Procedural Section

1.0 Scope and Application

1.1 The radiometric method is used to measure cholinesterase activity. The assay employs minimal dilution and short run times.

1.2 The method is the most accurate means for determining cholinesterase activity in tissues from animals that have been treated with reversible inhibitors.

1.3 The assay should be used for cholinesterase determinations in tissues containing hemoglobin,e.g., erythrocytes or high glutathione levels, e.g., liver.

2.0 Prerequisites

2.1 Equipment and Supplies

Water bath
Analytical balance
Vortexers
Stopwatch/Timer
Pipets
Scintillation counter
7 ml scintillation vials
Ice
Laboratory glassware

All equipment and supplies are considered standard laboratory devices or items and do not need to meet critical specifications.

2.2 Chemicals

0.2M Sodium Phosphate, monobasic (Sigma, S-9638): 27.6 g NaH_2PO_4 in1000 ml ddH2O
0.2M Sodium Phosphate, dibasic (Sigma, S-9763): 28.4 g Na_2HPO_4 in1000 ml ddH2O
0.1M Sodium Phosphate buffer, pH 8.0: 380 ml 0.2M Na_2HPO_4 (dibasic)
20 ml 0.2M NaH_2PO_4 (monobasic), adjust pH to 8.0, double volume by adding ddH2O and refrigerate
0.1M Sodium Phosphate buffer, pH 8.0 + 1% Triton (Sigma, T-6878)
Acetylcholine Iodide (Acetyl-3H) (New England Nuclear, NET-113)
24mM Acetylcholine Iodide (Sigma, A-7000): 65 mg Acetylcholine Iodide in10 ml 0.05M KPO_4 buffer & freeze
1M KH_2PO_4, monobasic (Sigma, P-5379): 136.1 g KH_2PO_4 in1000 ml ddH2O

1M K2HPO4, dibasic (Sigma, P-5504): 228.3 g K2HPO4 in1000 ml ddH20
1M Potassium Phosphate buffer, pH 7.0: 39 ml 1M KH2PO4 (monobasic) + 61 ml 1M K2HPO4 (dibasic), adjust pH to 7.0 and refrigerate
0.05M Potassium Phosphate buffer, pH 7.0: Dilute 10 ml of 1M stock with 190 ml ddH2O
Stop Solution: 23.6 g Chloroacetic acid (1M) (Sigma, C-0266)
5 g Sodium hydroxide (0.5M) (Fisher, S320-500)
29.2 g Sodium chloride (2M) (Sigma, S-9625)
Add ddH2O to a total volume of 250 ml
Scintillation cocktail: 5 g 2,5-Diphenyloxazole (Sigma, D-4630)
0.3 g 1,4-Bis[2-(5-Phenyloxazolyl)]benzene (Sigma, P-3754)
100 ml Isoamyl alcohol (Sigma, I-3643)
Add Toluene (Burdick & Jackson, 347-4) to a total volume of 1000 ml
Ultima Gold (Packard, 6013324)

2.3 Biological

Samples should be stored at -80°C in an alarm equipped freezer
Tissues will be received already appropriately diluted and homogenized in 0.1M Sodium Phosphate buffer, containing 1%Triton
Keep samples on ice during preparation

2.4 Personnel Training

Technical personnel should receive radiation safety training and be instructed and deemed capable by the Principle Investigator in performing the method prior to initiating the procedure alone. In many cases, the assay requires the skill of two technicians when short run times are employed.

3.0 Special Considerations

3.1 Avoid tissue volumes and lengthy run times which may use up the substrate (keep disintegrations per minute [DPM] $\leq$60,000).

3.2 If tissues are treated with a reversible inhibitor, short run times and minimal dilutions should be used to avoid reactivation. Historical data from our laboratory suggests the following guidelines:

Plasma-undiluted, 20 µl for 3 minutes
Erythrocytes-(1:10) dilution, 40 µl for 10 minutes
Brain-(1:50) dilution, 20 µl for 10 minutes
Heart-(1:10) dilution, 40 µl for 5 minutes

