TECHNIQUE

CATECHOLAMINE ASSAY BY RADIOENZYMATIC METHOD – A PROTOCOL

Nasim Karim[1], Joshua R. Berlin[2]

ABSTRACT

Catecholamines are a group of compounds identified by the catechol nucleus. The significant catecholamines found naturally in the body are epinephrine, nor- epinephrine and dopamine. They are found in various tissues of the body like brain, adrenals, GIT, blood, urine and cerebrospinal fluid in picogram quantities. Knowledge of catecholamines is important in the diagnosis and management of pheochromocytoma, CNS tumours like neuroblastoma, Diabetes mellitus, hypertension, coronary disease, angina pectoris, myocardial infarction etc. Analytical techniques for determination of catecholamines have been developed which are generally based upon the use of isolated enzyme catechol-O-methyltransferase (COMT) to transfer a radioactive methyl group from S-adenosyl-L-methionine (SAM) to an endogenous catecholamine acceptor molecule to form a radioactive O-methyl catecholamine derivative. This article gives a basic out line of the catecholamine assay along with a simplified protocol that can be easily performed in the laboratory.

Keywords: catecholamine; radioenzymatic assay,Catechol-o-methyltransferase (COMT).

INTRODUCTION

Catecholamines are best known as neurotransmitters and hormones which are produced and secreted by the central and autonomic nervous system.[1] Secretion from the adrenal medulla is part of the fight or flight reaction. Thus the perception or even anticipation of danger, fear, excitement, trauma, pain, hypovolemia, hypotension, anoxia, hypothermia, hypoglycemia and intense exercise causes rapid release of epinephrine and norepinephrine. Epinephrine secretion specifically increases in response to mild hypoglycemia, moderate hypoxia and fasting even through sympathetic nervous system activity may remain constant or may decrease.[2]

Concentration of nor-adrenaline (NA) and adrenaline (A) in plasma are widely used as an index of sympathoneuronal and/or sympatho-adrenomedullary activity.[3-6] Highly sensitive methods are needed in order to determine the very low catecholamine concentrations in small plasma samples. Analytical techniques have been developed with which circulating nor-epinephrine and epinephrine can be measured at the picomole level of sensitivity.[7-9] The difficulties met when estimating the minute amounts of catecholamines present in the blood of man and various animal species are reflected by the divergent data found in the literature.[10] Wide variations and factors concerned with techniques crucially affect the reliable measurement of the plasma catecholamine concentrations.[11]

An early method for measuring cateholamine levels in mammalian systems was spectrofluorometric assay of Von Euler and Floding in 1955 but this assay system was found to be relatively insensitive with poor accuracy.[12] Axelrod and Tomchick (1958) reported catechol – O –methyl transferase enzyme and later referred it to as COMT, an enzyme which transfers a methyl group from a donor molecule to a catechol nucleus thereby forming

1. Department of Pharmacology, Sindh Medical College, DUHS, Karachi, Pakistan.
2. Department of Pharmacology and Physiology, New Jersey Medical School, University of Medicine and Dentistry of New Jersey. New Ark, USA.
Correspondence: Dr. Nasim Karim, Assistant Professor, Department of Pharmacology, Sindh Medical College, DUHS, Karachi, Pakistan
E-mail: nsm_karim@yahoo.com
Received: July 28, 2008; accepted: November 27, 2008

a 3 – methyl moiety.[13] Later investigators used radiotracers and other techniques including chromatography but the basis were the same that is the use of COMT – enzyme and tritiated methyl donor, S – adenosyl –methionine to determine the catecholamine levels in the plasma and in the tissue supernatants.

The radioenzymatic assay of catecholamines therefore have an enzymatic aspect covered by COMT- enzyme and radioisotopic aspect being fulfilled by tritium labeled methyl donor S –adenosyl – L – methionine.

