Choline: Determination Using Gas Chromatography/Mass Spectrometry

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Overview

Choline is converted to a volatile derivative by forming the propionyl ester and then removing an *N*-methyl group using benzenethiolate. Internal standards, labeled with ^{13}C, ^{15}N, or ^{2}H, are used to correct for recovery. Choline must be extracted from tissues before the following analysis, and internal standards are usually added at the start of the extraction procedure. If choline, acetylcholine, phosphocholine, glycerophosphocholine, cytidine diphosphocholine, lysophosphatidylcholine, and phosphatidylcholine are to be determined, extraction and isolation of these metabolites can be accomplished using high pressure liquid chromatography.[1] If only choline and acetylcholine are to be measured, tissues can be homogenized in 9 volumes of cold 1 M formic acid in acetone, centrifuged at 1,500 × *g* for 10 minutes at 4°C, and an aliquot of the supernatant dried under vacuum for use in the choline determination (acetylcholine is measured simultaneously).

Reagents

5 mM Silver toluene sulfonate (Aldrich, Milwaukee, WI, USA) in Acetonitrile (HPLC grade: 139.54 mg/100 ml solvent. Dissolve completely before use.

Propionyl chloride (99%; Fluka Chemicals, Ronkonkoma, NY, USA). Must eliminate all traces of HCl: 0.5 L + 2 ml Tri-*N*-Octylamine (Aldrich), shake, and distill slowly.

Sodium benzenethiolate. This procedure should be done in a fume hood with gloves and appropriate laboratory apparel because thiophenol (benzenethiol) has an incredible stench. Also, toluene is harmful if inhaled. Use only glass labware because the smell will linger in other materials.

1. In an Erlenmeyer flask containing hexane, place 9.42 g sodium metal (Na°; Aldrich). Store Na° under hexane. Cut up under hexane or pentane. *Never* expose to air or water.
2. In a distillation apparatus, mix 67 g thiophenol (C_6H_5SH; Aldrich) and 250 ml methanol (HPLC grade).

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3. *Slowly* add the Na°, stirring vigorously until dissolved. Modest heating may be necessary.
4. Add toluene (300 ml) and heat the sample until the methanol evaporates at 63.8°C. The mixture will turn cloudy and the temperature will rise to 110°C (boiling point of toluene).
5. Add 200 ml cold toluene and keep distilling.
6. Add 1 L hot toluene in small aliquots to the distillation flask using the hot toluene that is collected in the condensor of the distillation apparatus (you may need to add additional boiling toluene).
7. Allow mixture to boil until you have reduced the volume to approximately 100 to 200 ml.
8. Under an atmosphere of nitrogen, pour the precipitate onto a filter paper (no. 2 Whatman paper in a Buchner funnel, Whatman; Maidstone, Kent, UK) and wash with 1 L boiling toluene. A white precipitate results, which is sodium benzenethiolate.
9. Immediately put the sodium benzenethiolate in an evaporating dish in vacuum desiccator over desiccant. Dry at least overnight (stir after a couple of hours to get everything dry).

50 mM Sodium benzenethiolate in 25 mM Thiophenol (97%; Aldrich) in 2-butanone (HPLC grade; Aldrich). 25 mM thiophenol (mol. wt., 110; d = 1.073) in butanone 25.6 µl/10 ml (or 27.5 mg/10 ml). Add 66 mg sodium benzenethiolate and dissolve under an atmosphere of nitrogen. We find that it is best to prepare this in advance and to store it in aliquots in sealed ampules under nitrogen atmosphere at −20°C. It is stable for at least 1 year.

0.5 M *Citric acid* (Mallinckrodt, Paris, KY, USA): 26.268 g/250 ml H_2O. Store at 4°C.

Pentane (reagent grade; EM Science, Cherry Hill, NJ, USA).

Ethyl ether (grade GR; EM Science).

2 M Ammonium citrate—7.5 M ammonium hydroxide buffer: 58% NH_4OH (Fisher Scientific, Medford, MA, USA; mol. wt., 35); ammonium citrate (dibasic; Aldrich; mol. wt. 226). Add 45.24 g ammonium citrate to 25 ml water and 50 ml NH_4OH. Mix using a stirring bar, then bring to 100 ml final volume using water. The buffer is usable as long as it retains a strong smell of ammonia.

