

**METHOD DEVELOPMENT AND VALIDATION TO DETERMINE
NEUROTRANSMITTERS BY USING HPLC**

**by
ÖZGE YETGİN**

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Prof. Dr. Yaşar DÜRÜST
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

Prof. Dr. Nedime DÜRÜST
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

Prof. Dr. Serpil YENİSOY KARAKAŞ
Supervisor

Examining Committee Members

1- Prof. Dr. Serpil YENİSOY KARAKAŞ

2- Prof. Dr. Adem ASAN

3- Prof. Dr. Hasan BASAN

ABSTRACT

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YETGİN, Özge

M. Sc., Department of Chemistry

Supervisor: Prof. Dr. Serpil YENİSOY KARAKAŞ

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In this study, two methods were developed for the determination of the neurotransmitters (gamma aminobutyric acid (GABA), glutamate, serotonin and noradrenaline) and the melatonin hormone in cerebrospinal fluid samples from the hippocampus, amygdala and raphe nuclei of the rat brain by using high-performance liquid chromatography (HPLC) with fluorescence detection and a C18 reversed-phase column. Samples were collected by using microdialysis technique. In the first method, pre-column derivatization with naphthalene-2,3-dicarboxaldehyde (NDA) in the presence of cyanide ion was performed for the simultaneous detection of GABA and glutamate. The derivatives were separated using isocratic elution and detected fluorimetrically at the excitation and the emission wavelengths of 420 and 480 nm, respectively. The second method involves the simultaneous detection of melatonin,

serotonin and noradrenaline without derivatization step. The compounds were separated using isocratic elution and detected fluorimetrically at the excitation and the emission wavelengths of 280 and 340 nm, respectively.

The methods were validated. The performance of the methods was evaluated in terms of linearity, method detection limit, limit of quantitation, recovery and precision. Standard addition method was used to obtain calibration curves. The method detection limit values were found as 28 ppb for GABA, 9 ppb for glutamate, 0.5 ppb for melatonin, 1.2 ppb for serotonin and 2.7 ppb for noradrenaline. The limit of quantitation values were found as 92 ppb for GABA, 30 ppb for glutamate, 1.7 ppb for melatonin, 3.8 ppb for serotonin and 8.8 ppb for noradrenaline. The average recoveries changed between 103 % and 118 % for GABA, 87 % and 99 % for glutamate, 88 % and 121 % for melatonin, 76 % and 117 % for serotonin and 72 % and 105 % for noradrenaline. The percentage relative standard deviation (RSD) values ranged from 0.78 to 1.80 for GABA, from 0.80 to 1.67 for glutamate, from 0.31 to 2.18 for melatonin, from 0.84 to 5.05 for serotonin and from 0.44 to 8.24 for noradrenaline.

Measurement uncertainties of all compounds were calculated by applying 'bottom up' method. The calculated percentage relative uncertainty values for the compounds changed between 10.1 and 16.7. The major sources of the uncertainty budget were calibration curves, stock solution and recovery.

Keywords: Neurotransmitter, GABA, Glutamate, Serotonin, Noradrenaline, Melatonin, Cerebrospinal Fluid, Microdialysis, HPLC, Fluorescence Detection, Method Validation, Uncertainty Budget

ÖZET

NÖROTRANSMİTTERLERİN TAYİNİ İÇİN HPLC'DE METOT GELİŞTİRİLMESİ VE METOT VALİDASYONU

YETGİN, Özge

Yüksek Lisans, Kimya Bölümü

Tez Danışmanı: Prof. Dr. Serpil YENİSOY KARAKAŞ

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Bu çalışmada, sıçan beyinlerinin hipokampus, amigdala ve rafe çekirdeği bölgelerinden mikrodializ yöntemi ile toplanan beyin omurilik sıvısı örneklerinde gama aminobütirik asit (GABA), glutamat, serotonin ve noradrenalin nörotransmitterleri ile melatonin hormonunun tayini için floresan dedektör, C18 ters-faz kolon ve yüksek performanslı sıvı kromatografisi (HPLC) kullanılarak iki farklı metot geliştirilmiştir. Birinci metotta, GABA ve glutamatın birlikte tayini için siyanür iyonunun varlığında 2,3-naftalindikarboksialdehit (NDA) ile ön-kolon türevlendirme yapılmıştır. Türevler izokratik yıkama ile ayrılmış ve 420 nm uyarma ile 480 nm emisyon dalga boylarında floresan detektör ile tayin edilmişlerdir. İkinci metot türevlendirme basamağı olmaksızın melatonin, serotonin ve noradrenalinin

birlikte tayinini içerir. Bileşikler izokratik yıkama ile ayrılmış ve 280 nm uyarma ile 340 nm emisyon dalga boylarında floresan detektör ile tayin edilmişlerdir.

Metotların validasyonu yapılmıştır. Metotların performansı lineer aralık, algılama ve tespit limiti, geri kazanım ve kesinlik parametreleri ile değerlendirilmiştir. Kalibrasyon doğruları için standart ekleme metodu kullanılmıştır. Algılama limitleri GABA için 28 ppb, glutamat için 9 ppb, melatonin için 0,5 ppb, serotonin için 1,2 ppb ve noradrenalin için 2,7 ppb olarak bulunmuştur. Tespit limitleri ise GABA için 92 ppb, glutamat için 30 ppb, melatonin için 1,7 ppb, serotonin için 3,8 ppb ve noradrenalin için 8,8 ppb'dir. Ortalama geri kazanım değerleri GABA için % 103 ile % 118, glutamat için % 87 ile % 99, melatonin için % 88 ile % 121, serotonin için % 76 ile % 117 ve noradrenalin için % 72 ile % 105 arasında değişmektedir. Yüzde bağıl standart sapma (RSD) değerleri ise GABA için 0,78 ile 1,80, glutamat için 0,80 ile 1,67, melatonin için 0,31 ile 2,18, serotonin için 0,84 ile 5,05 ve noradrenalin için 0,44 ile 8,24 aralıklarında bulunmuştur.

