



ELSEVIER

Journal of Chromatography B, 663 (1995) 83–89

JOURNAL OF  
CHROMATOGRAPHY B:  
BIOMEDICAL APPLICATIONS

## Determination of urinary valproylcarnitine by gas chromatography–mass spectrometry with selected-ion monitoring

Hideki Muro<sup>a</sup>, Tohoru Tatsuhara<sup>a</sup>, Tateo Sugimoto<sup>b,\*</sup>, Man Woo<sup>b</sup>, Naoki Nishida<sup>b</sup>, Kiyotaka Murakami<sup>c</sup>, Yukiharu Yamaguchi<sup>d</sup>

<sup>a</sup>Department of Pharmacy, Tottori University School of Medicine, 36-1 Nishi-Machi, Yonago, Tottori 683, Japan

<sup>b</sup>Kansai Medical University Otokoyama Hospital, Izumi 19, Otokoyama, Yawatashi Kyoto 614, Japan

<sup>c</sup>Nakano Children's Hospital, Shinmori 4-13-17, Asahiku, Osakashi, Osaka 535, Japan

<sup>d</sup>Bioassay Laboratory, Daicel Chemical Industries, 1239 Shinzaike, Aboshi-ku, Himeji, Hyogo 671-12, Japan

First received 17 May 1994; revised manuscript received 29 August 1994

### Abstract

A modified method for the determination of valproylcarnitine in urine samples of patients receiving sodium valproate by gas chromatography–mass spectrometry with selected-ion monitoring is described. The chemically analogous internal standard 2-ethylpentanoylcarnitine was added to the urine samples. Valproic acid and its metabolites were removed by extraction with chloroform at pH 5.0. The samples were then applied onto a C<sub>18</sub> Sep-Pak column. Inorganic and water soluble compounds were washed out with water. Valproylcarnitine and internal standard were eluted with methanol and were derivatized to the corresponding acyl-containing lactones by heating at 100°C for 60 min in dimethylformamide. Urinary valproylcarnitine levels of epileptic patients receiving valproate were determined according to the present method. The data obtained might be useful for diagnosis of carnitine deficiency.

### 1. Introduction

Valproic acid is a widely used anticonvulsant and has been associated with carnitine deficiency [1], hyperammonemia [2,3], but rarely with a Reye-like syndrome and hepatic failure [4–6]. Patients on chronic valproate therapy often show decreased levels of serum or muscle carnitine [1]. Ohtani et al. [1] reported that patients with valproate-induced carnitine depletion showed a restoration of normal serum carnitine and de-

creased hyperammonemia after carnitine replacement therapy. Although the mechanism of the development of carnitine deficiency is not clear, increased valproylcarnitine excretion [7] and renal loss of free carnitine [8] have been suggested. However, urine levels of valproylcarnitine have not been evaluated.

The zwitterionic, involatile nature of valproylcarnitine makes it unsuitable for direct gas chromatographic (GC) analysis. Only a method with fast-atom bombardment and thermospray liquid chromatograph–mass spectrometry [7] was successfully applied to the determination of

\* Corresponding author.

valproylcarnitine at therapeutic levels. However, the method cannot be routinely used for clinical studies because of the relatively high cost of the instrumentation.

Lowes and co-workers [9,10] developed a method for determining acylcarnitines, containing acetyl-, octanoyl-, 4-phenylbutanoyl- and palmitoyl-carnitines in urine by GC–mass spectrometry (GC–MS). The procedure involves a chemical derivatization in which the zwitterionic acylcarnitines are cyclized to volatile acyl-containing lactones.

In most of these procedures, sample clean-up involves double ion-exchange purification followed by freeze-drying for the removal of the eluent. As such, these methods are cumbersome and are not practical for clinical laboratories.

Isolation of acylcarnitines (from C<sub>2</sub> to C<sub>18</sub> acyl chains) by solvent extraction has been reported [11]. In that procedure, octanoylcarnitine, which is the isomer of valproylcarnitine, was extracted well into butan-1-ol at pH 2 with recoveries of approx. 85%. However, under the conditions where valproylcarnitine may be extracted with the solvent, many unfavorable impurities (valproic acid and its metabolites) are also extracted.

