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## Note

# Determination of total carnitine in human urine by high-performance liquid chromatography

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Carnitine, 4-(*N,N,N*-trimethylammonio)-3-hydroxybutanoate, is required for the oxidation of long-chain fatty acids by mitochondria [1,2]. Carnitine content in biological samples is usually determined radioenzymatically [3–8]. As an alternative to these radioenzymatic methods we have developed a high-performance liquid chromatographic (HPLC) method capable of determining total carnitine in human urine.

Derivatization of carboxylic acids for enhancement of sensitivity using conventional UV detectors after HPLC separation has been shown [9, 10]. We developed a new reagent for this purpose: 4′-bromophenacyl triflate [11]. We then demonstrated derivatization of carnitine, 4-(*N,N,N*-trimethylammonio)-butanoate (butyrobetaine), and 2-(*N,N,N*-trimethylammonio)acetate (betaine) standard solutions using this reagent followed by HPLC separation of the derivatives [12]. We now present results of the application of this analytical technique to the determination of total carnitine in human urine.

Carnitine is present as both non-acylated free carnitine and carnitinyl esters (acylcarnitines) in biological systems. Total carnitine is the term used to denote the molar sum of free carnitine and acylcarnitines. Total carnitine in biological specimens is determined by alkaline hydrolysis of the contained acylcarnitines and subsequent determination of all carnitine present. The quantification of total carnitine in human urine by HPLC and the comparison of these values with those determined in the same samples by an established radioenzymatic method [7] is the subject of this manuscript.

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## EXPERIMENTAL

### *Equipment*

The liquid chromatograph consisted of a Model 6000A pump, WISP-710B automatic sampler, RCM-100 radial compression module, and Model 440 fixed-wavelength detector (operated at 254 nm) purchased from Waters Assoc. (Milford, MA, U.S.A.). The chromatographic separation was accomplished using a $10 \times 0.5$ cm plastic cartridge containing Radial-Pak $C_{18}$ of 10 $\mu$m particle diameter (Waters Assoc.). The chromatographic column was protected by a $5 \times 0.4$ cm precolumn packed with Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.). The detector output signal was displayed on a Model 8373-00 chart recorder (Cole Parmer Instrument, Chicago, IL, U.S.A.). A Hewlett-Packard (Avondale, PA, U.S.A.) Model 3354C laboratory automation system was used for chromatographic peak identification based on relative retention time, peak-area integration, peak-height measurement, and calculations derived from those measurements.

### *Materials*

Acetonitrile (non-spectro grade) was purchased from Burdick & Jackson (Muskegon, MI, U.S.A.). The acetonitrile was filtered through nylon membranes of 0.45 $\mu$m pore diameter. Reagent-grade water was prepared by passage through a Milli-Q reagent-grade water system (Millipore, Bedford, MA, U.S.A.). 3-(Dimethylamino)-1,2-propanediol and *N,N*-diisopropylethylamine were purchased from Aldrich (Milwaukee, WI, U.S.A.). Sodium dodecylsulfate was purchased from Gallard Schlesinger (Carle Place, NY, U.S.A.). Phosphoric acid (85%) and sodium phosphate (monobasic) were purchased from Fisher Scientific (Cleveland, OH, U.S.A.).

The derivatization reagent 4′-bromophenacyl triflate and the internal standard 4-(*N,N*-dimethyl-*N*-(*n*-propyl)ammonio)-3-hydroxybutanoate were synthesized as described before [11, 12]. Dowex 1-X8 (200–400 mesh, $Cl^-$ form) and Dowex 50-X8 (200–400 mesh, $H^+$ form) were purchased from Sigma (St. Louis, MO, U.S.A.) and converted into the $OH^-$ and $NH_4^+$ forms, respectively, according to instructions published by Bio-Rad Labs. (Richmond, CA, U.S.A.). Conversion of the anion-exchange resin must be continued until tests for $Cl^-$ in the column effluent are negative.

