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DETERMINATION OF CARNITINE, BUTYROBETAINE, AND BETAINE AS 4'-BROMOPHENACYL ESTER DERIVATIVES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A method for determination of carnitine, 4-(N,N,N-trimethylammonio)butanoate (butyrobetaine), and 2-(N,N,N-trimethylammonio)acetate (betaine) is described. These ω-trimethylammonio carboxylates and the chemically analogous internal standards 4-(N,N-dimethyl-N-propylammonio)-3-hydroxybutanoate or 6-(N,N,N-trimethylammonio)hexanoate were derivatized by reaction with 4'-bromophenacyl triflate in the presence of N,N-diisopropylethylamine. The trialkylammonio carboxylate 4'-bromophenacyl ester derivatives were separated from other sample constituents by reversed-phase ion-pair high-performance liquid chromatography with spectrophotometric detection at 254 nm. Standard curves were linear over a sample concentration range of 10—100 nmol/ml. Quantities of 2.5 nmol of ω-trialkylammonio acid derivatives injected into the chromatograph were detected with signal-to-noise ratios greater than 50.

INTRODUCTION

The mitochondrial fatty acid acyltransferase cofactor carnitine [4-(N,N,N-trimethylammonio)-3-hydroxybutanoate] has been the object of various determination schemes. The first reported biological assay procedure related growth of the mealworm *Tenebrio molitor* to growth medium carnitine content [1]. Carnitine also has been determined by various spectrophotometric means based upon complex formation between its quaternary ammonium functionality and chromophoric anions. Periodide [2] and bromophenol blue [3, 4] complexes have been used for this purpose. Visualization of carnitine on thin-layer chromatograms has been accomplished through use of iodine [5], and by complex formation with iodoplatinate [6]. An alternative approach exploited

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enzymatic recognition of carnitine by carnitine acetyltransferase, leading to detection of a chromophoric coupled reaction product [7]. This concept was further modified by use of radioactively acetate-labeled acetyl coenzyme A and measurement of the production of acetate-labeled acetylcarnitine [8—12]. Of related interest is the determination of 4-(N,N,N-trimethylammonio)-butanoate (4-TMA-butanoate, butyrobetaine), which has been established as a biosynthetic precursor of carnitine [13—16]. This compound undergoes complex salt formation with the reagents used for spectrophotometric determination of carnitine [2, 5]. In addition, 4-TMA-butanoate has been determined indirectly by enzymatic conversion to carnitine in the presence of butyrobetaine hydroxylase and subsequent radioenzymatic determination of the carnitine product [16, 17]. 2-(N,N,N-Trimethylammonio)acetate (2-TMA-acetate, betaine) is an intermediate in the metabolism of choline [18, 19], and has been determined spectrophotometrically [2, 5], radioenzymatically [18, 19], and by high-performance liquid chromatography (HPLC) [20].

With the advent of HPLC and its application to analytical problems, we sought to use this new technique for our work related to the biosynthesis of carnitine. The carboxyl group is the most analytically accessible structural feature of carnitine and related ω-trimethylammonio acids (TMA acids). However, carboxyl groups are weakly chromophoric, and are neither fluorophoric nor electrophoric to any useful degree. Therefore, carboxylates with no other functionality permitting sensitive detection are derivatized for their determination at low concentration by HPLC. Among the most useful of reported acid derivatives are 4′-bromophenacyl esters [20—31]. Unfortunately, the four-carbon TMA acids proved to be both unreactive toward common carboxyl-O-alkylation reagents and thermally labile, making the usual conditions of 4′-bromophenacyl ester formation [21, 22, 32] entirely unsatisfactory. To solve this problem, we developed a new derivatizing agent with substantially increased alkylative reactivity, 4′-bromophenacyl triflate [33]. This reagent allows mild, rapid, and quantitative derivatization of carnitine, 4-TMA-butanoate, 2-TMA-acetate, and other trialkylammonio carboxylates. With this reagent, we developed a procedure for the determination of these trimethylammonio acids in standard solutions at concentrations of 10—100 nmol/ml.

This paper presents that method. Sample preparation by ion-exchange chromatography, derivatization by reaction with 4′-bromophenacyl triflate, separation from sample constituents by reversed-phase ion-pair HPLC, and spectrophotometric detection permit quantitative measurements of these compounds within a concentration range potentially useful for biological sample determinations.