The present study describes a modified GC–MS selected-ion monitoring (GC–MS–SIM) method [9] for the determination of urinary valproylcarnitine. The method includes a simple sample preparation step, which does not require ion-exchange purification. The method is rapid and of sufficient sensitivity and selectivity for the determination of valproylcarnitine in patient urine. Urinary valproylcarnitine levels of epileptic patients treated with valproate were determined according to the proposed method.

## 2. Experimental

### 2.1. Instrumentation and chromatographic conditions

GC–MS was carried out on a JMS-DX 300 instrument, equipped with a JEOL 2000H data analysis system (JEOL, Tokyo, Japan) under the

following conditions: ionization current, 30  $\mu$ A; ionization voltage, 70 eV; separator temperature, 210°C. GC analyses were performed in the splitless mode (injection temperature, 210°C) on a HR-1 fused-silica capillary column, 25 m  $\times$  0.32 mm I.D., 0.5  $\mu$ m film thickness (Shinwa Chemical Industries, Kyoto, Japan), with a carrier gas (helium) flow-rate of 1 ml/min. After the sample was injected at a column oven temperature of 110°C, the column temperature was raised immediately to 190°C at 8°C/min. The mass spectrometer was operated in the SIM mode for 10 min, and the following ions were selected:  $m/z$  172 (internal standard, I.S.) and  $m/z$  186 (valproylcarnitine).

### 2.2. Chemicals

The synthesis of pure valproylcarnitine hydrochloride and 2-ethylpentanoylcarnitine hydrochloride (I.S.) from DL-carnitine hydrochloride and, valproic acid and 2-ethylpentanoic acid was carried out as reported by Ziegler et al. [12]. The mass spectra of  $\beta$ -valproyloxy- $\gamma$ -butyrolactone and  $\beta$ -2-ethylpentanoyloxy- $\gamma$ -butyrolactone (see Fig. 1) resulting from the corresponding acylcarnitines were identical with those of the corresponding, independently synthesized, acyl-containing lactones; the preparation of  $\beta$ -valproyloxy- $\gamma$ -butyrolactone and  $\beta$ -2-ethylpentanoyloxy- $\gamma$ -butyrolactone was based on the synthesis of  $\beta$ -hydroxy- $\gamma$ -butyrolactone [13] followed by reaction with the corresponding acid chloride. Details of these procedures will be published elsewhere.

Dimethylformamide (DMF), chloroform and methanol were purchased from Wako Chemicals (Osaka, Japan). DMF was redistilled before use. Octanoylcarnitine was obtained from Aldrich (Gillingham, UK). All other chemicals and reagents were of analytical grade.

### 2.3. Urine specimens

Patient specimens were those submitted to the clinical laboratory for therapeutic drug monitoring. Urine samples were stored frozen at –40°C until assayed.

#### 2.4. Solid-phase extraction of urine samples and derivatization

Frozen urine was warmed to room temperature. After centrifugation (2000 g, for 5 min) a 0.5–1.0-ml aliquot was transferred into 3-ml vial and 0.3 ml of the I.S. solution (1  $\mu\text{g}/\text{ml}$ ), 0.2 ml of 0.33 M  $\text{NaH}_2\text{PO}_4$  (adjusted to pH 5.0) and 0.5 ml of chloroform were added. After vortex-mixing for 10 min, followed by centrifugation at 3000 g for 5 min, the upper layer was applied to a  $\text{C}_{18}$  Sep-Pak column (Millipore) at a rate of 2 ml/min. The sorbent was preconditioned using  $3 \times 1$  ml of methanol followed by  $3 \times 1$  ml of water. Urine interferences were removed by washing the cartridge with  $3 \times 1$  ml of water. For the elution into a 1-ml vial, 1 ml of methanol was used; the solvent was removed in vacuo using a centrifugal concentrator VC-360 (Taitec, Japan). The residue containing 0.25–10  $\mu\text{g}$  of valproylcarnitine was dissolved in 50  $\mu\text{l}$  of DMF. After sealing, the vial was heated to 100°C for 60 min under nitrogen. The reaction mixture was analysed for the resulting lactones by GC–MS–SIM. After cooling, aliquots of 0.1–0.2  $\mu\text{l}$  were injected into the GC–MS system.