### *Sample preparation*

Urine (250 $\mu$l), 4-(*N,N*-dimethyl-*N*-(*n*-propyl)ammonio)-3-hydroxybutanoate (internal standard, 250 $\mu$l, 200 nmol/ml), and 1 *M* potassium hydroxide (125 $\mu$l) were combined in a $12 \times 75$ mm polypropylene test tube. After allowing 10 min for acylcarnitine hydrolysis, 250 $\mu$l of this solution were applied to a double column consisting of a $3.5 \times 0.5$ cm column of Dowex 50 ($NH_4^+$ form) carefully layered above a $3.5 \times 0.5$ cm column of Dowex 1 ($OH^-$ form) anion-exchange resin, both contained in a single pasteur pipet. The column was eluted with 2.5 ml of 0.5 *M* ammonium hydroxide, and the effluent was collected and evaporated to dryness. Quantification standards were prepared by combining 250 $\mu$l of car-

TABLE I

STANDARD CURVE OF CARNITINE/INTERNAL STANDARD CHROMATOGRAPHIC PEAK-HEIGHT RATIOS VERSUS CARNITINE CONCENTRATION WITH 4-(*N,N*-DIMETHYL-*N*-(*n*-PROPYL)AMMONIO)-3-HYDROXYBUTANOATE AS INTERNAL STANDARD

| | Slope | *y*-Intercept | Linear regression coefficient ($r^2$) |
|---|---|---|---|
| Initial injection of standard solutions | $7.39 \cdot 10^{-3}$ | $5.59 \cdot 10^{-2}$ | 0.9946 |
| Repeat injection of standard solutions★ | $7.32 \cdot 10^{-3}$ | $5.43 \cdot 10^{-2}$ | 0.9949 |

★Injected after injections of 25 human urine specimens (prepared in duplicate); 24 h of continuous sampling separates the injections of these solutions.

nitine (50–500 nmol/ml), 250 μl of 4-(*N,N*-dimethyl-*N*-(*n*-propyl)ammonio)-3-hydroxybutanoate (200 nmol/ml), and 250 μl of 4-(*N,N,N*-trimethylammonio)butanoate (50–500 nmol/ml). A 300-μl aliquot of this solution was applied to a double column and treated as described above.

*Derivatization*

Each sample tube received 100 μl of *N,N*-diisopropylethylamine in acetonitrile solution ($3 \cdot 10^{-3}$ *M*) and was vortexed for 2 min. Next was added 100 μl of 4′-bromophenacyl triflate reagent in acetonitrile ($7.5 \cdot 10^{-3}$ *M*) followed by 2 min of vortexing.

*Chromatography*

The chromatographic eluent was prepared by dissolving 3.56 g of sodium dodecylsulfate, 2.21 g of sodium phosphate (monobasic), and 4.75 ml of 3-(dimethylamino)-1,2-propanediol in 2200 ml of water and adjusting the pH to 6.5 with concentrated phosphoric acid. This solution was filtered through a 0.45 μm pore diameter membrane filter and added to 5800 ml of filtered acetonitrile with thorough mixing. The flow-rate was 3.0 ml/min. The derivatized samples (20 μl) were injected directly into the HPLC system. The frequency of injection was every 25 min.

*Quantification*

Standard curves of carnitine/4-(*N,N*-dimethyl-*N*-(*n*-propyl)ammonio)-3-hydroxybutanoate peak-height ratios versus carnitine concentration were established. The carnitine concentration in experimental samples was interpolated from a least-squares regression line through the standard data points. All standards and experimental samples were analyzed in duplicate.

TABLE II

COMPARISON OF TOTAL CARNITINE VALUES DETERMINED BY RADIOENZYMATIC ANALYSIS (REA) AND BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Mean of the percentage error = 6.2 ± 3.9; least-squares fit of HPLC versus REA data: slope = 1.14, *y*-intercept = −24.2, linear regression coefficient = 0.955.

