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# Derivatization of isolated endogenous butyrobetaine with 4′-bromophenacyl trifluoromethanesulfonate followed by high-performance liquid chromatography

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ABSTRACT

A method for the isolation and chromatography of butyrobetaine from plasma, urine, and liver is described. The recovery of [$^3$H-methyl]butyrobetaine from spiked biological samples was from 76–80%. Spiked samples then were derivatized with 4′-bromophenacyl trifluoromethanesulfonate and the butyrobetaine 4′-bromophenacyl ester was isolated by high-performance liquid chromatography (HPLC). Radioactivity eluted in a single peak which co-chromatographed with authentic butyrobetaine 4′-bromophenacyl ester. Two identical liver specimens were treated according to this isolation procedure. Prior to derivatization, one specimen was treated with butyrobetaine hydroxylase. After derivatization, there was no butyrobetaine 4′-bromophenacyl ester peak in the specimen treated with butyrobetaine hydroxylase. The HPLC detection sensitivity to butyrobetaine 4′-bromophenacyl ester was 1 pmol injected with a signal-to-noise greater than 2:1.

## INTRODUCTION

Butyrobetaine (4-N,N,N,-trimethylammonio butanoate) (Fig. 1) is the immediate precursor of carnitine in the carnitine biosynthetic pathway [1,2]. In mammals, carnitine synthesis starts with protein-linked lysine, which is methylated to form protein-linked 6-N,N,N-trimethyllysine and subsequently released by protein turnover. Trimethyllysine is then converted to butyrobetaine by hydroxylation in the 3-position, glycine cleavage, and oxidation of the resulting aldehyde [3,4]. Butyrobetaine is then converted to carnitine by hydroxylation in the 3-position [5]. The conversion of butyrobetaine to carnitine is predominantly a hepatic function in the rat [6] and involves liver and kidney in humans [7]. For the study of carnitine biosynthesis in experimental animals and in humans, it is important to have a reliable method for the determination of butyrobetaine.

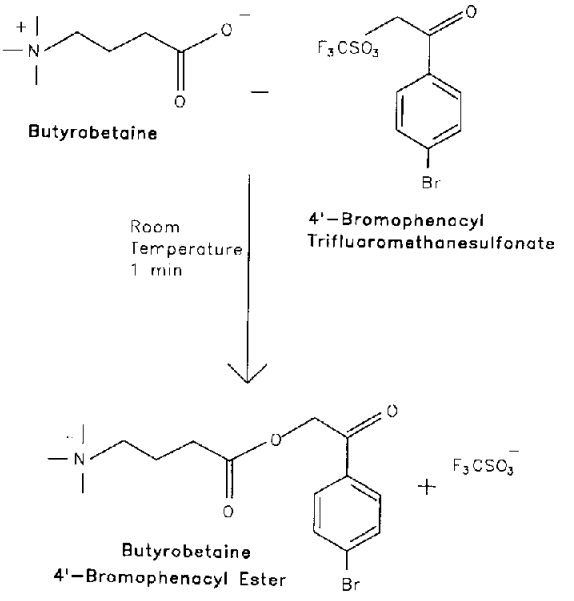


Fig. 1. Reaction scheme for the derivatization of butyrobetaine with 4′-bromophenacyl trifluoromethanesulfonate.

The published analytical methods for butyrobetaine begin by removal of carnitine from the specimen. The butyrobetaine is then converted to carnitine [8–10] and this carnitine is determined by a radioenzymatic method [11]. These procedures are complicated and time-consuming, and therefore they are not suitable as routine methods.

We present in this paper a sample isolation and derivatization procedure for butyrobetaine based on our experience with carnitine [12] and acylcarnitines [13]. We describe the isolation of butyrobetaine from small amounts of biological samples. This is followed by the quantitative derivatization of isolated butyrobetaine with 4′-bromophenacyl trifluoromethanesulfonate to form butyrobetaine 4′-bromophenacyl ester. Finally, we demonstrate the chromatographic separation of this ester from other sample constituents in various biological matrices.

