounds was about 1 h for preparation and 2 min per sample for mass spectrometric analysis. Recently, amino acids and acylcarnitines were analyzed in blood spots of neonatal mass screening in several countries [13].

Our findings indicate that the normal value of serum γ-butyrobetaine level is 1.80±0.23 nmol/ml, which is lower than that of the previously reported value [4], 4.66±0.80 nmol/ml. The reasons for the discrepancy of normal values were unclear, however contamination of other compounds with similar retention times is possible. γ-Butyrobetaine is a direct precursor of carnitine, and its availability is considered to be a limiting step for carnitine synthesis [14]. One of the reasons why the serum γ-butyrobetaine level increased after sports activity may be the increased supply of carnitine precursor from the muscle to the liver for carnitine synthesis to compensate for the decreased serum free carnitine level.

It has been reported that sports activity causes increased fatty acid oxidation and results in increased serum acylcarnitine levels and decreased serum free carnitine levels [15]. In this study, increased serum levels of acetylcarnitine, C4-and C8-acylcarnitines were observed, however propionylcarnitine and C5-acylcarnitine remained unchanged, suggesting that the energy source of fatty acid oxidation is mainly derived from even-chain saturated fatty acids.

The advantages of this method are summarized as follows: (1) a small sample volume is required, (2) accurate and specific measurement for each com-

Table 3
Effect of sports activity on serum metabolites (data are shown as mean±SD)

	Monitored ions (metabolite/I.S.)	Before exercise	After exercise	*P* value
Free fatty acid (m*M*)	–	0.385±0.129	1.416±0.449	<0.01
3-Hydroxybutyrate(nmol/ml)	–	13.8±6.4	295.5±155.0	<0.01
Lactate (mg/l)	–	7.0±2.67	5.6±1.42	n.s.
Butyrobetaine (nmol/ml)	*m/z* 160/169	1.80±0.23	2.21±0.32	<0.01
Free carnitine (nmol/ml)	*m/z* 176/185	52.38±7.80	47.12±9.52	<0.05
Acetylcarnitine (nmol/ml)	*m/z* 218/221	6.85±1.73	15.08±2.26	<0.01
Propionylcarnitine (nmol/ml)	*m/z* 232/235	0.37±0.19	0.47±0.12	n.s.
C4-acylcarnitine (nmol/ml)	*m/z* 246/253	0.19±0.07	0.28±0.11	<0.05
C5-acylcarnitine (nmol/ml)	*m/z* 260/269	0.11±0.06	0.15±0.11	n.s.
C8-acylcarnitine (nmol/ml)	*m/z* 302/305	0.13±0.19	0.31±0.19	<0.01

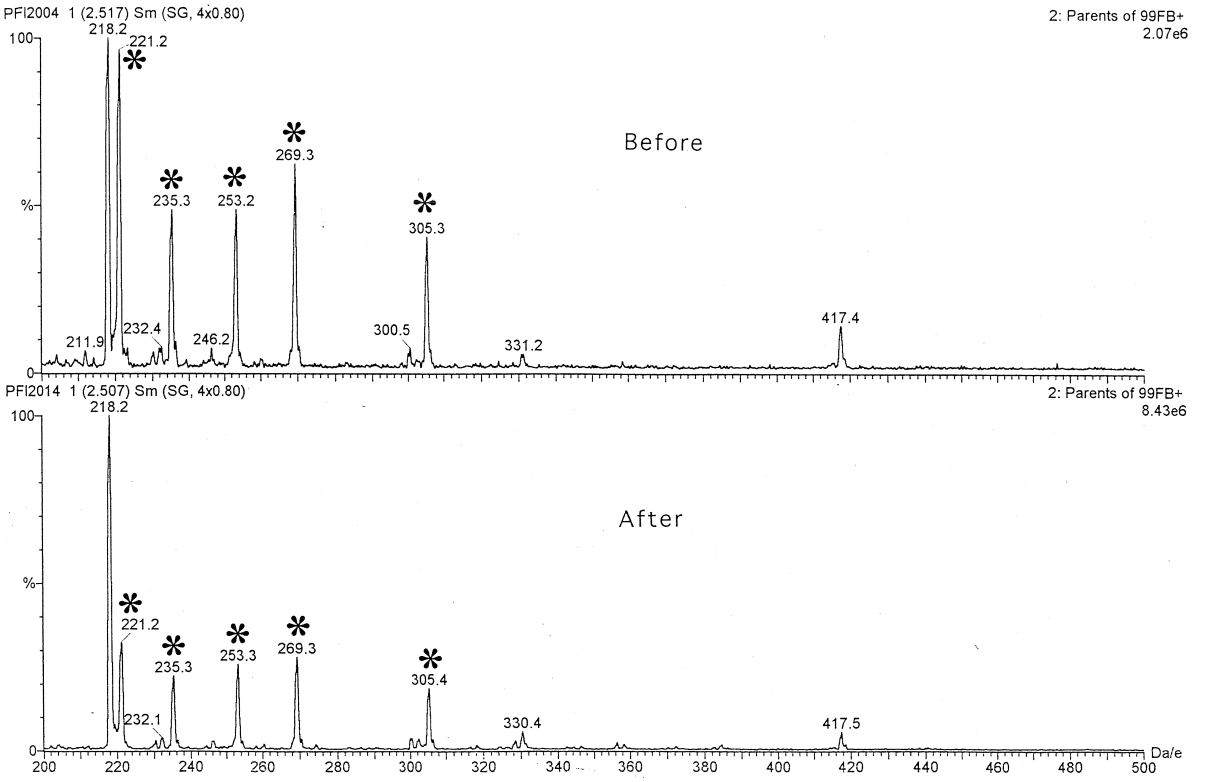

Fig. 2. Typical raw spectra of precursor ion scans of methyl esters of acylcarnitines before (upper panel) and after exercise (lower panel). Asterisks indicate stable isotope labeled internal standards. Peak of acetylcarnitine (m/z 218) increased markedly after exercise.

pound is achieved using stable isotope dilution analysis, (3) no time consuming step for sample preparation. Therefore, this method can be used for various studies concerning fatty acid oxidation and carnitine metabolism.

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