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PIVAMPICILLIN-PROMOTED EXCRETION OF PIVALOYLCARNITINE IN HUMANS

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Abstract—Pivampicillin treatment of seven children (five boys and two girls) for 7 days significantly reduced the amounts of total acid-soluble carnitine, free carnitine, and long-chain acylcarnitines and increased the amounts of acid-soluble acylcarnitine in plasma. The fasting plasma levels of 3-hydroxybutyrate at the end of treatment were 15% of the control value. The levels of free fatty acids were decreased, whereas triglyceride levels were unaffected, indicating impaired fat metabolism. Daily urinary excretion of total carnitine was four to five times higher than controls after the first day of treatment, although the amounts of free carnitine and acetylcarnitine were decreased. The urinary acylcarnitines were isolated and characterized by gas chromatography/electron impact mass spectrometry and fast-atom bombardment mass spectrometry. Pivaloylcarnitine was the predominant urinary acylcarnitine; it represented > 96% of the increased excretion of total carnitine and 75–80% of the total conjugated pivalic acid. The renal clearance of acylcarnitines was comparable to that of creatinine, indicating no reabsorption of pivaloylcarnitine. These data suggest a detoxification function of carnitine for pivalic acid in humans.

Although carnitine has a role in mitochondrial β-oxidation of long-chain fatty acids [1], several recent studies indicate that carnitine also has a role in the elimination of endogenous and xenobiotic carboxylic acids in human (see Refs. 2–5 for a review of the conjugation of xenobiotic carboxylic acids). Several examples of the conjugation of acyl moieties have been reported. They include: (1) excessive urinary excretion of specific acylcarnitines in some organic acidemias affecting the utilization of acyl-CoAs [6–10], (2) the presence of carnitine conjugates of branched-chain saturated and unsaturated medium-chain fatty acids in human urine [11], (3) the excretion of valproylcarnitine due to valproic acid therapy [12], (4) the presence of labeled pivaloylcarnitine in human urine following a single oral dose of the [1-^{14}C]pivaloyloxyethyl ester of methyldopa [13], and (5) the occurrence of *O*-(cyclopropylcarboxyl)-carnitine in tissues [14–17] following administration of hexadecyl cyclopropanecarboxylate.

Secondary carnitine deficiency can develop due to sustained high urinary excretion of specific acylcarnitines as occurs with certain organic acidemias [6–10]. It seems likely that xenobiotic acids, introduced in the form of drugs or prodrugs, might promote carnitine deficiency, if the acids were to undergo activation to the acyl-CoA derivatives, which are substrates for one or more of the carnitine acyltransferases. Many prodrugs are acyloxy derivatives of the therapeutically active drugs, in which the acyl moiety is one of the short-, straight- or branched-chain fatty acids. Following absorption, they undergo rapid enzymatic hydrolysis to produce the parent drug and free fatty acids [18–20]. For some, acyl activation and conjugation to carnitine occurs [21, 22]. Long-term therapy with such prodrugs could induce a secondary carnitine deficiency. The present study was undertaken to determine the effect of the pivalic acid (trimethylacetic acid) form of ampicillin (pivaloyloxymethyl-ampicillin, pivampicillin) on the carnitine status of humans.

MATERIALS AND METHODS

Patients. Five males and two females, with an age range of 8 to 15 years and a body weight range of 29 to 50 kg, were used. Both patients and parents were informed about the purpose of the study, and a written consent was obtained for each patient.

Although antibiotic treatment was justified for all patients, the clinical and laboratory data indicated no evidence of systemic infection. Five patients had pharyingitis with local colonization of β-hemolytic streptococci (3) and *Staphylococcus aureus* (2), while one had paronychia and one suffered from acute lymphoid leukemia. Even though the latter patient's disease had been in remission for the last 2 years, antibiotic treatment was necessary because of infection in the family.

Sample collection. Blood was taken after an overnight fast the day before treatment and 1 day after the treatment ended. Twenty-four-hour urine samples were collected throughout the study period, starting 1 day before treatment (control samples). The daily dose of pivampicillin was 2.0 g taken orally in four equal amounts (4 × 500 mg).

Plasma and urine samples were stored at −20° until analysis.