#### 2.5. Quantitation

Quantitation of valproylcarnitine was based on the peak-area ratios (valproylcarnitine/I.S.) of the  $[\text{M} - 42]^+$  ions. Calibration graphs (linear regression analysis) were obtained by analysing blank urine spiked with 0.10, 0.25, 0.50, 1.00, 5.00 and 10.00  $\mu\text{g}/\text{ml}$  of valproylcarnitine and 1.00  $\mu\text{g}/\text{ml}$  of the I.S. (aqueous solution). The extractions and derivatizations were carried out as described above.

#### 2.6. Precision

The between-day precision was determined by analysing three blank urine samples spiked with 0.50, 1.00 and 5.00  $\mu\text{g}/\text{ml}$  of valproylcarnitine and 1.00  $\mu\text{g}/\text{ml}$  of the I.S. Duplicate runs were conducted on six different days during a 2-week period. The extractions and derivatizations were performed as described above.

#### 2.7. Valproic acid

Valproic acid in patient urine was determined as total concentration after hydrolysis at 37°C for 60 min with  $\beta$ -glucuronidase according to the procedure described in the previous paper [14].

### 3. Results and discussion

#### 3.1. Derivatization

Valproylcarnitine and I.S. were successfully cyclized to give  $\beta$ -valproyloxy- $\gamma$ -butyrolactone and  $\beta$ -2-ethylpentanoyloxy- $\gamma$ -butyrolactone, respectively. However, the presence of a small amount of acidic substances, such as valproic acid and its metabolites, interfered with the derivatization. Therefore, these interfering acids should be removed prior to the cyclization as described later (see section 3.3). Several aprotic solvents, such as toluene, dioxane, pyridine and DMF, were evaluated for dissolving and cyclizing valproylcarnitine and I.S. DMF was found to be the most efficient solvent. The mass spectra of  $\beta$ -valproyloxy- $\gamma$ -butyrolactone and  $\beta$ -2-ethylpentanoyloxy- $\gamma$ -butyrolactone obtained in the electron-impact mode are shown in Fig. 1. All abundant ions were evaluated, but those indicated in the experimental section were selected in the final method by the criteria of favorable baseline and detection limit. The ion selected for the internal standard ( $m/z$  172) was 14 mass units lower than that of valproylcarnitine.

However, it has been reported that lactones may be converted to their corresponding free organic acid and unsaturated lactone by overheating or long reaction times [9]. Therefore, the conversion of valproylcarnitine to  $\beta$ -valproyloxy- $\gamma$ -butyrolactone was examined in DMF. For the establishment of optimal cyclization conditions, standard solutions of 5  $\mu\text{g}$  of valproylcarnitine and/or I.S. per 50  $\mu\text{l}$  of DMF were used. Fig. 2 shows the peak-area ratios of valproylcarnitine to dimethylphthalate (used as an external standard) versus time of cyclization at several heating conditions, where a ratio of 3 corresponds to the theoretical yield of the lactone.  $\beta$ -Val-