Tüm bileşikler için 'alttan-yukarı' yaklaşımı uygulanarak ölçüm belirsizlikleri hesaplanmıştır. Yüzde bağıl ölçüm belirsizlik değerleri 10,1 ile 16,7 aralığında değişim göstermiştir. Belirsizlik bütçesinin en büyük bileşenleri kalibrasyon eğrisi, stok çözelti ve geri kazanım olmuştur.

Anahtar Kelimeler: Nörotransmitter, GABA, Glutamat, Serotonin, Noradrenalin, Melatonin, Beyin omurilik sıvısı, Mikrodiyaliz, HPLC, Floresan Detektör, Metot Validasyonu, Belirsizlik Bütçesi

*Dedicated to my parents
and Altan ÇETİN...*

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CHAPTER 1

INTRODUCTION

The nervous system can be divided into two parts: the central nervous system (CNS) and the peripheral nervous system (Figure 1.1). The central nervous system consists of the brain and spinal cord. The peripheral nervous system includes all of the sensory and motor pathways outside the brain and spinal cord.

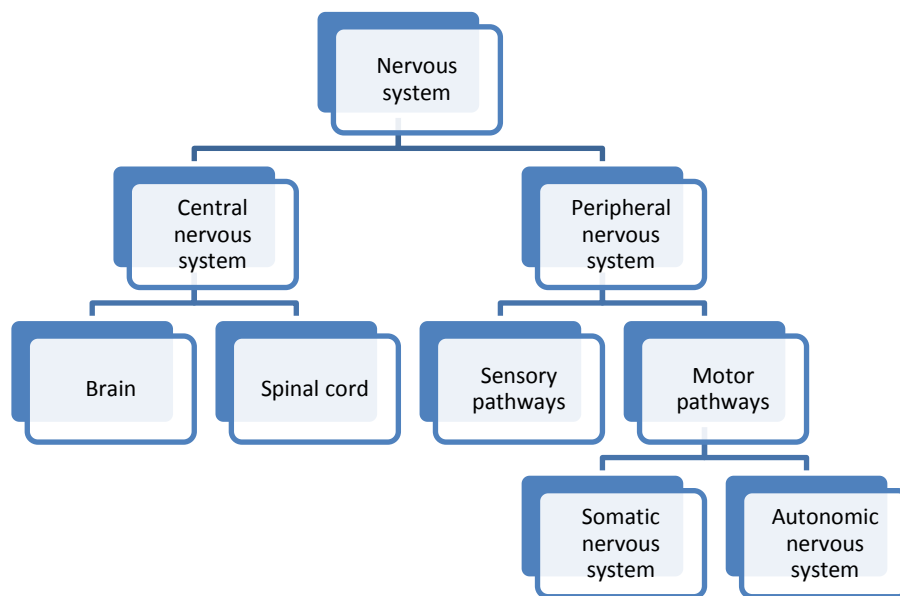


Figure 1.1. The components of the vertebrate nervous system (Raven, 1996)

Neurons (also called as nerve cells) are the basic unit of the nervous system. Neurons are specialized for the rapid transmission of information along long distances within the body. Although they have various appearances, all neurons possess the same functional structure. Each neuron consists of four major components: the cell body (soma), the dendritic tree, the axon and synaptic terminals (Figure 1.2). The cell body is an enlarged region containing the nucleus and various organelles and it is the metabolic and trophic (relating to nutrition) center of the neuron. Most nerve cells possess a profusion of highly branched dendrites that extend the cell body. The primary function of dendrites is to increase the surface area for receiving information from many different sources simultaneously and then they transmit this information to the cell body. The surface of the cell body integrates the information. If the resulting membrane excitation is great enough, an electrical impulse is sent out from the cell body along the axon. Each neuron generally has a single axon which may be quite long (Raven, 1996; Siegel, 2006).

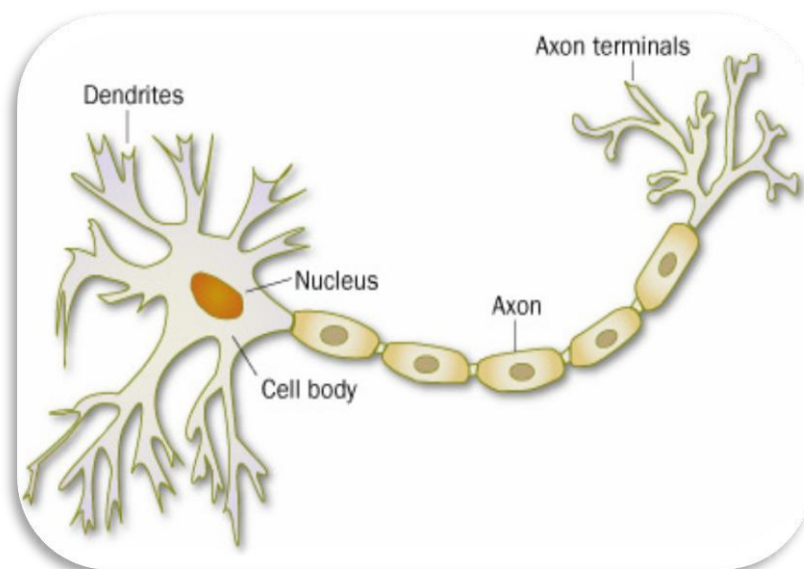


Figure 1.2. A schematic representation of a neuron (Anonymous 1, 2013)

The transmission of information from one cell to another is accomplished in two ways: electrically and chemically. Chemical transmission is the major mechanism of synaptic communication in the mammalian nervous system.