15% 1 N Formic acid in acetone: 1 N formic acid: bring 52.6 ml 88% formic acid to 1 L final volume with water. Add 15 ml 1 N formic acid to 85 ml acetone.

Dichloromethane (HPLC grade; Burdick & Jackson Laboratories, Muskegon, MI, USA).

1 M TAPS buffer, pH 9.2: 24.33 g *N*-tris[Hydroxymethyl]methyl-3-amino-propanesulfonic acid (TAPS; Sigma Chemicals, St. Louis, MO, USA) + 50 ml water. Adjust pH to 9.2 with 10 N NaOH. Make up volume to 100 ml. Store refrigerated.

1 mM Dipicrylamine (Pfaltz & Bauer, Waterbury, CT, USA) in dichloromethane. Add 43.9 mg dipicrylamine to 100 ml dichloromethane. Stir at room temperature for 1 hour.

Column packing for gas chromatography

4-Dodecyldiethylenetriamine succinamide (DDTS) is prepared as follows:

1. Add 125 ml toluene to 23.4 g 4-dodecyldiethylenetriamine (86 mmol; (Eastman Kodak, Rochester, NY, USA) in a distillation apparatus. Warm in an oil bath at 150°C until approximately 40 ml has distilled over a 30-minute period to remove traces of water.
2. Without cooling, slowly add 12.6 g dimethyl succinate (86 mmol; 1 ml = 1.12 g; Sigma Chemicals) in 100 ml toluene. Reflux for 1 hour.
3. After 80 ml solvent has distilled, add more dimethyl succinate (6.3 g in 50 ml toluene).
4. Heat mixture in oil bath at 150° to 160°C until no more distillation occurs, then heat under vacuum (1 mm mercury) for 20 minutes at 170°C.

5. Cool product and stir at 60 to 70°C for 1 hour with acetic anhydride (78 ml; Sigma Chemicals) and 7.8 g sodium acetate (Sigma Chemicals).
6. Cool. Add mixture to 150 ml ice-cold distilled water (use large beaker due to effervescence in next step). Let mixture stand for 16 hours at room temperature.
7. Potassium carbonate (140 g) (Sigma Chemicals) is slowly added and the aqueous phase (pH 11) is decanted after effervescence has ceased.
8. Dissolve residue in diethyl ether (200 ml). Dry with sodium sulfate (30 g; EM Science).
9. Filter through no. 2 paper (Whatman) and dry in a rotary evaporator (Haakebuchler Instruments, Saddlebrook, NJ, USA). Yield: 29 g (brown viscous oil with sweet, honey smell).

Column packing: 3% OV-17 on GC-22 precoated with 1% DDTS

1. Suspend 20 g GC-22 (Gas Chrom R, mesh 100/120; Alltech Associates; Deerfield, IL, USA) and 1 g DDTS in 100 ml chloroform. Swirl gently every few minutes for 2 hours.
2. Decant excess solution. Add 100 ml chloroform and swirl gently. Immediately decant.
3. Add OV-17 (0.6 g; Supelco, Bellefonte, PA, USA) to acetone to make a 3% solution and dry in a rotary evaporator (Haakebuchler Instruments).

Standard solutions

Choline standard curve. Choline chloride (Sigma Chemicals) is very hygroscopic. It should be recrystallized from methanolic solution and stored in a desiccator. Take 20 μl of the 14 mg choline chloride/10 ml methanol solution and dilute to 10 ml with methanol. Deliver 0, 10, 25, 50, 100, 200 μl (0, 0.2, 0.5, 1, 2, 4 nmol choline) into glass 12-ml screw cap centrifuge tubes.

Internal standard (20 μM *N,N,N*-trimethyl-d_9 choline chloride [MSD Isotopes, Montreal, Canada]). Take 20 μl of the 14.9 mg d_9-choline chloride/10 ml methanol solution and dilute to 10 ml with methanol.

Procedure

(Do not use plastic for any steps that involve organic solvents.)

