Clinica Chimica Acta 468 (2017) 187–194

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Matrix effect–corrected liquid chromatography/tandem mass-spectrometric method for determining acylcarnitines in human urine

Kazuki Abe [a], Hiroyuki Suzuki [b,1], Masamitsu Maekawa [b], Miki Shimada [a,b,2], Hiroaki Yamaguchi [a,b], Nariyasu Mano [a,b,*]

[a] Faculty of Pharmaceutical Sciences, Tohoku University, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
[b] Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan

ARTICLE INFO

Article history:
Received 7 December 2016
Received in revised form 18 January 2017
Accepted 1 March 2017
Available online 06 March 2017

Keywords:
Acylcarnitine
Hypocarnitinemia
LC/ESI-MS/MS
Matrix effect
Pivaloylcarnitine

ABSTRACT

Administration of pivalate-containing antibiotics decreases serum carnitine and increases urinary pivaloylcarnitine, resulting in hypocarnitinemia. Carnitine and acylcarnitines are important biomarkers in the diagnosis of carnitine deficiency, but the relationship between acylcarnitines and drug-induced hypocarnitinemia remains unclear. Quantification of acylcarnitines enables discovery of new biomarkers for prediction and diagnosis of drug-induced hypocarnitinemia. Here we describe a liquid chromatography/tandem mass-spectrometric method for simultaneously quantifying carnitine, 15 acylcarnitines, and cefditoren (the pivoxilated product of an antibiotic prodrug) in human urine. The matrix effect is corrected in 87.8–103% using deuterium-labeled internal standards ($^2H_9$-carnitine, $^2H_3$-hexanoylcarnitine, and $^2H_3$-stearoylcarnitine). The surrogate matrix method had an error of <13% in comparison with a standard addition method. Dynamic ranges were 0.1–100 μmol/l for acylcarnitines and 0.3–300 μg/ml for cefditoren. Both accuracy and precision were <19.7% at the lower limit of quantification and <14.8% for other quality controls. In an example application of this method, urine samples from eight healthy volunteers (five adults and three children) were analyzed, and individual differences were clearly observed.

1. Introduction

Antibiotics with a pivoxil (pivaloyloxymethyl) moiety, such as cefditoren pivoxil and cefcapene pivoxil, are prodrugs aimed at improvement of intestinal absorption efficiency. Following intestinal absorption, pivoxil-containing prodrugs are catabolized to yield active metabolites and pivalic acid, which is conjugated with carnitine (β-hydroxy-γ-trimethylazaniumylbutanoate) and excreted as pivaloylcarnitine into urine [1]. Excessive loss of carnitine induces hypocarnitinemia, whose symptoms include severe hypoglycemia and encephalopathy [2,3].

Carnitine plays an essential role in mitochondrial fatty acid β-oxidation by participating in intracellular transport of long-chain fatty acid into the mitochondrial matrix (Fig. 1). In addition, carnitine decreases the toxicity of accumulated acyl-CoA and fatty acid by generating acylcarnitines for excretion into urine [4]. Although the acylcarnitine profile of urine is significantly altered by carnitine deficiency [5], little is known about the acylcarnitine profile associated with drug-induced hypocarnitinemia. Techniques for quantifying and profiling acylcarnitines in urine would be helpful in this regard.

Because calibration standards are influenced by endogenous acylcarnitines in a blank matrix, the standard addition method (SAM) and surrogate matrix method (SMM) are often used for quantification of acylcarnitine. Several methods for quantification of acylcarnitines in human urine have been reported [6–9], but the matrix influences of endogenous analytes have not been quantitatively investigated. To quantify analytes accurately, the matrix effect should be evaluated quantitatively in a proper manner, and then minimized or corrected.

