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Tissue specificity of pivaloylcarnitine and short-chain acylcarnitine profiles after administration of pivalate-containing antibiotics in rat

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ABSTRACT

Pivalate causes hypocarnitinemia that may underlie pivalate-induced encephalopathy in human. To investigate the tissue distribution of free and short acylcarnitine and tissue-specific effect of long-term pivalate-containing antibiotics administration in rat, we analyzed free carnitine (FC) and acylcarnitine by HPLC-MS/MS.

Male Wistar rats (N = 16) were divided into treated and control groups. Cefditoren pivoxil (850 mg/kg body weight/day) was given to the treatment group. On day 30, brain, liver, heart, kidney, muscle, serum, and urine were collected from all rats. Organs were homogenized and their supernatants as well as serum and urine were analyzed by HPLC-MS/MS.

Pivaloylcarnitine was detected at the highest levels in kidney of treated rats (104.2 ± 33.8 nmol/g). FC was significantly reduced in all tissues, while the reduction rate of FC was highest in brain (53%). Ratio of acetylcarnitine (AC) to FC (AC/FC) in brain of treated group rats was reduced significantly compared with that of control ($P < 0.01$).

Pivaloylcarnitine was detected mostly in kidney but pivaloylcarnitine itself might not affect renal function. Although our data showed significant reduction of AC/FC in the brain, the effect of hypocarnitinemia in the brain is unknown. However, this may be a clue for our

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Abbreviations used: FC, free carnitine; AC, acetylcarnitine; CoA, coenzyme A; CDTR-PI, cefditoren pivoxil; IS, internal standard; ESI, electrospray ionization; LV, left ventricular

understanding of the pivalate-associated neurological symptoms.

Keywords: free carnitine; pivaloylcarnitine; cefditoren pivoxil; rat; HPLC-MS/MS

1. INTRODUCTION

Pivalate is used to generate prodrugs with high oral bioavailability. Many antibiotics containing pivalate are used, for example cefditoren pivoxil, cefteram pivoxil, cefcapene pivoxil, tebipenem pivoxil, and adefovir pivoxil, contain pivalate. Upon absorption, pivalate-bound drug is broken down into active drug and a pivalate molecule. The pivalate molecule is then conjugated to coenzyme A (CoA) through the activity of acyl-CoA synthetase to form pivaloyl-CoA. By conjugation with carnitine, pivaloyl-CoA is converted to pivaloylcarnitine, which is excreted in the urine (1). Thus long-term administration of pivalate-containing drugs causes hypocarnitinemia, which may affect fatty acid oxidation (2, 3).

Carnitine is derived from dietary source for 75% of the requirement whereas the remainder is biosynthesized from lysine and methionine (4). Carnitine is a molecule with important functions such as fatty acid transport into mitochondria, detoxification of potentially toxic metabolites, regulation of mitochondrial acyl-CoA/CoA ratio, and stabilization of cell membranes. Long-chain fatty acids are metabolized for beta oxidation in the mitochondria. First, they are converted into long-chain acyl-CoA by acyl-CoA synthetase. However, they cannot pass across the inner mitochondrial membrane (5). Long-chain acylcarnitine can pass across the inner mitochondrial membrane by carnitine-acylcarnitine translocase, which exchanges mitochondrial free carnitine (FC) for cytoplasmic acylcarnitine, after long-chain acyl-CoA is converted to acylcarnitine by carnitine palmitoyl transferase Ⅰ. In the mitochondria, acylcarnitine is converted to acyl CoA by carnitine palmitoyl transferase II and undergoes beta oxidation to acetyl CoA. Hence, carnitine is very important for tissues that use fatty acid as a fuel source or require CoA for cellular reactions.