THE RADIOENZYMATIC CATECHOLAMINE ASSAY

It brings together an aliquot of the supernatant of the deproteinized tissue homogenate, blood serum or plasma or biological fluid, with the enzyme catechol – O methyltransferase (COMT), the tritium labeled methyl donor S – adenosyl – L – methionine, a cation of oxidation number +2 which allows the methyl transfer to proceed, a compound which stabilizes the enzyme substrate system, and an agent which preferentially removes calcium ions from interference with the enzymatic reaction. It incubates together the components of "1" for a time, temperature and pH sufficient to O– methylate substantially all of the epinephrine and norepinephrine. It extracts the O – methylated epinephrine and norepinephrine with an organic solvent in which the O – methylated epinephrine and norepinephrine are preferentially soluble. It repartitions the O – methylated epinephrine and norepinephrine into an aqueous acid of sufficient strength to protonate the amine. It oxidizes O – methylated epinephrine and norepinephrine to vanillin. It extracts vanillin from the aqueous solution with an organic solvent in which the vanillin is preferentially soluble, and counts the radiation emitted from the vanillin.

PRE–REQUISITES AND BRIEF DESCRIPTION OF ASSAY

Various mammals in which catecholamines can be assayed using radioenzymatic assay include human, horse, cattle, dog, cat, rat, mouse, rabbit etc. Biological fluids (urine, CSF, lymphatic fluid), blood; any tissue with sympathetic innervation like vascular tissue, liver, adrenal, kidney or brain, can be used in this assay. Depending upon the picogram concentration, a general sample aliquot of the system to be assayed is from 10 to about 50 µl. Urine and tissue samples are diluted first and then used because of their high catecholamine content. The enzyme COMT is found in a variety of mammals like rat, cow, pig, mouse, cat, rabbit, human etc. Widely dispersed throughout the systems for example, liver, kidney, spleen, brain, intestines etc. The preferred species for isolation of this enzyme is rat and the preferred organ for its isolation is liver. S – adenosyl – L – methionine methyl is the labeled methyl donor employed in the enzymatic O – methylation. It is made by radiochemical methods and is commercially available from New England Nuclear Corporation. The tritium label is at the methyl position. Methyl donor should be present in the incubation mixture having specific activity between 5 – 15 Ci/mmol. For COMT enzymatic system to be active at least one of a number of cations of oxidation number and +2 must be present in the incubate. They are magnesium, cobalt and manganese but magnesium is preferred. It is used as magnesium chloride with a concentration range of 10 – 100 mM. Preferably about 25 – 35 mM should be present in the incubation mixture. Enzyme – substrate system should be stabilized by a compound which should maintain the integrity of the system by preventing oxidation of the catecholamines and assists in the continued activity of the enzyme during the incubation. Such compounds are glutathione (preferably), dithiothreitol, ascorbic acid, sodium metabisulphate etc. Its concentration range in the incubate ranges from 1 to about 10 mM preferably 2 – 4 mM. The incubate should have an agent which preferentially removes calcium ions from interference with the enzymatic conversion such agents are EGTA, sodium-oxalate etc. EGTA is preferred. Concentrations from 5 to about 25 mM of EGTA in the incubation mixture can be used. The enzymatic incubation is carried out in standard laboratory equipment for a time and at a pH and temperature which allows the enzymatic conversion to go to completion. The pH should be kept between 7-10, preferred pH range being 8 – 9. Buffer solutions suitable for the incubation are tris- phosphate etc. The temperature of the incubation should be 35^0C to 40^0C preferably 37^0C. Incubation is allowed to proceed for a period of 15 minutes to 2 hours preferably 60 minutes. The incubation is stopped by lowering or elevating the temperature or the pH can be raised or lowered. It is preferred to stop incubation by lowering of temperature and introduction of higher pH. The stopping solution should contain carrier quantities of metanephrine, normetanephrine and methoxytyramine.