Assays: Carnitine/Acylcarnitines. Urine was extracted with chloroform/methanol [23], and free carnitine was quantitated by a radiochemical assay

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[24] modified as described [25]. Acylcarnitines were calculated as the difference between total carnitine (obtained after alkaline hydrolysis) and free carnitine. Acetylcarnitine was quantitated using a coupled enzyme assay [26] following column chromatography of a urine aliquot on Dowex 2 × 8 column, (3 × 0.6 cm). Acylcarnitines other than acetyl were quantitated gas chromatographically [23] by pooling urine samples taken before and 24 hr after pivampicillin treatment. Positive identification of short-chain fatty acids derived from the acylcarnitines was made by using gas chromatography/mass spectrometry.*

Partial purification of pivaloylcarnitine from urine for fast-atom bombardment mass spectrometry* was achieved essentially as described [11], except that radioisotopic labeling was omitted and column chromatography on silicic acid was replaced by silica gel thin-layer chromatography [27]. Attempts to exchange radioactive carnitine into the unknown acylcarnitine fraction using commercial carnitine acetyltransferase as described previously [27] were not successful. Co-chromatography with authentic pivaloycarnitine was used to locate the ester on thin-layer plates.

Plasma free, acid-soluble acylcarnitine and long-chain acylcarnitine fractions were determined as above after perchloric acid extraction and alkaline hydrolysis [26].

Free fatty acids, triglycerides, glucose and ammonia/urea were quantitated using commercially available assay kits. Plasma 3-hydroxybutyrate was determined as referenced [28].

Urinary and plasma creatinine and inorganic phosphate levels were determined using the alkaline picrate method [29, 30] and the method of Morin and Prox [31] respectively.

Plasma amino acid analysis were done in the Central Laboratorium of Childrens Hospital, Budapest, Hungary.

Chemicals. Enzymes, coenzymes and other substrates were purchased from Boehringer and the Sigma Chemical Co. [1-^{14}C]Acetyl-CoA, specific radioactivity 57.7 mCi/mmol, was obtained from the Amersham Corp. L-Carnitine was a gift from Sigma-Tau, Rome. Pivaloyl-L-carnitine and valeryl-L-carnitine were synthetized from the respective acyl-chlorides as described [32]. Pivampicillin (Pondocillin) was a product of Leo Pharmaceutical Products, Ballerup, Denmark, and supplied by Dr. W. O. Godtfredsen.

Statistical analysis. Statistical significance of the data was evaluated by Student's *t*-test.

RESULTS

Serum levels, urinary excretion and renal clearance of carnitine and acylcarnitines. The average plasma levels of total acid-soluble, short-chain acyl, free and long-chain acylcarnitine before and after pivampicillin treatment of the seven patients are shown in Fig. 1. A daily dose of 2.0 g pivampicillin, administered for 7 days, markedly reduced the levels of all but short-chain acylcarnitines. Free carnitine was decreased an average of 75% and long-chain acylcarnitine levels were reduced by about 50%, while short-chain acylcarnitines were elevated 53% at the end of treatment.

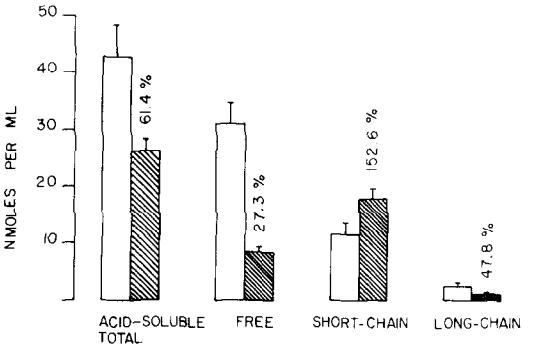

Fig. 1. Plasma levels of total acid-soluble, free, acid-soluble acyl (short-chain) and long-chain acylcarnitines before and 7 days after pivampicillin treatment. Key: after treatment (hatched bars); before treatment (open bars). The data represent means ± SE; N = 7. The numbers above the hatched bars represent the percentage of control values.

The daily urinary excretion (μmol/24 hr) of total, free, acetylcarnitine and other acylcarnitines is shown in Fig. 2. After 1 day of pivampicillin administration, the excretion of total carnitine was four to five times that of controls, which was not increased significantly by further drug treatment. Excretion of both free and acetylcarnitine decreased to less than 8% of the control values by day 3. Consequently, the approximate 5-fold increase in total carnitine excretion was due to enhanced increased excretion of acylcarnitine(s) that was not acetylcarnitine (see Fig. 2).