Our previous report revealed side effects of long-term administration of pivalate-containing drugs including carnitine-associated encephalopathy. In this case, the patient showed unconsciousness, seizure, and hypoglycemia, which former did not recover until initiation of carnitine administration even after normalization of blood glucose (6). We also previously investigated serum and urinary pivaloylcarnitine concentration after administration of pivalate-containing antibiotics (7). Very high levels of pivaloylcarnitine were detected in urine with low FC level over long term and we considered that pivalate, pivaloyl-CoA and/or pivaloylcarnitine might be present in the tissue after the pivalate-containing drugs were discontinued. Therefore, not only the effect of hypocarnitinemia caused by pivalate but also

that of pivaloylcarnitine or pivalate should be investigated as side effect of long-term pivalate administration.

Although tissue distribution of pivaloylcarnitine is important information to investigate the effect of pivaloylcarnitine in vivo, previous reports measured total short chain acylcarnitine levels for this purpose (8).

We measured pivaloylcarnitine itself, (FC, acetylcarnitine [AC], and propionyl carnitine) levels to investigate the tissue distribution and tissue-specific effect of long-term pivalate-containing antibiotics administration in rat.

2. MATERIALS AND METHOD

2.1 Animals.

Male Wistar rats weighing 120–146 g (N = 16) were supplied from Japan SLC, Inc. They were fed a standard laboratory animal chow, housed four in each cage and adapted for 7 days before the experiments. Rats were randomized into treatment and control groups. Control rats had free access to stock diet and water. Cefditoren pivoxil (CDTR-PI) 850 mg/kg body weight was given by stomach tube every day to treatment group. The quantity of CDTR-PI was set to contain almost the same pivalate as in the investigation of Diep et al (9). On day 30, all animals were anesthetized with pure sevoflurane. Blood was collected by cardiac puncture; the rats were decapitated and organs removed and frozen with liquid nitrogen and dry ice. The animals were handled with humane care according to the Guidelines of the Institutional Animal Care and Use Committee.

2.2 Materials

L-Carnitine hydrochloride was purchased from MP Biomedicals (Eschwege, Germany). AC, propionylcarnitine, and pivaloylcarnitine were prepared by reacting the corresponding acyl chloride with L-carnitine hydrochloride in trifluoroacetic acid. FC-d3, AC-d3, propionylcarnitine-d3, isobutyrylcarnitine-d3, isovalelylcarnitine-d3, 3-hydroxyisovalelyl carnitine-d3, and pivaloylcarnitine-d3 were synthesized in the Laboratory of Hospital Pharmacy, Graduate School of Pharmaceutical Sciences, Nagoya City University.

2.3 Sample preparation for carnitine in tissue

Approximately 50 mg of tissues (brain, heart, liver, kidney, and muscle) were placed in 1.5 ml micro centrifuge tubes, and the exact mass of the samples was determined. Methanol 300 μl and internal standard (IS) 300 μl were added to the samples and homogenized. IS included FC-d3 (100 μmol/L), AC-d3 (100 μmol/L), propionylcarnitine-d3 (100 μmol/L), isobutyrylcarnitine-d3 (100 μmol/L), isovalelylcarnitine-d3 (100 μmol/L), 3-

hydroxyisovalelylcarnitine-d3 (100 μmol/L), and pivaloylcarnitine-d3 (100 μM). After centrifugation at 15, 000 rpm and 4℃ for 15 min, supernatants were frozen at −80℃ until analysis.

2. 4 Sample preparation for creatinine in tissue

Approximately 50 mg of kidney was placed in 1. 5 ml Eppendorf vials and the exact mass of the sample determined. Water 300 μl was added to the sample and homogenized. The supernatants were frozen and at −80℃ until analysis.

