International Journal of Research and Review
[Indexed, Peer-Reviewed Journal]
Volume 12; Issue: 10; October 2025
Website: www.ijrrjournal.com
Review Paper *E-ISSN: 2349-9788; P-ISSN: 2454-2237*

Review on Simple Spectrofluorimetric Method for Neurotransmitters Estimation in Brain Tissues of Laboratory Animals

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DOI: https://doi.org/10.52403/ijrr.20251008

SUMMARY

Neurotransmitters are low-molecular weight compounds that chemically transfer the electrical impulse from one neuron to another. Neurotransmitters are responsible for the functioning of the central nervous system. Neurotransmitters estimation is one of the major parameters for neuroendocrinology research scientists. The neurotransmitters are good indicators of proper functioning and better assessment of the physiochemical functioning of brain towards acute or chronic intake of extraneous chemical or drug. If adverse effects caused by any exogenous factor on brain can be proven by the helping selection of specific neurotransmitters markers into the brain tissue homogenate. Therefore, this review article highlights firstly the impact of neurotransmitters and associated functions. Secondly, describe the different relevant methods related to neurotransmitters (γ-aminobutyric acid-GABA, dopamine-DA, noradrenaline-NA, adrenaline-A and serotonin-5HT) estimation in wet tissue of brain of laboratory animals.

Key Words: γ-aminobutyric acid, Dopamine, Noradrenaline, Adrenaline, Serotonin

INTRODUCTION

There are about 100 billion nerve cells/ neurons in the human or mammalian brain. These neurons vary in shape and size; basically, a neuron has a cell body with two types of projections in which dendrites (bringing information to the cell body) another is axon (carrying the information from the cell body) and over 99% of the communication between neurons are based on chemicals called neurotransmitters. When electrical impulses arrive at the nerve ending, neurotransmitters are released from the presynaptic neuron into synaptic cleft and bind to specific receptors of the postsynaptic neuron by active uptake mechanisms [1]. Also, neurotransmitters taken back to the presynaptic neuron, here metabolized or restored for a new neurotransmission process. Neurotransmitters have different structures and functions, they are divided into three chemical classes such as: 1) biogenic amines like catechol amines (dopamine-DA, noradrenaline-NA, adrenaline-A), indoleamine (serotonin-5HT), quaternary amine (acetylcholine-Ach) and histamine. 2) amino acids like γ-aminobutyric acid-GABA (neurotransmitter inhibitor), glutamate, aspartate, glycine, taurine, and tryptophan *etc.* and 3) neuroactive peptides (pituitary hormone releasing factors) like prostaglandins, angiotensin, opiate, endorphins and enkhephalins *etc* [2]. In which NA, DA, A and 5-HT are the most important activating biogenicamine neurotransmitters and while GABA is the major inhibitory

neurotransmitter within the nervous system [1]. The detail of these neurotransmitters as follows.