## EXPERIMENTAL

### *Equipment*

The high-performance liquid chromatographic (HPLC) pump was a Hewlett Packard 1050 Series quaternary pump (Avondale, PA, USA). A Waters Assoc. (Milford, MA, USA) WISP 710B automatic sampler and Model 440 fixed-wavelength detector (operated at 254 nm) were also used. The chromatographic column was a 300 mm × 3.9 mm I.D. 5 μm Resolve-Pak $C_{18}$ column (Waters). Between the automatic sampler and the analytical column was placed an in-line filter (Upchurch Scientific, Oak Harbor, WA, USA). A Hewlett-Packard Model 3354 laboratory automation system was used for the collection of chromatographic data. Liquid scintillation counting was performed by a PRIAS scintillation spectrometer (Packard Instruments, Downers Grove, IL, USA). For recovery studies employing [$^3$H-methyl]butyrobetaine, a U6K syringe loading injection valve (Waters) was used in place of the WISP autosampler. In addition, an Ultra Rac II fraction collector (LKB Scientific) was used for collection of the HPLC effluent fractions.

### *Materials*

Burdick & Jackson HPLC-grade methanol was purchased from Baxter Scientific (Obetz, OH, USA). HPLC-grade acetonitrile was purchased from Fisher Scientific (Cleveland, OH, USA). Reagent-grade water was prepared by passage through a Milli-Q reagent-grade water system (Millipore, Bedford, MA, USA). N,N-Diisopropylethylamine and triethylamine were purchased from Aldrich (Milwaukee, WI, USA) and these compounds were distilled from sodium hydroxide before use. Phosphoric acid was purchased from Aldrich. Acetic acid was purchased from Fisher. Silica gel 60 (230–400 mesh) was purchased from Curtin Matheson Scientific (Broadview Heights, OH, USA). Solid-phase extraction columns (Superclean LC-18, 1 ml) were purchased from Supelco (Bellefonte, PA, USA). Glycolic acid was obtained from Sigma (St. Louis, MO, USA). The scintillation cocktail was prepared by combining 1 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, 33 g of 2,5-diphenyloxazole, 4 l of toluene and 2 l of Triton X-100 (all from Research Products International, Elk Grove Village, IL, USA). Dowex-1 $Cl^-$ and Dowex-50 $H^+$ were purchased from Sigma. Dowex-1 $Cl^-$ was converted to Dowex-1 $OH^-$ by exhaustive washing with 2 *M* sodium hydroxide.

Butyrobetaine was synthesized from 4-(dimethylamino)butyric acid hydrochloride and iodomethane (both from Aldrich) and [$^3$H-methyl]butyrobetaine was synthesized with [$^3$H-methyl]iodomethane as described [14]. *l*-Carnitine was a generous gift from Sigma-Tau (Rome, Italy). Butyrobetaine 4′-bromophenacyl ester and 4′-bromophenacyl trifluoromethanesulfonate were synthesized as described [12,15].

### *Sample preparation*

Plasma (100 μl) or urine (25 μl) was placed in a microcentrifuge tube, and 1 ml of acetonitrile–methanol (3:1, v/v) was added. Liver was homogenized in acetonitrile–methanol (3:1, v/v) at a concentration of 50 mg tissue per 1 ml of acetonitrile–methanol; 500 μl of this homogenate (corresponding to 25 mg of original tissue) were used for the butyrobetaine determination. Plasma, urine or liver preparations were centrifuged for 2.5 min at 13 600 *g*, and the supernatant was evaporated to dryness under a stream of compressed air. The residue was dissolved in 1 ml of

water, centrifuged again for 2.5 min at 13 600 *g*, and the supernatant was applied to a $C_{18}$ solid-phase extraction column. The column was washed with 2 ml of water and the collected effluent was evaporated under a stream of compressed air. The residue was dissolved in 1 ml of methanol, centrifuged, and the supernatant was applied to a column containing 0.5 ml of silica gel in a Pasteur pipet. The column was washed with 2 ml of methanol followed by 3 ml of 0.5% acetic acid in methanol (v/v). Butyrobetaine was eluted from the column with 4 ml of 2% acetic acid and 2% triethylamine in methanol (v/v/v). The effluent (4 ml) was evaporated to dryness under a stream of compressed air, reconstituted in 1 ml of methanol, transferred to a microcentrifuge tube and evaporated to dryness under a stream of air.

