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Short communication

Enzymatic determination of acetylcarnitine for diagnostic applications

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Abstract

An enzymatic method was proposed for determination of acetylcarnitine (AcCar), even when carnitine (Car), non-acetylated form, co-exists. The method is consisted of four enzymatic reactions: First, AcCar is hydrolysed by acylcarnitine hydrolase to yield acetate; followed by the other three reactions coupled with three enzymes, respectively, acetate kinase, pyruvate kinase and lactate dehydrogenase; finally, the acetate formation causes a decrease in NADH. The amount of AcCar is then evaluated as the change in absorbance at 340 nm. The reagent composition of the reaction mixture was determined, and the characteristics of the method were investigated. The dilution test showed a good linearity over a wide range. The precision and accuracy tests produced satisfactory results. The co-existence of Car gave no effect on the measurement. The present method was found to be used easily, simply and rapidly for the selective determination of AcCar. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

In 1905, L-carnitine (3-hydroxy-4-*N*-trimethylaminobutyrate) (Car) was discovered in beef [1], and it has been proved to play an important role in fatty acid metabolism, e.g., the transport of fatty acid into mitochondria and the modulation of intramitochondrial CoA/acyl-CoA ratio [2]. Acylcarnitine (ACar), in connection with Car, also attracts a keen interest, has been regarded as a transient substance of fatty acid metabolism in mitochondria, and is also found to have some important pharmacological properties [3].

In the recent 10 yr, there has been markedly increasing recognition of the chronic fatigue syndrome (CFS). It was named by a working group of American public health epidemiologists, academic researchers and clinicians in 1988 [4], and characterized by prolonged general fatigue, slight fever, muscle weakness, myalgia, postexertional

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malaise, arthralgia and neuropsychiatric symptoms [4,5]. Recently, Kuratsune et al. [6] reported that the amount of serum ACar has a close relation to CFS, and the possibility was suggested for making a diagnosis of CFS by the measurement of serum ACar. The relationship between the amount of serum ACar and exhaustive exercise [7] or starvation [8] was also reported. Moreover, serum ACar is known to be mostly comprised of acetylcarnitine (AcCar) [9], and much interest has been focused on the biological and medical significance of AcCar itself in many fields [10–13] besides CFS.

The measurement of AcCar was performed by the methods belonging to high performance liquid chromatography [14,15]. It required expensive instruments, and was complicated and tedious. Therefore, they are not suitable for routine medical analysis, and limited to the basic research. As described above, the necessity for the measurement of serum AcCar is growing in recent years, but to date, little attention has been paid to the convenient method of simplicity and easiness. As for the determination of total carnitine consisting of Car (non-acylated form) and ACar (acylated form), Takahashi et al. [16] announced a simple enzymatic method using acylcarnitine hydrolase (ACH) and carnitine dehydrogenase as key enzymes. However, the method did not make it possible to measure ACar alone, much less AcCar alone, when Car co-exists.

In this paper, an enzymatic method is proposed for measuring serum AcCar not influenced by the co-existence of Car. This method is rapid and convenient, and does not require any special or expensive instruments.

2. Experimental

2.1. Material

Acylcarnitine hydrolase (from *Alcaligenes* sp.) was supplied by Asahi Chemical Industry Co., Tokyo, Japan. Acetate kinase (AK, EC 2.7.2.1, from *Bacillus stearothermophilus*) was supplied by Unitika Co., Osaka, Japan. Pyruvate kinase (PK, EC 2.7.1.40, from rabbit muscle), lactate dehydrogenase (LDH, EC 1.1.1.27, from hog muscle), ATP and NADH were obtained from Roche Diagnostics Co., Mannheim, Germany. Acetylcarnitine was obtained from Sigma Chemical Co., St. Louis, MO. The other chemicals were commercial products of analytical grade.

2.2. Instrumentation

The absorbance at 340 nm was measured with a Shimadzu UV 2200 spectrophotometer. A cuvette for measuring the absorbance was of a semi-micro type (1 cm path-length) and made of quartz.

2.3. Principle

The analytical principle is schematically shown in Fig. 1. That is to say, the principle consists of four enzymatic reactions: first, AcCar is hydrolyzed by ACH to yield acetate, and finally, the acetate formation causes a decrease in NADH. The amount of AcCar is then evaluated as the change in absorbance at 340 nm.

2.4. Procedure

A 0.8 ml volume of the reaction mixture was pre-incubated at 30°C for 5 min, and 0.2 ml of AcCar aq solution was added as a sample. The composition of the reaction mixture was as follows (final concentration): K-phosphate buffer (pH 7.2, 0.1 M); 2.5 mM ATP; 0.5 mM PEP; 0.3 mM NADH; 7.0 mM MgSO4; 50.0 mM KCl; 20.0 U ml^{-1} AK; 9.0 U ml^{-1} PK; 4.0 U ml^{-1} LDH; 0.5 U ml^{-1} ACH. Each concentration including pH was determined in a preliminary experiment, considering the concentration range

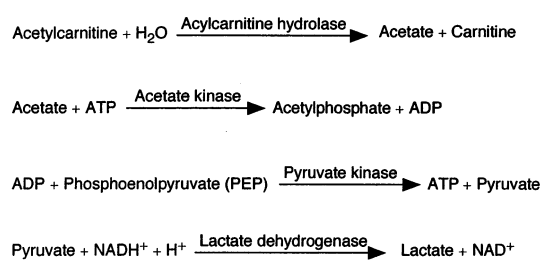

Fig. 1. Schematic principle of the determination of AcCar.