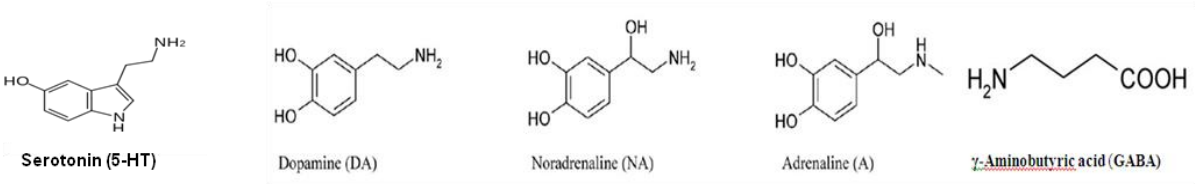

Figure 1: Chemical structure of different neurotransmitters

γ-Aminobutyric acid/ gamma-aminobutyric acid/GABA is chief inhibitory–modulator of the activating neurotransmissions (adrenergic, serotoninergic, dopaminergic, glutamatergic, *etc*) and significantly diminishes cerebral excitability, inhibits anxiety responses and modulates the thymic mood [1]. Although chemically it is an amino acid, but it is never incorporated into a protein like alpha amino acid. It is synthesized from glutamate using the enzyme L-glutamic acid decarboxylase and cofactor pyridoxal phosphate (active vitamin B_6). This process converts glutamate, the principal excitatory neurotransmitter, into the principal inhibitory neurotransmitter (GABA) [3,4]. In general, endogenous GABA does not cross the blood–brain barrier [5] such as the periventricular nucleus but systematically injected exogenous GABA is reached [6]. In vertebrates, GABA acts at inhibitory synapses in the brain by binding to specific transmembrane receptors in the plasma membrane of both pre- and postsynaptic neuronal processes. Two general classes of GABA receptor are known: GABA-A (ligand-gated ion channel) and GABA-B (G protein-coupled receptors). GABA-A receptors are ligand-activated chloride channels; that is, when activated by GABA, they allow the flow of chloride ions across the membrane of the cell. These binding causes the opening of ion channels to allow the flow of either negatively charged chloride ions into the cell or positively charged potassium ions out of the cell, usually causing hyperpolarization. When net chloride flows out of the cell, GABA is excitatory or depolarizing; when the net chloride flows into the cell, GABA is inhibitory or hyperpolarizing. When the net flow of chloride is close to zero, the action of GABA is shunting. GABA is synthesized by neurons and acts both as an autocrine (acting on the same cell) and paracrine (acting on nearby cells) signalling mediator [7]. GABA regulates the proliferation of neural progenitor cells the migration and the elongation of neuritis and the formation of synapses [8]. GABA also regulates the growth of embryonic and neural stem cells [9]. GABA activates the GABA-A receptor, causing cell cycle arrest in the S-phase, limiting growth [10].

Serotonin/5-hydroxytryptamine/5-HT is a monoamine neurotransmitter which is biochemically derived from tryptophan, serotonin is primarily found in the gastrointestinal (GI) tract, platelets, and in the central nervous system (CNS) of animals including humans. It is popularly thought to be a contributor to feelings of well-being and happiness. Approximately 90% of the human body's total serotonin is located in the enterochromaffin cells in the alimentary canal (gut), where it is used to regulate intestinal movements. The remainder is synthesized in serotonergic neurons of the CNS, where it has various functions. These include the regulation of mood, appetite, and sleep [11,12].

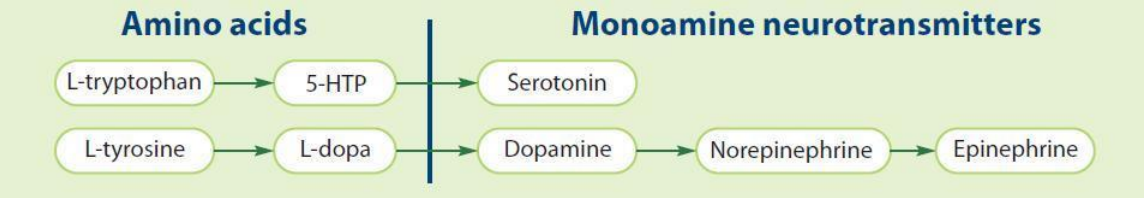

Figure 2: Biochemical origin of monoamine neurotransmitters from amino acids

Dopamine (DA) is chemically known as 4-(2-aminoethyl)-benzene-1,2-diol, and is one of the crucial catecholamine neurotransmitters widely distributed in the CNS for message transfer. DA, a simple organic chemical in the catecholamine family, is a monoamine neurotransmitter, which consists of an amine group (NH2) linked to a catechol structure, called dihydroxyphenethylamine, the decarboxylated form of dihydroxyphenylalanine (acronym DOPA). In the brain, dopamine functions as a neurotransmitter—a chemical released by nerve cells to send signals to other nerve cells. The human brain uses five known types of dopamine receptors, labeled D1, D2, D3, D4, and D5. As a chemical messenger, DA affects brain processes that control movement, emotional response, and ability to experience pleasure and pain. It plays a pivotal role in the function of cardiovascular, hormonal, renal and CNS [13]

Noradrenaline/NE/noradrenaline/NA

Norepinephrin-NE or noradrenaline-NA is a catecholamine, released from the adrenal medulla as hormone by secretory granules of the medullary chromaffin cells (synthesized from dopamine-by-dopamine β-hydroxylase in the secretory granules of the medullary chromaffin cells) and by noradrenergic neurons as neurotransmitter in the CNS. NA plays an important role in sexual behavior via alpha- adrenergic receptor while alpha-2 adrenergic receptors inhibit sexual behavior [14,15].