*Derivatization*

N,N-Diisopropylethylamine in methanol (50 μl, 0.1%, v/v) was added to the evaporated samples. The samples were vortex-mixed and 100 μl of 4′-bromophenacyl trifluoromethanesulfonate in acetonitrile (15.3 mg per ml, 45 m*M*) was added. The mixture was vortex-mixed for 5 s and excess 4′-bromophenacyl trifluoromethanesulfonate was quenched after 1 min by addition of 10 μl of 100 m*M* glycolic acid (8 mg/ml) in methanol containing 0.1% (v/v) N,N-diisopropylethylamine (total volume = 160 μl). Derivatized samples (25 μl) were injected directly into the HPLC system.

*High-performance liquid chromatography*

Four chromatographic eluents were used. Eluent A contained 800 ml acetonitrile and 200 ml water; eluent B contained 200 ml of acetonitrile and 800 ml of water; eluent C contained 5 ml of triethylamine, 4 ml of phosphoric acid, 200 ml of acetonitrile and 800 ml of water; eluent D contained 5 ml of triethylamine, 4 ml of phosphoric acid, 800 ml of acetonitrile and 200 ml of water. Initially, 100% eluent A was pumped at a flow-rate of 1.00 ml/min. At 1.01 min after sample injection, eluent A was replaced by 100% eluent B. At 5.00 min after sample injection, eluent B was replaced by 100% eluent C. At this time there also began a linear gradient over 45 min of 100% eluent C to 70% eluent C and 30% eluent D. At 50.00 min after sample injection, the eluent was switched to 100% eluent D and at 60.00 min to 100% eluent A. The total run time was 65 min per injection.

*Recovery of butyrobetaine from plasma, urine and liver samples*

Replicate representative rat liver, urine and plasma specimens each received $5 \cdot 10^4$ dpm of [$^3$H-methyl]butyrobetaine. These specimens then underwent the above described butyrobetaine isolation, derivatization and HPLC procedure. Radioactivity was determined in three replicate experiments with each biological matrix after extraction with acetonitrile–methanol, after $C_{18}$ solid-phase extraction chromatography, after silica gel chromatography and after methanol extraction of the residue. Derivatized samples then were injected into the HPLC system and 1-ml (approximately 1-min) fractions of the HPLC effluent were collected. Fractions were evaporated to dryness and the contained radioactivity was determined after reconstitution in 0.5 ml of water and addition of 3 ml of scintillation cocktail.

*Conversion of butyrobetaine to carnitine*

Butyrobetaine was isolated from two identical liver specimens as described above. After sample isolation, butyrobetaine in one of these liver samples was treated with butyrobetaine hydroxylase under conditions which lead to more than 99% conversion of butyrobetaine to carnitine [10]. The second liver sample was treated identically, but water was substituted for butyrobetaine hydroxylase. After conversion, the samples were acidified by the addition of 100 μl of 0.1 *M* hydrochloric acid and applied to a Dowex-50 $H^+$ column (0.5 ml of resin contained in a Pasteur pipet). The column was washed with 2 ml of water and stacked over a Dowex-1 $OH^-$ column (0.5 ml contained in a Pasteur pipet). Quaternary ammonio carboxylates were eluted from the column with 3 ml of 15% ammonium hydroxide (v/v). The eluent was evaporated to dryness with a stream of compressed air, derivatized with 4′-bromophenacyl trifluoromethanesulfonate and analysed by HPLC.

*HPLC detection sensitivity to butyrobetaine 4'-bromophenacyl ester*

A solution containing 1 μmol/ml butyrobetaine 4'-bromophenacyl ester was prepared (0.0026 g in 10 ml of acetonitrile). Serial dilutions of this standard solution were made to prepare butyrobetaine 4'-bromophenacyl ester solutions of 100 nmol/ml, 10 nmol/ml, 1 nmol/ml and 100 pmol/ml. All these solutions were injected into the HPLC system (10 μl) and the resulting chromatograms of 10 nmol, 1 nmol, 100 pmol, 10 pmol and 1 pmol injected butyrobetaine 4'-bromophenacyl ester were recorded.