In this study, we developed an analytical method for quantifying urinary carnitine, acylcarnitines, and cefditoren (the active form of cefditoren pivoxil) using liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS). For precise quantification, the matrix effect and recovery were quantitatively monitored. The influences of endogenous compounds on calibration standards were eliminated using a surrogate matrix, and the matrix effect was corrected

* Corresponding author at: Department of Pharmaceutical Sciences, Tohoku University Hospital, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan.
E-mail address: mano@hosp.tohoku.ac.jp (N. Mano).
[1] Present address: Department of Clinical Pharmaceutics, Tohoku Medical and Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan.
[2] Present address: Division of Pharmacy, Tottori University Hospital, 36-1 Nishi-cho, Yonago, Tottori 683-8504, Japan.

http://dx.doi.org/10.1016/j.cca.2017.03.001
0009-8981/

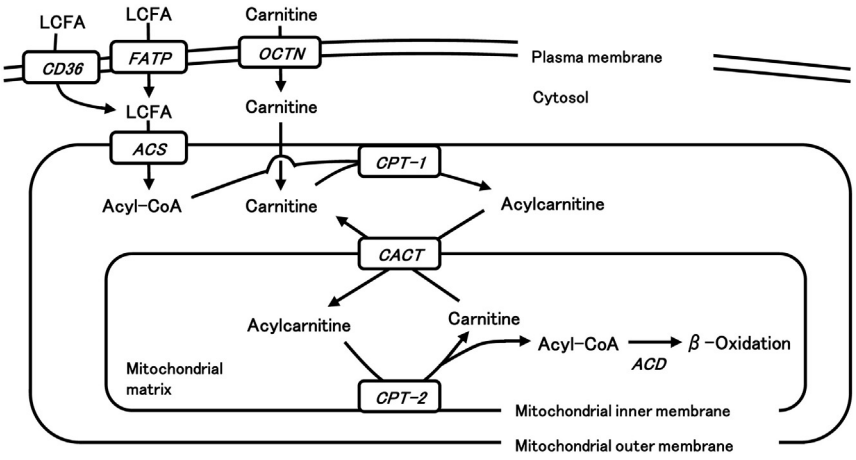

Fig. 1. The mechanism of the carnitine cycle ACD: acyl-CoA dehydrogenase; ACS: acyl-CoA synthase; CACT: carnitine acylcarnitine translocase; CD36: cluster of differentiation 36; CPT: carnitine palmitoyl transferase; FATP: fatty acid transport protein; OCTN: organic cation/carnitine transporter.

using deuterium-labeled internal standards (ISs). This method was successfully validated and used to analyze urine samples from healthy adults and children.

2. Materials and methods

2.1. Chemicals

L-Carnitine (C0) was purchased from Wako (Osaka, Japan), and acetyl-L-carnitine (C2) hydrochloride was purchased from Enzo Life Science (Farmingdale, NY, USA). Propionyl-L-carnitine (C3), isobutyryl-L-carnitine (C4i), butyryl-L-carnitine (C4), isovaleryl-L-carnitine (C5i), valeryl-L-carnitine (C5st), lauroyl-L-carnitine (C12) hydrochloride, and stearoyl-L-carnitine (C18) were purchased from Larodan (Stockholm, Sweden). 2-Methylbutyrylcarnitine (C5m) was purchased from Medical Isotopes (Pelham, NH, USA), and hexanoyl-DL-carnitine (C6), octanoyl-DL-carnitine (C8), decanoyl-DL-carnitine (C10), myristoyl-DL-carnitine (C14), and palmitoyl-DL-carnitine (C16) were purchased from Tocris Bioscience (Minneapolis, MN, USA). $^{2}H_{9}$-DL-Carnitine (d9-C0) hydrochloride was purchased from Toronto Research Chemicals (Toronto, Canada), and $^{2}H_{3}$-hexanoyl-L-carnitine (d3-C6) and $^{2}H_{3}$-stearoyl-L-carnitine (d3-C18) were purchased from CDN isotopes (Quebec, Canada). Cefditoren (CDTR) was purchased from TOKU-E (Tokyo, Japan). Pivaloylcarnitine (C5) was synthesized from pivaloyl hydrochloride and L-carnitine according to the method reported by Todesco et al. [10]. Chemical structures of target analytes are shown in Fig. 2.

