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Research Article

BIOCHEMICAL AND IN-VIVO ANTIOXIDANT PARAMETERS FOR EVALUATION OF MEMORY ENHANCING ACTIVITY

Nazima Ameen and Sabeeha Shafi*

Department of Pharmaceutical Sciences, University of Kashmir, Hazratbal, Srinagar, Kashmir, Jammu & Kashmir, India.

ABSTRACT

Memory is the ability of an individual to record sensory stimuli, events, information etc., retain them for short or long periods of time and recall the same later when needed **.** Memory loss, also referred to as amnesia, dementia or memory impairment, is an abnormal degree of forgetfulness and/or inability to recall past events. It is a disorder characterized by loss of intellectual ability sufficiently severe as to interfere with one's occupational or social activities. Memory deficits have long been recognized as severe and consistent neurological disorders associated with numerous psychiatric and neurodegenerative diseases, such as Alzheimer's disease, Senile dementia, Parkinson's disease, Huntington's disease, Trauma, Chronic insomnia, Epileptic disorder and Attention deficit disorders etc. However, the most common cause of memory loss has been found to be Alzheimer's disease. These Neurodegenerative diseases associated with dementia are characterized by cognitive deficits and memory impairment,thus stimulating research for memory enhancing drugs.

Keywords: Memory, amnesia, nootropic drugs, biochemical, antioxidant.

INTRODUCTION

Cognitive disorders such as amnesia, attention deficit and Alzhiemers disease are emerging nightmares in the field of medicine because no exact cure exists for them, as existing nootropic agents (piracetam, tacrine etc) have several limitations. The Indian system of medicine is replete with medicinal plants claimed to promote learning, memory and intelligence. A number of experimental procedures are being used to evaluate the memory enhancing potential of new drugs and the biochemical estimations and in-vivo antioxidant methods are the most important parameters which are used for the evaluation of these nootropic drugs.[1,2]

1. BIOCHEMICAL PARAMETERS FOR EVALUATION OF MEMORY ENHANCING ACTIVITY

The important biochemical parameters used for screening memory enhancing activity are as under:

- Estimation of Brain Acetylcholinesterase (AChE) Activity
- Estimation of Brain Monoamine Oxidase (MAO) Activity
- Estimation of Brain Corticosterone Levels

➢ ESTIMATION OF BRAIN ACETYLCHOLINESTERASE (AChE) ACTIVITY

Principle

The assay is based on measurement of the change in absorbance at 412 nm. The assay uses the thiol ester acetylthiocholine instead of the oxy ester acetylcholine. AChE hydrolyses the acetyl-thiocholine to produce thiocholine and acetate. The thiocholine in turn reduces the dithiobis-nitrobenzoic acid (DTNB) liberating nitro-benzoate, which absorbs at 412 nm [3]. The reaction is shown below:

Acetylthiocholine $\xrightarrow{\text{enzyme}}$ **thiocholine + acetate**

Thiocholine + dithiobisnitrobenzoate $\longrightarrow$ **yellow color**

The time frame of cholinesterase activity estimation is similar to behavioral tests. At the end of the behavioral tests or after 60 minutes after administration of inducing agent the animals are killed by cervical dislocation carefully to avoid any injuries to the tissue and the whole brain is dissected out as described by Glowinsky and Iverson and suspended in phosphate buffer and weighed accurately[4] . The whole brain AChE activity is measured using the Ellman method [3]. The end point is the formation of yellow color due to the reaction of thiocholine with dithiobisnitrobenzoate ions.

Procedure

The brain cholinesterase activity was measured by the method of Ellman[3]. A 0.4ml aliquot of the prepared homogenate was added to a cuvette containing 2.6ml of sodium phosphate buffer (pH 7.2, 0.1M). To this, 100µl of Ellman's reagent (DTNB) was added and taken into a photocell. The absorbance was set to zero at 412 nm when the fluctuations stopped. Of the substrate (Acetyl thiocholine iodide) 20µl was added. A change in the absorbance per minute was noted

Calculations

AChE activity is calculated using the following formula

$$R = \frac{\Delta A}{1.36 \times 10^4} \times \frac{1}{\left(\frac{400}{3120}\right) C_0} = 5.74(10^{-4}) \frac{\Delta A}{C_0}$$

Where,

ΔA = Change in absorbance per minute

C_0 = Original concentration of the tissue (mg/ml)

R = Rate in moles substrate hydrolyzed per minute per gram of tissue

- **ESTIMATION OF BRAIN MONOAMINE OXIDASE (MAO) LEVELS**

Principle and Procedure

In this method the whole brain tissue samples are sonicated with a measured volume of 20% 0.4 N perchloric acid containing 100 ng/ml of the internal standard, DHBA followed by centrifugation at 4°C, 15 000×g for 10 min. The supernatant procured after filtration through a 0.22-µm filter unit (Millipore, Bedford, USA), is directly injected (20 µl) into the HPLC system with an electrochemical detector. The mobile phase consists of 0.1 M potassium phosphate (pH 4.0), 10% methanol and 1.0 mM heptane sulphonic acid. Samples are separated on a C18 column using a flow rate of 1.0 ml/min. The concentrations of catecholamines are then calculated using a standard curve generated by determining the ratio between three samples of known amounts and with the internal standard in triplicate [5].