A concentration from 2 – 4 mM can be employed. The O – methylated catecholamines are removed from the incubate by solvent extraction. Any organic solvent which is immiscible with water can be used like butanol, isoamylalcohol, hexanol, toluene etc. Better preferred are certain mixtures of organic solvents specifically a 35 – 75 % volume to volume mixture of toluene:isoamyl alcohol. The organic extract of O – methylated catecholamines is repartitioned into an aqueous acid of sufficient strength to protonate the amine function of the catecholamines. Formic, acetic, hydrochloric and sulphuric acids can be used for this purpose. Because aqueous phase is denser than the organic phase, separation of the two phases is facilitated by freezing the aqueous phase. Freezing shortens the assay time and allows for a cleaner, more precise separation of the organic phase from the aqueous phase. The aqueous acid extract is preferentially dried down under reduced pressure and taken up in basic solution. The metanephrine and normetanephrines are oxidized to vanillin. The oxidation of the beta–hydroxyl-O–methylated catecholamines is accompanied by contacting them with an oxidizing agent as sodium metaperiodate at a pH 7 – 12, at a temperature 0– 50C for a period of 2 – 30 minutes. The time of the reaction is preferably 2–10 minutes. The metanephrines and normetanephrines should be quantitatively oxidized to vanillin. Oxidation is completed or stopped generally by a glycerol solution. The pH of the system should be at or below 7. The vanillin is extracted into an organic solvent immiscible with the aqueous fraction.This organic solvent should be relatively non-polar so as to effect a good separation between vanillin and relatively polar side products. Examples are benzene, toluene, diethylether etc. Aqueous phase is freezed up and organic phase is removed into a scintillator vial. The scintillator is solubilized within an organic solvent, the combination of scintillator and solvent is referred to as liquid scintillation medium. The organic solvent containing vanillin is added to the liquid scintillation medium and counted. The specificity of the assay is further improved by contracting the vanillin containing organic solvent with an aqueous acid, thereby removing additional water soluble impurities. It is preferred to have the aqueous acid phase in contact with the liquid scintillation medium. In this way the number of separate transfers and phase separation is minimized. When vanillin is added to the liquid scintillation medium, the vanillin remains in the organic layer and is counted by the scintillator. However any tritium labeled contaminant extracted into the aqueous acid phase is not counted since the scintillator is in the organic phase and the tritium emission beta ray is stopped by the aqueous phase and interface. Various radioemission counting techniques can be employed but liquid scintillation counting technique is preferred.

The sensitivity of the assay is determined as the level of catecholamines which has double the counts of the blanks. The standard curve for the assay is obtained by assaying known quantities of epinephrine. norepinephrine and dopamine alongside the unknown and blank. The counts obtained with the known concentration of epinephrine, norepinephrine or dopamine is plotted against the concentration. The unknown concentration of catecholamine is then determined by the number of counts emitted with reference to the standard curve.[14]

PROTOCOL OF CATECHOLAMINE RADIOENZYMATIC ASSAY BEING DONE IN LABORATORY

Take 50 µl of plasma, add 50 µl of methylation mixture. Incubate in water bath at 37C for 60 minutes with gentle shaking. After 60 minutes add 50 µl of carrier solution. Extract into 2 ml of Toluene – isoamylalcohol liquid by vigorous shaking with vortex for 30 seconds. Centrifuge at 4000 rpm at 5^0C for 5 minutes. Freeze the aqueous phase at -70^0C using dry ice and acetone till the lower part freezes. Decant (pour out) the organic phase into tube having 100 µl 0f 0.1 N Acetic acid. Vigorously shake at the vortex for 30 seconds. Centrifuge again at 4000 rpm at 5C for 5 minutes. Freeze and discard the upper organic layer using the dry ice and acetone. Dry the acetic acid layer under reduced pressure for 90 minutes at medium speed. Dissolve the catecholamines in 1 ml of 1 N NH_4OH. Vigorously shake at the vortex for 30 seconds. Add 50 µl of sodium periodate ($NaIO_4$ 4% w/v). Shake at the vortex for 10 seconds. 5 minutes later add 50 µl of glycerol 10% v/v solution. Shake at the vortex for 10 seconds. Add 0.3 ml (300 µl) of pure glacial acetic acid. Shake at the vortex for 10 seconds. Add 10 ml of toluene – scintillation fluid 10:0.5. Vigorously shake at the vortex for 30 seconds. Centrifuge again at 4000 rpm at 5^0C for 5 minutes. Freeze the aqueous phase with dry ice and acetone. Pour out the organic layer into scintillation vial having 2 ml of 0.1 N acetic acid. Count the radioactivity with the liquid scintillation counter using program number 10 for a period