HPLC-grade acetonitrile was obtained from Kanto Chemical (Tokyo, Japan), and heptafluorobutyric acid (HFBA) was obtained from GL Science (Tokyo, Japan). Methanol, acetone, diethylether, and trifluoroacetic acid (TFA) were obtained from Wako. Water was purified using a PURELAB Ultra Genetic system (Organo, Tokyo, Japan).

2.2. Instrument

A NANOSPACE SI-2 HPLC system was equipped with 3202 and 3001 pumps, a 3133 auto sampler, and a 3014 column oven (Shiseido, Tokyo, Japan). Analytes were separated with an Inertsil ODS-80A (2.1 mm i.d. × 150 mm, 5 μm, GL Science, Tokyo, Japan) coupled with a guard column (ODS-80A, 3.0 mm i.d. × 10 mm, 5 μm). The analytical column was kept at 40 °C, and flow rate was 0.2 ml/min: for analysis, a 1-μl aliquot of a mixture of carnitine, acylcarnitines, and CDTR was injected onto the column. The mobile phases consisted of elute A (0.1% HFBA in water) and elute B (0.1% HFBA in acetonitrile). The flow rate of methanol (as a post-column additive) was 0.2 ml/min. The gradient started at 5% B, and then proceeded as follows: 0–1 min, gradient to 13% B; 1–20 min, gradient to 15% B; 20–27 min, gradient to 19% B; 27–40 min, gradient to 90% B; 40–45 min, hold at 90% B; 45–45.1 min, gradient back to 5% B; 45.1–55 min, hold at 5% B to recondition the column.

Selected reaction monitoring (SRM) was performed on an AB SCIEX API 5000 triple quadrupole mass spectrometer (Framingham, MA, USA) equipped with an ESI interface under positive-ion detection mode. The interface heater was maintained at 300 °C, and ion spray voltage was 4500 V. The nebulizer gas (GS1), heater gas (GS2), curtain gas (CUR), and collision-activated dissociation gas (CAD) were set at 50 psi, 80 psi, 30 psi, and 6, respectively. The dwell time of each analyte was 70 ms. Optimization of the parameters, mass transitions, declustering potential (DP), collision energy (CE), and collision exit potential (CXP) were performed by flow injection analysis of a standard solution. Data was analyzed using the Analyst® software, version 1.4.1 (AB SCIEX).

2.3. Calibration curve

Standard stock solutions were prepared at concentrations of 4 mmol/l for C0, C2, C3, C4i, C5, C5i, C5st, C6, C8, C10, C12, and C14; 2 mmol/l for C4 and C18; 1 mmol/l for C5m; 500 μmol/l for C16; and 4 mg/ml for CDTR in water/acetonitrile 1:1 (v/v). Stock solutions of carnitine and acylcarnitines were diluted with water/acetonitrile 1:1 (v/v) to 0.1, 0.3, 1, 3, 10, 30, and 100 μmol/l (STD1), and the stock solution of CDTR was diluted with water/acetonitrile 1:1 (v/v) to 0.3, 1, 3, 10, 30, 100, and 300 μg/ml (STD2). IS stock solutions were prepared at concentrations of 500 μmol/l for d9-C0 and 1 mmol/l for d3-C6 and d3-C18, all in water/acetonitrile 1:1 (v/v). Before use, the stocks were mixed and diluted with water/acetonitrile 1:1 (v/v) to a final concentration of 10 μmol/l (ISTD1).

2.4. Sample preparation

Fifty microliters of human urine was diluted with 175 μl pure water in a micro tube, and then 50 μl ISTD1 and 225 μl acetonitrile were added. The sample was mixed by vortexing for 5 s, and then centrifuged at 15,000 × *g* at 4 °C for 5 min. The supernatant was transferred to a new micro tube, and then 1 μl was injected into the analytical system.

The concentration of creatinine in urine was determined using the CRE-L kit (Serotec, Sapporo, Japan).

2.5. Matrix effect and recovery

Matrix effect and recovery were evaluated in eight urine samples from different individuals (five adults and three children). For this purpose, four kinds of samples were prepared as described briefly below.