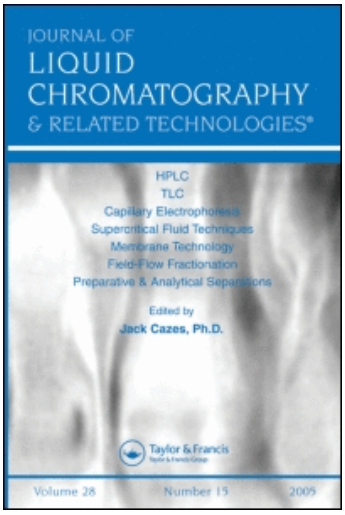

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HPLC OF ACETYL-*L*-CARNITINE IN HUMAN PLASMA BY DERIVATIZATION WITH p-BROMOPHENACYL BROMIDE

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ABSTRACT

A high performance liquid chromatographic method is developed for the determination of acetyl-*L*-carnitine (ALC), an endogenous carnitine derivative, in human plasma. e-Carnitine, which is the structural analogue of ALC, was used as an internal standard. The human plasma samples were treated with ninhydrin solution for the removal of amino acids and then extracted with methanol. After solid phase extraction on silica columns, samples were derivatized with p-bromophenacyl bromide in the presence of 18-crown-6.

The derivative of ALC was quantified by high-performance liquid chromatography with UV detection at 260 nm. The retention time of ALC and e-carnitine was about 27.5 and 22.3 min, respectively. The limit of quantitation was 0.22 nmol/mL, based on signal to noise ratio of 3. The accuracy deviation of assay was less than 12.86%, and the intra-day and inter-day coefficients of variation (CV, %) were lower than 5.18 and 5.06%, respectively.

INTRODUCTION

Acetyl-*L*-carnitine (ALC) is a carnitine derivative, which is endogenous material participating in the disorder of organic acid metabolism.[1] Recently, ALC was clinically employed in the treatment of dementia type Alzheimer disease (Nicetile®, Sigma Tau, Italy). A method of quantitative determination of ALC in biological samples, including urine, plasma, and tissues, has been developed for the diagnosis and management of ALC related diseases.

To determine ALC and carnitine derivatives, radioisotope exchange high performance liquid chromatography,[2] fast atom bombardment ionization coupled with mass spectrometry (FAB-MS),[3] on-column N-demethylation gas chromatography followed by mass spectrometry (GC-MS),[4] precolumn acylcarnitine deamination GC-MS,[5] and precolumn chemical derivatization[6–9] have been reported. However, most of these methods have some limits of clinical application due to their sensitivity, specificity, ease of analysis, reproducibility, and costs. Among the precolumn derivatization method, phenacyl derivatization is most useful in the derivatization of acid, including carnitine derivatives for HPLC and UV detection.[7–9]

In this paper, p-bromophenacyl bromide as derivatizing reagent and e-carnitine as internal standard was used for the determination of ALC in human plasma. A quick and simple derivatization procedure was developed for HPLC determination with UV detection. This analytical method was applied to determine the concentration of ALC in human plasma.

EXPERIMENTAL

Materials and Apparatus

p-Bromophenacyl bromide was obtained from Merck (Darmstadt, Germany). 18-Crown-6 (1,4,7,10,13,16-hexaoxacyclooctadecane) and ninhydrin solution were purchased from Sigma (St. Louis, MO, USA). Acetyl-*L*-carnitine·HCl was purchased from Flamma (BG, Italy). 4-(N,N-dimethyl-N-ethylammonio)-3-hydroxybutanoate (e-carnitine) ·HCl was synthesized from L-carnitine with a method proposed by Minkler et al.[10] Acetonitrile was of HPLC grade form Fisher Sci. (Fair Lawn, NJ, USA). All other chemicals were of GR grade and used without further purification. Water was deionized, distilled, and degassed in house.

The HPLC system consisted of a L-6200 intelligent pump, L-4200 UV detector and L-7200 autosampler (Hitachi, Tokyo, Japan).

Preparation of Reagents

p-Bromophenacyl bromide solution (p-BPB solution) was prepared by dissolving 0.025 M p-bromophenacyl bromide and 0.0025 M 18-crown-6 in acetonitrile. ALC and e-carnitine standard solutions were prepared by dissolving ALC and e-carnitine in water, and then successively diluted with water.

Sample Preparation and Derivatization

To 100 μL of human plasma or standard solutions of ALC, 50 μL each of internal standard solution and ninhydrin solution were added and kept for 10 min at room temperature to remove amino acid in normal plasma. The reaction mixture was then added to 1 mL of methanol followed by vortex mixing and centrifugation at 3000 g for 10 min. Supernatant was evaporated to dryness under N_2 stream at 30°C. The residue was reconstituted with 1 mL of methanol and transferred to silica gel solid phase extraction column (Lichrolute®Si 60, Merck, Darmstadt, Germany) and washed with 1 mL of methanol and 0.7 mL of 1% acetic acid in methanol, successively.

