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Determination of Highly Soluble L-Carnitine in Biological Samples by Reverse Phase High Performance Liquid Chromatography with Fluorescent Derivatization

Qing-Ri Cao, Shan Ren, Mi-Jin Park, Yun-Jaie Choi[1], and Beom-Jin Lee

National Research Laboratory for Bioavailability Control, College of Pharmacy, Kangwon National University, Chuncheon 200-701, Korea and [1]College of Agricultural Life Science, Seoul National University, Seoul 151-742, Korea

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This study was performed in order to validate an effective high performance liquid chromatograpy (HPLC) method to determine L-carnitine in biological samples such as plasma, milk and muscle in cows. An L-carnitine derivative for fluorescence absorption was synthesized with 1-aminoanthracene (16 mg/mL in acetone) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC; 160 mg/mL in 0.01 M NaH_2PO_4 buffer) as a precolumn fluorescent derivative reagent. γ-Butyrobetaine HCl was used as an internal standard. A reversed-phase column with fluorescence detection at the excitation and emission wavelengths of 248 and 418 nm were used. The mobile phase consisted of 30% acetonitrile with 0.1 M ammonium acetate in water (pH 3.5) adjusted with acetic acid and delivered at a flow rate of 1.5 mL/min. The L-carnitine concentration in plasma, milk and muscle samples of cows after oral feeding with 24 g L-carnitine/day for 2 months was then determined. All biological samples were deproteinated by barium hydroxide and zinc sulfate heptahydrate before the derivative reaction. Blank cow plasma was dialyzed using cellulose membrane for standard calibration. The calibration curve showed good linearity (r^2 >0.999) over the concentration range of 50 to 5000 ng/mL. The precision and accuracy were also satisfactory with less than 15% intra- and inter-day coefficiency of variations. The peaks of L-carnitine and internal standard in HPLC chromatography were successfully separated in plasma, milk and muscle samples of cows. The current derivatization method of L-carnitine for fluorescence detection was simple and adequately sensitive and could be applied to determine L-carnitine in biological samples.

Key words: L-carnitine, In vivo biological samples, HPLC analysis, Fluorescence derivatization, Cows

INTRODUCTION

L-carnitine, (R)-3-carboxy-2-hydroxy-N,N,N-trimethyl-1-propanaminium hydroxide, inner salt, is a biological substances localized in various tissues of mammals. It is an amino acid like and vitamin B-like substance, essential for fatty acid oxidation and energy production in humans and in ruminants (Bremer, 1983; Siliprandi *et al.*, 1989). Currently, it is clinically used for the treatment of carnitine deficiency or as a dietary supplement for people with various chronic diseases (Brass, 1995).

Quantitative analysis of L-carnitine using reversed-phase high performace liquid chromatography (HPLC) is challenging due to its high polarity and lack of chromophore. Many methods for assaying L-carnitine in biological fluids have been published by various authors. The most common methods are those employing enzymes (Takeyama *et al.*, 1989) and those using HPLC with precolumn derivatization and UV or fluorimetric detection (Yoshida *et al.*, 1988; Minker *et al.*, 1993; Longo *et al.*, 1996; Marzo *et al.*, 1997). Some HPLC methods based on pre-derivatization of L-carnitine have been developed to analyze the amount of L-carnitine in human plasma (van Kempen and Odle, 1992; Longo *et al.*, 1996).

Longo *et al.* described a new sensitive method for the simultaneous determination of L-carnitine in human plasma, based upon the synthesis of a fluorescent derivative of the carnitines. Since plasma was purified by solid phase extraction prior to derivatization, the extraction process

Correspondence to: Beom-Jin Lee, National Research Lab. for Bioavailability Control, College of Pharmacy, Kangwon National University, Chuncheon, Korea
Tel: 82-33-250-6919, Fax: 82-33-242-3654
E-mail: bjl@kangwon.ac.kr

was time-consuming and expensive (Longo *et al.*, 1996). Furthermore, the limit of quantitation (LOQ) was unsatisfactory in some cases.