EXPERIMENTAL

Equipment

The liquid chromatograph consisted of a Model 6000A pump, a U6K syringe loading sample injection valve, an RCM-100 radial compression module, and a Model 440 fixed-wavelength spectrophotometric detector purchased from Waters Assoc. (Milford, MA, U.S.A.). The chromatographic separation was

accomplished on a 10 × 0.8 cm plastic cartridge containing Radial-Pak C_{18} of 5 μm nominal particle diameter (Waters Assoc.). The chromatographic column was protected against particulate sample contaminants by a 5 × 0.4 cm column constructed from zero-dead-volume chromatographic unions (Crawford Fitting, Solon, OH, U.S.A.) and packed with the pellicular reversed-phase medium Co:Pell ODS (Whatman, Clifton, NJ, U.S.A.). The detector was operated at 254 nm, and its output signal was recorded by a Linear Instruments (Irvine, CA, U.S.A.) Model 291 chart recorder. A Hewlet-Packard (Avondale, PA, U.S.A.) Model 3354C laboratory automation system was used for chromatographic peak area integration, peak height measurement, and calculations derived from those measurements. A Waters Assoc. WISP-710A automatic sampler was used in experiments related to establishment of standard curves. Liquid scintillation counting was performed with a Packard Instruments (Downers Grove, IL, U.S.A.) PRIAS scintillation spectrometer. ^1H Magnetic resonance spectra were acquired with a Bruker Instruments (Billerica, MA, U.S.A.) WH-270 pulsed Fourier transform nuclear magnetic resonance (NMR) spectrometer. A Kraft Apparatus (Mineola, NY, U.S.A.) Big Vortex unit was used for continuous mixing of samples during derivatization procedures.

Materials

Acetonitrile (OmniSolv, non-UV grade) was purchased from MCB (Cincinnati, OH, U.S.A.) and filtered through nylon membranes of 0.45 μm nominal pore size before use in chromatographic mobile phases. Acetonitrile intended for use in derivatization reactions was distilled from calcium hydride. Water was prepared for use as a chromatographic mobile phase constituent by passage through a Milli-Q water purification unit (Millipore, Bedford, MA, U.S.A.). Sodium dodecyl sulfate (SDS, electrophoresis grade) was purchased from Gallard-Schlesinger (Carle Place, NY, U.S.A.). Sodium dihydrogen phosphate and phosphoric acid (85%), N,N-dimethylformamide (DMF), and ethyl acetate were purchased from Fisher Scientific (Cleveland, OH, U.S.A.). Triethylamine, N,N-diisopropylethylamine, 3-(N,N-dimethylamino)-1-propanol, and 3-(N,N-dimethylamino)-1,2-propanediol were purchased from Aldrich (Milwaukee, WI, U.S.A.).

(*l*)-Carnitine (chloride) was a generous gift of the Otsuka Pharmaceutical Factory (Naruto, Tokushima, Japan). 2-TMA-acetate and hydroxyacetic (glycolic) acid were purchased from Sigma (St. Louis, MO, U.S.A.). 4-Aminobutanoic acid was purchased from Nutritional Biochemicals (Cleveland, OH, U.S.A.). 6-Aminohexanoic acid was purchased from Chemical Procurement Labs. (College Pt., NJ, U.S.A.). 2,4′-Dibromoacetophenone was purchased from Aldrich. 1-Iodopropane was purchased from Eastman Organic Chemicals (Rochester, NY, U.S.A.). Dowex 1-X8 (200–400 mesh, Cl^- form) anion-exchange resin was purchased from Sigma and converted to the OH^- form according to instructions published by Bio-Rad Labs. (Richmond, CA, U.S.A.).

Synthesis of authentic compounds

4′-Bromophenacyl triflate was synthesized as described previously [33]. Authentic TMA-carboxylates were synthesized from the corresponding ω-amino acids according to modifications [16] of the method of Linstedt and

Lindstedt [14]. 4-(N,N-Dimethyl-N-propylammonio)-3-hydroxybutanoate (N-propylcarnitine) was prepared by N-demethylation of carnitine [34] and alkylation of the resultant 4-(N,N-dimethylamino)-3-hydroxybutanoic acid by 1-iodopropane. Product identity was confirmed by ^{1}H NMR spectroscopy. [^{14}C-Methyl]trimethylammoniobutanoate was synthesized and purified as described [16], as was [^{14}C-methyl]carnitine [34].

Authentic 4′-bromophenacyl esters of TMA-carboxylates were synthesized by slight modifications of the method of Cooper and Anders [32]. Thus, a TMA acid (Cl^- salt) was applied in aqueous solution to a column of Dowex 1-X8 (OH^- form) anion-exchange resin of excess ion-exchange capacity and eluted with 4 column vols. of deionized water. The effluent was collected, evaporated to dryness, and redissolved in DMF. To this were added 1.1 molar equivalents of both triethylamine and 2,4′-dibromoacetophenone, and the solution was heated to 40°C with stirring. After 3 h, the product was precipitated by addition of ethyl acetate, collected by filtration, and recrystallized from ethanol—acetone. Product identities were confirmed by ^{1}H NMR spectroscopy, and the products were shown to be of greater than 99% chromatographic purity at 254 nm.