ALC and e-carnitine were eluted with 4 mL of eluting solvent, containing acetic acid, water, and methanol (3:2:95, v/v). The elute was evaporated under N_2 stream and derivatized in 50 μL of water and 300 μL of p-BPB solution for 40 min at 70°C. After cooling to room temperature, an aliquot of reaction mixture (50 μL) was analyzed on HPLC.

Chromatographic Condition

Chromatography was performed using reverse-phase C_8 column (Hypersil MOS-1, 3 μm, 4.6 × 150mm, Alltech, Deerfield, IL) and gradient elution at UV 260 nm. The solvent system for gradient elution contained 80% acetonitrile (eluent A), 100% water (eluent B), and 80% acetonitrile containing 0.8% of TEA and 0.64% of phosphoric acid (eluent C). Elution was carried as follows. Initially, 100% eluent A was pumped at a flow rate of 1 mL/min. At 0.2 min after sample injection, eluent A was replaced with 100% eluent B and continued to 2.9 min.

At 3 min after sample injection, linear gradient over 12 min was applied with 20% eluent C and 80% eluent B, followed by 30% eluent C and 70% eluent B. At 15 min after injection, linear gradient over 8 min was applied with 38% eluent C and 62% eluent B, followed by 40% eluent C and 60% eluent B, and then maintained for 4 min. At 30 min after injection, the eluent was switched to 100% eluent A and eluted for further 15 min.

Recovery

ALC·HCl standard solutions were added to aliquots of human normal plasma, and samples were prepared and derivatized as described above. The peak height ratios of spiked plasma samples were compared to that of standards, which were directly derivatized without preparation procedure. Aliquots of human normal plasma without spiked ALC were also prepared to determine the concentration of endogenous ALC. A concentration of endogenous ALC was subtracted from peak height ratios of plasma spiked with ALC prior to the calculation of assay recoveries.

Method Validation

Derivatization of ALC·HCl standard solutions (0.5, 1.39, 4.17, 8.34, 16.69 nmol/mL in water) was carried out through the above procedure and then the linear calibration curve was constructed based on the concentration to peak height ratios. The ALC concentration in human plasma was interpolated from this standard curve. The detection limit of ALC was determined as the concentration of drug giving a signal-to-noise ratio greater than 3:1. The precision (defined as the coefficient of variation of replicate analysis) and the accuracy (defined as the absolute deviation between added and calculated concentrations) of assay were evaluated.

In addition, 100 μL of blank human plasma was derivatized by the above method for three replicates in a day for three consecutive days. The results thus obtained were used to calculate precision and accuracy of the procedure. Moreover, various concentrations of ALC (0.1, 2.0, 6.0 nmol/mL) were spiked to 100 μL of human blank plasma to ascertain intraday (n=3) variation in assay results.

Preparation of Biological Samples

After oral administration of Nicetile® tablet (equivalent to 500 mg ALC) to 3 healthy males, aged 21–25 years, blood samples (3 mL) were collected from the individual subject at designated time intervals. The blood samples were kept in an ice bath for 30 min and were then centrifuged at 2000 g for 10 min. The plasma was collected and stored at −20°C until use for HPLC analysis as described above. Maximum plasma concentration of ALC (C_{max}) and time to reach the peak concentration (t_{max}), were compiled from the plasma concentration of ALC-time curve.

RESULTS AND DISCUSSION

Derivatization

Derivatization of ALC and e-carnitine (internal standard) was completed under mild reaction conditions. Figure 1 shows the effect of reaction temperature (A) and time (B) on the derivatization of ALC and e-carnitine. Reaction was equilibrated at higher than 50°C and almost terminated within 40 min. In this report, the reaction was performed for 40 min at 70°C for the assurance of derivatization. The reaction mixture was stable at least 24 h at room temperature.

Method Validation

Figure 2 shows typical chromatograms of ALC standard (A), human blank plasma (B), and spiked plasma (C). The retention time of ALC and e-carnitine was about 27.5 and 22.3 min, respectively. No interference from endogenous substances was observed in any chromatograms of human plasma samples.