2. 5 Analysis

FC and acylcarnitines were extracted from each organ, serum, and urine using an Oasis MCX 30 mg/ml SPE cartridge (Waters, Milford, MA, USA). The cartridge was conditioned using 1 mL methanol and 1 mL water. Samples of serum or urine (100 μL) and IS solution (100 μL) were loaded, washed with 1 mL 0. 1% phosphoric acid then 1 mL acetonitrile, and eluted with 2 mL of 100 mmol/L pyridine in water/acetonitrile (1 : 1). The eluent was evaporated under nitrogen at 60℃ and the residue dissolved in 100 μL water/acetonitrile (95 : 5). FC and acylcarnitines were measured by HPLC/MS/MS method as reported by Maeda et al (10). A binary pump HPLC system (Waters) consisting of a gradient pump, vacuum degasser, and auto-sampler was used. Quattro Premier XE (Waters) and Scherzo SM-18 column (2. 0 × 150 mm; Imtakt, Kyoto, Japan) were used. A 10-μL mixture of FC and acylcarnitines was injected onto the column and eluted at a flow rate of 0. 2 mL/min using a step gradient alternating between acetonitrile (A) and 0. 08% aqueous ion-pairing reagent (IPCC-MS3; GL Sciences, Tokyo, Japan) (B). The gradient began with 5% A then was programmed as follows: 0−1min, gradient to 15% A; 1−6 min, hold at 15% A; 6−7 min, gradient to 20% A; 7−10 min, hold at 20% A; 10−11 min, gradient to 25% A; 11−14 min, hold at 25% A; 14−15 min, gradient to 30% A; 15−18 min, hold at 30% A; 18−19 min, gradient to 35% A; 19−22min, hold at 35% A; 22−23 min, gradient to 40% A; 23−25 min, hold at 40% A; 25−25. 1 min, gradient back to 5 %A; 25. 1−33 min, hold at 5% A to re-equilibrate column. Quattro Premier XE triple quadrupole mass spectrometer (Waters) equipped with an electrospray ionization (ESI) source was used for MS/MS analysis. Nitrogen was used as nebulizing gas and argon as collision gas at a pressure of 0. 15 Pa. The source temperature was 120℃; capillary voltage used was 3. 2 kV. FC and acylcarnitines were analyzed in positive ion MRM mode.

Creatinine in kidney, serum, and urine was analyzed by TBA-40FR (Toshiba, Tochigi, Japan).

2. 6 Statistical analysis

Results are reported as means ± SD. We compared two groups by Mann-Whitney *U*-test; differences with $P<0.05$ were considered statistically significant.

3. RESULTS

3. 1 Free carnitine in various tissues

Figure 1 shows the detection values of FC. In the CDTR-PI-treated group, FC content was high in heart (53. 5 ± 31. 0 nmol/g) and skeletal muscle (52. 1 ± 16. 1 nmol/g). In the control group FC was highest in heart (202. 1 ± 34. 2 nmol/g). In brain, FC level was low in treated and control groups (4. 8 ± 1. 1 and 9. 1 ± 1. 5 nmol/g, respectively). In the treated group the highest content of FC was detected in heart followed by skeletal muscle then kidney; this order of FC level was the same as that of control group. FC level of treated group organs was significantly lower than that of control group organs ($P<0.01$). Reduction rate of FC content in tissue of CDTR-PI administration group versus control was high in brain (53%) and lowest in heart (26%) (Table 1).

3. 2 Acetylcarnitine in various tissues

Figure 2 shows detection values of AC. AC in all samples of treated rat was significantly

