Carnitine: Determination of Total Carnitine Using a Radioenzymatic Assay

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Overview

Acetylcarnitine and acylcarnitine are hydrolyzed by alkaline hydrolysis. The total carnitine is measured by incubation of the sample with ^{14}C-acetylCoA and carnitine acetyltransferase. The radioactive acetylcarnitine is separated from the unreacted radioactive acetylCoA using a Dowex 2 × 8 anion exchange column. The radioactive acetylcarnitine fraction is quantitated by counting in a liquid scintillation counter.

Reagents

Potassium hydroxide (1 N)

Dissolve 56.11 g of potassium hydroxide (Fisher Scientific Company Medfield, MA, USA, catalog #P-250) in 700 to 800 ml of deionized H_2O and bring the final volume to 1,000 ml with deionized H_2O. Store at room temperature.

Potassium hydroxide (4 N)

Dissolve 224.44 g of potassium hydroxide in 700 to 800 ml of deionized H_2O and bring the final volume to 1,000 ml with deionized H_2O. Store at room temperature.

Perchloric acid (0.6 M)

Add 193.5 ml of 70% perchloric acid (Fisher Scientific Company, catalog #P-229) to 3,556.5 ml of deionized H_2O. Store at room temperature.

Potassium phosphate dibasic (K_2HPO_4) (1 M)

Dissolve 174.18 g of potassium phosphate dibasic (Fisher Scientific Company, catalog #P-288) in 700 to 800 ml of deionized H_2O and bring the final volume to 1,000 ml with deionized H_2O in a volumetric flask.

Modified from Cederblad, G., and Lindstedt, S. (1972). A method for the determination of carnitine in the picomole range. *Clin. Chim. Acta* **37**:35.

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Potassium phosphate monobasic (KH_2PO_4) (1 M)

Dissolve 136.09 g of potassium phosphate monobasic (Fisher Scientific Company, catalog #P-285) in 700 to 800 ml of deionized H_2O and bring the final volume to 1,000 ml with deionized H_2O in a volumetric flask.

Potassium phosphate buffer, pH 7.0 (1 M)

Mix 1 M potassium phosphate monobasic with 1 M potassium phosphate dibasic until the pH is 7.0. Store at 0 to 5°C.

N-ethylmaleimide (24 mM)

Dissolve 21 mg of N-ethylmaleimide (Sigma Chemical Company, St. Louis, MO, USA, catalog #E3876) in 7 ml of 1 M potassium phosphate buffer, pH 7.0. Make fresh on the day of the assay and discard the unused portion.

Phenol red (0.1%)

Dissolve 0.1 g of phenol red (Fisher Scientific Company, catalog #P-74) in 100 ml of 100% ethanol. Add one drop of the solution to 10 × 75 mm glass test tubes, allow to dry, and store at room temperature.

[1-^{14}C] Acetyl coenzyme A

Dissolve 50 μCi of ^{14}C-acetylCoA (Amersham Radio Chemicals, catalog #CFA, 452.50 mCi/mmol) in 150 ml of cold deionized H_2O. Dispense 15-ml aliquots into plastic scintillation vials and freeze at −70°C. Stable for at least 6 months.

Potassium bicarbonate ($KHCO_3$) (1 M)

Dissolve 1 g of potassium bicarbonate (Fisher Scientific Company, catalog #P-184) in 7 to 8 ml of deionized H_2O and bring the final volume to 10 ml with deionized H_2O. Make fresh on the day of the assay and discard the unused portion.

Acetic anhydride (0.1 M)

Add 0.05 ml of acetic anhydride (Fisher Scientific Company, catalog #A-10) to 4.95 ml of cold deionized H_2O, mix, and use immediately.

Acetyl coenzyme A (0.1 mM)

Dissolve 40 mg of coenzyme A lithium salt (P. L. Biochemicals, catalog #6200) in 2.0 ml of cold deionized H_2O and mix. Add 0.4 ml of 1 M potassium bicarbonate and mix. Add 0.8 ml of 0.1 M acetic anhydride and mix immediately. Bring the final volume to 320 ml with deionized H_2O. Dispense 15 ml into plastic vials and freeze at −20°C. Stable for at least 6 months.

Carnitine acetyltransferase

Dilute the $(NH_4)_2SO_4$ solution of carnitine acetyltransferase (Boehringer Mannheim Biochemicals, catalog # 103241) with deionized H_2O to a concentration of 25 U/ml. Store at 2 to 5°C.