| Patient No. | REA (nmol/ml) | HPLC (nmol/ml) | Percentage error (mean deviation) |
|---|---|---|---|
| 1 | 177.9 | 179.2 | 0.4 |
| 2 | 322.1 | 320.7 | 0.2 |
| 3 | 340.0 | 398.9 | 7.8 |
| 4 | 110.2 | 121.2 | 4.6 |
| 5 | 304.4 | 338.2 | 5.3 |
| 6 | 286.1 | 288.9 | 0.5 |
| 7 | 87.2 | 97.7 | 5.7 |
| 8 | 68.7 | 86.9 | 11.7 |
| 9 | 88.4 | 82.8 | 3.3 |
| 10 | 147.0 | 114.7 | 12.3 |
| 11 | 204.4 | 159.2 | 12.4 |
| 12 | 97.8 | 87.9 | 5.4 |
| 13 | 63.5 | 54.1 | 8.0 |
| 14 | 95.4 | 93.0 | 1.3 |
| 15 | 228.0 | 258.0 | 3.7 |
| 16 | 168.3 | 164.4 | 1.2 |
| 17 | 306.9 | 358.7 | 7.8 |
| 18 | 189.4 | 199.2 | 2.5 |
| 19 | 286.1 | 339.9 | 8.6 |
| 20 | 61.7 | 52.5 | 8.1 |
| 21 | 138.6 | 118.5 | 7.8 |
| 22 | 114.3 | 89.6 | 12.1 |
| 23 | 125.6 | 100.9 | 10.9 |
| 24 | 144.5 | 125.9 | 6.9 |
| 25 | 92.2 | 83.0 | 5.3 |

## RESULTS AND DISCUSSION

The current radioenzymatic methods available for the determination of carnitine [4–8] depend upon the recognition of carnitine by the enzyme carnitine acetyltransferase in the presence of [$^{14}$C] acetyl coenzyme A, resulting in the formation of [$^{14}$C] acetylcarnitine. This species is then quantified by scintillation counting. These methods are accurate and precise, but must be performed with strict attention to reaction time and conditions for the enzyme to perform predictably. Additionally, this enzyme-based strategy is not directly applicable to quantification of either the biosynthetic precursor of carnitine [4-(*N,N,N*-trimethylammonio)butanoate, butyrobetaine] or acylcarnitines. We have chosen to pursue a more general analytical approach: sensitivity enhancement by chemical derivatization followed by HPLC separation and quantification with UV detection. Using 4′-bromophenacyl triflate as the dervatization reagent, we have shown in standard solutions that this approach is rapid, convenient, and suffi-


Fig. 1. Chromatogram of a water blank carried through the analytical procedure. The chromatographic separation was accomplished using a $10 \times 0.5$ cm plastic cartridge containing Radial-Pak $C_{18}$ of 10 $\mu$m particle diameter. The eluent was $1.58 \cdot 10^{-3}$ *M* sodium dodecylsulfate, $2 \cdot 10^{-3}$ *M* sodium phosphate (monobasic), and $5 \cdot 10^{-3}$ *M* 3-(dimethylamino)-1,2-propanediol in water-acetonitrile (27.5:72.5, v/v). The aqueous component of the eluent was adjusted to pH 6.5 with concentrated phosphoric acid before addition to acetonitrile. The eluent flow-rate was 3.0 ml/min, the injection volume was 20 $\mu$l, and the absorbance detector was operated at 254 nm. Full-scale absorbance is 0.1 absorbance units.

ciently sensitive for determination of carnitine in biological specimens [12]. Also, since this approach relies only on the presence of a carboxylate functional group within the species of interest, it should be equally applicable to carnitine, butyrobetaine, and acylcarnitines.

As a preliminary step we chose to examine the simplest case: determination of total carnitine in human urine. Our results using radioenzymatic methods indicated that the concentration of total carnitine in urine is typically 50–500 nmol/ml, which is well above the spectrophotometric detection sensitivity limit for carboxylate 4′-bromophenacyl ester derivatives. Since butyrobetaine is also a part of the biological sample matrix, it was our hope to determine both total carnitine and butyrobetaine simultaneously. Therefore, standard solutions containing both of these compounds were prepared for experimental quantification. To internally