4.0 Procedure

1. Set water bath temperature to 26 °C-temperature verified with mercury thermometer
2. Prepare substrate as follows:
    a. 500 µl of 24mM Acetylcholine Iodide stock solution
    b. 1.5 ml 0.05M Potassium Phosphate buffer, pH 7.0
    c. 20µl 3H-Acetylcholine Iodide
    d. Vortex. Transfer 20 µl to a 7 ml scintillation vial and add 5 ml Ultima Gold. Vortex. Count on Easy Count program on scintillation counter. Need a minimum of 220,000 DPM.
3. Perform a trial run to determine the appropriate amount of tissue and length of the run time to be used for the assay by:
    a. Pipet several varying amounts of tissue, in duplicate, into 7 ml vials. Record in notebook.
    b. Add 0.05M Potassium Phosphate buffer, pH 7.0, so that the tissue volume + buffer volume=80µl
    c. Pipet 80 µl of 0.05 Potassium Phosphate buffer, pH 7.0 in a separate 7 ml vial to run a blank
    d. Place vials into the 26°C water bath
    e. Add 20 µl of 3H-Acetylcholine Iodide substrate to each vial; vortex
    f. When a set run time has expired, add 100 µl of stop solution to each vial; vortex
    g. Add 5 ml of scintillation cocktail to each vial
    h. Cap and invert vials before placing on scintillation counter to count

NOTE: Lowest value should be at least 2-3 times the blank and the highest value should be ≤ 60,000 DPM. If counts are too high, reduce the sample volume and/or the length of the run time. If counts are too low, first try increasing the sample volume; then, increase the run time if necessary.

4. Prepare substrate/buffer solution according to the following formula:
    (100-a)/20=X
    X*b=Y
    Y-b=Z
    Where: a=µl of sample used for assay
        b=ml of substrate originally prepared
        Y=final volume of substrate/buffer solution in ml
        Z=ml of 0.05M Potassium Phosphate buffer, pH 7.0 to be added to the substrate that was originally prepared
    Example: 2 ml of substrate has been prepared and it has been determined from the pre-run that 20 µl of sample will be assayed
        (100-20 µl)/20=4
        4*2 ml=8 ml
    Therefore, 6 ml of 0.05M Potassium Phosphate buffer, pH 7.0, will be added to the 2 ml of substrate already prepared for a total of 8 ml.
    Rationale: By diluting the substrate with the buffer in this manner, the technician can

eliminate a pipetting step that can save time and improve assay accuracy.

5. Pipet the appropriate amount of tissue, in duplicate, into 7 ml vials
6. Pipet the same amount of 0.05M Potassium Phosphate buffer, pH 7.0 into 2 vials for substrate blanks
7. Place all vials in the 26° C water bath
8. Add the appropriate volume of substrate/buffer solution to each vial (tissue volume + substrate/buffer solution volume=100 µl); vortex
9. After the appropriate run time has elapsed, stop the reaction by adding 100 µl of solution; vortex
10. Add 5 ml of scintillation cocktail to each vial, cap and invert
11. Prepare 2 total count vials by placing the same volume of substrate/buffer solution that was used in the assay into (2) 7 ml vials and adding 5 ml of Ultima Gold
12. Count on the scintillation counter within 24 hours

Quality Control Section

1.0 All samples are run in duplicate.

1.1 The assay uses the analysis of blanks.

1.2 Calculations:

a. Data should be expressed in nmol/minute
b. Scintillation counter reports results in DPM
c. Correct the averaged DPM value for dilution, µl of tissue and run time used for the assay
d. Complete the conversion by taking the corrected average DPM value/(total counts/specific activity constant of the radioisotope), where specific activity constant is determined by the following calculation: Take the specific activity expressed in Ci/mmol from the product sheet that accompanies the radioisotope, convert to mCi/µmol (/1000), convert toμCi/nmol (/1000), multiply the number by 2,200,000 DPM (DPMs=1 μCi) to get DPM/nmol, and complete conversion to DPM/pmol (/1000)

Example: 40 µl of erythrocytes diluted at (1:10) were run for 10 minutes. The mean DPM value was 16582.92. Total counts for the assay were 101026.5 DPM. The specific activity constant of the radioisotope was 121.8 DPM/nmol.

Mean DPM	DPM/min(/10)	dilution correction(*10)	nmol/min(TC/SA)	nmol/min/ml(/40*1000)
16582.92	1658.29	16582.92	19.99	499.82

1.3 Warnings

3H-Acetylcholine Iodide is a radioisotope. Laboratory personnel should wear the

appropriate personal protective equipment. Avoid spills, contamination and discard waste in accordance with the US EPA Radiation Safety Manual.

1.4 Record keeping Requirements

All sample inventories, correspondence, etc. for a study are kept in a labeled hanging file folder in the laboratory's file cabinet. As assays are run, notations on procedure, including any problems, are documented in the technician's laboratory notebook. Resulting data is organized in Excel (Microsoft Office '97) files. Hard copies are printed and placed in the study file.

References

1. Johnson, CD and Russell, RL. A rapid, simple radiometric assay for cholinesterase suitable for multiple determinations. *Anal. Biochem.,* 1975, 64, 229-238.