of 1 minute for each vial. Subtract the blank CPM (counts per minute) from the standards and samples and plot the Log graph.[15,16]

CALCULATIONS

Total catecholamines

The data summarized below provided an example for the calculation of total NA and A in the total catecholamines

Sample No.	Description	Radioactivity (cpm) Total NA + A
1.	Blank	106
2.	Blank	86
3.	50 µl plasma # 1	880
4.	50 µl plasma # 1	907
5.	50 µl plasma # 1 + Std	7464
6.	50 µl plasma # 1 + Std	7668

All standards are 200 picograms (sum of concentration of NA and A).

Description	Average radioactivity (cpm) Total NA + A
Blank	96
Plasma # 1	894
Plasma # 1 + Std	7566

Catecholamine concentration (pg/ml) =

$$\frac{\text{cpm (sample)-cpm blank}}{\text{cpm (sample + std) – cpm sample}} \times \frac{\text{pg standard}}{\text{ml sample vol**}}$$

Plasma sample #1 (total catecholamines NA + A)

$$NA \frac{894-96}{7566-894} \times \frac{200}{0.05} = 478 \text{ picograms/ml}$$

In this calculation standard is the sum of NA and A which is 200 pg.[17]

assay.

SOLUTIONS REQUIRED IN LABORATORY FOR CATECHOLAMINE ASSAY:

1. Plasma Additive Solution Preparation

For each ml of blood 20 µl of plasma additive solution is required. Plasma additive is 950 mg EGTA, adjusted to pH 6.5 with 1 N NaOH (approx. 0.2 gms of NaOH pellets). Final volume is 30 ml. To each 10 ml of this solution, 600 mg reduced glutathione is added on the day of working and is stored in refrigerator at 2-4°C.

2. Methylation Mixture preparation

100 ml 0.3 M Tris buffer at pH 8.2 adjusted with concentrated HCl is required. For each 200 ml of 0.3 M Tris buffer use 7.268 gTris base. To make 100 ml of 0.3 M Tris buffer, are 99 ml of 0.3 M Tris buffer, 1.10 g of EGTA, 4.6 mg Benzyloxyamine (1 ml from stock solution of 23 mg/5 ml equal to 4.6 mg/ml), and 1.77 g $MgCl_2$. Recheck and adjust the pH to 8.2 if different and store in refrigerator at 2-4°C.

3. [3H] S–ADENOSYLMETHIONINE (SAM); specific activity: 5 – 15 Ci/mmol 500 µCi/ml

For each sample, 5 µl is required which is equal to 2 – 2.5 µCi. Store in refrigerator at 2-4°C.

4. Catechol–O–Methyl Transferase Enzyme (COMT)

Dissolve in COMT reconstitution solution. Aliquots are made and stored at -20°C.
For a 50 µl plasma sample, 10 µl of COMT solution is required which contains 6 U of commercially prepared COMT per sample in our Laboratory. The quantity of COMT in units needs to be evaluated through experiments in each laboratory but the volume used is fixed to 10 µl per 50 microliters of sample.