Ingredient mixture

Prepare fresh on the day of the assay and prepare only enough for the day's assay (100 μl per assay tube). Mix two volumes of [1-^{14}C] acetyl coenzyme A, 1 volume of 24 mM N-ethylmaleimide, and 1 volume of 0.1 mM acetyl coenzyme A.

Dowex columns

Stuff the constricted area that begins the formation of the tip of a 5¾-inch Pasteur pipet with glass wool to form support for the resin. Wash Dowex 2-X8, 200 to 400 mesh (BioRad Laboratories, catalog #745-2451) with distilled water until all the fines have been removed.

Add deionized H_2O to the resin until a slurry is formed that can just be pipetted with a Pasteur pipet (about two parts resin to one part H_2O). Fill the columns with the resin to a height of 35 mm. Store the slurried resin and the filled columns at 2 to 5°C.

ACS aqueous counting scintillant (Amersham Corporation, catalog #196290)

Standards

L-*Carnitine hydrochloride (500 nmol/ml)*

Carnitine chloride is very hygroscopic and should be stirred in a dessicator. Dissolve 9.88 mg of L-carnitine hydrochloride (Sigma Chemicals) in 70 to 80 ml of deionized H_2O and bring the final volume to 100 ml with deionized H_2O in a volumetric flask. Dispense 1 ml aliquots into 12 × 75 mm plastic test tubes and store frozen at −70°C. Stable for at least 1 year.

Procedure

1. A standard curve is performed in quadruple and consists of 10 aliquots of carnitine ranging in concentration from 0 to 15 nmoles of carnitine. Unknowns are run in triplicate. A standard curve is run at the beginning of each incubation batch. One incubation batch contains 60 tubes.
2. Bring the volume of all tubes to 225 µl with deionized H_2O.
3. Add 25 µl of 1N KOH, mix by vortexing and incubate at 50°C for 30 minutes. Cool to room temperature.
4. Add 750 µl of 0.6 M perchloric acid, mix by vortexing, and centrifuge at 2,000 to 3,000 rpm for 10 minutes.
5. Filter the supernatant of sample that is cloudy (this often occurs with samples that have a high fat content, such as milk) through a 0.2-µm Gelman Acrodisc Filter (Fisher Scientific Company, catalog #09-730-218).
6. Pipet 500 µl of each supernatant into a corresponding phenol red tube. Neutralize with 4N KOH by drop-wise addition (stop adding KOH when tubes turn pink).
7. Store in the cold room overnight (0 to 5°C).
8. Centrifuge the phenol red tubes at 2,000 to 3,000 rpm for 10 minutes.
9. Pipet 100 µl of each supernatant into a corresponding 10 × 75 mm glass test tube. Add 100 µl of ingredient mix to each tube.
10. Add 40 µl of carnitine acetyltransferase to each tube at 30-second intervals and incubate each tube at 37°C for exactly 30 minutes.
11. At the end of exactly 30 minutes at 37°C, remove 200 µl of each reaction mixture at 30-second intervals and place on individual drained Dowex columns which are resting in individual 20-ml plastic scintillation vials.
12. Wash each column with two 0.5-ml aliquots of deionized H_2O. Add 9 ml of ACS aqueous counting solution to each vial and count in a liquid scintillation counter.

Discussion

The above procedure measures total carnitine. Many investigators have used the method to approximate the concentration of free carnitine, short chain acyl carnitine, and long chain acyl carnitine. Deletion of the alkaline

hydrolysis step (step 3) will give an approximation of the free carnitine concentration. Although the assay conditions have been designed to favor the formation of the acetylcarnitine, the reaction is still somewhat reversible and the presence of short chain carnitine will influence the reaction. If the perchloric acid precipitation (step 4) is performed before the alkaline hydrolysis step, most of the long chain acyl carnitine will be precipitated and the free carnitine and short chain carnitine will be acid-soluble. Assay of the alkaline hydrolysate of the supernatant will approximate the concentration of the free and short acyl carnitine. Alkaline hydrolysis of the well-washed perchloric acid precipitate followed by the radioenzymatic assay will approximate the long chain acyl carnitine. The fractionation of the acyl carnitines using perchloric acid can be influenced by the profile of acyl carnitines present in the sample. Some acyl carnitines may also be more resistant to alkaline hydrolysis and require hydrolysis at 60°C for several hours to be hydrolyzed.

Alternative methods exist for the assay of carnitine. The colormetric method that depends on the reaction of the sulfydryl group with DTNB[1] is difficult to use in samples that have a high concentration of sulfydryl groups, such as most biologic tissue samples. High-pressure liquid chromatography[2-8] and GC-MS[9] methods have the advantage of allowing the determination of specific acyl carnitines, but are not practical for the assay of large numbers of samples.

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