To determine which specific acylcarnitine(s) contributed to the elevated acylcarnitine excretion, gas chromatographic analyses were performed on the fatty acids derived from the acylcarnitine fraction of pooled urine samples collected 24 hr before and after the treatment started. The gas chromatographic

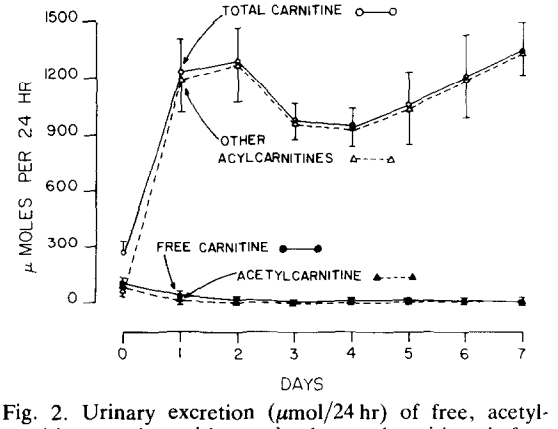

Fig. 2. Urinary excretion (μmol/24 hr) of free, acetylcarnitine, total carnitine and other acylcarnitines before and during the treatment period. The values are the means ± SE; N = 7.

* The authors gratefully acknowledge the use of the mass spectrometry facilities at Michigan State University under the supervision of Dr. J. T. Watson.

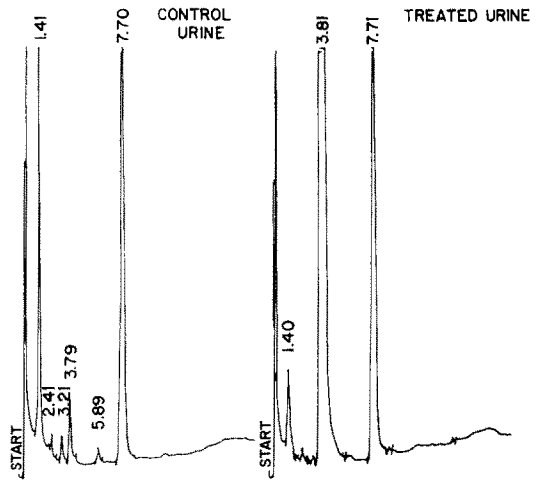

Fig. 3. Gas chromatography of the volatile fatty acids obtained from urinary acylcarnitines. Left panel: before treatment; right panel: 24 hr after treatment. Pooled urine samples from the seven patients were used for the analyses. Retention times, in minutes, represent the internal standard from valeryl-L-carnitine = 7.71, acetic acid = 1.41, propionic acid = 2.41, isobutyric acid = 3.21, butyric acid = 3.79, pivalic acid = 3.81, and isovaleric acid = 5.89.

tracing in Fig. 3 shows that pivampicillin administration caused the appearance of a new fatty acid with retention time of 3.81 min. It represented more than 96% of the total acylcarnitines. The fatty acid had properties identical to pivalic acid (trimethylacetate), using the criteria of combined gas chromatography and electron impact mass spectrometry (Fig. 4). The presence of pivaloylcarnitine in urine was confirmed by fast-atom bombardment mass spectrometry of a partially purified acylcarnitine from urine; it had a spectra-like authentic pivaloylcarnitine (see Fig. 5).

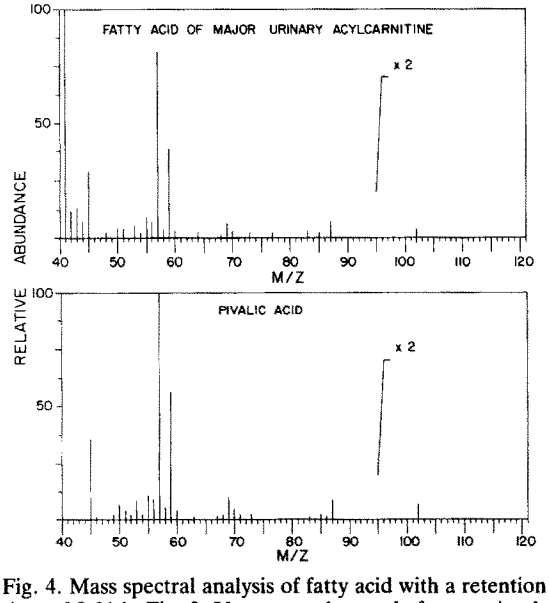

Fig. 4. Mass spectral analysis of fatty acid with a retention time of 3.81 in Fig. 3. Upper panel: sample from patient's urine; lower panel: standard pivalic (trimethylacetic) acid.