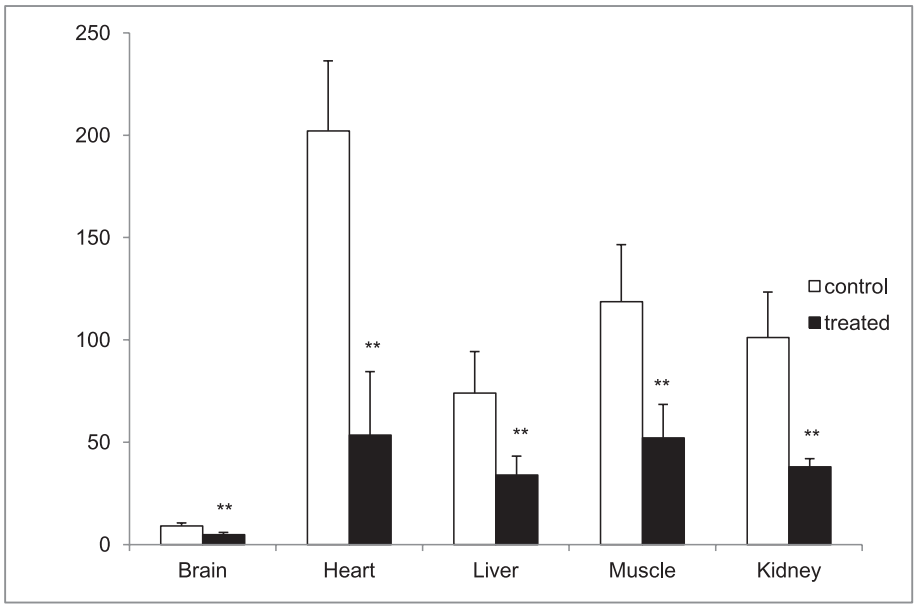

Fig. 1 Free carnitine (FC) content in various tissues in rats. Values are mean ± SD for eight rats. FC levels of treated group organs were significantly lower than those of control ($P<0.01$). Statistical significance represented as different from control: $^{**}P<0.01$.

Table 1. Free, acetyl, and pivaloyl carnitine content in rat organs (control group N = 8; treated group N = 8)

		Freecarnitine (FC)	Acetylcarnitine (AC)	Propionylcarnitine	Pivaloylcarnitine (PC)	AC/FC
Brain	Control	9.07 ± 1.46	2.15 ± 0.47	ND	ND	0.25 ± 0.07
	Treated	4.83 ± 1.09 **	0.63 ± 0.13 **	ND	1.65 ± 0.20	0.13 ± 0.03 **
Heart	Control	202.14 ± 34.21	104.24 ± 33.71	1.96 ± 0.63	ND	0.55 ± 0.28
	Treated	53.48 ± 31.01 **	26.77 ± 14.99 **	0.65 ± 0.29 **	26.84 ± 16.80	0.50 ± 0.16
Liver	Control	73.96 ± 20.33	15.06 ± 6.66	1.18 ± 0.51	ND	0.20 ± 0.07
	Treated	33.99 ± 9.22 **	4.48 ± 2.33 **	0.16 ± 0.11 **	9.58 ± 1.64	0.13 ± 0.05
Kidney	Control	101.19 ± 22.20	13.86 ± 3.89	ND	ND	0.14 ± 0.03
	Treated	38.00 ± 3.95 **	3.07 ± 0.55 **	ND	77.57 ± 13.72	0.08 ± 0.02 *
Muscle	Control	118.71 ± 27.83	116.66 ± 32.80	4.62 ± 1.51	ND	0.98 ± 0.16
	Treated	52.11 ± 16.37 **	53.64 ± 18.47 **	1.67 ± 0.61 **	1.62 ± 0.47	1.03 ± 0.16

Free, acetyl, and pivaloyl carnitine content (mean ± SD, nmol/g).
**$P<0.01$compared with and control.
*$P<0.05$compared with control.
ND, not detected

reduced compared with control ($P<0.01$). In control animals, skeletal muscle and heart contained high levels of AC (116.7 ± 32.8 and 104.2 ± 33.8 nmol/g, respectively). In the treated group, AC content in skeletal muscle was high (53.6 ± 18.5 nmol/g). In the treated group the highest content of AC was detected in skeletal muscle followed by heart then liver; this order of AC level was the same as that of control group. AC content of each organ of treated group was significantly lower than that of control group ($P<0.01$) (Figure 2). Reduction rate of AC content in tissue of CDTR-PI administration group versus control was high in muscle (46%) and lowest in kidney (22%) (Table 1).