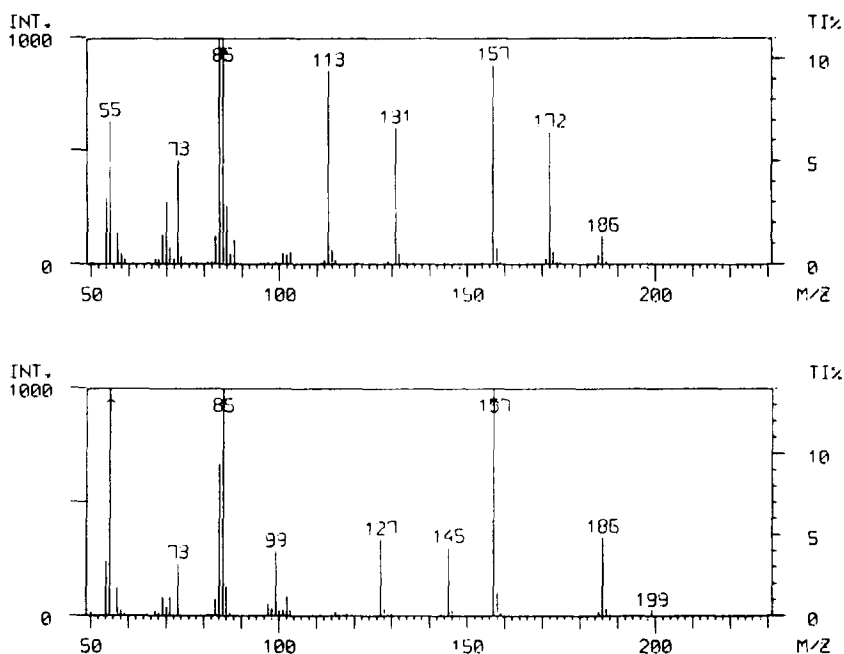


Fig. 1. Electron-impact mass spectra of  $\beta$ -2-ethylpentanoyloxy- $\gamma$ -butyrolactone (upper spectrum) and  $\beta$ -valproyloxy- $\gamma$ -butyrolactone (lower spectrum) derived from 2-ethylpentanoylcarnitine (I.S.) and valproylcarnitine, respectively.

proyloxy- $\gamma$ -butyrolactone was formed rapidly at 110°C but also disappeared quickly.

The increase in yield of the lactone at 100°C was somewhat slower than that at 110°C. The maximum peak-area ratio was obtained after 50 min at 100°C, and thereafter remained nearly constant up to 100 min, as shown in Fig. 2. The

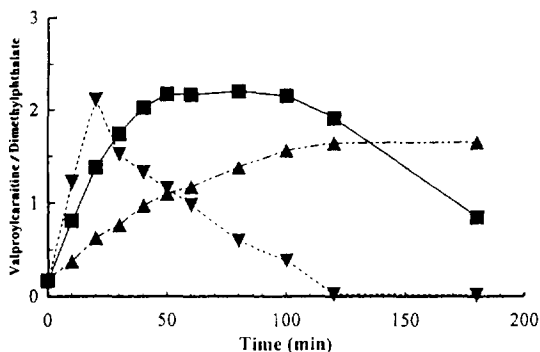


Fig. 2. Derivatization of valproylcarnitine in dimethylformamide. Peak-area ratios of valproylcarnitine to dimethylphthalate as an external standard versus time of derivatization at several temperatures: 110°C (▼), 100°C (■), 90°C (▲).

maximum peak-area ratio was obtained after 180 min at 90°C. In the experiments, however, no other peaks, e.g. valproic acid and unsaturated lactone, were detected on either the total-ion monitoring or SIM chromatogram. It is likely that valproic acid and unsaturated lactone were either absorbed on the liquid phase of the GC column or eluted in the solvent front.

The profiles for the cyclization of the I.S. were quite similar to that of valproylcarnitine and excellent linear calibration curves for valproylcarnitine were obtained using the I.S.

On the basis of the above data, subsequent experiments were carried out at 100°C for 60 min using DMF as the cyclization solvent.

### 3.2. Separation

Fig. 3 shows the total-ion monitoring chromatogram of a standard mixture containing I.S., valproylcarnitine and octanoylcarnitine. The isomers valproylcarnitine and octanoylcarnitine, which is an abnormal acylcarnitine, are well resolved by GC under the conditions employed.