- **ESTIMATION OF PLASMA CORTICOSTERONE LEVELS**

Principle and Procedure

The fluorimetric method is used for estimation of plasma corticosterone, as an index of hypothalamo–pituitary–adrenal axis (HPA) function. The plasma is stored at −70 °C overnight and processed for the estimations. The reaction mixture consisting of 1 ml plasma, 7.5 ml dichloromethane is shaken for 2 min, centrifuged in order to separate the phases and the plasma layer removed. At 0 time, 2.5 ml fluorescence reagent (7 volumes of concentrated sulphuric acid+3 volumes of ethanol) is added and after shaking for 2 s, the supernatant is removed and exactly at 12 min, the acid extract is transferred to separate cuvettes for reading. The readings are taken at 530 nm with activation at 470 nm. The concentration is calculated as nmol/l and expressed as µg/100 ml of blood [5].

2. IN-VIVO ANTI-OXIDANT METHODS FOR EVALUATION OF MEMORY ENHANCING ACTIVITY

The main methods used for the evaluation of in-vivo antioxidant activity are as under:

- Estimation of Brain Malonaldehyde (MDA) Levels Or Thio-Barbituric Acid Reactive Substances (TBARS) Assay
- Estimation of Brain Reduced Glutathione (GSH) Activity
- Estimation of Brain Glutathione Reductase (GR) Activity
- Estimation of Brain Glutathione Peroxidase (GPx) Activity
- Estimation of Brain Glutathione-S-Transferase (GSt) Activity
- Estimation of Brain Superoxide Dismutase (SOD) Activity
- Estimation of Brain Catalase (CAT) Activity

- **ESTIMATION OF BRAIN MALONDIALDEHYDE (MDA) LEVELS OR THIO-BARBITURIC ACID REACTIVE SUBSTANCES (TBARS) ASSAY**

Principle

Lipid peroxidation is a free radical induced event. The primary products of such damage are a complex mixture of peroxides which then breakdown to produce carbonyl compounds. The malondialdehyde (MDA) is one such compound which forms a characteristic chromogenic adduct with two molecules of thio-barbituric acid (TBA) to give a pink colour, the

absorbance of which is determined at 540 nm. The colorimetric reaction of TBA with MDA, a secondary product of lipid peroxidation has been widely accepted for measuring lipid peroxidation. It is also known as thio-barbituric acid reactive substances (TBARS) assay [6].

Procedure

In this method, 1 ml of the supernatant is taken from the centrifuged homogenate (10 %) and to it 0.5 ml of 30 % TCA is added, followed by 0.5ml of 0.8 % TBA reagent. The tubes are then covered with aluminum foil and kept in shaking water bath for 30 minutes at 80°C. After 30 minutes tubes are taken out and kept in ice-cold water for 30 minutes. These were then centrifuged again at 3000 rpm for 15 minutes. The absorbance of the supernatant is read at 540 nm at room temperature against blank[6] .

Calculations

The amount of MDA present in a sample was calculated according to the following equation and the result was expressed as nanomole of MDA/mg of protein.

$$\frac{\text{Nanomole of MDA}}{\text{mg protein}} = \frac{V \times OD_{540}}{0.156} \times \text{mg of protein}$$

Where;

V = Final volume of the test solution
OD = Optical density at 540 nm
0.156 = Extinction coefficient/M/cm

➢ **ESTIMATION OF BRAIN REDUCED GLUTATHIONE (GSH) ACTIVITY**

Glutathione is a cysteine-containing peptide found in most forms of aerobic life [7]. It is not required in the diet and is instead synthesized in cells from its constituent amino acids. Glutathione has antioxidant properties since the thiol group in its cysteine moiety is a reducing agent and can be reversibly oxidized and reduced. In cells, glutathione is maintained in the reduced form by the enzyme glutathione reductase and in turn reduces other metabolites and enzyme systems, such as ascorbate in the glutathione-ascorbate cycle, glutathione peroxidases and glutaredoxins, as well as reacting directly with oxidants [8].Due to its high concentration and its central role in maintaining the cell's redox state, glutathione is one of the most important cellular antioxidants [7].

Principle

Reduced Glutathione estimation in brain homogenate is measured according to the Ellman method. This method is based on the development of a yellow color when 5, 5′-dithio-bis-2-nitrobenzoic acid (DTNB) is added to the compound containing the sulfhydryl groups .