3.3 Propionylcarnitine

Figure 3 shows propionylcarnitine content in rat tissues. In the control group skeletal

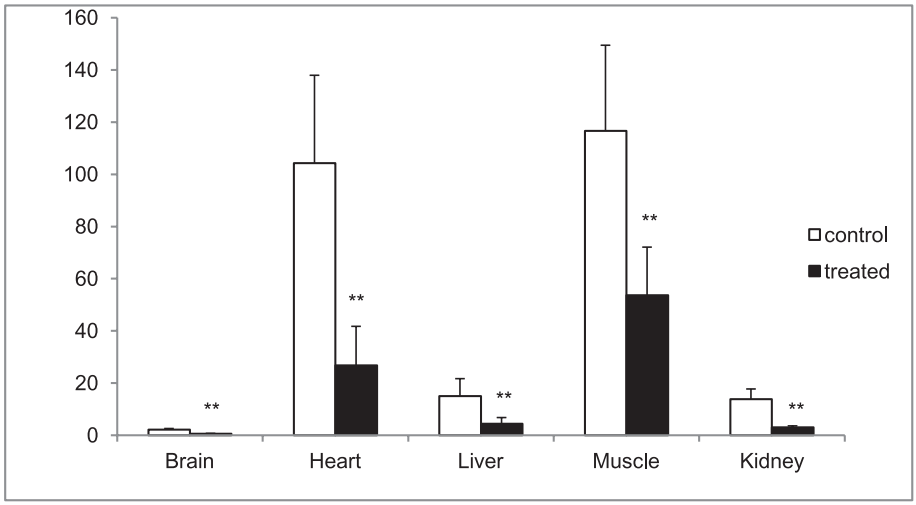

Fig. 2 Acetylcarnitine (AC) content in various tissues in rats. Values are ± SD for eight rats. AC levels of treated group organs were significantly lower than those of control ($P<0.01$). Statistical significance represented as different from control: $^{**}P<0.01$.

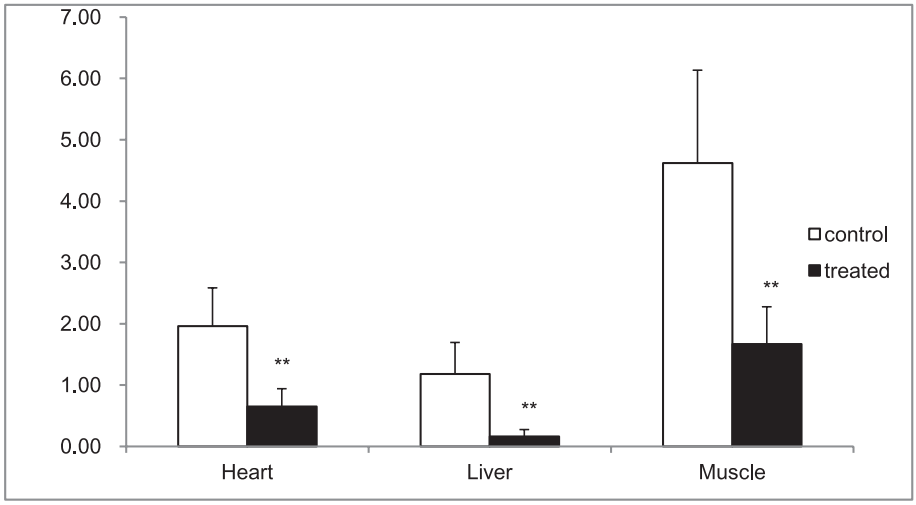

Fig. 3 Propionylcarnitine content in various tissues in rats. Values are ± SD for eight rats. Propionylcarnitine in brain and kidney was not detected in both groups. Propionylcarnitine levels of treated group organs were significantly lower than those of control ($P<0.01$). Statistical significance represented as different from control: $^{**}P<0.01$.

muscle contained the highest propionylcarnitine content (4.6 ± 1.5 nmol/g). In the treated group, propionylcarnitine content in skeletal muscle was decreased to 1.6 ± 0.6 nmol/g. In both groups, propionylcarnitine were not be detected in brain and kidney. Propionylcarnitine content of heart, liver, and muscle of treated group was significantly lower than control ($P<0.01$) (Table 1).