Sample preparation

Standard solutions of carnitine, 4-TMA-butanoate, and 2-TMA-acetate were prepared in water at concentrations of 10—100 nmol/ml. Aqueous working solutions of N-propylcarnitine and 6-TMA-hexanoate were prepared at concentrations of 1 μmol/ml. The former compound was used as a procedural internal standard for carnitine determination, while the latter was used for determinations of 4-TMA-butanoate and 2-TMA-acetate. In a 1.5-ml (13 × 39 mm) polypropylene Eppendorf tube were combined 600 μl aqueous TMA-carboxylate standard solution and 60 μl of the appropriate trialkylammonio carboxylate internal standard working solution. The tube was vortexed, and 550 μl of the contents were applied to a 7 × 0.5 cm column of Dowex 1-X8 (OH^- form) anion-exchange resin contained by a Pasteur pipet. Excluded species were eluted from the column with 2 ml of deionized water. The effluent was collected in a 13 × 100 mm polypropylene test tube and the contents evaporated to dryness by a gentle stream of oil-free compressed air.

Derivatization

The dry residue from the isolation procedure was reconstituted in 100 μl of $2 \cdot 10^{-3}$ *M* N,N-diisopropylethylamine in distilled acetonitrile and vortexed for 2 min. To this were added 100 μl of a $5 \cdot 10^{-3}$ *M* solution of 4′-bromophenacyl triflate in acetonitrile, and the sample was vortexed for 10 min. The excess alkylating agent was destroyed by addition of 100 μl of $1 \cdot 10^{-2}$ *M* hydroxyacetic acid N,N-diisopropylethylammonium salt in acetonitrile and vortexing for 2 min. Sample aliquots of 15 μl were injected directly into the chromatograph.

Chromatography

Two chromatographic eluents were used. The mobile phase used for chro-

matography of carnitine 4′-bromophenacyl ester derivatives was $5.0 \cdot 10^{-4}$ *M* SDS, $2.0 \cdot 10^{-3}$ *M* sodium dihydrogen phosphate, and $5.0 \cdot 10^{-3}$ *M* 3-(N,N-dimethylamino)-1,2-propanediol in acetonitrile—water (75:25, v/v). This was prepared by dissolving 0.070 g ($2.5 \cdot 10^{-4}$ mol) of SDS, 0.140 g ($1 \cdot 10^{-3}$ mol) of sodium dihydrogen phosphate (monohydrate), and 0.30 ml (0.30 g, $2.5 \cdot 10^{-3}$ mol) of 3-(N,N-dimethylamino)-1,2-propanediol in 125 ml water, adjusting the pH to 6.5 with 85% phosphoric acid, and filtering this solution through a 0.2-μm pore diameter cellulose nitrate membrane. This was combined with 375 ml of filtered acetonitrile with thorough magnetic stirring. The mobile phase used for chromatography of 2-TMA-acetate and 4-TMA-butanoate 4′-bromophenacyl esters was $1.0 \cdot 10^{-3}$ *M* SDS, $1.0 \cdot 10^{-3}$ *M* sodium dihydrogen phosphate, and $1.0 \cdot 10^{-2}$ *M* 3-(N,N-dimethylamino)-1-propanol in acetonitrile—water (70:30, v/v). This was prepared analogously. The eluents in both cases were pumped at 5.0 ml/min. Absorbance of the effluent stream was monitored at 254 nm.

Verification of sample recovery through sample preparation columns

The recovery of TMA-carboxylates from the Dowex 1-X8 anion-exchange resin columns was demonstrated by application of aliquots of [^{14}C-methyl]-carnitine to several columns and collection of 0.5-ml eluent fractions directly in 5.5-ml scintillation vials. Scintillation cocktail was added, and the radioactivity of each fraction was determined by liquid scintillation counting. Recovery of applied radioactivity was determined by comparison of eluted radioactivity with the radioactivity found in aliquots of labeled material equal in volume to that applied to the sample preparation column.

Reaction completion verification

The extent of completion of the reaction of 4′-bromophenacyl triflate with the TMA-carboxylates under the recommended conditions was evaluated by subjecting an aliquot of [^{14}C-methyl]carnitine to the entire procedure. The resulting reaction mixture was chromatographed as described, but at an eluent flow-rate of 1.0 ml/min to permit collection of 1.0-ml eluent fractions in 5.5-ml scintillation vials. Scintillation cocktail was added and the contained radioactivity determined by liquid scintillation counting. Recovery of the sample radioactivity from the chromatograph was established by comparison of the radioactivity found within the eluent fractions and that detected in a reaction mixture aliquot equal in volume to that chromatographed.

Quantitation

Standard curves of carnitine:N-propylcarnitine, 2-TMA-acetate:6-TMA-hexanoate, and 4-TMA-butanoate:6-TMA-hexanoate were established over a sample concentration range of 10—100 nmol/ml. All standard solutions were determined in duplicate for standard curve generation. Linearity of detector response over this sample concentration range was established by algebraic least-squares fit of determined chromatographic peak height ratios and their respective sample concentrations to a linear equation.