1.1. Chemical Transmission

A synapse is described as specialized intercellular junctions where one neuron communicates with another one (Figure 1.3). The neuron whose axon transmits action potentials to the synapse is called to as the presynaptic cell. Furthermore, the cell on the other side of the synapse is known as the postsynaptic cell (Raven, 1996). The cells do not actually make contact. Instead, they are separated by synaptic cleft which are fluid-filled narrow gaps (20-50 nm). The presynaptic and postsynaptic membranes adhere to each other through the matrix of extracellular fibrous protein in the synaptic cleft. The presynaptic terminal contains numerous synaptic vesicles that are filled with several thousand molecules of a specific chemical substance, the neurotransmitter (Siegel, 2006).

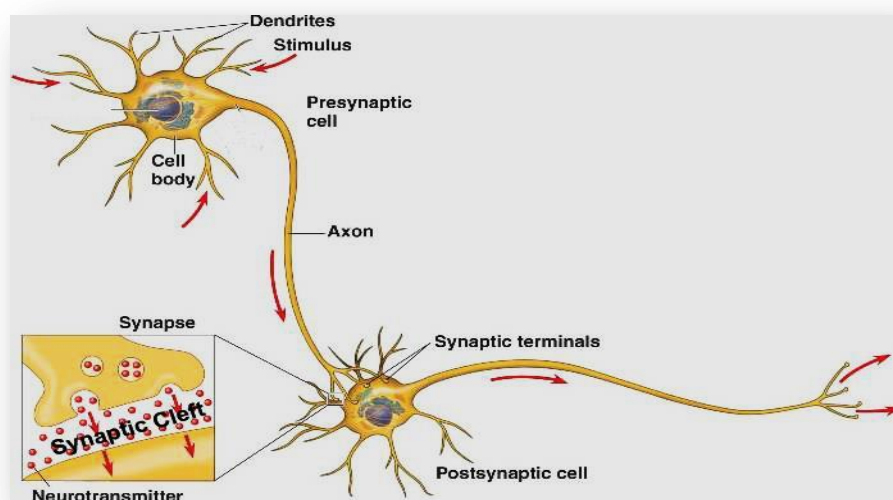
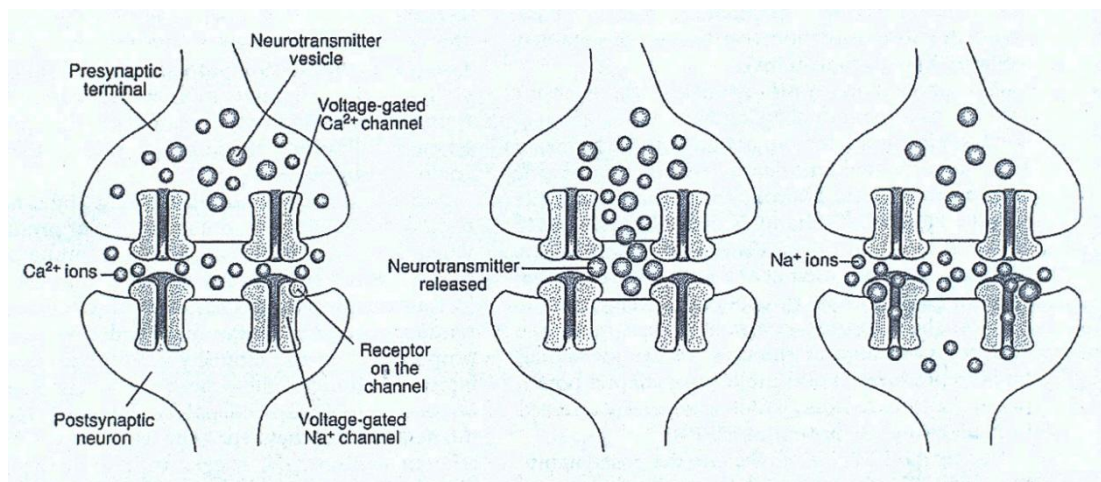


Figure 1.3. A schematic representation of a synapse (Anonymous 6, 2013)

An action potential signal depolarizes the presynaptic nerve terminal. This depolarization causes voltage-gated Ca^{2+} channels located in the presynaptic terminal membrane to open. Ca^{2+} enters the terminal because its concentration is greater outside the cell than inside. These events cause vesicles filled with neurotransmitter to migrate towards the presynaptic membrane. The vesicles fuse with the presynaptic membrane and a continuous membrane is formed, then the neurotransmitter is released into the synaptic cleft. This process of transmitter release is called exocytosis. The neurotransmitter diffuses through the synaptic cleft to the membrane of the postsynaptic neuron and binds with its specific receptors (Siegel, 2006; Anonymous 5, 2013). This process is depicted in Figure 1.4.



Action potential depolarizes the presynaptic terminal membrane, Ca^{2+} channels open and Ca^{2+} ions enter the terminal.	Ca^{2+} ions in the terminal promote fusion of vesicular and terminal membranes and the neurotransmitter is released in the synaptic cleft.	Neurotransmitter binds with the receptor on the channel which opens, Na^{+} ions enter the postsynaptic neuron, depolarize it and excite it.
--	--	---

Figure 1.4. Mechanism of transmitter release (Siegel, 2006)

1.2. Neurotransmitters

Neurotransmitters are synthesized in presynaptic terminals and stored in synaptic vesicles. Neurotransmitters are the primary chemical messengers that are released at a synapse following depolarization of the nerve terminal and relay, amplify and modulate signals to other cells. Neurotransmitters are present throughout the body and various biological fluids including serum, plasma, platelets, cerebrospinal fluid, saliva and urine. Neurotransmitters serve specific biological functions throughout the body; however, the important role of neurotransmitters is in regulating neurological function in the brain (Siegel, 2006; Marc et al., 2011).