## RESULTS

Plasma, urine and liver samples were spiked with [$^3$H-methyl]butyrobetaine and the recovery of radioactivity was determined after each step of the isolation procedure. As illustrated in Table I, 75–80% of the radioactivity initially added to the samples was recovered before derivatization. This value is similar to the recovery of [$^{14}$C-methyl]carnitine from urine by methanol extraction and silica gel chromatography [13]. Specimens were then derivatized and separated by HPLC. Fractions (1 ml) were collected and the sample radioactivity was eluted in a single peak (fractions 31–34). This peak co-chromatographed with authentic butyrobetaine 4'-bromophenacyl ester (Fig. 2). After correction for background radioactivity, the radioactivity found in the 4'-bromophenacyl butyrobetaine peak in rat liver, urine and plasma was 93.3, 90.8 and 90.4% of the total radioactivity injected, respectively. Underivatized butyrobetaine eluted earlier in the chromatogram (fractions 10–11) and accounted for 1.6, 1.0 and 2.8% of the total determined radioactivity. When corrected for the injection volume, the radioactivity in the HPLC butyrobetaine 4'-bromophenacyl ester peak represents 70–80% of the original radioactivity committed to the experiment.

Representative chromatograms of human urine, rat liver and rat plasma prepared using this procedure are shown in Figs. 3–5. Retention times for carnitine 4'-bromophenacyl ester and butyrobetaine 4'-bromophenacyl ester were determined by derivatization and injection of authentic carnitine and butyrobetaine.

The identity of the butyrobetaine peak was further investigated by conversion of butyrobetaine to carnitine with butyrobetaine hydroxylase. Duplicate rat liver samples (before derivatization) were treated with butyrobetaine hydroxylase (converted sample) or water (control sample).

TABLE I

RECOVERY OF [$^3$H-METHYL]BUTYROBETAINE THROUGH THE PROCEDURE

Biological samples (rat liver, rat plasma, rat urine) were spiked with [H$^3$-methyl]butyrobetaine prior to sample isolation and then prepared as described in the text. Radioactivity was determined in three samples of each matrix after each step of the butyrobetaine isolation procedure.

| Step | Recovery | | | | | |
|---|---|---|---|---|---|---|
| | Liver | | Plasma | | Urine | |
| | dpm | % | dpm | % | dpm | % |
| [$^3$H-methyl]Butyrobetaine aliquot | 53 730 | 100 | 53 730 | 100 | 53 730 | 100 |
| Extraction/protein precipitation | 50 108 | 93.4 | 50 710 | 94.4 | 52 430 | 97.6 |
| (mean ± S.D., $n$ = 3) | ± 1010 | | ± 1210 | | + 1180 | |
| SPE column | 49 730 | 92.6 | 47 900 | 89.2 | 50 830 | 94.6 |
| (mean ± S.D., $n$ = 3) | ± 3450 | | ± 860 | | ± 4020 | |
| Silica gel column | 46 390 | 86.3 | 45 410 | 84.5 | 47 150 | 87.8 |
| (mean ± S.D., $n$ = 3) | ± 350 | | ± 1480 | | ± 2000 | |
| Methanol reconstitution | 41 360 | 77.0 | 40 560 | 75.5 | 42 660 | 79.4 |
| (mean ± S.D., $n$ = 3) | ± 1190 | | ± 1520 | | ± 2650 | |