Time course of the reaction of carnitine and 4-TMA-butanoate with 2,4'-dibromoacetophenone (Fig. 1)

In 15-ml conical screw-capped test-tubes were combined 200 µl of a 10^{-4} *M* solution of carnitine or 4-TMA-butanoate in ethanol, $2 \cdot 10^4$ dpm of the corresponding ^{14}C-labeled substrate, and 3 µl of $6 \cdot 10^{-2}$ *M* N,N-diisopropylethylamine in methanol. The tubes were vortexed, and the solvents were evaporated under a compressed air stream. The reaction was started by addition of 500 µl of $7 \cdot 10^{-3}$ *M* 2,4'-dibromoacetophenone in isopropanol. The tubes were capped, placed in a water bath at 70°C, and shaken continuously during the reaction period. At the indicated time intervals, one tube was cooled to room temperature, opened, and 300 µl of water were added to the reaction mixture. The solution was extracted with 6 ml of *n*-butyl acetate, and a 75 µl-aliquot of the aqueous phase was injected into the liquid chromatograph.

Chromatography was performed with the apparatus described under *Equipment*. A 30 × 0.39 cm steel column packed with µBondapak C_{18} reversed-phase medium (10 µm nominal particle diameter) was used. The mobile phase was $5 \cdot 10^{-3}$ *M* sodium heptanesulfonate in acetonitrile—water (62:38, v/v), and was pumped at 1.5 ml/min. Eluent fractions of 0.5 ml were collected, and the contained radioactivity was determined as described. The extent of reaction was calculated by comparison of the radioactivity which co-chromatographed with the carnitine or 4-TMA-butanoate derivative peak with that contained in a second 75-µl aliquot of the same reaction solution extract. These derivatization and chromatographic conditions were found to be unsuited for determination of TMA-carboxylates at lower sample concentrations.

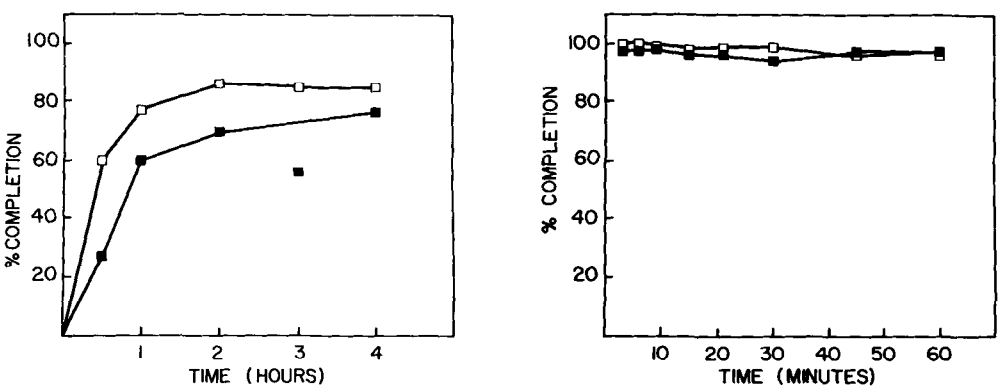

Fig. 1. Extent of reaction of carnitine (▪) and 4-TMA-butanoate (▫) with 2,4'-dibromoacetophenone versus time. Details are given in the Experimental section. The derivatization and chromatographic conditions were found to be unsuited for determination of TMA-carboxylates at low concentrations.

Fig. 2. Extent of reaction of carnitine (▪) and 4-TMA-butanoate (▫) with 4'-bromophenacyl triflate versus time. Details are given in the Experimental section. Data points are the average of two determinations.

Time course of the reaction of carnitine and 4-TMA-butanoate with 4'-bromophenacyl triflate (Fig. 2)

In 15-ml conical screw-capped test tubes were combined 2 µl of a 10^{-1} *M* ethanolic solution of carnitine or 4-TMA-butanoate, and 2 µl of a solution of

the corresponding ^{14}C-labeled substrate containing about $8 \cdot 10^4$ dpm of radioactivity. The samples were neutralized by addition of 10 μl of a solution of $8.4 \cdot 10^{-2}$ *M* tri-*n*-butylamine in acetonitrile, and then were evaporated to dryness. The reaction was started by addition of 150 μl of $8.6 \cdot 10^{-3}$ *M* 4′-bromophenacyl triflate in acetonitrile and 50 μl of $2.1 \cdot 10^{-2}$ tri-*n*-butylamine in acetonitrile. The tubes were vortexed, capped, and allowed to stand at room temperature. At the indicated time intervals, a pair of tubes were immersed in a dry-ice—acetone bath, the frozen solvent was removed under vacuum, and the contents reconstituted in 500 μl of 10^{-2} *M* hydrochloric acid. A 50-μl aliquot was injected directly into the chromatograph without extraction. Chromatography, fraction collection, and liquid scintillation counting were performed as described for trials with 2,4′-dibromoacetophenone, although no spectrophotometric detector was used. The extent of reaction was determined by comparison of the radioactivity isolated in fractions containing the carnitine or 4-TMA-butanoate derivative peaks with the sum of the radioactivity isolated in both those fractions and fractions containing unreacted labeled substrate. Overall recovery of radioactivity was calculated by comparison of total radioactivity isolated from the chromatographic column with that contained in a second 50-μl aliquot of the reconstituted residue from the derivatization reaction. These recoveries were 95% (± 7%, n = 16) in experiments with carnitine, and 89% (± 7%, n = 16) in experiments with 4-TMA-butanoate.