Adrenaline-A is derived from tyrosine amino acid and sometimes referred to as a catecholamine as it contains the catechol moiety (this is a part of the molecule that contains the group $C_6H_4(OH)_2$). Adrenaline is also involved in the fight or flight response in humans occurs when a person is subject to a threat. This causes a signaling process to occur, which causes the body to react to the potential danger. Specifically, once a threat is perceived, a signal is sent to the brain and then brain sends nerve impulses to the adrenal gland in the kidneys. When the nerve signal reaches the adrenal gland, chromaffin cells, in the medulla of the adrenal gland, release adrenaline. Adrenaline then enters the bloodstream. It is thus carried around the body to cells in various locations, where it initiates several responses. Despite initiating several different responses, adrenaline's effects have a collective purpose – to provide energy so that the major muscles of the body can respond to the perceived threat. Physiological responses to adrenaline by body organs as increases heart rate, increases respiratory rate, vasoconstriction and vasodilatation, stimulates glycogenolysis, triggers lipolysis and muscle contraction [15].

Aim of this review

1. A comprehend method for neurotransmitters estimation in in brain tissue with its reference value.
2. Review on the different neurotransmitters with its significance.

Table 1: The values of neurotransmitters in the different brain areas of healthy albino rat, *Rattus norvegicus*.

Neurotr-ansmitter (µg/g)	Cerebellum mean±SE	Striatum mean±SE	Cerebral cortex mean±SE	Hypothalamus mean±SE	Brain stem mean±SE	Hippocampus mean±SE	References mean±SE
DA	301.10±4.22	733.85±8.91	105.17±1.19	665.18±9.60	175.18±7.65	621.86±6.78	Waggas and Hasan, 2009 [21]
DA	146 ± 0.082	473.9 ± 0	60.48 ± 0.05	754 ± 2.1	452.29 ± 0.63	243.14 ± 086	Bawazir, 2011 [22]
GABA	300.95±9.94	691.27±15.82	127.54±1.24	736.24±9.20	151.05±10.83	715.94±5.66	Waggas and Hasan, 2009 [21]
GABA	-	-	192.6±0.2613	312.8±0.3140	211.4±0.3038	-	Verma *et al.,* 2011 [22]
NA	306.91±9.13	719.83±8.59	107.64±0.82	650.72±7.57	157.31±12.41	613.66±9.34	Waggas and Hasan, 2009 [21]
NA	95.382±0.85	511.47 ± 1.8	56.44 ± 0.22	596.99 ± 3.24	390.49 ± 0.48	292.54 ± 1.54	Bawazir, 2011 [22]
5HT	306.62±6.30	742.92±11.53	108.02±0.51	666.38±9.60	155.06±10.57	667.61±4.23	Waggas and Hasan, 2009 [21]
5HT	192.46±0.79	171.65 ± 0.45	57.25 ± 0.39	432.03 ± 0.32	118.16 ± 0.20	214.79 ± 1.32	Bawazir, 2011 [22]

Table 2: The values of neurotransmitters in whole brain of albino Rat, *Rattus norvegicus* and Mice, *Mus musculus.*

UNIT	ANIMAL model	GABA mean±SE	DA mean±SE	NA mean±SE	A mean±SE	5-HT mean±SE	REFERENCES
µg/mg	Albino Rat.	1.49±0.12	0.74±0.04	0.287±0.01	0.16±0.01	0.5±0.12	Khalifa *et al.,* 1997 [24]
µg/mg	Albino Mice	0.32±0.12	0.51 ± 0.12	0.09 ± 0.12	0.08±0.03	0.29±0.22	Dilip, 2009 [25]
pg/mg	Albino Rat.	274±1.64	624.32±3.26	756±2.06	-	184±4.52	Senthil and Rajkapoor, 2010 [26]

Method evaluation

There are several methods for neurotransmitter estimation, in which Shellenberger and Gordon (1971) [16] method for neurotransmitter estimation is one of them. All these methods are good with high specificity and high precision, but difficult because of its complicated procedure and time-consuming. The methods which used in review are relatively simple and precise.