COMT – reconstitution solution

Dissolve 0.154 g Dithiothrietol (DTT) into 10 ml Deionized H2O. Make reconstitution solution with 0.0121 g Tris Base, 0.0372 g EDTA and 0.01g Bovine Serum Albumin (BSA), pH 7.5 (with NaOH) in a final volume of 9.9 ml. For final 10 ml reconstitution solution for COMT, add 100 µl of DTT to this salt solution. From this 10 ml solution, take out ½ ml and inject into the vial having COMT powder. Shake and mix well. Make aliquots 50 µl each and store at -20°C. Solution preparation and aliquoting should be done on ice.

5. CARRIER SOLUTION

Ten ml of 0.8 M Borate buffer pH 10.0 is adjusted with 1 N NaOH. Make 100 ml of 0.8 M Borate Buffer at pH 10.0. 4.94 gm Boric acid and 3.2 gm NaOH pellets dissolved into 100 ml Deionized H_2O having a pH of 11. To each 10 ml of borate buffer on the day of working add

0.234 g EDTA, 0.002 g Normetanephrine, and 0.002 g methoxytyramine. The final pH is 10.0. Keep on ice.

Procedure:

Weigh out the boric acid required for 100 ml of D-H^2O. Add it to D-H2O less than 100 ml. Stir it with the metal stirrer. Heat the solution (without metal stirrer) for 40–50 seconds in microwave. Again stir it with metal stirrer till it is completely dissolved and solution is clear. Add D-H^2O to make it 100 ml in total. Now add NaOH pellets and dissolve it completely by stirring. Check the pH. It should be 11 because when ETDA will be added it will fall down to 10. Store this solution in refrigerator at 4C. When required for use take out 10 ml and heat it in the microwave for 30 seconds. Add EDTA to it. Check the pH to be 10. Add the required amount of normetanephrine and methoxytyramine to this solution. Dissolve them and take out the solution in a test tube. Keep this test tube immediately in the ice bucket. Take out the required amount of borate buffer solution needed for the assay in a separate test tube. Cap it and place in the ice bucket. Discard the rest of the solution.

Precautions:

Plain boric acid solution can be made and kept in the refrigerator for 1 week or till it does not dissolve on heating any more. Heating is necessary in making the borate buffer so as to keep the boric acid in solution in dissolved form otherwise precipitation will occur. EDTA, normetanephrine and methoxytyramine should be added only on the day of use. Before adding EDTA 10 ml of borate solution should always be heated to help dissolving the EDTA otherwise precipitation will occur. Once normetanephrine and methoxytyramine is added the solution should be kept in the ice bucket till use. Fresh solution should be made when required for use.

6. Toluene – Isoamylalcohol (3:2 v/v)

To each 60 ml of toluene add 40 ml of isoamylalcohol in a glass bottle with plastic cap. Keep bottle in the hood covered in aluminum foil.

7. Acetic Acid 0.1 N

750 µl of glacial acetic acid added in 99.25 ml of deionized H_2O to make 100 ml of 0.1 N acetic acid. Keep in the hood, in a glass bottle with plastic cap.

8. Heparin Solution (10 U/ml)

Stock 1: 0.01 g heparin powder and 2 ml deionized H_2O.Working solution is made of 120 µl of stock 1 + 9.88 ml deionized H_2O. Store in refrigerator at 2 – 4°C. Add 0.16 ml of heparin to the syringe prior to each ml of blood drawn.

9. 10% v/v Glycerol Solution

It is made with1 m l of glycerine (99 %) + 10 ml of deionized H_2O. Store in refrigerator at 2-4°C in a plastic tube with cap.

10. 4% Sodiumperiodate $NaIo_4$ Solution

0.4 mg $NaIO_4$ is added to 10 ml of deionized H_2O and stored in refrigerator at 2-4°C in a plastic tube with cap.

11. Toluene – Scintillation Fluid Solution (10:0.5)

100 ml of toluene and 5 ml of scintillation fluid are kept in a glass bottle with plastic cap in the hood covered with aluminum foil to protect from light degradation.

12. 1 N Ammonium Hydroxide is commercially available as such.