RESULTS AND DISCUSSION

Our work related to the biosynthesis of carnitine necessitated the development of an analytical method for the determination of several TMA-carboxylates with a measurement limit of 10 nmol/ml. These compounds are not amenable to detection by any of the usual methods employed for HPLC without prior derivatization. 4′-Bromophenacyl ester derivatives have proved useful for highly sensitive determinations of carboxylic acids. These derivatives are intensely chromophoric (λ_{max} = 250—260 nm, log ϵ = 3.8–4.5 [21, 22]), the required alkylating agent (2,4′-dibromoacetophenone) is commercially available and inexpensive, and the methodology of the derivatization procedure apparently has been well developed [20—31]. Two important general methods have been employed for preparation of 4′-bromophenacyl esters on the analytical scale. Durst et al. [22] reacted 10^{-3} *M* carboxylate potassium salts with a two-fold excess of 2,4′-dibromoacetophenone and 5—10 mol% of the macrocyclic polyether phase-transfer catalyst 18-crown-6 in acetonitrile at 80°C for periods of 15—30 min. Cooper and Anders [32] prepared the analogous 2-naphthacyl esters by reaction of carboxylate trialkylammonium salts with similar equivalent proportions of 2-bromoacetonaphthone in DMF at 40°C in periods of hours. Variations of both procedures have been used for determination of carboxylates [20—31].

We applied modifications of both procedures to [^{14}C-methyl]carnitine and [^{14}C-methyl]4-TMA-butanoate with 2,4′-dibromoacetophenone as the alkylating agent. All attempts under reaction conditions similar to the crown-ether phase-transfer catalyzed system failed to produce derivatives, while some suc-

cess was achieved in experiments with variations of the latter mentioned procedure. Eventually, we found that yields of derivatives as great as 90% could be prepared by extension of reaction times and through use of forcing temperature and concentration conditions (Fig. 1). Interestingly, similar yields of homologous two-, five-, and six-carbon TMA-carboxylates could be prepared in shorter reaction times. The resistance of the four-carbon TMA-acids to carboxyl-O-alkylation by 2,4′-dibromoacetophenone may arise from their assumption of a folded conformation permitting strong ionic interaction of the large onium head and carboxylate tail of the molecule, thereby limiting carboxylate approach to electrophiles. Examination of framework models suggests that the folded conformation allowing close proximity of both ends of the molecule are accessible and preferred only in the four-carbon TMA-acid homologues.

The low rate of carboxylic acid O-alkylation by 2,4′-dibromoacetophenone in dilute solution and at elevated temperatures [21, 22, 25, 32] suggested that there is a significant activation barrier to displacement of bromide from the alkylating agent. We reasoned that a more reactive derivatization reagent would provide better derivative yields at lower sample concentrations and at lower temperatures. We therefore designed a synthetic route to 4′-bromophenacyl triflate [33]. This new alkylating agent proved highly reactive toward samples of ^{14}C-labeled carnitine and ^{14}C-labeled 4-TMA-butanoate in acetonitrile solution, with complete reaction occurring within a few minutes at room temperature. Fig. 2 represents a progress curve for the reactions of [^{14}C-methyl]carnitine and [^{14}C-methyl]4-TMA-butanoate with 4′-bromophenacyl triflate in acetonitrile at room temperature.

The derivatization reaction with the new alkylating agent occasionally failed. This problem was solved completely by inclusion of trialkylamines in the reaction solution. Although the TMA-carboxylates probably do not form intimate ion pairs with trialkylammonium ions in solution, the added base does neutralize any residual acid present in the sample. Thus, the reaction succeeds uniformly when the carboxylate conjugate bases are the species actually present in the reaction medium. Among bases tested, the sterically hindered N,N-diisopropylethylamine was alkylated least rapidly by the derivatization reagent [33], and therefore was used for sample neutralization.

The formation of side-reaction products during derivatization of organic acids by 2,4′-dibromoacetophenone has been noted [23]. One of these by-products was identified spectroscopically as 2-chloro-4′-bromoacetophenone by Patience and Thomas [31], who showed that Cl^- ions present in the reaction solution compete successfully with carboxylates for available 2,4′-dibromoacetophenone. Since 4′-bromophenacyl triflate readily underwent the same reaction, a method for removal of Cl^- from samples of TMA-acid (Cl^- salts) was required. The strong anion exchanger Dowex-1 (OH^- form) quantitatively exchanges Cl^- for OH^-, and does not retain TMA-carboxylates as their dipolar ions present in solution at neutral pH. Columns of Dowex-1 were used to prepare samples of the TMA-carboxylates for both semi-preparative and microscale syntheses of their 4′-bromophenacyl ester derivatives. Recovery of carnitine from these columns was investigated by application of [^{14}C-methyl]-carnitine to a 7 × 0.5 cm column of Dowex 1-X8 (200—400 mesh, OH^- form)

anion-exchange resin contained by a Pasteur pipette. Elution of the columns with 2 ml of deionized water allowed the recovery of 95% (± 1%, $n = 4$) of the applied radioactivity in the collected effluent.