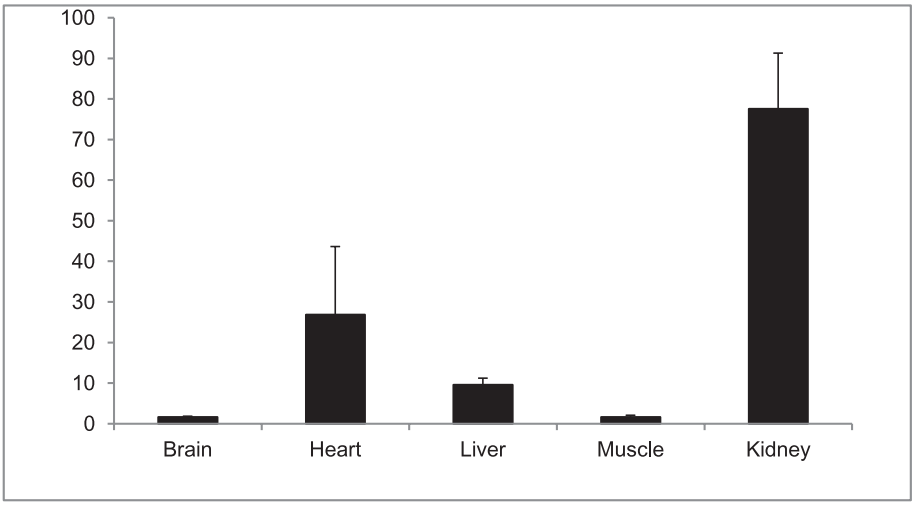

Fig. 4 Pivaloylcarnitine content in various tissues in rats of treated group. Values are ± SD for eight rats. Pivaloylcarnitine in various tissues of control rats was not detected.

3. 4 Pivaloylcarnitine in various tissues

In the control group pivaloylcarnitine was not detected in all tissues. In treated rats pivaloylcarnitine content was highest in kidney (104. 2 ± 33. 8 nmol/g) followed by heart then liver (26. 8 ± 16. 8 and 9. 6 ± 33. 8 nmol/g, respectively) (Figure 4). Pivaloylcarnitine in the kidney of treated rats was significantly higher than other organs ($P<0.01$) (Table 1).

3. 5 Ratio of acetylcarnitine to free carnitine

Ratio of AC to FC (AC/FC) was high in heart (control group, 0. 55 ± 0. 28; treated group, 0. 50 ± 0. 16) and skeletal muscle (control group, 0. 98 ± 0. 16; treated group, 1. 03 ± 0. 16). AC/FC in treated rat kidney and brain was significantly higher than control (kidney: treated group, 0. 08 ± 0. 02; control group, 0. 14 ± 0. 03; $P<0.05$; brain: treated group, 0. 13 ± 0. 03; control group, 0. 25 ± 0. 07; $P<0.01$) (Figure 5, Table 1).

3. 6 Free and acylcarnitine concentration

Table 2 shows the concentration of FC, AC, propionylcarnitine, and pivaloylcarnitine in serum and urine. Pivaloylcarnitine was not detected in serum and urine of control group. Serum FC concentration of treated rats was significantly reduced compared with that of controls ($P<0.01$). On the other hand, FC concentration in urine did not show any significant difference. AC concentration of treated rat serum was significantly lower than control ($P<0.01$), whereas there was no difference of either concentration in urine between the two groups.