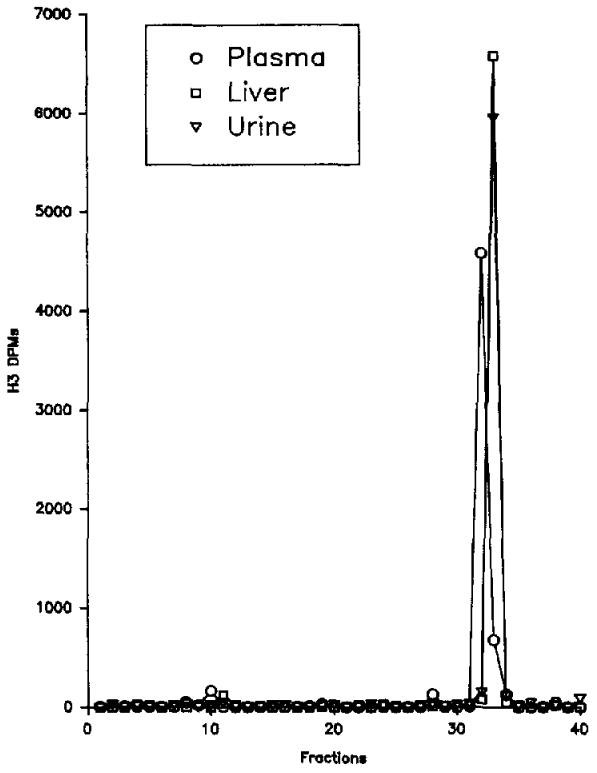


Fig. 2. Figure representing three chromatograms. [$^3$H-methyl]Butyrobetaine was added to rat liver, rat plasma and rat urine and then recovered using the described sample isolation procedure. Radioactivity was determined in 1-ml fractions of the HPLC effluent following derivatization of the isolated samples. Underivatized butyrobetaine eluted in fractions 10–11.

These were then subjected to ion-exchange chromatography and derivatized as described above. As illustrated in Fig. 6, the sample treated with butyrobetaine hydroxylase had no butyrobetaine peak and the carnitine peak was higher when compared to the carnitine in the control sample.

The limit of detection to butyrobetaine 4′-bromophenacyl ester experiment resulted in an observable peak at 1 pmol injected with a signal-to-noise ratio of greater than 2:1.

## DISCUSSION

The isolation of butyrobetaine from biological matrices began with an extraction/precipitation using a combination of acetonitrile–methanol. When compared to extraction/precipitation with methanol alone, this acetonitrile–methanol mixture results in precipitation of more material (protein and salts) from biological samples without impairing the recovery of butyrobetaine (Table I). The samples were applied next to a $C_{18}$ solid-phase extraction column. This removed apolar organic substances which interfered with the derivatization and separation of butyrobetaine by HPLC. Lastly, samples were applied to a silica gel column. This column was washed with methanol, methanol plus acetic acid, and then eluted with a methanol eluent containing acidified triethylamine. The fact that butyrobetaine was not eluted with methanol or methanol plus acetic acid, yet eluted with methanol containing acidified triethylamine, implied that the silica gel acted as a cation exchanger [16]. In our experience, the precise volumes and concentrations of acidified triethylamine were not critical; butyrobetaine was extensively retained on the silica gel

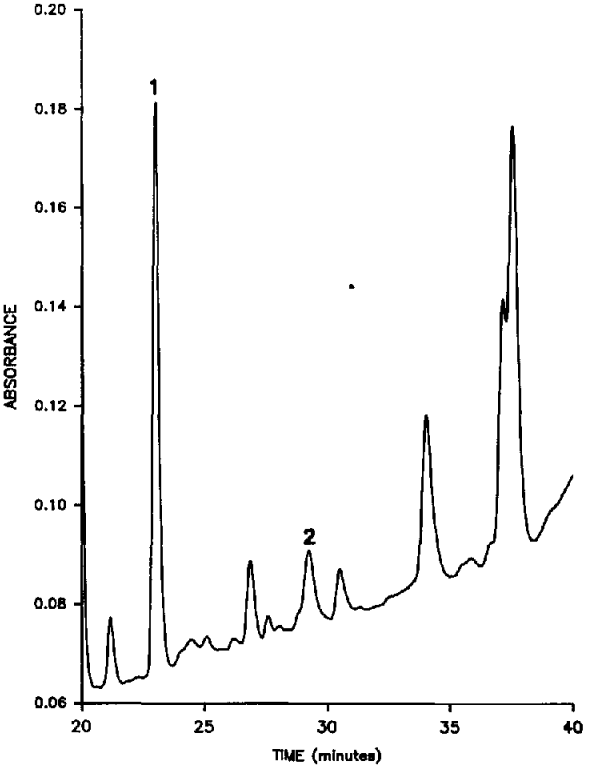


Fig. 3. HPLC profile of a representative human urine which underwent the described butyrobetaine determination procedure. Sample isolation, derivatization and chromatographic conditions are described in the text. Peaks: 1 = carnitine; 2 = butyrobetaine.