Instead of time-consuming and labor intensive organic extractions, Sandra *et al.* described a simple and rapid quantitative determination of acetaminophen in plasma. The analysis method involved a single plasma protein precipitation step followed by liquid chromatographic determination of drug in the clear supernatant. The plasma proteins were denatured and precipitated using 0.3 M $Ba(OH)_2$ and 5% $ZnSO_4$ solutions (O'connell *et al.*, 1982). Until now, there has been little information regarding the analysis of L-carnitine in biological samples of cows. A simple analysis method with a high detection limit available for biological samples including plasma, milk and muscle is necessary for the quality validation in food industry and animal sciences.

In this paper, we describe an improved HPLC method, purifying samples by protein precipitation prior to derivatization, for the quantitative analysis of L-carnitine in biological samples such as plasma, milk and muscle in cows.

MATERIALS AND METHODS

Materials

L-carnitine, (3-carboxypropyl) trimethylammonium chloride (γ-butyrobetaine hydrochloride), dialysis tubing cellulose membrane [molecular weight cut-off (MWCO) of 12000 or greater] and Krebs-Ringer bicarbonate buffer were purchased from Sigma (Sigma Chemical Co. St. Louis, U.S.A.). 1-Aminoanthracene was from Aldrich (Aldrich Chem. Co. Milw., WI) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC) was obtained from Sigma-Aldrich Co. (St. Louis, U.S.A.). Ammonium acetate, acetic acid and zinc sulfate heptahydrate were purchased from SHOWA chemical Co. Ltd. (Tokyo, Japan) and barium hydroxide was from Yakuri Pure Chemicals Co., Ltd. (Osaka, Japan). All other chemicals used were of reagent grade were used without further purification.

Apparatus

The HPLC system consisted of a Waters 600E Multisolvent Delivery System (Waters 600E System Controller; Milford, MA, USA), a Waters TM 717 Plus auto sampler, and a Jasco FP-920 intelligent Fluorescence Detector (Hachioji, Tokyo, Japan). Data were analyzed with an Autochro 2000 1.0 chromatographic data collection station (Young Jin, Seoul, Korea).

Optimal HPLC conditions

The HPLC mobile phase was prepared by mixing 700 ml of 0.1 M ammonium acetate in water (pH 3.5 adjusted with acetic acid) with 300 mL of acetonitrile. Chromatographic separation was performed at a flow rate of 1.5 mL/min, using LUNA C18, 150 × 4.6 mm I.D., particle size 5 μm column (Phenomenex, Torrance, CA, U.S.A.). The excitation and emission wavelengths of the spectrofluorimeter were 248 and 418 nm, respectively. 20 μL of the sample was injected into the HPLC system. The peak area ratios of L-carnitine and internal standard (2 μg/mL γ-butyrobetaine hydrochloride) were used to measure L-carnitine concentration. Retention times of L-carnitine and IS were 4.0 and 4.9 min, respectively.

Preparation of calibration standards

Dialysis of plasma

Cow plasma was dialyzed using a membrane mode Spectrapor MWCO: 12,000 dialyzer. The dialyzing buffer was a Krebs-Ringer phosphate solution. The plasma (20 mL), closed in the membrane, was exposed to the buffer solution (500 mL) for about 36 h at 4°C. During this period, the buffer solution was refreshed at least 3 times, at intervals of about 8-10 h. No protein denaturalization phenomenon was observed from the plasma sample in the course of dialysis process, showing relatively good stability. This dialysis process was just required with blank plasma to remove the endogenous L-carnitine for the construction of standard calibration curves.