To facilitate the development of an HPLC system for separation of the TMA-carboxylate esters from other species in the reaction mixture, authentic 4′-bromophenacyl esters of carnitine, 2-TMA-acetate, and 4-TMA-butanoate were synthesized. These cations are candidates for chromatography by a reversed-phase ion-pair separation mechanism. Preliminary experimentation with the μBondapak C_{18} medium led to the development of chromatographic conditions adequately selective for separation of derivatization reaction products in concentrated samples. However, these conditions proved unsuitable for work with samples of biologically representative concentrations owing to chromatographic interference. We therefore experimented with the considerably more retentive reversed-phase medium Radial-Pak C_{18}. Initially, an eluent system containing $1 \cdot 10^{-2}$ *M* SDS and $1 \cdot 10^{-2}$ *M* sodium dihydrogen phosphate in acetonitrile–water (50:50, v/v) was tried. The TMA-carboxylate ester derivatives were retained extensively under these conditions, and the chromatograms exhibited poor peak shapes. These problems were solved by the inclusion of triethylamine in the eluent. It has been proposed that amine modifiers in chromatographic eluents used for reversed-phase chromatography on silica-bonded reversed-phase media masks residual silanol groups which otherwise interact strongly with the basic functional groups of some solutes [35, 36]. This led us to investigate several tertiary amines as possible chromatographic mobile phase constituents. Experimentation with authentic esters and later with micro-scale synthetic mixtures led to the development of the eluents actually used. N-Propylcarnitine and 6-TMA-hexanoate were selected as procedural internal standards on the basis of their retention relative to other sample constituents under these chromatographic conditions. The inversion of the expected reversed-phase ion-pair elution order of 2-TMA-acetate and 4-TMA-butanoate was confirmed by ^{1}H NMR spectroscopy of the authentic compounds.

We found it necessary to remove excess 4′-bromophenacyl triflate from the sample matrix to prevent chromatographic interference by slower forming side-reaction products. This was accomplished by the addition of a molar excess of a carboxylic acid–tertiary amine solution (1:1 molar) to the reaction mixture after allowing a few minutes for TMA acid ester derivative formation. Hydroxyacetic acid–N,N-diisopropylethylamine was suitable for this purpose. The resultant reaction product was nearly unretained under these chromatographic conditions.

To demonstrate the extent of the derivatization reaction, [^{14}C-methyl]-carnitine was subjected to the described reaction conditions and chromatographic system. Of the radioactivity committed to the experiment, 96% was recovered from the chromatographic column, and 98% of the recovered radioactivity appeared in a peak which co-chromatographed with the chromatographic peak of injected carnitine 4′-bromophenacyl ester.

Fig. 3 is a chromatogram of an aqueous blank specimen carried through the analytical scheme and chromatographed with the eluent used for determination of carnitine. A chromatogram of a processed 10 nmol/ml carnitine stan-