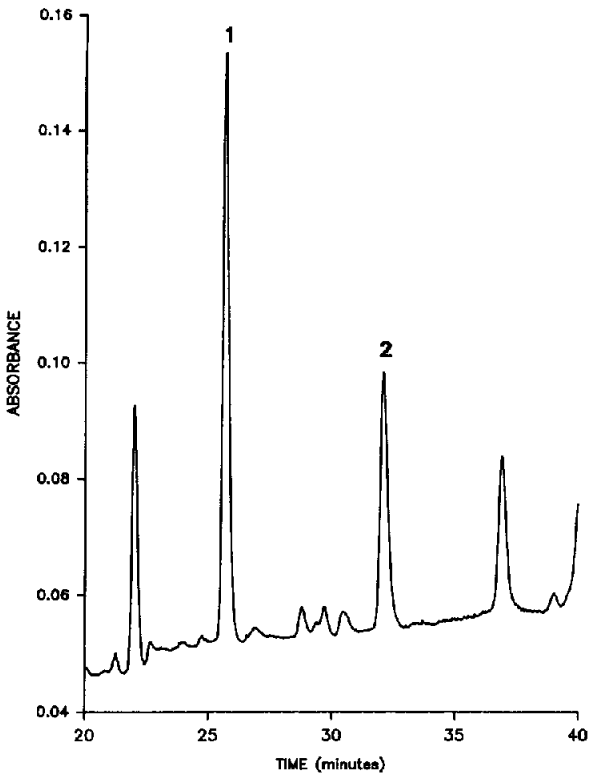


Fig. 4. HPLC profile of a representative rat plasma which underwent the described butyrobetaine procedure. Conditions are described in the text. Peaks: 1 = carnitine; 2 = butyrobetaine.

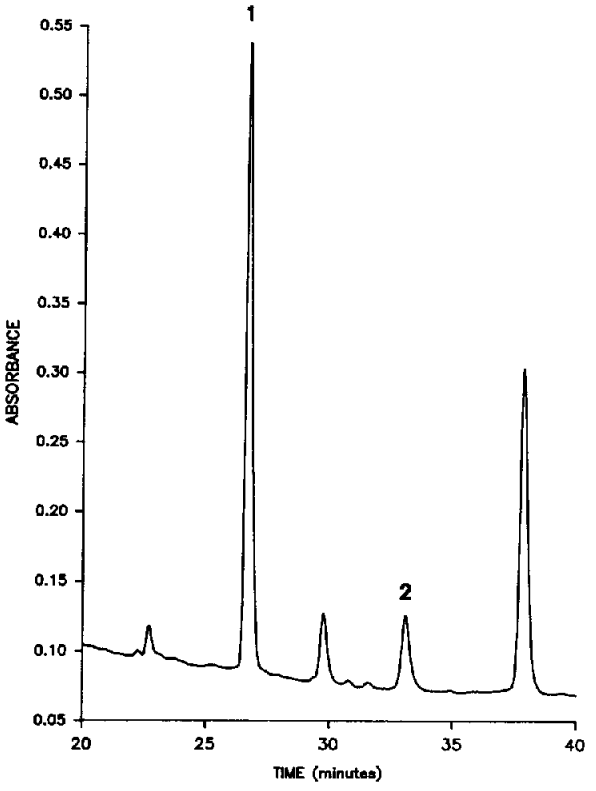


Fig. 5. HPLC profile of a representative rat liver which underwent the described butyrobetaine procedure. Conditions are described in the text. Peaks: 1 = carnitine; 2 = butyrobetaine.

until it was displaced by the acidified triethylamine. The recovery of butyrobetaine using this sample isolation procedure was demonstrated using [$^3$H-methyl]butyrobetaine and shown to be greater than 85% (Table 1).

The HPLC column (Resolve-Pak $C_{18}$) was a high-carbon-load non-end-capped column designed to retain solutes by a combination of hydrophobic and silanophilic mechanisms [17]. We exploited the chromatographic characteristics of this column, using the initial eluent of 80% acetonitrile and 20% water to extract the positively charged quaternary ammonio carboxylate 4′-bromophenacyl esters (which are strongly retained) from the much larger quenched reagent peak (which elutes after 3 min). The quaternary ammonio 4′-bromophenacyl esters were presumably retained by ion exchange and, despite the high acetonitrile concentration, not eluted. This initial eluent was then abruptly changed to 20% acetonitrile and 80% water. This was necessary to prepare the column for the gradient elution of the quaternary ammonio 4′-bromophenacyl esters. At 5.00 min after injection, the acetonitrile concentration remained at 20% but then there was included acidified triethylamine in the eluent. At this time there also began a linear gradient of increasing acetonitrile concentration. With the inclusion of the amine modifier (triethylamine), the silanophilic retention of the quaternary ammonio 4′-bromophenacyl esters was disrupted and they eluted in reversed-phase retention order.