MATERIAL AND METHOD

For these neurotransmitters estimation the collective spectrofluorimetric methods have been established in which all estimations were expressed in easy, simple and conventional way in a single review paper which give best result in lesser time comparatively to other methods. The obtained excitation and emission spectra at different wavelengths were read from the spectrofluorimeter. What principle behind, preparation of regents and procedures are expressed in very easy and systematic manner discussed below of each estimation.

Estimation of GABA

Method: By Ninhydrin reagent test according to the method of Lowe *et al.,* 1958 [17].

Principle

The determination of endogenous GABA concentration is based on a fluorimetric assay that depends on the formation of a fluorescent product from the reaction between GABA and ninhydrin at alkaline pH and in the presence of glutamate.

The reaction can be defined as:

GABA + Ninhydrin ▼ **Alkaline Medium)** **Fluorescent product**

Reagents

A. 0.01 N HCl: 0.085 ml HCl dissolved in 100 ml of distilled water.
B. 10% Trichloroacetic acid (TCA): 10 g trichloroacetic acid dissolved in 100 ml of distilled water.
C. 0.05 M Glutamate solution (pH =6.4): 0.736 g Glutamic acid dissolved in 100 ml 0.2M Sodium phosphate buffer.
D. 0.2M Sodium Phosphate Buffer (pH=6.4):
a. 3.12 g NaH_2PO_4 (Di-basic) dissolved in 100 ml distilled water.
b. 5.364 g Na_2HPO_4 (Mono-basic) dissolved in 100 ml distilled water.
For 100 ml Solution add 38 ml of a. and 62 ml of b.
E. 14mM Ninhydrin solution (pH 9.9-10): 0.25g Ninhydrin dissolved in 100 ml 0.5M sodium carbonate buffer.
F. 0.5M sodium carbonate buffer (pH 9.9-10): 5.3g sodium carbonate dissolved in 100 ml distilled water.
G. Cupper tartrate reagent: Prepared by mixing 1.6g sodium carbonate, 329mg tartaric acid and 300mg $CuSO_4.5H20$, all made in l liter of distilled water.
H. GABA standard: 50 µg or 100 µg GABA dissolved in 1 ml TCA.

Preparation of tissue homogenate

1. Animals were sacrificed by cervical dislocation and brains were rapidly removed, and separated forebrain region.
2. It was blotted, weighed and placed either in 5ml of chilled 10% TCA (it precipitate the proteins), then homogenized and centrifuged at 10,000rpm for 10min at 0°C, to obtain a clear filtrate/supernatant. (All steps were carried out at 0^0C).

Procedure of estimation

1. Test sample is prepared by 0.25 ml of supernatant was diluted with 0.25 ml of 0.01 M HCl and 0.5 ml of 10% TCA.
2. Blank is prepared by without 0.25 ml of brain homogenate and remain same as above.
3. Standard is prepared by 0.25 ml GABA standard instead of 0.25 ml of brain homogenate and remain same as above.
4. Then, mixture was centrifuged at 10,000 rpm for 10 min at 0°C.
5. After centrigugation, 100 µl aliquots of the supernatant were removed and add 15

µl of Glutamate solution and 200 µl of 14mM Ninhydrin solution.
6. This mixture was kept in water wath for 60ºC for 30 minutes and then cooled before the addition of 5 ml Copper tartrate reagent.
7. 15 minutes later; the fluorescence of the solution was adjusted at 380 or 450 nm by spectofluorimeter.

Calculation:

$GABA$ (µg/g Brain Tissue) $= \frac{O.D.\ OF\ TEST\ SAMPLE}{O.D.\ OF\ STANDARD} X$ CONC. OF GABA STANDARD X DILUTION FACTOR

Estimation of 5-HT

Method: By O-phthaldialdehyde (OPT) reagent test and according to the methods of Schlumpf *et al.,* 1974; Ciarlone 1978; and Rahman and Eswaraiah, 2009 [18-20].