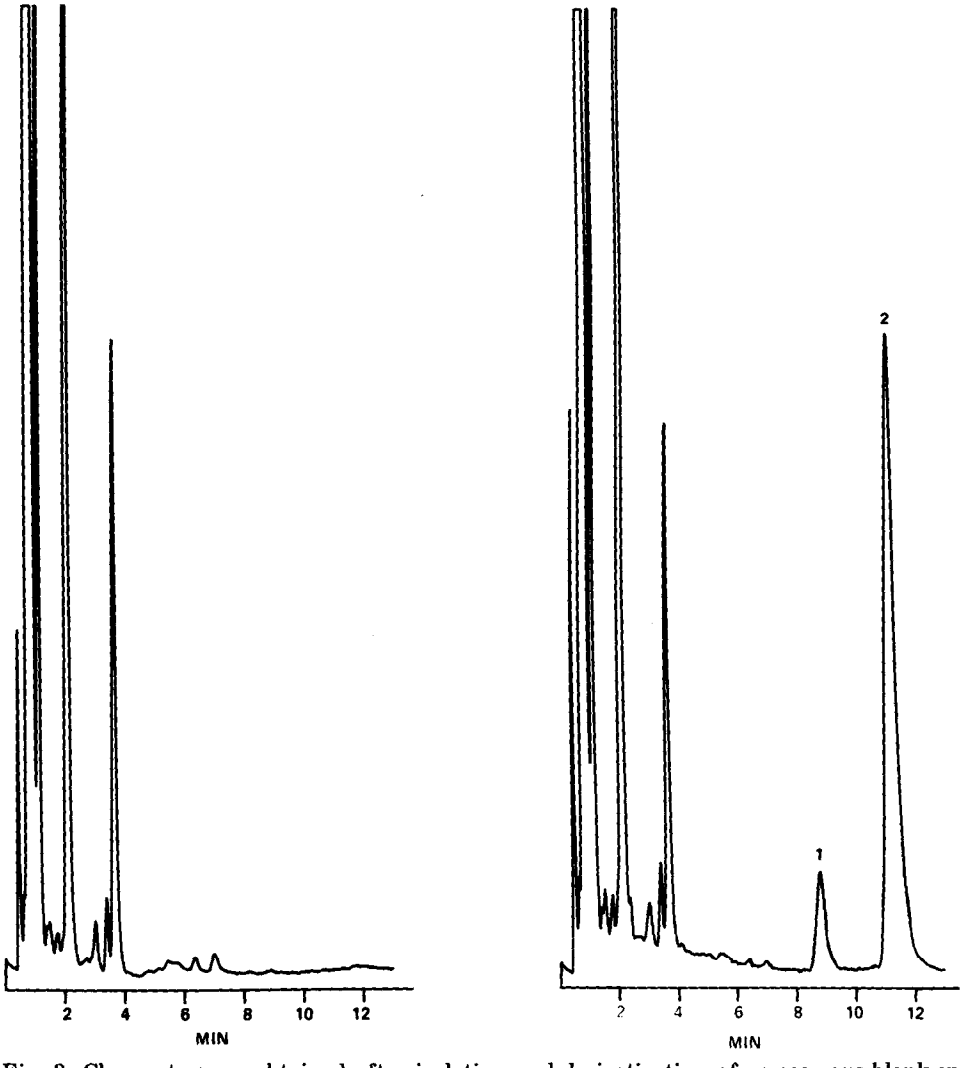

Fig. 3. Chromatogram obtained after isolation and derivatization of an aqueous blank specimen according to the procedure described in the text and chromatography with the eluent used for carnitine determination. The chromatographic column was a 10 × 0.8 cm plastic cartridge containing Radial-Pak C_{18} (5 μm nominal particle diameter). The eluent was $5 \cdot 10^{-4}$ *M* SDS, $2 \cdot 10^{-3}$ *M* sodium dihydrogen phosphate, and $5 \cdot 10^{-3}$ *M* 3-(N,N-dimethylamino)-1,2-propanediol in acetonitrile—water (75:25, v/v). The sample aliquot injected was 15 μl. The pump was operated at a flow-rate of 5 ml/min. The absorbance detector was operated at 254 nm. Full scale of the ordinate is 0.02 absorbance units (a.u.).

Fig. 4. Chromatogram obtained after isolation and derivatization of 5 nmol of carnitine and 50 nmol of N-propylcarnitine. The chromatographic conditions were as described under Fig. 3. A 15-μl aliquot of the reaction mixture was injected into the chromatograph. Peaks: 1 = carnitine 4′-bromophenacyl ester; 2 = N-propylcarnitine 4′-bromophenacyl ester.

dard solution containing N-propylcarnitine internal standard is shown in Fig. 4. Fig. 5 is a chromatogram of a processed aqueous blank carried through the entire analytical scheme and chromatographed with the eluent used for determination of 2-TMA-acetate and 4-TMA-butanoate. Chromatograms of pro-