We attempted pre-HPLC isolation of butyrobetaine 4′-bromophenacyl ester using liquid–liquid extraction and small-column chromatography. However, the isolated butyrobetaine 4′-bromophenacyl ester appeared to hydrolyze under our conditions. Therefore, we developed the above described on-line HPLC extraction proce-

dure. This approach replaces a pre-HPLC, labor-intensive isolation step with a precise, rapid, automated, on-line sample simplification step.

There was some day-to-day variability of retention time of the butyrobetaine 4′-bromophenacyl ester (29–33 min) as a function of eluent batch and age of the eluent (Figs. 2–5). However, peak retention within batch runs of standards and samples over a 24-h period were very consistent. We injected standard solutions daily to verify the retention time of butyrobetaine 4′-bromophenacyl ester.

Fig. 2 demonstrates the quantitative derivatization of butyrobetaine isolated from rat liver, urine and plasma. The complete derivatization of contained butyrobetaine was the result of using the reagent 4′-bromophenacyl trifluoromethanesulfonate. 4′-Bromophenacyl bromide (2,4′-dibromoacetophenone) *cannot* be substituted for 4′-bromophenacyl trifluoromethanesulfonate in this procedure. Reaction conditions used for derivatization with 4′-bromophenacyl bromide (*e.g.* 70°C for 2 h) result in incomplete derivatization [12] and destruction of butyrobetaine (unpublished studies). On the other hand, reaction with 4′-bromophenacyl trifluoromethanesulfonate resulted in near instantaneous, quantitative alkylation of butyrobetaine at room temperature. 4′-Bromophenacyl bromide does appear to derivatize $10^{-4}$ *M* standard solutions of butyrobetaine (ten fold excess reagent at 70°C for 2 h; 85% derivatization [18]). However, this does not simulate the interference and concentrations of butyrobetaine found in biological matrices. This study reports quantitative derivatization of butyrobetaine isolated from biological matrices at concentrations of approximately $10^{-6}$ *M*. Comparable reaction conditions using 4′-bromophenacyl bromide would result in little if any reaction [15]. This low rate of carboxylic acid alkylation in dilute solution at elevated temperatures by 4′-bromophenacyl bromide has been extensively reported [18–21].

The chromatograms resulting from this procedure (Figs. 3–5) display the butyrobetaine (and free carnitine) in the various biological samples. These chromatograms demonstrate adequate sensitivity for detection of the endogenous butyrobetaine within small amounts of biological samples (25 μl of urine, 50 mg of liver and 100 μl of plasma). Comparable radioenzymatic assays sometimes require ten-fold more sample [9,10].

The carnitine within these samples probably is isolated quantitatively and it should not contain carnitine from hydrolyzed acylcarnitines [13]. However, recovery and derivatization studies were not performed using carnitine. Such studies are required before extending this procedure to free carnitine evaluation.

The chromatogram resulting from treating isolated rat liver with the enzyme butyrobetaine hydroxylase is shown in Fig. 6. The complete disappearance of the butyrobetaine peak and enlargement of the carnitine peak in the liver specimen treated with butyrobetaine hydroxylase (when compared to the untreated liver specimen) is consistent with the enzyme's action. This is strong evidence of the identities of the butyrobetaine and carnitine 4′-bromophenacyl ester peaks.