There are several criteria for accepting a chemical substance as a neurotransmitter.

1. The substance must be synthesized in the neuron and the biosynthetic enzymes for the synthesis of the substance must be present in the neuron.
2. The substance must be released by stimulation of the presynaptic neuron in a Ca^{2+} dependent manner and in sufficient quantity to elicit a response from the postsynaptic neuron or cell located in the effector organ.
3. A specific mechanism for removal or inactivation of the substance from the synaptic cleft must exist.
4. Its effect on a neuron is the same whether released exogenously (i.e., from outside the organism as a drug) or endogenously (from the presynaptic terminal) (Suszkiw, 2001; Siegel, 2006; Anonymous 6, 2013).

There are over 60 distinct chemicals used as neurotransmitters by the nervous system. Acetylcholine is the first neurotransmitters to be discovered (Raven, 1996). The most important monoamine neurotransmitters are serotonin, dopamine,

noradrenaline and adrenalin. Dopamine is the most abundant of the four monoamine neurotransmitters (Perry et al., 2009). Classification of neurotransmitters in the nervous system is shown in Table 1.1. There are three major categories: small molecule transmitters, neuroactive peptides and gaseous neurotransmitters.

Table 1.1. Classification of neurotransmitters (Siegel, 2006)

Small Molecule Neurotransmitters	Neuropeptides	Gaseous Neurotransmitters
Acetylcholine	Opioid peptides	Nitric oxide
Excitatory amino acids	β -endorphin	
Glutamate	Methionine-enkephalin	
Aspartate	Leucine-enkephalin	
Inhibitory amino acids	Endomorphins	
GABA	Nociceptin	
Glycine		
Biogenic amines	Substance P	
Catecholamines	This substance is an	
- Dopamine	undecapeptide (it is	
- Norepinephrine	composed of 11 amino	
- Epinephrine	acids).	
Indoleamine		
- Serotonin		
Imidazole amine		
- Histamine		
Purines		
- Adenosine triphosphate		
- Adenosine		

1.2.1. Gamma Aminobutyric Acid (GABA)

GABA is the major inhibitory neurotransmitter in the mammalian CNS. Full acceptance of GABA as a neurotransmitter occurred at the end of 1960s and early 1970s (Bowery and Smart, 2006).

The steps involved in the synthesis of GABA and its removal from the synaptic cleft are as follows:

1. Glutamine is converted into glutamate by glutaminase enzyme.
2. GABA is formed by α -decarboxylation of glutamate. This reaction is catalyzed by L-glutamic acid-1-decarboxylase (GAD).
3. Synthesized GABA is taken up into vesicles and stored there.
4. It is released into synaptic cleft by exocytosis.
5. After its release, GABA is taken up into presynaptic terminal via GABA transporters and repackaged into vesicles for subsequent use. Also, GABA is taken up into the glia via GABA transporters.
6. In glia, GABA is converted to glutamate by GABA transaminase enzyme.
7. Glutamine synthetase enzyme converts glutamate into glutamine which is then transported into the neighboring nerve terminals where it is processed to synthesize glutamate (Siegel, 2006).

GABA, which is a ubiquitous, non-protein aminoacid neurotransmitter, is found in high concentrations in the brain and spinal cord but is absent in peripheral nerves or peripheral tissues. GABA is heterogeneously distributed throughout the brain and brain GABA content is greater than most other neurotransmitters (30-40% of all synapses) (Siegel, 2006; Zeyden et al., 2008). Unlike glutamate, GABA is not an essential metabolite and is not incorporated into a protein (Siegel, 2006).

Numerous studies indicate that alteration of GABAergic circuits has been implicated in neurological and psychiatric disorders such as Huntington's chorea, Parkinson's disease, senile dementia, Alzheimer's disease and schizophrenia (Siegel, 2006; Zeyden et al., 2008). A deficit GABA is responsible for certain types of anxiety (Raven, 1996; Zeyden et al., 2008). Elevated GABA levels were observed in the CSF of meningitis patients (Zhang and Beyer, 2006). Decrease in the neuronal inhibition effect of GABA in neurons causes epileptic discharges resulting in seizure development (Eroğlu, 2012).

1.2.2. Glutamate

Glutamate is the main excitatory neurotransmitter in the mammalian CNS. Full acceptance of glutamate as an amino acid neurotransmitter firstly occurred at the end of the 1970s (Zeyden et al., 2008).

Glutamate synthesis in the brain exists by using two processes. In one process, glucose enters the neuron by facilitated diffusion and is metabolized via the Krebs cycle. α -oxoglutarate forms during the Krebs cycle and it is transaminated by α -oxoglutarate transaminase to form glutamate. In the second process: glial cells and nerve terminals reuptake the glutamate released from the nerve terminals via glutamate transporters located in their cell membranes. In the glia, glutamate is converted into glutamine by glutamine synthetase enzyme. Glutamine is transported out of the glia into the neuronal terminal via glutamine transporters located in the glial and neuronal terminal membranes. Glutamine is converted into glutamate by glutaminase enzyme in the neuronal terminal. Glutamate is taken up into the vesicles by active transport, stored and subsequently released by exocytosis. Then released glutamate is actively taken up by the glia and neuronal terminals via glutamate

transporters. It is repackaged into the vesicles for subsequent reuse. Glutamate is taken up by a high affinity, sodium dependent, reuptake mechanism into the nerve terminals and glial cells via glutamate transporters which are located on the plasma membrane of the presynaptic nerve terminal and on glial cells (Siegel, 2006).