Principle

The determination of endogenous Serotonin concentration is based on a fluorimetric assay that depends on the formation of a fluorescent product from the reaction between Serotonin and OPT reagent in acidic medium. The reaction can be defined as:

Serotonin + OPT reagent ▼ (Acidic Medium) fluorescent product

Reagents

1. HCl – Butanol solution: 0.085 ml HCl dissolved in 100 ml of *n*-Butanol.
2. n-Heptane
3. 0.2 N acetic acid: 1.14 ml of Glacial acetic acid upto 100 ml with distilled water.
4. O-phthaldialdehyde (OPT) reagent: 20 mg OPT dissolved in 100 ml conc. HCl.
5. Conc. HCl
6. 5-HT standard: 500 µg 5-HT dissolved in 1 ml mixture of distilled water and HCl-butanol solution (1:2 ratio).

Preparation of tissue homogenate

1. Mice were sacrificed by cervical dislocation, the whole brain was dissected out and separated the forebrain, weighed and kept at -80ºC until analyzed.
2. Weighed quantity of tissue was homogenized in glass homogenizer submerged in ice and containing 5 ml of HCl – Butanol solution (Note: 50-75 mg of tissue for homogenate with 5 ml of HCl-Butanol in correlation of same tissue concentration 1.5-5 mg/0.1 ml of HCl-butanol).
3. The mixture was homogenized and placed on a vortex mixer for 30 seconds.
4. The crude homogenate was centrifuged for 10 min at 2,000 rpm by using bench top centrifuge.
5. 2.5ml of the supernatant fluid transferred to glass tubes containing 1.6 ml of 0.2 N acetic acid and 5 ml n-Heptane.
6. After 10 min of vigorous shaking or thoroughly mixed by using vortex mixer, then centrifuged 5 min at 2,000 rpm by using bench top centrifuge and see clear separation of two phases.
7. The supernatant or overlaying organic phase was discarded and 0.20 ml of the aqueous phase was transferred to a tube for 5-HT assay (All steps were carried out at 0^0C).

Procedure of estimation

1. Test sample is prepared by taking 0.20 ml of aqueous extract and add 0.25 ml of OPT reagent.
2. Blank is prepared by taking 0.20 ml conc. HCl instead of tissue aqueous extract and add 0.25 ml of OPT reagent.
3. Standard is prepared by taking 0.20 ml GABA standard and add 0.25 ml of OPT reagent.
4. The fluorophore was developed by heating in boiling water bath at 100°C for 10 min.
5. Then cool under tap water, after the samples reached equilibrium with the ambient temperature.

6. Readings were taken in micro cuvette at 360-470 nm by spectofluorimeter.

Calculation

$5-HT$ (µg/g Brain Tissue) $= \frac{O.D.\ OF\ TEST\ SAMPLE}{O.D.\ OF\ STANDARD} X$ CONC. OF 5-HT STANDARD X DILUTION FACTOR

Estimation of catecholamines (DA, NA AND A)

Method: By tri-hydroxide method and according to the methods of Schlumpf *et al.,* 1974; Ciarlone 1978; and Rahman and Eswaraiah, 2009 [18-20].

Reagents:

1. HCl – Butanol solution: 0.085 ml HCl dissolved in 100 ml of *n*-Butanol.
2. n-Heptane
3. 0.2 N Acetic acid: 1.14 ml of Glacial acetic acid upto 100 ml with distilled water.
4. 0.4 M HCl: 3.4 ml conc. HCl is added into 100 ml Distilled water.
5. Sodium acetate buffer (pH 6.9): 2.88 ml of 1M acetic acid is added into 27.33 ml of 0.3M sodium acetate and volume is made up to 100 ml with distilled water (pH is adjusted with sodium hydroxide sol).
6. 5M Acetic acid: 28.5 ml of Glacial acetic acid upto 100 ml with distilled water.
7. 0.3M Sodium acetate: 4.08 g of sodium acetate 100 ml with distilled water)
8. 0.1M Iodine solution: 4 g of KI +2.6 g of Iodine dissolved in ethanol volume is made up to 100 ml.
9. 10M Acetic acid: 57 ml of glacial acetic acid dissolved in distilled water up to 100 ml.
10. Na_2SO_3 solution: 0.5 g Na_2SO_3 in 2 ml H2O and18 ml 5 M NaOH.
11. DA/NA/A standard: 500 µg DA/NA/A dissolved in 1 ml mixture of distilled water and HCl-butanol solution (1:2 ratio).