Procedure

0.5 ml of brain homogenate is mixed with 1.5 ml of 0.2 M Tris buffer (pH-8.2) and 0.1 ml of 0.01 M DTNB and this mixture is brought to 10.0 ml with 7.9 ml of absolute methanol. The above reaction mixture is centrifuged at approximately 300 g at room temperature for 15 minutes. The absorbance of supernatant is read in a spectrophotometer against reagent blank (without sample) at 412 nm. Tissue protein is than estimated using Lowry method of protein assay [9].

Calculations

$$\frac{\text{GSH}}{\text{mg Protein}} = \frac{OD_{412} \times \text{Dilution factor}}{1.36 \times 10^4} \times 100$$

Where;

OD = Optical density at 412 nm
1.36 × 10^4 = Extinction coefficient/M/cm

➢ **ESTIMATION OF BRAIN GLUTATHIONE REDUCTASE (GR) ACTIVITY**

Principle

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases and glutathione S-transferases[7] . This system is found in animals, plants and microorganisms [10]. Glutathione peroxidase is an enzyme containing four selenium-cofactors that catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals. Glutathione peroxidase I is the most abundant, dispensable and is a very efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is most active with lipid hydroperoxides[11]

Procedure

Glutathione reductase (GR) activity is assayed by the method of Sharma *et al.*, 2001. The assay mixture consists of 1.6 ml sodium phosphate buffer, 0.1 ml of 1 mM ethylenediamine tetra acetic acid disodium salt (EDTA, Amresco), 0.1 ml nicotinamide adenine dinucleotide phosphate reduced (NADPH) and 0.1 ml oxidized glutathione as well as PMS (0.1ml) in total volume of 2ml. The enzyme activity is measured at 340 nm and calculated as nanomole NADPH oxidized/min/mg of protein [12].

Calculations

$$\text{Nmol NADPH oxidized} = \frac{\text{OD} \times \text{Volume of assay}}{6.22 \times 10^3 \times \text{Volume of enzyme}} \times \text{mg of protein}$$

Where;

OD = Optical density
6.22 × 10³ = Extinction coefficient/M/cm

➢ **ESTIMATION OF BRAIN GLUTATHIONE PEROXIDASE (GPx) ACTIVITY**

The glutathione system includes glutathione, glutathione reductase, glutathione peroxidases and glutathione S-transferases [7]. This system is found in animals, plants and microorganisms [10]. Glutathione peroxidase is an enzyme containing four selenium-cofactors that catalyzes the breakdown of hydrogen peroxide and organic hydroperoxides. There are at least four different glutathione peroxidase isozymes in animals. Glutathione peroxidase 1 is the most abundant, dispensable and is a very efficient scavenger of hydrogen peroxide, while glutathione peroxidase 4 is most active with lipid hydroperoxides [11].

Principle

The activities of GPx are determined by quantifying the rate of oxidation of the reduced glutathione to the oxidized glutathione by hydrogen peroxide (H_2O_2) catalyzed by GSH-Px. The assay is based on the reduction of H_2O_2 by GPx through consumption of reduced glutathione (GSH) that is restored from oxidized glutathione (GSSG) in a coupled enzymatic reaction by GR. GR reduces GSSG to GSH using NADPH as a reducing agent. The decrease in absorbance at 340 nm due to NADPH consumption is then measured spectrophotometrically. One unit of GPx is defined as the amount of enzyme that catalyzes the oxidation of 1mmol of NADPH to $NADP^+$ per minute.

Procedure

GPx activity is measured using the method of [12]. The assay mixture consists of 1.49ml of sodium phosphate buffer (0.1M), 0.1ml EDTA (1mM), 0.1ml sodium azide (1mM), 0.1ml GSH (1mM), 0.1ml NADPH (0.021mM), 0.01mlH_2O_2 and 0.1ml of sample in a total volume of 2ml. oxidation of NADPH is recorded spectrophotometrically at 340 nm and the enzyme activity is calculated as nmoles NADPH oxidized/min/mg of protein, using extinction coefficient of 6.22 × 10^3 M/cm [12]

Calculations

$$\text{Nmol NADPH oxidized} = \frac{\text{OD}_{340} \times \text{Volume of assay}}{6.22 \times 10^3 \times \text{Volume of enzyme}} \times \text{mg of protein}$$

Where;

OD = Optical density at 340 nm
6.22 × 10³ = Extinction coefficient

➢ **ESTIMATION OF BRAIN GLUTATHIONE-S-TRANSFERASE (GST) ACTIVITY**

Principle

Glutathione-S-transferases (GSTs) catalyze the conjugation of reduced glutathione - via a sulfhydryl group - to electrophilic centers on a wide variety of substrates [13]. This activity detoxifies endogenous compounds such as peroxidised lipids [14], as well as breakdown of xenobiotics. GSTs may also bind toxins and function as transport proteins[15] .