Glutamate is found throughout the mammalian brain and takes part in many metabolic pathways (Platt, 2007). Glutamate is used by nearly 50-60 % of all terminals in the CNS as a neurotransmitter. Over half of the 100 billion neurons in the brain produce glutamate and also 90 % of all neurons display sensitivity to glutamate (Zeyden et al., 2008). The concentration of glutamate in the mammalian brain is high with an average value of 12 mM which is greater than GABA (5-10-fold smaller) (Eckstein et al., 2008).

In addition to its role as a neurotransmitter in the CNS, glutamate is an intermediate in energy metabolism of the CNS, detoxifies ammonia via the formation of glutamine, is an important building block in the synthesis of proteins and peptides, is involved in fatty acid synthesis and is a precursor for GABA (Zeyden et al., 2008).

Glutamate has been implicated as a neurotransmitter in most aspects of normal brain functioning, including learning and memory processes (Siegel, 2006; Platt, 2007; Zeyden et al., 2008). Also, it has a major role in neuron-development and synaptic plasticity of the CNS, including the migration, differentiation and death of cells, and in the induction and elimination of synapses (Zeyden et al., 2008).

Glutamate may be a potent neurotoxin. Prolonged stimulation of neurons by excitatory amino acids causes neuronal death or injury (excitotoxicity) (Siegel, 2006). Excitotoxic action of glutamate has been linked to a variety of neurodegenerative and psychiatric disorders including epilepsy, motor neuron disease, traumatic brain injury, Huntington's chorea, Parkinson's, Alzheimer's

disease, amyotrophic lateral sclerosis and stroke (Platt, 2007; Zeyden et al., 2008). Decreased concentrations of glutamate have been reported in the prefrontal cortex and hippocampus of schizophrenic patients (Shah et al., 2002).

1.2.3. Serotonin (5-hydroxytryptamine; 5-HT)

Serotonin is well known as a neurotransmitter in the control and regulation of many brain functions and has been strongly implicated in several pathological conditions (Ishida et al., 1998). Serotonin-containing neurons have been identified in the midline raphe nuclei of medulla, pons, and upper brainstem (Siegel, 2006).

Serotonin is an indoleamine that is biologically synthesized from the essential amino acid tryptophan. Indoleamines are substituted indole compounds that contain an amino group (Anonymous 10, 2014). The chemical structure of serotonin is shown in Figure 1.5.

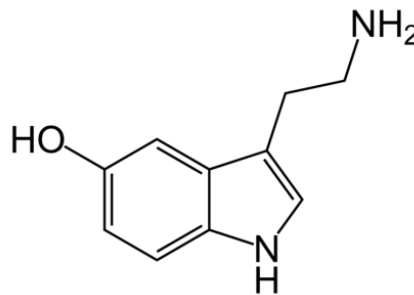


Figure 1.5. Chemical structure of serotonin

Serotonin does not cross the blood-brain barrier. Therefore, brain cells must synthesize their own serotonin. Dietary tryptophan serves as a substrate for serotonin synthesis. Plasma tryptophan enters the brain by an active uptake process and is hydroxylated by tryptophan hydroxylase to form 5-hydroxytryptophan. Formation of

serotonin occurs by decarboxylation of 5-hydroxytryptophan. The reaction is catalyzed by aromatic L-amino acid decarboxylase. Then, serotonin is actively taken up and stored in vesicles where it is ready for release. After its release, serotonin is removed from synaptic cleft by the mechanisms of reuptake and metabolism. Reuptake involves entry of serotonin into the neuronal terminals by active transport via serotonin transporter proteins. Serotonin is metabolized via oxidative deamination (Siegel, 2006).

Serotonin has been implicated in the regulation of a wide range of brain functions including the control of mood, aggression, sex and sleep (Waggoner et al., 1998). Serotonin-containing cells in the raphe regions of the brainstem are considered to play a role in decreasing pain-control systems. There may be a role of other serotonin-containing neurons in mediating affective processes like aggressive behavior and arousal. Also, serotonin synthesized in the pineal gland serves as a precursor for the synthesis of melatonin (Siegel, 2006).

Abnormalities in the serotonin-related processes lead to various pathological conditions. Aberrations in its CNS function are thought to be involved in anorexia, anxiety, depression and schizophrenia. Degeneration of serotonergic neurons have been noted in Alzheimer's and Parkinson's diseases. Peripheral aberrations in serotonin-related processes have been implicated in hypertension, migraine, genesis of cardiac arrhythmias, Raynaud's disease and some symptoms of carcinoid syndrome (Antmen, 2011). Decreased levels of the neurotransmitter serotonin in blood platelets is a clinical symptom characteristic of individuals with Down's syndrome (Schickler et al., 1989).

1.2.4. Noradrenaline (Norepinephrine)

Noradrenaline is one of the major endogenous catecholamines. The catecholamines are a group of compound bearing a dihydroxyphenyl moiety (Mishra et al., 2009). The chemical structure of noradrenaline is shown in Figure 1.6.

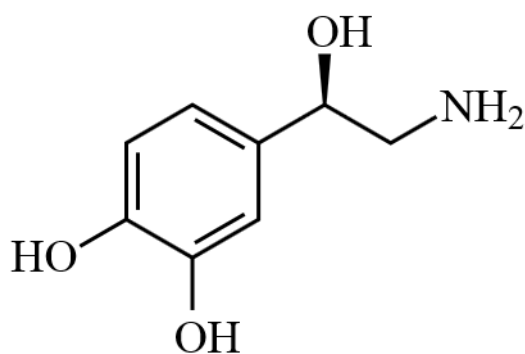


Figure 1.6. Chemical structure of noradrenaline

The natural stereoisomer is L-(-)-(R)-noradrenaline. The prefix *nor-* indicates that noradrenaline is the next-lower homolog of adrenaline. The two structures differ only in that adrenaline has a methyl group attached to its nitrogen, whereas the methyl group is replaced by a hydrogen atom in noradrenaline. The prefix *nor-* is derived as an abbreviation of the word "normal", used to indicate a demethylated compound (Anonymous 11, 2014).