Standard solutions

A stock solution of L-carnitine was prepared by dissolving approximately 10 mg in 10ml deionized Milli-Q filter water. Further standard solutions were obtained by serial dilutions of the stock solution with 0.5, 1, 5, 10 and 50 μg/mL L-carnitine. A five-point calibration curve was prepared by adding standard solutions to dialyzed plasma to obtain the following concentrations: 50, 100, 500, 1000 and 5000 ng/mL. Each calibration plasma sample was processed as described below.

Plasma protein precipitation and extraction procedure

30 μL of the internal standard solution (20 μg/mL) were added to 300 μL of plasma samples containing the amounts of L-carnitine needed for calibration and mixed. 300 μL of saturated 0.3 M $Ba(OH)_2$ solution was added and vortexed for 2 min on a multiple tube vortex mixer at a sufficient speed to ensure thorough mixing. 300 μL of 10% $ZnSO_4$ solution was then added to each sample which should have a milky opaque appearance. Each sample was vortexed at high speed for 1 min. The tubes were then centrifuged at 10,000 g for 10 min. The supernatant was separated from the precipitate and used for the derivatization reaction.

Derivatization procedure

1-Aminoanthracene (fluorescent reagent) was dissolved

in acetone (16 mg/mL) and EDC in 0.01 M NaH_2PO_4-H_2O pH 3.5 (160 mg/mL). 500 μL of the supernatant was mixed with 500 μL of 0.01M NaH_2PO_4-H_2O (pH 3.5). 20 μL of 1M HCl, 100 μL of 1-aminoanthracene solution and 100 μL of EDC solution were then sequentially added to the sample with continuous vortex mixing. The mixture was incubated at 25°C for 20 min and the excess reagent was removed by washing the sample with 5 mL of diethlether. A 300 μL aliquot of the aqueous phase was then transferred to a plastic tube, 700 μL of 0.01 M Na_2HPO_4-$2H_2O$, pH 9.1 was added to adjust the pH of the samples to about 7 and the mixture was washed with 5 mL of chloroform. 500 μL aliquots of the final aqueous phase were diluted with 500 μL of 0.01 M NaH_2PO_4-H_2O, pH 3.5 and 20 μL of this solution was injected into the HPLC system.

Analytical validations

The linearity of the assay was determined over the concentration range 50-5000 ng/mL. The precision and accuracy of the method were evaluated by testing five replicates of five concentrations (50-5000 ng/mL) of plasma sample constituents for within-day and by assaying five concentrations of sample constituents over 5 different days for inter-day. The precision and accuracy were defined as the relative standard deviation (RSD) and as the error from the theoretical nominal concentration, respectively.

The LOQ was defined as the lowest concentration at which the precision expressed by the RSD was lower than 15% and the accuracy expressed by the relative difference of the measured and the value was also lower than 15%.

Recoveries of L-carnitine during precipitation were assessed at the lowest and the highest concentrations of the calibration range. Each sample was analyzed in five replicates and the results were compared with those obtained analyzing standard aqueous samples containing the same concentrations of the analytes.

Applications to biological samples

Fresh biological samples of plasma, milk and muscle were collected after 24 g L-carnitine was given daily for two months to five cows. Plasma and milk samples were analyzed by the method described above. Muscle sample (approximately 0.5 g) was weighed and homogenized with 2 mL of saline buffer for 30 sec in an ice bucket. The homogenized tissue was then diluted 5 times with saline buffer and centrifuged at 3000 g for 15 min. 300 μL supernatant was analyzed by the protein precipitation and extraction procedure described previously.

RESULTS AND DISCUSSION

Typical HPLC chromatograms of undialyzed blank plasma, dialyzed blank plasma and dialyzed plasma spiked with L-carnitine (5 μg/mL) and internal standard (IS, γ-butyrobetaine hydrochloride 2 μg/mL) following precipitation and derivatization procedures are shown in Fig. 1. No interfering peaks were observed at the retention times corresponding to the compounds of interest in dialyzed plasma while the endogenous L-carnitine peak appeared in blank plasma at the retention time of 4.0 min. The retention times for L-carnitine and internal standard were 4.0 and 4.9 min, respectively. The total time consumed for sample analysis was less than 6 min for each sample.