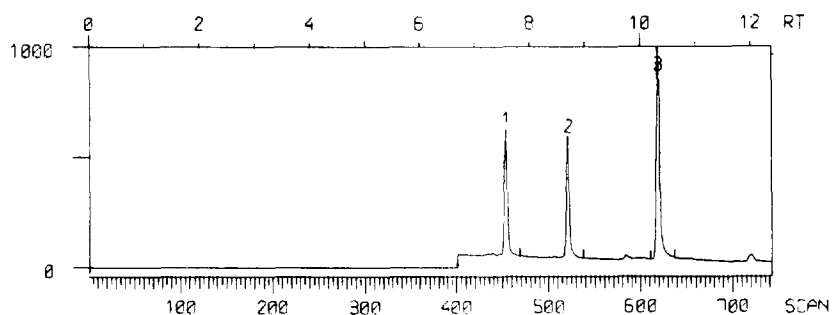


Fig. 3. Total-ion monitoring chromatogram from a standard mixture of acylcarnitines, containing I.S. (0.1 mg/ml in DMF), valproylcarnitine (0.1 mg/ml) and octanoylcarnitine (0.2 mg/ml) after derivatization to the acyl-containing lactones. Peaks: 1 = I.S., 2 = valproylcarnitine, 3 = octanoylcarnitine.

### 3.3. Extraction and recovery

The sample clean-up in the first step, i.e. chloroform extraction at pH 5.0, removed valproic acid and its metabolites which interfere with the cyclization of valproylcarnitine; when valproylcarnitine (0.25–10  $\mu\text{g}$ ), valproic acid (12–250  $\mu\text{g}$ ) and its metabolites (*cis*-2-en-VPA, 0.3–7  $\mu\text{g}$ ; *trans*-2-en-VPA, 0.2–3  $\mu\text{g}$ ; 3-en-VPA, 0.1–2  $\mu\text{g}$ ; 4-en-VPA, 0.1–2  $\mu\text{g}$ ; 3-OH-VPA, 2.5–50  $\mu\text{g}$ ; 4-OH-VPA, 1.4–28  $\mu\text{g}$ ; 5-OH-VPA, 1–20  $\mu\text{g}$ ; 3-keto-VPA, 15–300  $\mu\text{g}$ ; 4-keto-VPA, 1–23  $\mu\text{g}$ ; propylglutaric acid, 1–25  $\mu\text{g}$ ) were added to 1 ml of blank urine, more than 90% of valproic acid and its metabolites were removed by a single chloroform wash. The aqueous phase was applied onto a  $\text{C}_{18}$  Sep-Pak column. Inorganic and water soluble compounds were washed out with water. The standardized extraction procedure does not need any ion-exchange procedures which usually result in low recovery (approximately 30%) [9] and which might cause hydrolysis of the unstable valproylcarnitine. The SIM chromatogram in Fig. 4 of a urine extract from a patient undergoing valproate therapy shows that most of the interfering endogenous matrix could be eliminated. No other peaks other than those from both valproylcarnitine and I.S. were detected using the total-ion monitoring mode.

The absolute recovery of the extraction step was checked by comparing the valproylcarnitine/I.S. peak-area ratios after extraction with those

obtained by direct injection after derivatization. The recoveries of valproylcarnitine throughout the procedure at the 0.25, 0.50, 1.00, 5.00 and 10.00  $\mu\text{g}/\text{ml}$  levels were found to be  $95.0 \pm 6.5$  (mean recovery  $\pm$  standard deviation,  $n = 6$ ),  $98.7 \pm 5.5$ ,  $97.3 \pm 3.8$ ,  $98.5 \pm 5.9$ , and  $96.5 \pm 3.8\%$ , respectively. Thus, since the I.S. showed the same chromatographic behavior on a  $\text{C}_{18}$  Sep-Pak column as valproylcarnitine, it could be added at the initial stage of the procedure.

### 3.4. Calibration

The calibration graphs of valproylcarnitine against I.S. were drawn using 0.5 ml of control urine spiked with several known amounts of valproylcarnitine as described in Experimental. The calibration graphs constructed for the peak-area ratios of valproylcarnitine to I.S. were linear over the range 0.10–10.00  $\mu\text{g}/\text{ml}$  of valproylcarnitine with a correlation coefficient of 0.998 and passed through the origin when extrapolated.