Noradrenaline is mainly synthesized by the locus ceruleus neurons that is located in the pons. Noradrenaline is released in the hypothalamus, hippocampus, septum, cerebral cortex and cerebellum (Custodio et al., 2010). The noradrenergic neurons contain dopamine β -hydroxylase enzyme and it converts dopamine into noradrenaline. The synthesis of noradrenaline takes place in the storage vesicles. Thus, the noradrenaline is ready to be released by exocytosis (Siegel, 2006).

1.3. Melatonin

Melatonin (5-methoxy-N-acetyltryptamine) is an indoleamine hormone that was first isolated in 1958 by Lerner and his coworkers from bovine pineal gland (Tanaka et al., 2007; Mubashshir et al., 2011). The chemical structure of melatonin is shown in Figure 1.7.

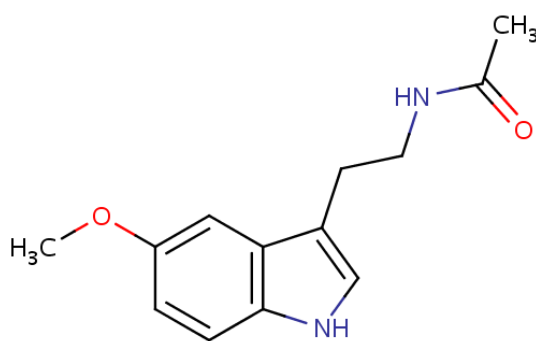


Figure 1.7. Chemical structure of melatonin

Melatonin does exist in bacteria, eukaryotic unicellular organisms, macroalgae, plants, fungi and various taxa of invertebrates and vertebrates (Aguzzi et al., 2009). Melatonin is secreted mainly by the pineal gland (In humans, the pineal gland lies in the center of the brain, behind the third ventricle.), but synthesized also in many other tissues and cells, including the retina, human and murine bone marrow cells, platelets, the gastrointestinal tract, skin or lymphocytes (de Almeida et al., 2011).

Melatonin is synthesized from the amino acid tryptophan. Serotonin is an intermediate product in this synthesis. Serotonin converts into N-acetylserotonin under the action of N-acetyltransferase. The subsequent enzymatic methylation of N-acetylserotonin under the action of hydroxyindole-o-methyltransferase leads to

melatonin (Andreeva et al., 1999; Harumi and Matsushima, 2000). Melatonin secretion is controlled by noradrenaline (Andreeva et al., 1999).

Melatonin is predominantly secreted into the blood and is capable of readily penetrating through the histohematic barriers. The hormone exists in cerebrospinal and amniotic fluids, lymph, saliva, etc. (Andreeva et al., 1999).

The biotransformation of the melatonin occurs in the liver. The hormone is rapidly metabolized by hydroxylation with sulfuric and glucuronic acids. The metabolites are excreted from the organism in urine (Brezezinski, 1997; Andreeva et al., 1999).

The secretion of melatonin in the organism decreases as a result of aging. The hormone level in senile humans is about half the amount of young. Also, the level of melatonin exhibits both circadian and seasonal variations. Melatonin is more intensively synthesized overnight (in the dark). The concentration of melatonin in the blood during nighttime is ten times higher than during daytime. However, even a short-time exposure to intensive light at night sharply decreases the hormone secretion (Andreeva et al., 1999).

Melatonin is involved in the regulation of circadian rhythm (sleep-wake cycle) and the alleviation of sleep disorders, such as insomnia due to jetlag and shift work (Pothinuch and Tongchitpakdee, 2011). Also, melatonin exhibits direct and indirect antioxidant properties. There is strong evidence that melatonin can counteract the deleterious effects of reactive oxygen and nitrogen species in different systems (de Almeida et al., 2011). Melatonin is involved in the regulation of essential biological rhythms, puberty, and the seasonal sexual activity cycle, participates in the programming and prevention of aging, immune reactions, and

redox processes, and controls the organism's adaptation to weather changes, alteration of seasons, and changing time zones (Andreeva et al., 1999).

Melatonin is also involved in many biological and neuronal functions. Melatonin can modulate hippocampal synaptic transmission and inhibit the induction of long-term potentiation. These findings suggest that melatonin can directly affect synaptic transmission as a neuromodulator in a non-hormonal role (Tanaka et al., 2007)

1.4. Cerebrospinal Fluid (CSF)

Cerebrospinal fluid (CSF) is a clear, colorless ultrafiltrate of plasma with low protein content and few cells (Di Terlizzi and Platt, 2006). CSF circulates through subarachnoid space, around the brain and spinal cord, and through the ventricles of the brain (Figure 1.8).