The analytical conditions were further validated. The linearity of the assay was determined over the concen-

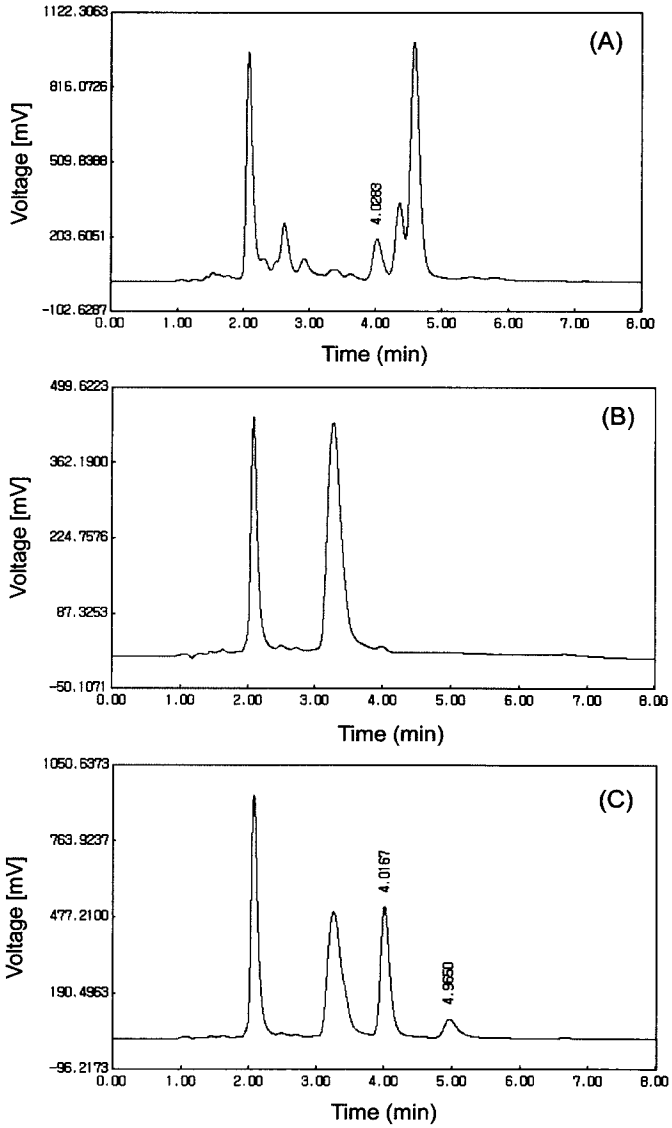

Fig. 1. HPLC chromatograms of undialyzed blank plasma (A), dialyzed blank plasma (B) and dialyzed plasma spiked with L-carnitine (5 μg/mL) and internal standard (IS, γ-butyrobetaine hydrochloride, 2 μg/mL) (C).

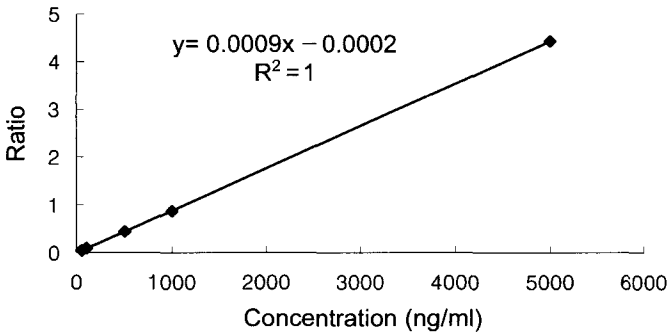

Fig. 2. Linearity of the standard calibration curve of L-carnitine in plasma samples