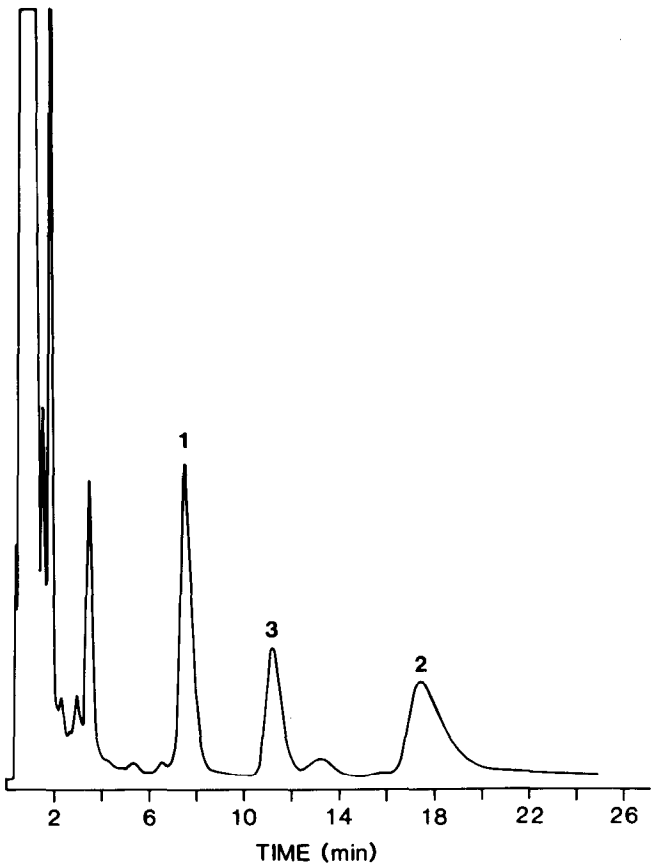


Fig. 2. Chromatogram of a derivatized standard solution containing carnitine (200 nmol/ml), internal standard (200 nmol/ml), and butyrobetaine (200 nmol/ml). Chromatographic conditions are described under Fig. 1. Peak identities: 1 = carnitine; 2 = butyrobetaine; 3 = internal standard.

standardize the procedure, we synthesized 4-(*N,N*-dimethyl-*N*-(*n*-propyl)ammonio)-3-hydroxybutanoate [12] and added an appropriate quantity of this compound to the standard solutions and human urine specimens.

The use of small columns of ion-exchange resin for sample simplification has been very useful to us in the past [7, 12–14]. Successful carnitine derivatization required the complete removal of large accompanying quantities of inorganic salts from the urine sample matrix. These salts otherwise interfered with the derivatization of $\omega$-trialkylammonio acids, both by occlusion of the compounds of interest within the solid residue from sample evaporation and by side-reactions of nucleophilic anions with the derivatization reagent. The recovery of $\omega$-trialkylammonio carboxylates from this small column system was demonstrated by the addition of a 10-$\mu$l aliquot of [$^{14}$C-methyl]butyrobetaine (2000 dpm) to 300 $\mu$l of an aqueous solution containing standard carnitine, butyrobetaine, and internal standard. This solution was applied to a double column, the eluent collected, and the contained radioactivity determined, demonstrating a recovery of $104 \pm 3\%$ ($n=5$).