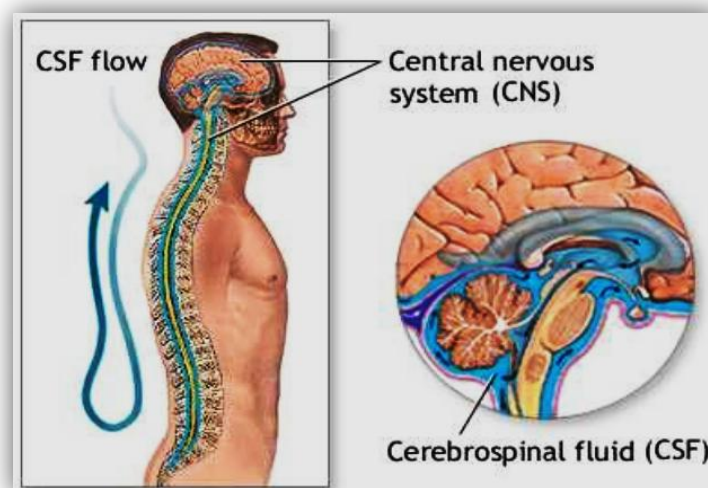


Figure 1.8. Circulation of cerebrospinal fluid (Anonymous 12, 2014)

The ventricles are cavities in the brain that are connected to each other with the central canal of the spinal cord and the subarachnoid space (Figure 1.9) (Anonymous 13, 2014).

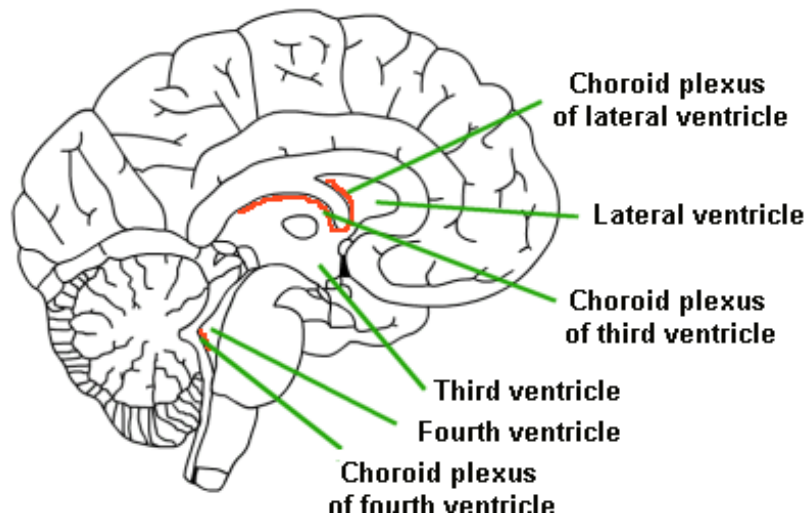


Figure 1.9. Ventricles of the brain (Anonymous 13, 2014)

The daily production rate in people is about three to four times as much as the total volume of fluid in the entire system of CSF (Di Terlizzi and Platt, 2006). About 70 % of the CSF presents in the brain and spinal cord and it is produced by the choroid plexuses at the rate of approximately 0.002 mL/min in rats and 0.35 mL/min in people. The rate of formation is predominantly related to the weight of the choroid plexuses and to the rate of sodium and bicarbonate ion exchange (Di Terlizzi and Platt, 2006; Siegel, 2006). The remaining 30 % of CSF, which is secreted by the parenchyma of the brain, crosses the ependyma and enters the ventricles. The CSF formation is an active, energy-dependent process involving the enzyme carbonic anhydrase and specific transport mechanisms (Siegel, 2006).

1.4.1. Functions of Cerebrospinal Fluid

The CSF has a protective role. The brain and spinal cord float in the CSF. This buoyant effect of the CSF results in reduction of traction exerted upon the nerves and blood vessels connected with the CNS. The CSF dampens the effects of trauma. The CSF also has a role in the excretion of the potentially toxic by-products of cerebral metabolism. Under normal conditions, the CSF provides a stable ionic environment for the CNS. The CSF has a filtration function which provide for movement of water-soluble substances from brain parenchyma into the CSF. The CSF serves as a vehicle for the intracerebral transport of biologically active substances. The CSF is also known to carry neurotransmitters and neuropeptides (Di Terlizzi and Platt, 2006; Siegel, 2006).

1.4.2. Composition of Cerebrospinal Fluid

CSF is a product of plasma filtration and membrane secretion. Its composition is different from that of plasma. Normally, CSF is clear, colorless, nearly acellular. Protein concentration in the normal CSF is very low. Varied ions, enzymes, neurotransmitters and other substances are also found in normal CSF. Large molecules cannot pass from blood into CSF thanks to blood-CSF barrier. The CSF composition is maintained comparatively constant by the various membrane interfaces, but some fluctuation can occur with fluctuations in plasma composition (Di Terlizzi and Platt, 2006).

Glucose is derived essentially from the plasma and carried into the brain by facilitated transport or diffusion. Sodium is the most abundant ion in the CSF. It is

important in transport and osmoregulation. The sodium concentrations of CSF and plasma are closely related. Potassium concentration is crucial for neuronal function and the release of neurotransmitters. Potassium concentration is lower in CSF than in plasma and even with very high potassium plasma concentrations, the CSF potassium concentration remains within the normal range (Di Terlizzi and Platt, 2006). CSF is sterile and so the results of microbiological studies on normal CSF should be negative. CSF normally does not contain red blood cells and contains up to 5 lymphocytes/ μL . Thereby, in CSF an increased white blood cell count indicates disease (Siegel, 2006).

Table 1.2. Composition of cerebrospinal fluid (Siegel, 2006)

Constituent	CSF
Protein (g/L)	0.15-0.45
Glucose (mmol/L)	2.2-3.9
Ca^{2+} (mmol/L)	1-1.35
K^{+} (mmol/L)	2.8-3.2
Na^{+} (mmol/L)	147-151
Cl^{-} (mmol/L)	118-132
Mg^{2+} (mmol/L)	0.78-1.26

1.5. Amygdala, Hippocampus and Raphe Nuclei

The amygdala and hippocampus are situated within the temporal lobe. The amygdala is a prominent structure that lies just anterior to the hippocampus

(Figure 1.10). Studies involving tumors of amygdala region have demonstrated increases in rage behavior, impulsivity and loss of emotional control. Electrical stimulation of the amygdala in both animals and humans does not produce aggressive responses. In humans, it has been reported that stimulation can evoke different types of emotional feelings such as relief, relaxation, detachment and a general pleasant sensation (Siegel, 2006).