tration range of 50-5000 ng/mL. Linearity of the calibration standard curve of L-carnitine in dialyzed cow plasma is shown in Fig. 2. There was a good linear relation, with a regression coefficient (r^2) of 1.0. The intra-day and inter-day precision and accuracy of L-carnitine measurement in plasma samples are shown in Tables I and II. Precision, expressed as % RSD, was in the range of 0.60-6.68%. Accuracy, expressed as % error, was always lower than 6.96% with a relative error of 2.88 to 6.96%. The accuracy and precision were highly satisfactory for the analysis of biological samples. The LOQ for plasma samples was below 50 ng/mL of L-carnitine where the relative standard deviations for inter- and intra-day assays were less than 15% with acceptably low relative errors. The LOQ was significantly lower than 5 nmol/mL (approximately 806 ng/mL) in human plasma, which was prepared by the solid phase extraction and derivatization procedure (Longo *et al.*, 1996). However, our results showed that precipitation and extraction procedure used in this study was more efficient than reported solid phase extraction procedure. A high-performance liquid chromatographic method was also developed for the determination of carnitine in human urine sample, which treated with a disposable cation-exchange column to extract carnitine before derivatization. However, the lower limit of quantitation was not satisfied yet, showing 103 nmol/mL (Kamimori *et al.*, 1994). Although a lower detection limit for carnitine was reported as 0.24 nmol in human plasma, a costly and time-consuming cation-exchange column was also used to prepare the analytes suitable for derivatization (Kuroda *et al.*, 1996).

Table I. Intra-day precision and accuracy of L-carnitine in cow plasma

Spiked concentration (ng/mL)	Calculated concentration (mean ± SD, n=5, ng/mL)	Precision RSD (%)	Accuracy Error (%)
50	52.44 ± 1.26	2.40	4.89
100	105.00 ± 1.48	1.41	5.00
500	489.89 ± 4.71	0.96	-2.02
1000	978.67 ± 7.39	0.75	-2.13
5000	4999.56 ± 65.32	1.31	-0.01

* Samples were analyzed by five replicates for each concentration per day

Table II. Inter-day precision and accuracy of L-carnitine in cow plasma

Spiked concentration (ng/mL)	Calculated concentration (mean ± SD, n=5, ng/mL)	Precision RSD (%)	Accuracy Error (%)
50	52.82 ± 0.83	1.56	5.64
100	106.96 ± 7.14	6.68	6.96
500	497.31 ± 9.40	1.89	-0.54
1000	971.20 ± 12.85	1.32	-2.88
5000	4925.29 ± 29.32	0.60	-1.49

* Samples were analyzed every day for 5 days

Recovery of L-carnitine during precipitation is also shown in Table III. Mean drug recovery at the lowest and the highest L-carnitine concentrations of the calibration ranges were 81.44% and 84.85%, respectively. Although mean recovery of L-carnitine was almost in agreement with the 82.6-84.80% obtained by Longo *et al.* (1996), the accuracy and precision were highly satisfactory as shown in Table I and II.

The current HPLC analytical conditions were also applied to milk and muscle samples. The HPLC chromatogram of L-carnitine in plasma, milk and muscle from a drug distribution study performed two months after oral administration of 24 g L-carnitine/day to cows is shown in Fig. 3. The peaks of L-carnitine and internal standard were well separated as shown in the HPLC chromatogram while there was a big unknown peak between drug and internal standard peaks. Concentrations of L-carnitine in plasma, milk and muscle are shown in Table IV. Drug concentrations in plasma and milk were 2.96 mg/mL and 26.09 mg/mL, and that in muscle was 1.91 mg/g. The concentrations of L-carnitine in milk and muscle samples were much higher than in the plasma samples. Most of the total body carnitine is stored in skeletal muscle due to its larger size and higher L-carnitine concentration. The results also suggest that carnitine in blood is much less concentrated than in muscle tissues (Campos *et al.*, 1993; Di Donato, 1994). The current assay method was selective as demonstrated by the lack of interfering peaks in the dialyzed samples following a visual inspection of the chromatogram. The advantages of this improved HPLC method are mainly its lower LOQ, the relatively low cost of