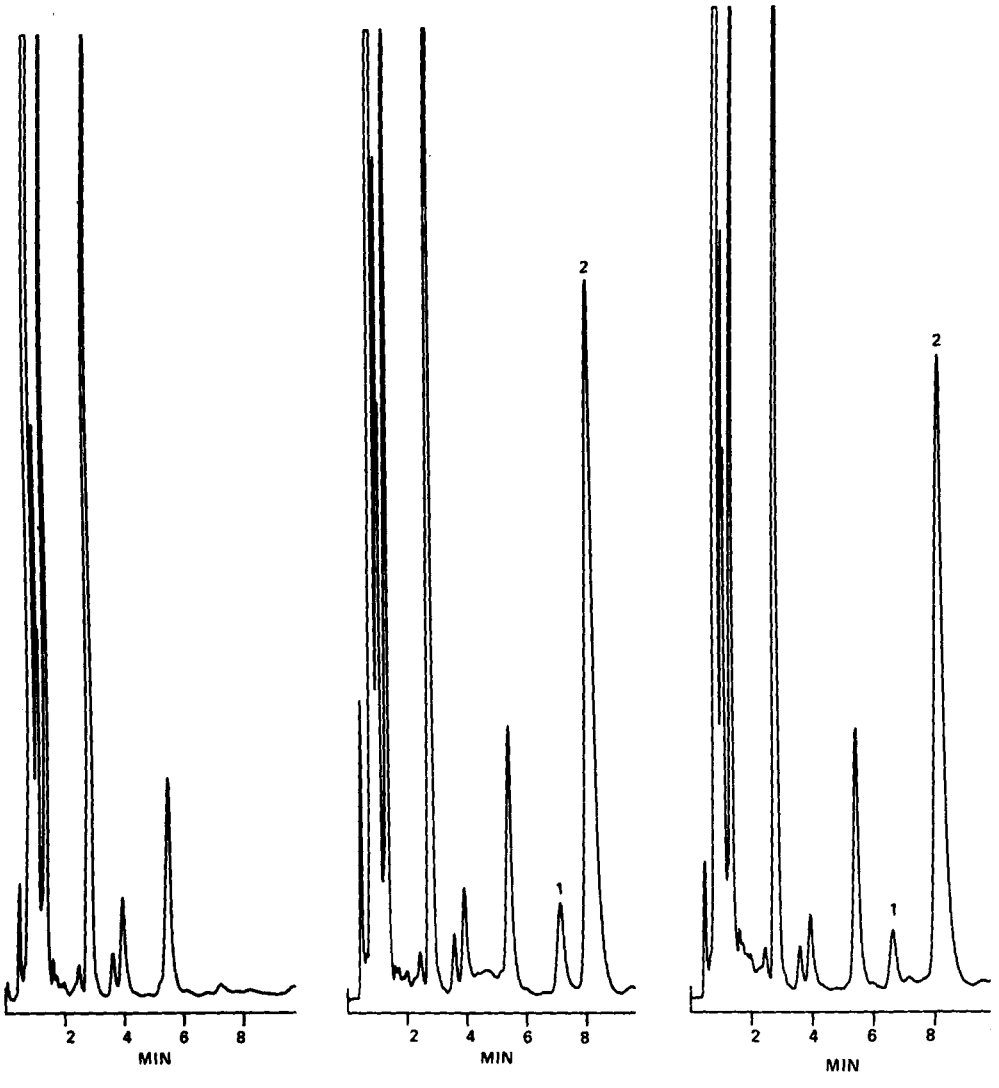

Fig. 5. Chromatogram obtained after derivatization of an aqueous blank specimen and chromatography with the eluent used for 2-TMA-acetate and 4-TMA-butanoate determinations. The apparatus used was that described under Fig. 3. The eluent was $1 \cdot 10^{-3}$ *M* SDS, $1 \cdot 10^{-3}$ *M* sodium dihydrogen phosphate, and $1 \cdot 10^{-2}$ *M* 3-(N,N-dimethylamino)-1-propanol in acetonitrile—water (70:30, v/v). The sample aliquot injected was 15 μl. The pump was operated at a flow-rate of 5 ml/min. The absorbance detector was operated at 254 nm, and the full scale of the ordinate is 0.02 a.u.

Fig. 6. Chromatogram obtained after derivatization of 5 nmol of 2-TMA-acetate and 50 nmol of 6-TMA-hexanoate, internal standard, isolated according to the described procedure. The chromatographic conditions were as described under Fig. 5. Peaks: 1 = 2-TMA-acetate 4′-bromophenacyl ester; 2 = 6-TMA-hexanoate 4′-bromophenacyl ester.

Fig. 7. Chromatogram obtained after derivatization of 5 nmol of isolated 4-TMA-butanoate and 50 nmol of isolated 6-TMA-hexanoate. The chromatographic conditions were as described under Fig. 5. Peaks: 1 = 4-TMA-butanoate 4′-bromophenacyl ester; 2 = 6-TMA-hexanoate 4′-bromophenacyl ester.

cessed aqueous standard solutions containing 10 nmol/ml of 2-TMA-acetate or 4-TMA-butanoate, with included 6-TMA-hexanoate internal standard, are shown in Figs. 6 and 7, respectively.

Standard curves of carnitine:N-propylcarnitine, 2-TMA-acetate:6-TMA-hexanoate, and 4-TMA-butanoate:6-TMA-hexanoate peak height ratios versus sample concentration all were found to be linear over a sample concentration range of 10—100 nmol/ml. The linear regression coefficients were, respectively, 0.9983, 0.9952, and 0.9908; slopes were 0.0152, 0.0115, and 0.0084; *Y*-intercepts were —0.0055, 0.0234, 0.0113. These *Y*-intercept values are less than 18% of the chromatographic peak height ratios obtained at the 10 nmol/ml concentration point.

The method as presented is suitable for determination of the specific radioactivity and radiochemical purity of labeled carnitine and TMA-butanoate. We also have extended the procedure to the determination of carnitine in urine. This application requires complete removal of large accompanying quantities of inorganic salts from the urine sample matrix. These salts otherwise interfere with the derivatization of TMA acids, both by occlusion of the compounds of interest within the salt residue and by the side-reaction of nucleophilic anions with the derivatization reagent [33]. Furthermore, some functionally polar sample constituents not removed by the usual desalting techniques were found to interfere chromatographically, necessitating changes in the composition of the chromatographic eluent. A separate manuscript describing the determination of total carnitine in human urine is in preparation.

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