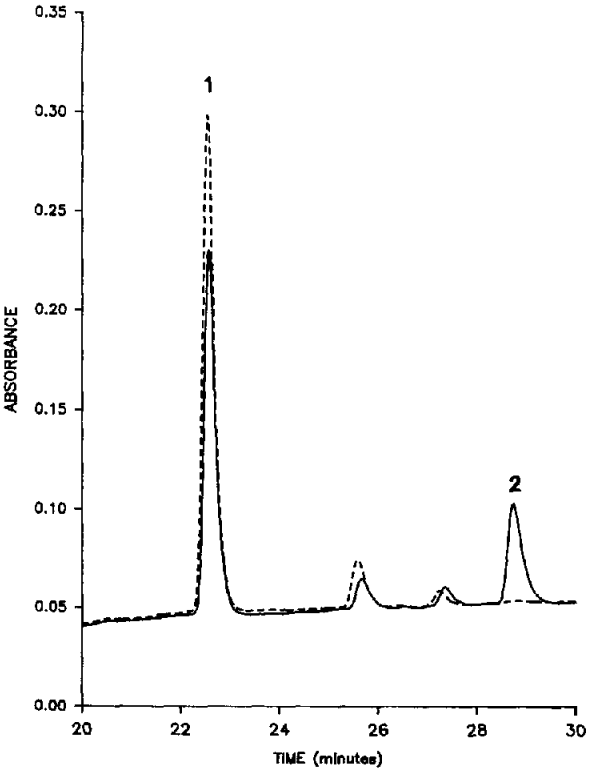


Fig. 6. HPLC profiles of replicate isolated rat liver, one treated (– – –) and one not treated (——) with the enzyme butyrobetaine hydroxylase. Conditions are described in the text. Peaks: 1 = carnitine; 2 = butyrobetaine.

We attempted to evaluate the potential for detecting butyrobetaine using this HPLC procedure. We were able to detect 1 pmol of butyrobetaine 4′-bromophenacyl ester injected with a signal-to-noise ratio greater than 2:1. This sensitivity is more than sufficient for conventional carnitine biosynthesis studies.

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REFERENCES

1 J. Bremer, *Phys. Rev.*, 63 (1983) 1420.
2 C. L. Hoppel and A. T. Davis, *Biochem. Soc. Trans.*, 14 (1986) 673.
3 D. S. Sachan and C. L. Hoppel, *Biochem. J.*, 188 (1980) 529.
4 C. L. Hoppel, R. A. Cox and R. F. Novak, *Biochem. J.*, 188 (1980) 509.
5 G. Lindstedt, S. Lindstedt and M. Tofft, *Biochemistry*, 9 (1970) 4336.
6 R. A. Cox and C. L. Hoppel, *Biochem. J.*, 142 (1974) 699.
7 C. J. Rebouche and A. G. Engel, *Biochim Biophys. Acta*, 630 (1980) 22.
8 R. Parvin and S. V. Pande, *J. Biol. Chem.*, 254 (1979) 5423.
9 H. Noel, R. Parvin and S. V. Pande, *Biochem. J.*, 220 (1984) 701.
10 A. Sandor, P. E. Minkler, S. T. Ingalls and C. L. Hoppel, *Clin. Chim. Acta*, 179 (1988) 17.
11 C. L. Hoppel, in F. A. Hommes (Editor), *Techniques in Diagnostic Human Biochemical Genetics: A Laboratory Manual*, Wiley-Liss, 1991, p. 309.
12 P. E. Minkler, S. T. Ingalls, L. S. Kormos, D. E. Weir and C. L. Hoppel, *J. Chromatogr.*, 336 (1984) 271.
13 P. E. Minkler, S. T. Ingalls and C. L. Hoppel, *Anal. Biochem.*, 185 (1990) 29.
14 R. A. Cox and C. L. Hoppel, *Biochem. J.*, 136 (1973) 1083.
15 S. T. Ingalls, P. E. Minkler, C. L. Hoppel and J. E. Nordlander, *J. Chromatogr.*, 299 (1984) 365.
16 B. A. Bidlingmeyer, J. K. Del Rios and J. Korpi, *Anal. Chem.*, 54 (1982) 442.
17 *Waters Chromatography Columns and Supplies Catalogue*, Waters Assoc., Milford, MA, 1991, pp. 6 and 19.
18 E. Grushka, H. D. Durst and E. J. Kitka, Jr., *J. Chromatogr.*, 112 (1975) 673.
19 H. D. Durst, M. Milano, E. J. Kitka, Jr., S. A. Connelly and E. Grushka, *Anal. Chem.*, 47 (1975) 1797.
20 R. L. Farhi and C. Monder, *Anal. Biochem.*, 90 (1978) 58.
21 M. J. Cooper and M. W. Anders, *Anal. Chem.*, 46 (1974) 1849.