All solutions should be made weekly. If any solution becomes turbid before this period it should be discarded and prepared again.

PLASMA SAMPLE PREPARATION

The rat is anaesthetized with sodium pentobarbital (Nembutal) given through intraperitoneal injection in a dose of 50 mg / kg body weight. Blood is then taken from the external jugular vein using polyethylene (PE- 50) catheter in a heparinized syringe and also through direct cardiac puncture using 3 ml heparinized syringe with 20 gauge needle. Heparin is used in a concentration of 10 U/ml and for each ml of blood 0.16 ml of heparin is used. To each ml of heparinized blood 20 ml of plasma additive solution is added to a glass test tube & is mixed by a gentle tilting of the glass test tube 3-4 times. Plasma was

immediately separated by centrifugation in a refrigerated centrifuge at 4000 rpm for 15 minutes at 4°C . Aliquots of plasma sample are made and stored at -70°C until analysis is done. Glass tubes used should be pre-chilled and sample should be constantly kept in ice.[18]

ACKNOWLEDGEMENT

This work was done by the author as a part of her post doctoral fellowship under the supervision of Dr. Berlin.

REFERENCES

1. Jonathan NB , Porter JC. A sensitive radioenzymatic assay for dopamine, norepinephrine and epinephrine in plasma and tissue. Endocrinol 1976; 98:1497-507.

2. Landsberg L, Young JB. Catecholamines and the adrenal medulla. In: Wilson JD, Foster DW (eds). Williams textbook of endocrinology 9th ed , Philadelphia, WB Saunders 1998.

3. Lake CR, Ziegler MG, Kopin IJ. Use of plasma norepinephrine for evaluation of sympathetic neuronal function in man. Life Sci 1976; 18: 1315-26.

4. Koplin IJ, Lake RC, Ziegler MG. Plasmas levels of norepinephrine. Ann Int Med 1978; 88: 671-80.

5. Watson RD, Hamilton CA, Reid JL et al. Changes in plasma norepinephrine, blood pressure and heart rate during physical activity in hypertensive man. Hypertension 1979; 1: 341-6.

6. Dimsdale JE, Moss J. Plasma catecholamine in stress and exercise. JAMA 1980; 243: 340-2.

7. Coyle JT, Henry D. Catecholaminies in fetal and new born rat brain. J Neurochem 1973; 21: 61-7.

8. Cuello AC, Hiley R, Iversen LL. Use of catechol-O-methyl transferase-chemical assay of dopamine. J Neurochem 1973; 21: 1337-40.

9. DeChamplain J, Farley L, Cousiheau D et al. Circ Res 1976; 38: 109-14.

10. Callingham BA. Catecholamines in blood. In : Handbook of Physiology. Blaschko H, Sayer G, Smith AD (edi), Vol VI, Washington DC, American Physiology Society 1975; 427-45.

11. Callingham BA, Barrand MA. Catecholamine in blood. J Pharm Pharmac 1976; 28: 356-60.

12. VonEuler and Floding. Acta Physiol Scand 1955; 33 : 118, 45.

13. Axelrod J, Tomchick R. Enzymatic O-methylation of epinephrine and other catechols. J Biol Chem 1958; 233: 702-5.

14. Radioenzymatic assay of catecholamines patent 4284587 freepatentosonline 2007. http://freepatentsonline.com/4284587html

15. Appel E, Bayer P, Hajdu P et al. Determination of plasma catecholamines by means of radioenzymatic labeling and high pressure liquid chromatographic separation. Arch Pharmacol 1981; 315: 233-9.

16. Peuler JD, Johnson GA. Simultaneous single isotope radioenzymatic assay of plasma norepinephrine, epinephrine and dopamine. Life Sci 1977; 21: 625-36.

17. Biotrak Cellular Communication assays 2007. Catecholamine research assay system.

18. Department of Pharmacology and Physiology, New Jersey Medical School, University of Medicine and Dentistry New Jersey (UMDNJ), USA. 2007.