### 3.5. Precision and detection limit

The precision of the present method is shown in Table 1. For a signal-to-noise ratio of 5, the detection limit for valproylcarnitine was 0.01  $\mu\text{g}/\text{ml}$  using 0.5-ml samples.

The between-day precision measured at low (0.25 and 0.50  $\mu\text{g}/\text{ml}$ ), medium (1.00  $\mu\text{g}/\text{ml}$ )

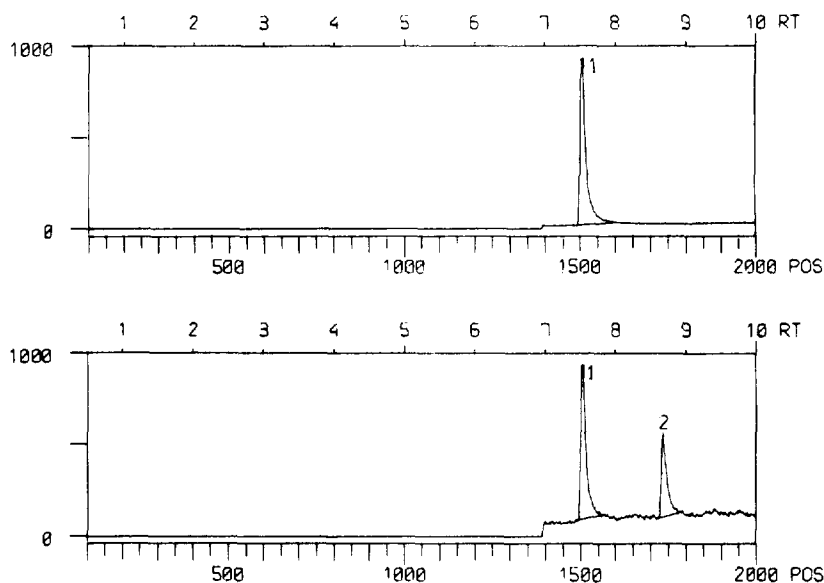


Fig. 4. Selected-ion monitoring chromatograms of I.S. ( $1 \mu\text{g/ml}$ ) (upper chromatogram,  $m/z = 172$ ) and valproylcarnitine ( $0.57 \mu\text{g/ml}$ ) (lower chromatogram,  $m/z = 186$ ) from a urine sample of an epileptic patient treated with valproate. Peaks: 1 = I.S., 2 = valproylcarnitine.

and high ( $5 \mu\text{g/ml}$ ) concentration levels is summarized in Table 2.

### 3.6. Stability

The stability of valproylcarnitine in urine samples spiked with valproylcarnitine at the levels of  $0.50$ ,  $1.00$ ,  $10.00 \mu\text{g/ml}$  and stored at  $-40^\circ\text{C}$  for a period of 14 days was investigated. No decomposition of valproylcarnitine could be observed as shown in Table 3.

Table 1  
Accuracy and precision of the assay applied to human urine spiked with valproylcarnitine

Amount added ( $\mu\text{g/ml}$ )	Amount found (mean; $n = 6$ ) ( $\mu\text{g/ml}$ )	Precision (C.V., %)
0.10	0.10	10.0
0.50	0.49	6.4
1.00	1.02	6.7
10.00	10.26	6.5

### 3.7. Application to urine samples from epileptic patients

In order to demonstrate the applicability of the present method to biological materials, the amount of valproylcarnitine in urine of epileptic patients taking valproic acid was analysed. The relationship between urinary valproylcarnitine and valproic acid concentrations in patients who are not taking carnitine is shown in Fig. 5. Although the number of clinical examples is still limited, valproylcarnitine concentrations seemed to be correlated with the valproate concentra-