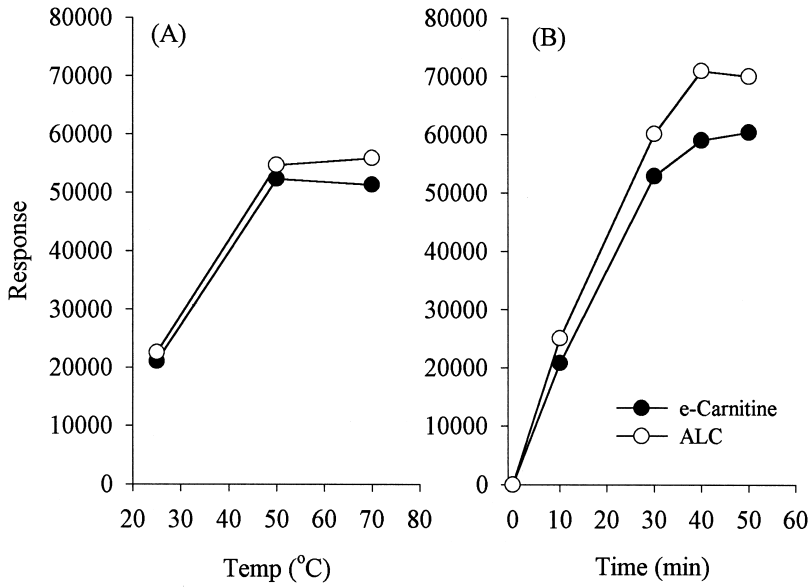

Figure 1. Effect of reaction temperature (A) and reaction time (B) on the derivatization of ALC and e-carnitine (internal standard) with p-BPB.

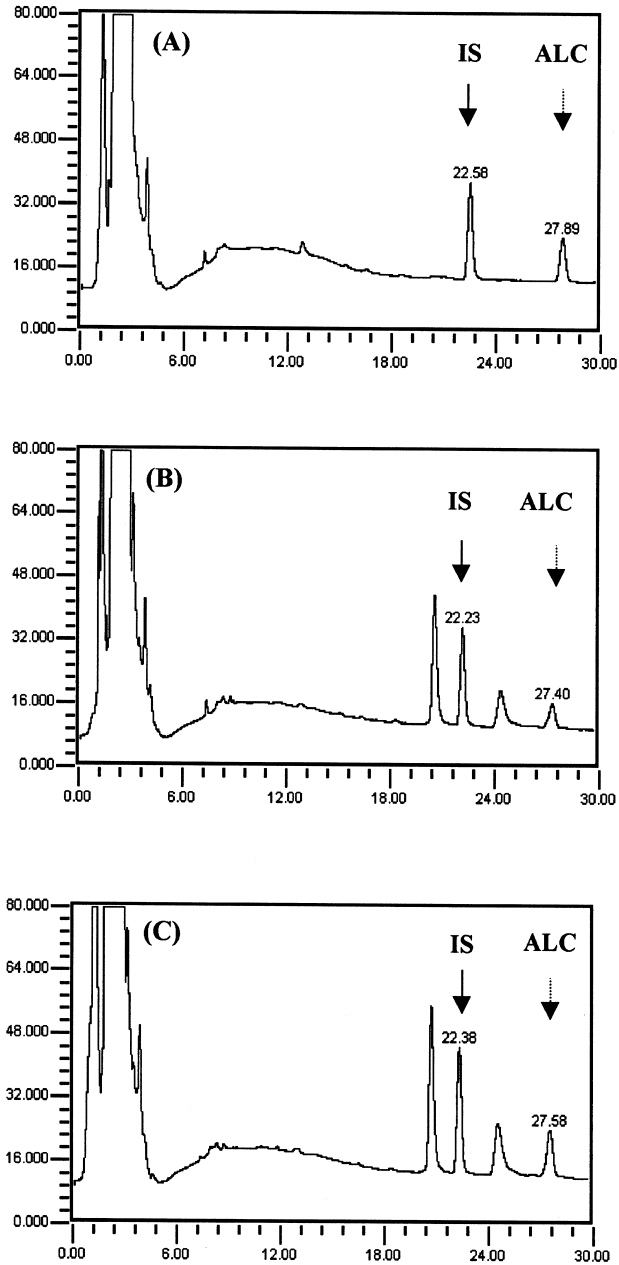

Figure 2. Chromatograms of ALC standard solution (A), blank plasma spiked with internal standard (B), and plasma sample from a human subject at 5 hour after oral administration of Nicetile® 500 mg. (C). Peak: ALC, Acetyl-*L*-carnitine; IS, internal standard (e-carnitine).