Preparation of tissue homogenate

1. Mice were sacrificed by cervical dislocation, the whole brain was dissected out and separated the forebrain, weighed and kept at -80ºC until analyzed.
2. Weighed quantity of tissue was homogenized in glass homogenizer submerged in ice and containing 5 ml of HCl – Butanol solution.
3. The mixture was homogenized and placed on a vortex mixer for 30 seconds.
4. The crude homogenate was centrifuged for 10 min at 2,000 rpm by using bench top centrifuge.
5. 2.5ml of the supernatant fluid transferred to glass tubes containing 1.6 ml of 0.2 N acetic acid and 5 ml n- Heptane.
6. After 10 min of vigorous shaking or thoroughly mixed by using vortex mixer, then centrifuged 5 min at 2,000 rpm by using bench top centrifuge and see clear separation of two phases.
7. The supernatant or overlaying organic phase was discarded and 0.2-1ml of the aqueous phase was transferred to a tube for catecholamine assay (All steps were carried out at 0^0C).

Procedure of estimation

1. Take 0.2 ml of aqueous phase of tissue homogenate, 0.05 ml 0.4 M HCl and 0.1 ml of EDTA/Sodium acetate buffer (pH 6.9) were added, followed by 0.1 ml 0.1 M iodine solution for oxidation of catecholamines.
2. The reaction was stopped after 2 min. by addition of 0.1 ml Na_2SO_3 solution. After 1.5 min. 0.1 ml of 10M Acetic acid is added.
3. Blanks were prepared by adding the reagents of the oxidation step in reversed order (sodium sulphite before iodine).
4. Standards were prepared by taking 0.2 ml DA/NA/A standard instead of aqueous phase of tissue homogenate.

5. Before heating adrenaline fluorescence was read immediately at 410nm and 500nm.
6. The solution was then heated to 100°C for 2 minutes in boiling water bath and when the sample reached room temperature noradrenaline fluorescence was read at 380 and 480nm.
7. After determination of norepinephreine, again heating for exactly 5 minutes and when the sample reached room temperature dopamine was read at 320 and 375nm for dopamine (DA).

Calculation

DA (µg/g Brain Tissue) $= \frac{O.D.\ OF\ TEST\ SAMPLE}{O.D.\ OF\ STANDARD} X$ CONC. OF DA STANDARD X DILUTION FACTOR

NA(µg/g Brain Tissue) $= \frac{O.D.\ OF\ TEST\ SAMPLE}{O.D.\ OF\ STANDARD} X$ CONC. OF NOR-ADRENALINE STANDARD X DILUTION FACTOR

A (µg/g Brain Tissue) $= \frac{O.D.\ OF\ TEST\ SAMPLE}{O.D.\ OF\ STANDARD} X$ CONC. OF ADRENALINE STANDARD X DILUTION FACTOR

Concluding Remarks:

These spectrofluorimetric methods of neurotransmitter estimations are simple, rapid and economical for the analysis of levels of neurotransmitters *viz.* GABA, DA, NA and 5-HT in wet tissue of brain of laboratory animals like mice, rat *etc.*

Declaration by Authors

Acknowledgements: I was most grateful to the following for the generous contribution of ideas, suggestion, and references which have been invaluable in the development of this review: Prof. Vinoy K Shrivastava, HOD, Bioscience Department, Barkatullah University, Bhopal, Madhya Pradesh, India.

Source of Funding: None

Conflict of Interest: The author declares that no conflict of interest pertaining to this review.

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How to cite this article: Neha Jain. Review on simple spectrofluorimetric method for neurotransmitters estimation in brain tissues of laboratory animals. *International Journal of Research and Review*. 2025; 12(10): 68-76. DOI: *https://doi.org/10.52403/ijrr.20251008*

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