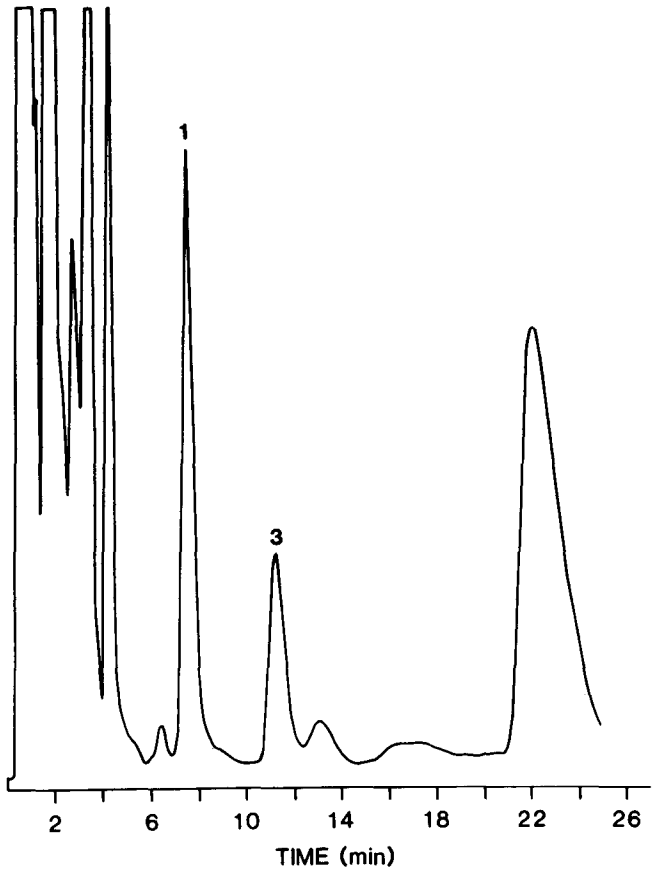


Fig. 3. Chromatogram of 250 $\mu$l of human urine prepared as described in the text. Conditions are described under Fig. 1. Peak identities: 1 = carnitine; 3 = internal standard. Carnitine quantification: 398.9 nmol/ml.

Human urine specimens of measured carnitine concentration (by radioenzymatic determination [7]) were selected for the HPLC total carnitine determination experiment. Aqueous standard solutions, 25 urine specimens, and 1 aqueous blank were carried through the entire determination procedure in duplicate. The standard curve samples were injected first and reinjected after chromatography of the entire experimental sample set. Carnitine concentrations were calculated as the mean of sample duplicates. Table I represents the results of two serial injections of the standard curve specimens. Agreement of the slope and $y$-intercept values was excellent despite the 24-h period between experimental trials. These data showed that derivatized samples are stable for 24 h at least. The average mean deviation of all duplicate standard curve and urine specimens was $1.9 \pm 1.5\%$, and in no case exceeded 5.5.%. Table II displays the total carnitine values determined by both the radioenzymatic method [7] and the HPLC method described here. The small mean percentage error (mean deviation of both measurements) values and the good least-squares correlation of the results of these two fundamentally different determination methods strengthened our confidence in the accuracy of both techniques.

Butyrobetaine was included in the standard solutions used to generate the carnitine standard curve. Its standard curve was also excellent: slope = 0.0023, $y$-intercept = 0.0139, and linear regression coefficient = 0.9934. However, butyrobetaine cannot be determined in human urine with this procedure due to chromatographic interference and/or insufficient concentration (less than 50 nmol/ml, therefore below the described standard curve) of butyrobetaine. Butyrobetaine could be determined by this method if the chromatographic eluent were optimized for its resolution and detection. Alternatively, butyrobetaine can be determined radioenzymatically [15, 16].

We have shown that the conversion of carnitine into the 4′-bromophenacyl ester is complete under these reaction conditions [12]. However, in some urine samples we observed that the height of the internal standard peak was reduced; overall there was internal standard peak-height variability of 30%. We interpreted this to mean that the added quantity of derivatization reagent was not sufficient to derivatize all reactive species in these samples. Despite incomplete derivatization noted in some samples, the quantification of carnitine should be correct since the procedure is internally standardized by addition of a close chemical analogue of carnitine to all samples. Calculation of sample carnitine concentration is based on carnitine/internal standard peak-height ratios, rather than on absolute carnitine derivative peak heights. This claim of quantitative accuracy is supported by the good agreement between values obtained using HPLC and those obtained by the radioenzymatic method.

Fig. 1 is a chromatogram of an aqueous sample blank carried through the small column procedure, derivatization, and HPLC. Fig. 2 is a chromatogram of a standard curve solution containing 200 nmol/ml carnitine, 200 nmol/ml internal standard, and 200 nmol/ml butyrobetaine after small column isolation, derivatization, and injection into the HPLC system. Fig. 3 is a chromatogram of a typical human urine specimen carried through the analytical procedure.

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