Table III. Recovery of L-carnitine during precipitation

Concentration (ng/mL)	Recovery (mean ± SD) (%)	C.V. (%)
50	81.44 ± 1.94	2.38
5000	84.85 ± 1.13	1.33

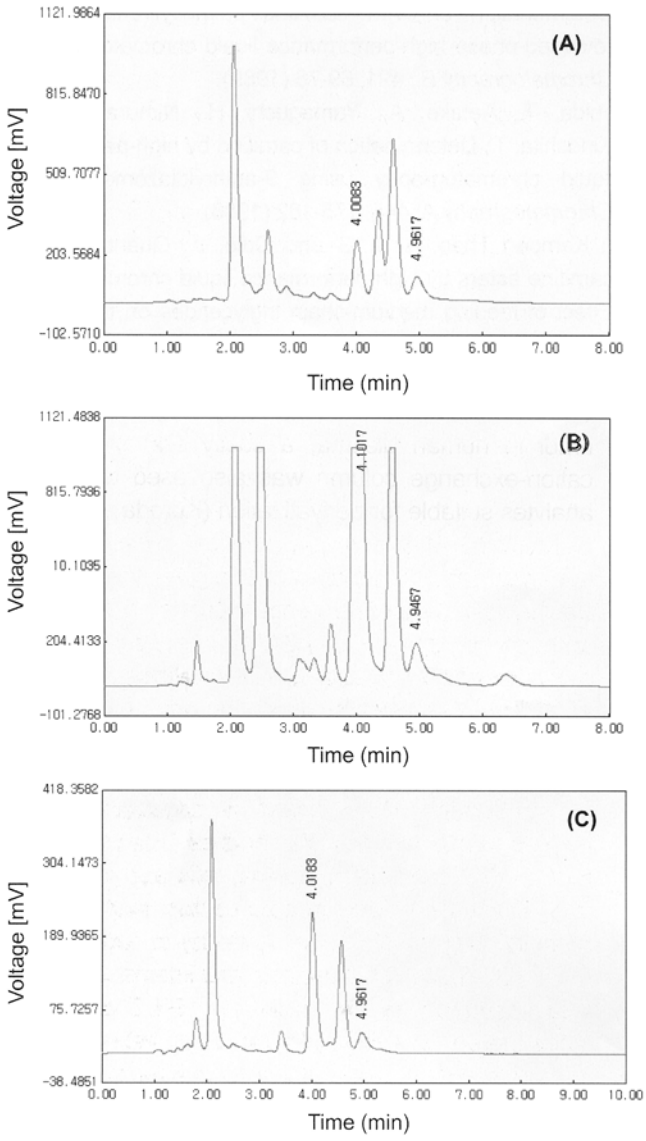

Fig. 3. HPLC chromatograms of L-carnitine in the biological fluids two months after oral administration of 24 g L-carnitine/day. Plasma (A), milk (B) and muscle (C).

Table IV. Concentrations of L-carnitine in plasma, milk and muscle two months after oral administration of 24 g L-carnitine/day to five cows

Sample	Plasma (μg/mL)	Milk (μg/mL)	Muscle (mg/g)
Drug concentration in biological sample (mean ± SD)	2.96 ± 0.91	26.09 ± 9.27	1.91 ± 0.31

the analytical process and its potential application to different types of biological samples.

CONCLUSIONS

A new and sensitive HPLC method for the determination of L-carnitine in biological samples such as plasma, milk and muscle in cows was successfully established based upon the synthesis of fluorescent derivatives. This method demonstrated good specificity, reproducibility, linearity, accuracy and precision. It could be applied to the detection of L-carnitine concentration in various biological samples of animals over a suitable calibration range (50-5000 ng/mL).

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