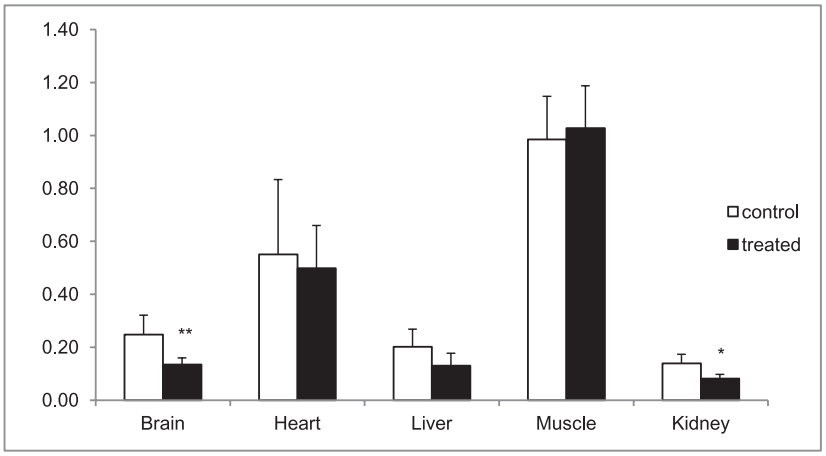

Fig. 5 Acetyl carnitine/free carnitine in various tissues in rats. Values are ± SD for eight rats. AC/FC in treated rat kidney and brain was significantly lower than control (kidney, $P<0.05$; brain, $P<0.01$).

Table 2. Serum and urine concentration of free, acetyl and pivaloyl

	Serum (μM)				**Urine (μmol/g creatinine)**			
	Free carnitine	Acetyl carnitine	Propionyl carnitine	Pivaloyl carnitine	Free carnitine	Acetyl carnitine	Propionyl carnitine	Pivaloyl carnitine
Treated group (n=8)	50.0±4.0[a]	4.7±0.7[a]	N.D[a]	3.8±0.9[a]	21.5±6.8	3.0±1.6	N.D	2250±1090
Control group (n=8)	97.0±18.8	18.3±4.1	0.08±0.03	N.D	15.5±1.5	5.0±0.4	N.D	N.D

mean ± SD, serum μM, urine μmol/g creatinine
[a]$P<0.01$ compared with the controls

3.7 Free and acylcarnitines corrected by creatinine

Table 3 shows FC and AC concentrations corrected by creatinine concentration in kidney and urine. In both groups FC and AC in kidney was significantly higher than in urine ($P<0.01$). In treated group pivaloylcarnitine concentration in kidney was significantly higher than in urine ($P<0.01$).

Table 3. Kidney, urine and serum concentration of free, acetyl and pivaloyl carnitine and fractional excretion of free and acylcarnitine.

	Control group (n=8)			Treated group (n=8)		
	Free carnitine	Acetylcarnitine	Pivaloylcarnitine	Free carnitine	Acetylcarnitine	Pivaloylcarnitine
Kidney (μmol/g creatinine)	8650±3510**	1150±480**	ND	3310±1020**	282±112**	6230±2070**
Urine (μmol/g creatinine)	15.5±1.5	5.0±0.4	ND	21.5±6.8	3.0±1.6	2250±1090
Serum (μM)	97.0±18.8	18.3±4.1	ND	50.0±4.0	4.7±0.7	3.8±0.9
Fractional excretion (%)	8.65±1.02	9.04±0.74	ND	27.6±2.85	73.0±11.1	38600±10100

Mean ± SD, μmol/g creatinine.
**$P<0.01$compared with urine.
ND, not detected.
Fractional excretion = carnitine concentration of urine · creatinine concentration of serum/ carnitine concentration of serum · creatinine concentration of urine.