Since pivampicillin treatment caused a several-fold increase in acylcarnitine excretion, renal handling of carnitine and acylcarnitines was determined by calculating renal clearance rates for free and acylcarnitines (Table 1). Renal clearance for free carnitine was reduced due to treatment, whereas that for acylcarnitines was increased. The latter was equal to that of creatinine. Values of tubular phosphorous reabsorption were unaltered, indicating unimpaired renal function.

Since low plasma levels of free carnitine could indicate a partial carnitine deficiency and, possibly, impaired lipid metabolism, plasma levels of 3-hydroxybutyrate, an indicator of hepatic mitochondrial fatty acid oxidation, free fatty acids, triglycerides and glucose were determined (Table 2). After 7 days of treatment, fasting levels of 3-hydroxybutyrate and free fatty acids were decreased by 85 and 36%, respectively, whereas triglyceride levels were unaffected. Plasma glucose was elevated slightly at the end of the treatment. Plasma levels of urea (Table 2), as well as ammonia and amino acids, and urinary excretion of urea and ammonia were determined to assess whether the nitrogen metabolism was also affected. Except for decreased levels of plasma urea, asparagine, α-aminobutyric acid and lysine, the levels of the other above-mentioned metabolites were unchanged (data not shown).

Search for non-carnitine pivaloyl conjugate. Although considerable quantities of pivaloyl-L-carnitine were found in the urine, it represented only 35% of the total pivampicillin administered. Therefore, the presence of other conjugates of pivalic acid in urine merited consideration. Samples of pooled urine were passed over Dowex 50 H^+ columns and washed with 8–10 column volumes of 20% ethanol.

The acidic effluent did not contain free carnitine or acylcarnitines (determined by analyses for total carnitine). This effluent should contain neutral, as well as negatively charged, water-soluble material. Carnitine and carnitine esters were then eluted with 6–8 column volumes of 1.0 N NH_4OH in 20% ethanol. Aliquots of the acidic (initial column wash) and the NH_4OH–20% ethanol effluent were hydrolyzed by three different treatments. Samples of each were: (1) made to 0.5 N KOH and let stand for 14 hr at room temperature = mild alkaline hydrolysis; (2) made to 5 N KOH and hydrolyzed for 14 hr at 100° = strong alkaline hydrolysis; and (3) made to 5 N HCl and hydrolyzed for 14 hr at 100° = strong acid hydrolysis. Mild alkaline hydrolysis of the initial wash of the column contained 16% of the total conjugated pivalic acid. Both the strong acid and strong base hydrolysates of the initial column wash contained 23% of the total conjugated pivalic acid, although this fraction did not contain any carnitine or acylcarnitines. Thus, a maximum of 23% of the total conjugated pivalic acid was not conjugated to carnitine. Presumably, the 16% found in the initial wash represents pivaloylglucuronide, and the difference between the 23% found by strong acid and base hydrolysis represents pivalic acid conjugated to amino acids. These latter identifications must be considered as tentative because the materials were not rigorously characterized.

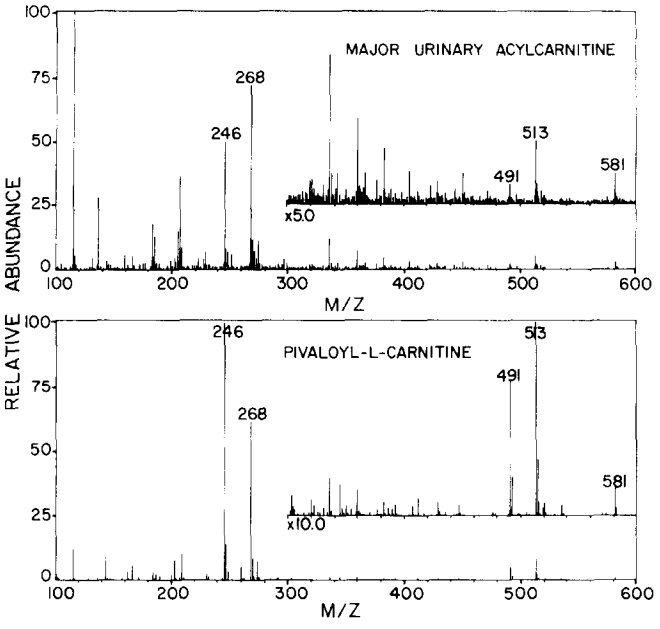

Fig. 5. FAB analysis of the partially purified acylcarnitine(s) from urine (upper panel) and authentic pivaloyl-L-carnitine (lower panel). Values for m/z: 246 represents protonated pivaloylcarnitine; 268 represents the sodium adduct of pivaloylcarnitine. The ions at m/z 491 and 513 represent the protonated dimer and the sodium adduct of the dimer of pivaloylcarnitine respectively.