Procedure

Glutathione-S-transferase (GST) activity is assayed using the method of Haque *et al*., 2003. The reaction mixture consists of 1.675 ml sodium phosphate buffer, 0.2 ml of 1mM GSH, 0.025 ml of 1mM 1-chloro-2,4-dinitrobenzene (CDNB) and 0.1ml of homogenate in a total volume of 2 ml. The change in absorbance is recorded at 340 nm and the enzyme activity calculated as nmol CDNB conjugates formed/min/mg protein using extinction coefficient (ϵ) of 9.6x10^3/M/cm[16].

Calculations

$$\text{CDNB conjugates formed} = \frac{\text{OD}_{340} \times \text{Volume of assay}}{9.6 \times 10^3 \times \text{Volume of enzyme}} \times \text{mg of protein}$$

Where;

OD = Optical density at 340 nm
9.6 × 10³ = Extinction coefficient

➢ **ESTIMATION OF BRAIN SUPEROXIDE DISMUTASE (SOD) ACTIVITY**

Superoxide dismutases (SODs) are a class of closely related enzymes that catalyze the breakdown of the superoxide anion into oxygen and hydrogen peroxide [17].These are present in almost all aerobic cells and in extracellular fluids [18] and contain metal ion co-factors that, depending on the isozyme, can be copper, zinc, manganese or iron. In humans, the copper/zinc SOD is present in the cytosol, while manganese SOD is present in the mitochondrion [19]. There also exists a third form of SOD in extracellular fluids, which contains copper and zinc in its active sites, but the mitochondrial isozyme

seems to be the most biologically important of these three[20] .In plants, SOD isozymes are present in the cytosol and mitochondria, with an iron SOD found in chloroplasts [21].

Principle

The activities of SOD are assayed based on the mechanism that the oxidation of oxymine by the xanthine–xanthine oxidase system could be inhibited by SOD and the hydroxylamine nitrite produced by the oxidation of oxymine had an absorbance peak at 550 nm. One unit (U) of SOD activity is defined as the amount that reduces the absorbance at 550 nm by 50%. The SOD activity is expressed as units per microgram of brain protein.

Procedure

In this method, the reaction mixture consists of 0.5ml supernatant, 1ml of 50mM Sodium carbonate, 0.4ml of 25µM NBT, 0.2ml of 0.1mM EDTA. The reaction is then initiated by the addition of 0.4ml of 1mM hydroxylamine hydrochloride. The change in absorbance is recorded at 560 nm using a UV spectrophotometer. The control is simultaneously run without homogenate. Units of SOD activity are expressed as the amount of enzyme required to inhibit the reduction of NBT by 50 % [22].

Calculations

$$\% \text{ Inhibition} = \frac{\text{Ab.C} - \text{Ab.S}}{\text{Ab.C}} \times 100$$

Where;

Ab. C = Absorbance of Control
Ab. S = Absorbance of Sample

➢ **ESTIMATION OF BRAIN CATALASE (CAT) ACTIVITY**

Principle

Catalases are enzymes that catalyze the conversion of hydrogen peroxide to water and oxygen, using either an iron or manganese cofactor [23]. This protein is localized to peroxisomes in most eukaryotic cells. Catalase is an unusual enzyme since, although hydrogen peroxide is its only substrate and follows a ping-pong mechanism. Its cofactor is oxidised by one molecule of hydrogen peroxide and then regenerated by transferring the bound oxygen to a second molecule of substrate [24]. Despite its apparent importance in hydrogen peroxide removal, humans with genetic deficiency of catalase — "acatalasemia" — or mice genetically engineered to lack catalase completely, suffer few ill effects.

Procedure

Catalase is assayed by the method of [25]. The mixture consists of 1.95 ml of phosphate buffer (0.05 M, pH- 7), 1 ml of H_2O_2 (0.019 M) and 0.05 ml sample (10 % w/v) in a final volume of 3 ml. control cuvette contains all the components except substrate. Change in absorbance is then recorded at 240 nm and the catalase activity is calculated.

Calculations

$$\frac{\text{CAT U}}{\text{mg Protein}} = \frac{\text{OD}_{240} \times \text{Volume of assay}}{0.081 \times \text{Volume of enzyme}} \times \text{mg of protein}$$

Where;

OD = Optical density at 240 nm
0.081 = Extinction coefficient

CONCLUSION

From the past few decades the use of phytochemicals, neutraceuticals and other herbal products have increased dramatically due to their low level of side effects and better therapeutic efficacy in providing health benefits expanding research and a anecdotal reports on neutraceuticals and related herbal products have convinced the health care community and the general population.The present study provides a platform for the evaluation of new plant/herbal drugs for the treatment of different cognitive defects.

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