1. Usually, an extract of tissue has been prepared that contains d_9-internal standard; if not, 100 μl (2 nmol) of the d_9-internal standard must be added to each tube. The dried sample extract is resuspended in 300 μl water. Add 0.5 ml TAPS buffer and 2.5 ml 1 mM dipicrylamine. Cap and mix vigorously for 2 minutes. Centrifuge at 1,500 × *g* for 2 minutes at room temperature. Aspirate and discard upper phase (aqueous), transfer lower phase (organic) to 12-ml glass screw cap centrifuge tubes and dry under nitrogen *completely*. Samples can be stored dry indefinitely.
2. Add 0.5 ml silver toluene sulfonate solution and 50 μl propionyl chloride.
3. Mix and let stand at room temperature for 5 minutes. Dry *completely* under nitrogen.
4. Add 0.5 ml 50 mM sodium benzenethiolate and cap tube under nitrogen.
5. Mix briefly and incubate at 80°C for 45 minutes.
6. Cool; add 100 μl 0.5 M citric acid. Mix briefly.
7. Wash with 2 ml ethyl ether. Mix and centrifuge at 300 × *g* for 1 minute and aspirate and discard supernatant. Then wash twice with pentane (mix and centrifuge at 300 × *g* for 1 minute and aspirate and discard supernatant). Under a stream of nitrogen evaporate any remaining pentane.
8. Add 50 μl dichloromethane and 100 μl ammonium citrate buffer.
9. Cap, mix, and centrifuge at 300 × *g* for 2 minutes.

10. Inject 1 μl of bottom phase (dichloromethane phase-cloudy) onto gas chromatograph.
11. Gas chromatography–mass spectrometry (GC-MS):

    Hewlett Packard GC-MSD (model 5890/5970; Andover, MA, USA).

    Packed Column: 6′ × 2 mm ID glass packed with 3% OV-17 on GC-22 precoated with 1% DDTS. Inlet temperature, 170°C; jet separator temperature, 155°C; transfer line and detector temperature, 215°C; and oven temperature isothermal at 100 to 110°C. Helium carrier gas flow, 45 ml/min. Typical retention times are 2.3 and 3.7 minutes for acetylcholine and choline derivatives, respectively.

    Electron impact voltage, 70 eV; electron multiplier, 400 relative emV. The fragment at m/z 58 is used to calculate choline (or acetylcholine) concentrations (the fragment at m/z 64 for trimethyl-d_9 choline).

Discussion

Choline concentration increases in tissues stored at room temperature; therefore, it is important that they be frozen as quickly as possible (we collect tissue by freeze-clamping between tongs cooled in liquid nitrogen). Storage at −20°C is acceptable for a brief period but for more prolonged periods, store at −60°C or colder. Once derivatized, samples are only good for *1 to 2 days*. The sodium benzenethiolate is extremely sensitive to moisture, and care should be taken that it is stored in sealed vials. Water also damages the gas chromatography column.

Our assay could detect 200 pmol of choline (equivalent to 4 pmol applied to the GC-MS) and remained linear up to 20 nmol. We normally used a sample size of 50 μl plasma or serum, or 100 mg of liver.

Alternative methods exist for the assay of choline. The assay we have described can be used with a nitrogen-phosphorus or a flame ionization detector instead of with the mass spectrometer.[2] Other methods for measurement of choline include a biologic assay using the thermophilic enteric yeast *Torulopsis pintolopessi*.[3] A chemiluminescence method for assay of choline has also been reported.[4] A radioenzymatic method in which choline is isolated by liquid cation exchange and converted to choline-^{32}P in a reaction catalyzed by choline kinase is widely used.[5–7] Other methods isolate choline using high-pressure liquid chromatography. It is possible to use a post-column reaction, converting the choline to betaine and hydrogen peroxide which is detected in the effluent using an electrochemical detector.[8–12] Alternatively, choline can be converted to the 3,5-dinitrobenzoate derivative, then analyzed by paired-ion high-performance liquid chromatography with UV detection at 254 nm.[13] Choline can also be measured using field desorption mass spectrometry.[14] Each of these methods has advantages. For example, the radioenzymatic method permits analysis of 100 samples in 2 days and does not require the purchase of expensive equipment. The mass spectrometric assay that we describe is unique in that it permits the use of internal standards labeled with stable isotopes.

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