DISCUSSION

Following treatment with pivampicillin, the daily total urinary carnitine excretion increased approximately 5-fold, with more than 96% of the increase due to pivaloylcarnitine. The sustained high excretion of this carnitine conjugate is consistent with a role for carnitine in detoxification, as suggested previously [33]. The elimination of pivalate as a carnitine conjugate found in this study and also found by Vickers *et al.* [20] is similar to the role for carnitine in detoxification of cyclopropanecarboxylic acid [14–17]. Presumably, pivalate is converted to its CoA ester *in vivo*, which, because of its structure (trimethylacetic acid), should not be further metabolized except for transfer of the acyl group to acyl acceptors such as carnitine. High intracellular levels of pivaloyl-CoA could be produced, thereby reducing both free CoASH and other CoA esters. The transfer of the pivaloyl moiety to carnitine, catalyzed by one (or more) of the carnitine acyltransferase(s), should relieve the acyl pressure and produce free CoASH. This intracellular acyl-CoA buffering function of carnitine [23] is presumably accompanied by increased outward transport of the carnitine esters from the organ(s) with subsequent urinary elimination. The elevated acylcarnitine/carnitine ratio in plasma (0.37 vs 2.1 before and 7 days after treatment) and the increased renal acylcarnitine clearance (12.67 vs 56.98 ml × min^{-1}) are consistent with the proposed detoxification mechanism. More than 96% of the urinary acylcarnitine is pivaloylcarnitine,

Table 1. Renal clearance of free and acylcarnitine and tubular phosphorous reabsorption before and 6 days after pivampicillin treatment

	Clearance*			Tubular phosphorous reabsorption† (%)
	Creatinine (ml/min)	Free carnitine (ml/min)	Acylcarnitine (ml/min)	
Before treatment	60.07 ± 5.16	2.07 ± 0.55	12.67 ± 4.38	99.84 ± 0.04
After treatment	61.92 ± 6.35	0.39 ± 0.11	56.98 ± 7.28	99.83 ± 0.95
Significance	NS‡	$P < 0.005$	$P < 0.005$	NS

Values represent means ± SE; N = 6.

* Clearance = $U \times V/P$, where U and P indicate urine and plasma concentrations and V is urine flow (ml/min).

† Tubular phosphorous reabsorption: $\left(1 - \frac{UP \times SCR}{SP \times UCR}\right) \times 100$ where UP and SP represent urine and plasma phosphorous concentrations, and UCR and SCR represent urine and plasma creatinine concentrations.

‡ Not significant.

Table 2. Plasma levels of glucose, 3-hydroxybutyric acid, free fatty acids, triglycerides and urea before and after 7 days of pivampicillin treatment

	Glucose (mM)	3-HB (μM)	FFA (μM)	TG (mM)	Urea (mM)
Before treatment	5.24 ± 0.11	271.8 ± 62.3	408.6 ± 54.4	1.24 ± 0.21	4.61 ± 0.25
After treatment	6.76 ± 0.3	41.9 ± 18.5	263.5 ± 29.4	1.41 ± 0.17	3.74 ± 0.18
Significance	NS*	$P < 0.05$	$P < 0.05$	NS	$P < 0.05$

Values represent means ± SE; N = 7. Abbreviations: FFA, free fatty acids; TG, triglycerides; and 3-HB, 3-hydroxybutyric acid.
* Not significant.

whose renal clearance is in the range of that of creatinine. This suggests that pivaloylcarnitine is not reabsorbed.

Approximately 35% of the daily dose of pivalate was excreted as the acylcarnitine during 24 hr (data not shown). After a single oral dose of the [1-^{14}C]pivaloyloxyethyl ester of methyldopa to humans, Vickers *et al.* [13] found that after 48 hr more than 90% of the radioactivity was excreted in urine primarily as a carnitine ester. Whether other detoxification reactions, such as conjugation with glycine or glucuronic acid, are involved in elimination of pivalate in humans has not been rigorously determined. However, the studies of differential hydrolysis of Dowex 50 eluates indicate that about 20% of the conjugated pivalic acid was not esterified to carnitine. The sustained high excretion of carnitine may have depleted some of the tissue carnitine stores. The low plasma levels of free carnitine and possibly 3-hydroxybutyrate indicate such a depletion. The cause for the reduced free fatty acid levels is not known, although low tissue levels of carnitine have been implicated in impaired lipolysis [34].

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