Table 1
Method validation[a]

Linearity ($n = 12$; 0–500 μM)
Slope ± SD = 1.0192 ± 0.0025; RSD = 0.24%
Intercept ± SD = 0.0002 ± 0.0005
$r = 0.9998$
Limit of detection
LOD = 5.02 μM
Precision ($L = 20$, $M = 50$, $H = 100$ μM)
Within-day ($n = 8$)
RSD = 4.32% (L), 3.07% (M), 2.36% (H)
Between-day ($k = 8$)
RSD = 4.48% (L), 3.17% (M), 2.35% (H)
Accuracy ($L = 20$, $M = 50$, $H = 100$ μM; $n = 5$) (95% confidence limits)
19.27 ± 1.11 μM (96.4 ± 5.6%)
50.95 ± 1.90 μM (101.9 ± 3.8%)
99.26 ± 3.26 μM (99.3 ± 3.3%)

[a] Conditions as described in text. S.D. – standard deviation; RSD – relative standard deviation.

not having an influence on the measurement. After addition of AcCar, the change in absorbance at 340 nm was measured by an end-point method within 10 min.

3. Results

3.1. Linearity

The sample with a high AcCar concentration (500 μM) was diluted in the concentration range 0–500 μM. A linear relationship existed over a full range up to 500 μM with a high correlation coefficient (r) of 0.9998 (Table 1). The limit of detection (LOD) was calculated from the intercept of the regression line, assuming that it is equal to three times the standard deviation (SD) of the blanks. LOD was 5.02 μM, which reached a practical level.

3.2. Precision

The results of within-day and between-day precision tests using samples with three AcCar concentrations are shown in Table 1. The former test was carried out eight times within one day, and the latter eight consecutive days. Precision parameters were satisfactory for medical analysis, and not very different from each other.

3.3. Analytical recovery

AcCar solutions were added to normal control serum at 20, 50 and 100 μM concentrations. As shown in Table 1, the method was found to be sufficiently accurate. The biological fluid did not affect the measurement significantly. The comparison between within-run and between-run assays did not make a statistical difference. $F_{obs} = 2.49 < F_{0.05}(2,12) = 3.89$.

3.4. Effect of co-existents

An interference study was performed involving the addition of the following substances that may co-exist in serum: lactate (0–2.5 g l^{-1}), glucose (0–1 g l^{-1}), urate (0–1 g l^{-1}), glutathione (0–1 g l^{-1}) and ascorbate (0–1 g l^{-1}). No significant interference was observed.

3.5. Effect of non-acetylated carnitine

Non-acetylated carnitine (free carnitine, Car) (0–5 g l^{-1}) was added to samples. Any effect was not observed.

3.6. Effect of anticoagulants

EDTA (1.0 g l^{-1}) , sodium citrate (5.0 g l^{-1}), heparin (100 mg l^{-1}), sodium fluoride (10 g l^{-1}), and sodium oxalate (1.25 g l^{-1}) were added to the reaction mixture individually. The former three had no effect, on the other hand sodium fluoride led to a small positive error and sodium oxalate led to a small negative error.

4. Discussion

A simple enzymatic method was developed for the determination of AcCar. As mentioned in Section 2.4, the concentration of each component of the reaction mixture was determined in a preliminary experiment. On performing it, the con-

centration of AcCar in the sample was fixed at 150 μM in order to save a lot of time and labor. The highly satisfactory results of linearity showed that this setting well served our purpose in this work.

The present method was based on using ACH and AK as key enzymes. Both enzymes have been used as analytical tools for measuring components in serum, that is to say, the former for measuring total Car consisting of Car and ACar [16] and the latter for measuring cholinesterase activity [17], which suggested that a serum sample did not interfere with the present method. ACH has broad substrate specificity for ACar [16], but AK has high substrate specificity for acetate [17].The present method is regarded as a suitable one for measuring AcCar alone.

In the reaction catalyzed by dehydrogenases such as LDH in this work, 1 mol of NADH reacts with 1 mol of substrate to be converted to 1 mol of NAD stoichiometrically [18]. Moreover, a molar extinction coefficient of NADH at 340 nm is hardly affected by a variation in the condition of measurement such as pH and so on [19]. The present method was based on the reaction involving NADH and the measurement of its absorbance at 340 nm, which made us expect high reliability of the measurement. The reaction proceeded stoichiometrically, and a dilution test showed a good linearity over a wide range. A good precision and accuracy were observed. None of co-existents including reductants, which are apt to interfere with signal reactions based on oxidoreductase such as oxidase, influenced the determination of AcCar, and especially, the co-existence of Car had no influence, which shows the inertia to reductants and the high selectivity or specificity of the present method for AcCar. Two among the five anticoagulants had some influence on the measurement. The problem can be solved with a choice of other three.

The present method was the first one for measuring the amount of AcCar itself, and produced a satisfactory result. It was also noteworthy that the method was found to be used easily, rapidly and simply for the determination of AcCar. Medical or clinical application remains to be investigated.

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