Table 2  
Between-day precision of assays for valproylcarnitine in urine

Amount added ( $\mu\text{g/ml}$ )	Amount found (mean $\pm$ S.D., $n = 6$ ) ( $\mu\text{g/ml}$ )	C.V. (%)
0.25	$0.25 \pm 0.02$	8.0
0.50	$0.48 \pm 0.04$	7.3
1.00	$0.98 \pm 0.09$	6.2
5.00	$5.01 \pm 0.14$	2.8

Table 3  
Stability of valproylcarnitine in urine stored at  $-40^{\circ}\text{C}$

Concentration of valproylcarnitine ( $\mu\text{g}/\text{ml}$ )	Residual amount (%)				
	0	Day 1	Day 2	Day 5	Day 14
0.50	100.0	98.2	96.0	98.3	99.1
1.00	100.0	99.5	95.7	97.4	98.6
10.00	100.0	101.0	99.7	100.8	102.3

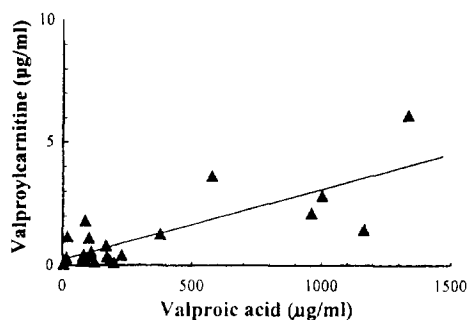


Fig. 5. Relationship between urine valproic acid and valproylcarnitine concentrations in patients receiving sodium valproate.

tions ( $p < 0.001$ ). Further studies on the excretion of valproylcarnitine in patients taking sodium valproate concomitant with or without carnitine by means of the proposed method are in progress, and the results will be reported elsewhere.

In conclusion, the method has demonstrated that valproylcarnitine can be rapidly, accurately and precisely determined by GC-MS-SIM as  $\beta$ -valproyloxy- $\gamma$ -butyrolactone without cumbersome and time-consuming procedures. The assay might be useful for the diagnosis of carnitine deficiency.

## References

- [1] Y. Ohtani, F. Endo and I. Matsuda, *J. Pediatr.*, 101 (1982) 782.
- [2] D. Haidukewych, G. John, J.J. Zielinski and E.A. Rodin. *Ther. Drug Monit.*, 7 (1985) 290.
- [3] T. Sugimoto, A. Yasuhara, T. Matsumura, K. Hara, Y. Sakane and K. Taniuchi, in H. Akimoto, H. Kazamatsuri, M. Seino and A. Ward (Editors), *Advances in Epileptology, XIIIth Epilepsy International Symposium*, Raven Press, New York, NY, 1982, pp. 297–300.
- [4] H.U. Zimmerman and K.G. Ishak, *Hepatology*, 2 (1982) 591.
- [5] W. Kochen, A. Schneider and A. Ritz, *Eur. J. Pediatr.*, 141 (1983) 30.
- [6] H. Bohles, K. Richter, E. Wagner-Thiessen and H. Schafer, *Eur. J. Pediatr.*, 139 (1983) 185.
- [7] D.S. Millington, T.P. Bohan, C.R. Roe, A.L. Yergey and J.J. Liberato, *Clin. Chim. Acta*, 145 (1985) 69.
- [8] I. Matsuda, Y. Ohtani and N. Ninomiya, *J. Pediatr.*, 109 (1986) 131.
- [9] S. Lowes and M.E. Rose, *Analyst*, 115 (1990) 511.
- [10] S. Lowes, M.E. Rose, G.A. Mills and R.J. Pollitt, *J. Chromatogr.*, 577 (1992) 205.
- [11] R.J. Morrow and M.E. Rose, *Clin. Chim. Acta*, 211 (1992) 73.
- [12] H.J. Ziegler, P. Brukner and F. Binon, *J. Org. Chem.*, 32 (1967) 3989.
- [13] S. Henrot, M. Larcheveque and Y. Petit, *Synth. Commun.*, 16 (1966) 183.
- [14] T. Tatsuhara, H. Muro, Y. Matsuda and Y. Imai, *J. Chromatogr.*, 399 (1987) 183.