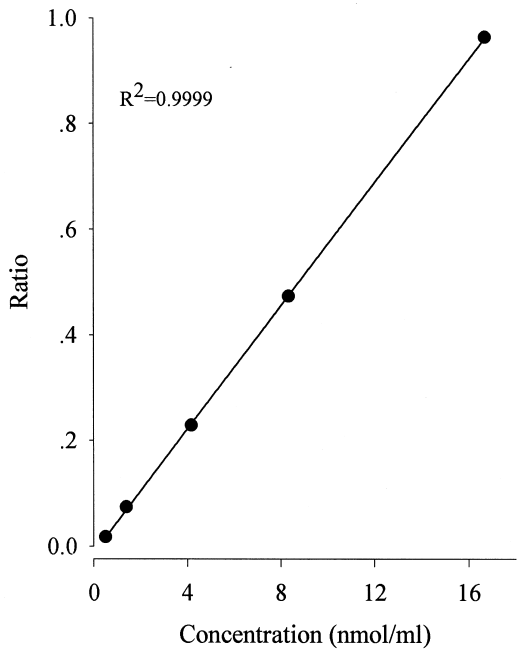

Figure 3. Calibration curve for ALC after derivatization with p-BPB.

Table 1. Reproducibility of ALC Determination in Standard Solution and Human Plasma

Conc.	Precision (CV, %)		Accuracy (Absolute Error, %)	
(nmol/mL)	Inter-day (n = 5)	Intra-day (n = 3)	Inter-day (n = 5)	Intra-day (n = 3)
Standard solution				
0.50	2.21	4.12	7.14	9.95
1.39	5.06	3.45	5.40	12.86
4.17	2.56	0.84	2.00	0.99
8.34	1.01	2.81	0.82	3.59
16.69	0.19	2.26	0.23	2.02
Blank plasma	1.34	2.02	1.93±0.05 (nmol/mL)	1.91±0.04 (nmol/mL)
Spiked plasma[a]				
2.01		5.18		4.46
2.91		1.42		1.04
6.91		0.53		0.15

[a]Concentration = concentration of blank plasma (1.91 nmol/mL) + concentration of spiked standard solution.

Recoveries of the procedure were between 88.2% and 85.4% for ALC and e-carnitine, respectively, at various concentrations examined (data not shown). Correlation of ALC concentration with peak height ratios showed good linearity within the concentration range examined (R^2=0.9999, Figure 3). The limit of quantitation (LOQ), based on signal to noise ratios (S/N) of 3, was 0.22 nmol/mL. Table 1 shows the reproducibility of the assay. In standard solutions, the interday and intraday accuracy deviations were less than 7.14 and 12.86%, respectively, and the interday and intraday coefficients of variation (CV, %) were lower than 5.06 and 4.12%, respectively.

Endogenous ALC in blank human plasma used in this experiment was determined to be 1.91 nmol/mL by our method. Coefficients of variation (CV, %) of intraday assay on three spiked plasma samples were lower than 5.18 %, while accuracy deviation was lower than 4.46 %.

From the above results, our reported method is adequate to determine the concentration of ALC in human plasma with respect to simple and mild derivatization conditions.

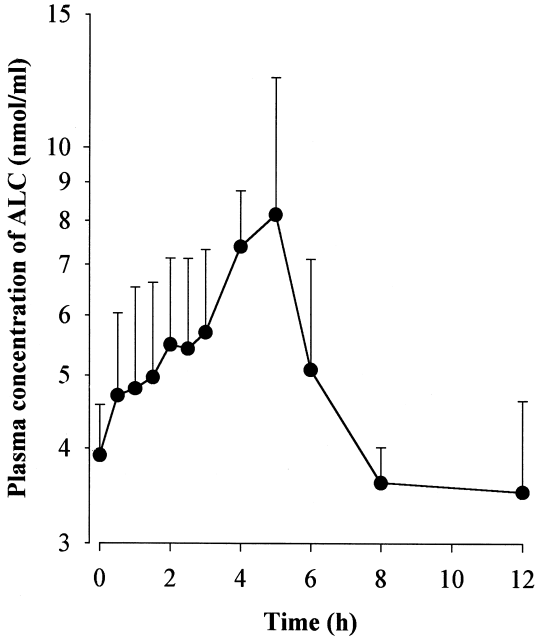

Figure 4. Mean plasma concentration versus time plot of ALC in human subjects after oral administration of Nicetile® 500 mg. Each point represents the mean ± standard deviation of three human subjects.

Application

Figure 4 shows a mean plasma concentration versus time plot of ALC following oral administration of Nicetile® 500 mg to human subjects. The basal concentration of ALC at 0 h after administration, was 3.92 ± 0.65 nmol/ml. The C_{max} of ALC was 9.81 ± 2.81 nmol/mL at 4.33 ± 0.58 h after the dose, which was 2.5-times higher than basal concentration, and returned to basal concentration at 8 h post administration.

This observation strongly suggests that our analytical method to determine the concentration of ALC in human plasma to be a quick and simple derivatization procedure.

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