4. DISUCUSSION

Diep et al. (9) reported reduction of FC in rats after long-term administration of pivalate-containing antibiotics. Furthermore, high levels of pivaloylcarnitine were observed in rat kidney and heart after bolus administration (8). They detected pivaloylcarnitine by radioactive HPLC method after injection of L-[^{3}H] butyrobetaine, a precursor of carnitine. However, using this method quantitative performance is insufficient because a certain amount of endogenous carnitine might exist. The focus of our research was to investigate the effect on various organs of long-term administration of oral pivalate-containing antibiotic by analyzing FC, AC, and pivaloylcarnitine concentration by LC-MS/MS. Although the mechanism for species difference on the pivalate metabolism was reported (11), these result might be helpful for investigation of the effect of the pivalate containing drugs for the human. In our study the highest pivaloylcarnitine content was observed in kidney. FC, AC, and pivaloylcarnitine levels corrected by creatinine in kidney were markedly higher than in urine.

Ohnishi et al (12) reported that pivaloylcarnitine inhibited FC reabsorption in the perfused rat kidney. However, because FC and AC concentration corrected by creatinine in kidney was significantly higher than in urine, pivaloylcarnitine might not affect FC and AC concentration and renal function.

In heart tissue we detected very high levels of pivaloylcarnitine compared with in skeletal muscle and even in liver. Low content of FC and acylcarnitine in heart was previously reported (13) as well as left ventricular (LV) dysfunction associated with hypocarnitinemia induced by pivalate (14). In human, transient reduction of LV mass after 7–8-week administration of pivampicillinam was reported (15). Because the energy source in heart is mainly by beta-oxidation, the influence of pivaloylcarnitine might be easily detected there. It was reported that pivalate reduced carnitine level but did not cause severe metabolic change in rat liver (16). In our study pivaloylcarnitine content was not high in liver and heart.

Reduction of AC was observed in all organs. FC plays a primary role in regulation of acyl-CoA/CoA and acetyl-CoA/CoA ratios by converting to acylcarnitine or AC in tissues. Therefore we compared AC/FC ratio in each tissue between control and treated groups; AC/FC was significantly reduced in kidney and brain. In these two organs, reduction of AC was more remarkable than that of FC. As previously mentioned, a certain amount of pivaloylcarnitine was produced in the kidney; however, because kidney is one of the main organs of carnitine production the reduction of FC level might be mild.

Although FC is little synthesized in brain (17) both it and acylcarnitine can cross the blood-brain barrier (18, 19). In brain AC has some roles such as energy metabolism, membranes protection from excitotoxicity, and antioxidant and antiapoptotic function (5). We observed a case of loss of consciousness and encephalopathy induced by hypocarnitinemia after long-term administration of pivalate-conjugated antibiotics. Symptoms included hypoglycemia and disturbance of consciousness, which were not improved by administration of glucose but of carnitine (6). Hypocarnitinemia associated with pivalate were reported in Japan, including following＜2-week administration of pivalate-containing antibiotics (20).

In the present study only a small amount of pivaloylcarnitine was detected in brain but AC reduction was significant compared with in other organs. Therefore when giving pivalate-conjugating antibiotics especially to children, it is important to pay attention to their consciousness and to investigate whether they previously received other pivalate-conjugating drugs.

There is no report of heart failure induced by administration of pivalate-containing drugs, even in the loss-of-consciousness and encephalopathy cases. Broderick et al (14) assessed LV dysfunction in rats after taking sodium pivalate. Checking LV function may be considered in patients with hypocarnitinemia induced by pivalate-containing antibiotics, even

if they have no symptoms of heart failure.

Our study showed the pivalate-containing drugs caused accumulation of pivaloylcarnitine itself and decrease of the FC and AC in the each organ, and decrease of AC/FC ratio in the brain. These results cannot show the effect of the pivalate to the organs directly because toxicity of pivaloylcarnitine is not reported. To investigate the effect of pivalate to the each organ, CoA and acyl CoA should be investigated and it might clear the effect of pivalate. But the some cases of carnitine associated encephalopathy or unconsciousness with hypocarnitinemia caused by pivalate are reported in Japan. The present study suggests that the brain is one of the most affected organs by pivalate-containing antibiotics.

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