The hippocampus (it is part of the hippocampal formation) can be viewed as a primitive form of three-layered cortical tissue. The hippocampal formation includes in the modulation of aggressive behavior, autonomic and endocrine functions and certain forms of learning and memory (Siegel, 2006).

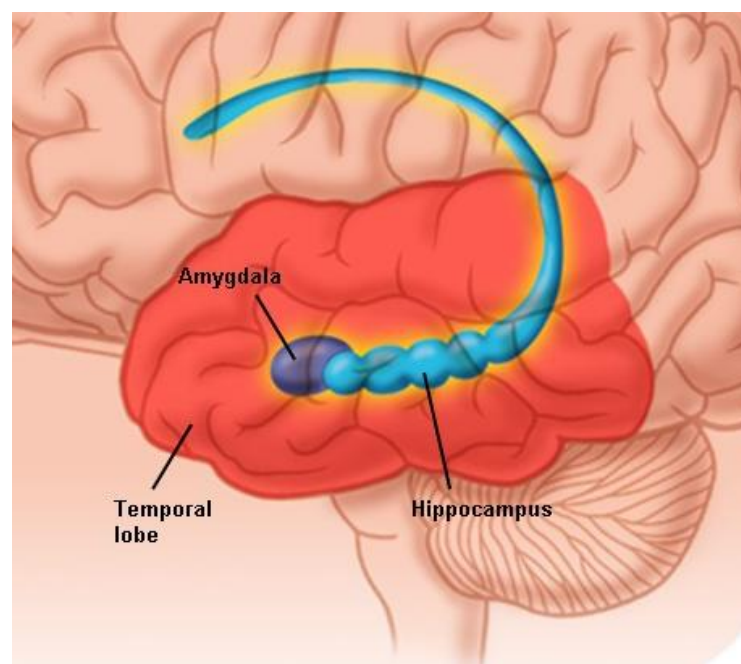


Figure 1.10. Location of the amygdala and hippocampus in the brain (Anonymous 14, 2014)

The raphe nuclei are a moderate-size cluster of nuclei found in the brain stem. Their main function is to release serotonin to the rest of the brain (Figure 1.11) (Anonymous 16, 2014).

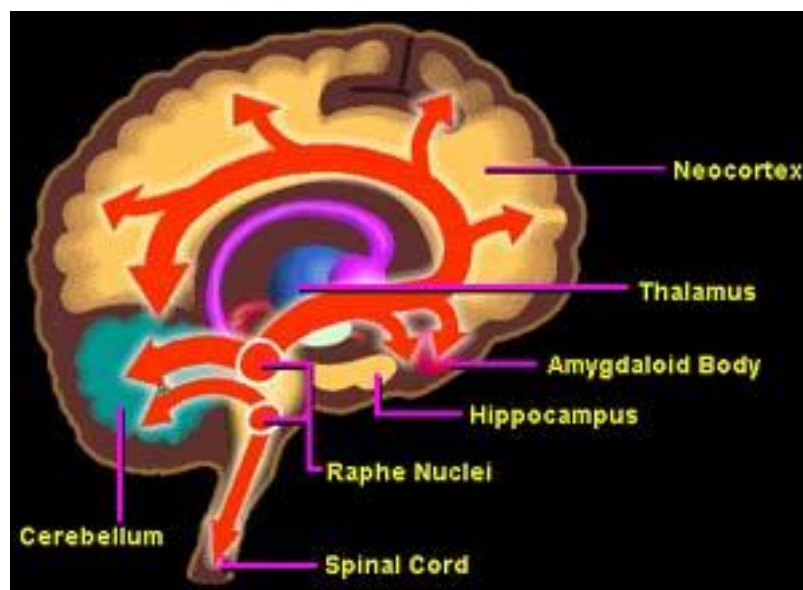


Figure 1.11. Serotonergic pathway: The axons of serotonergic neurons originate in the raphe nuclei of the brain stem and project to the cerebral cortex, the limbic system, cerebellum, and spinal cord (Anonymous 15, 2014)

1.6. Microdialysis

Microdialysis is an *in vivo* bioanalytical sampling technique which is used for continuously monitoring the chemical constituents of the extracellular space in living tissue systems (Zhang and Beyer, 2006; Anonymous 7, 2013). Furthermore, *in vivo* microdialysis enables to deliver drugs directly into the specified brain structures (Orlowska-Madjak, 2004).

In vivo microdialysis is a widely used technique since the late 1960s and early 1970s. The utility of microdialysis technique was first described in the area of neuroscience, but at the present day this technique is common in pharmacokinetic, pharmacodynamic and, now more recently, in clinical study designs. At the result of commercialization, numerous research groups employed the technique in a variety of

freely moving, unanesthetised animals including mice, rats, guinea pigs, monkeys and humans. Since fluid balance is unchanged during the microdialysis experiments, the subject's health is maintained and even small animals can be continuously sampled. Currently, in vivo microdialysis systems can be fully automated in terms of the collection and injection of dialysis samples (Zhang and Beyer, 2006).

In principle, microdialysis is based on the diffusion of molecules through small-diameter pores of a semipermeable membrane tubing connected to a probe that is stereotactically implanted into a determined brain region (Orlowska-Madjak, 2004). A principal component of microdialysis is the probe used to capture molecules from the extracellular space. A microdialysis probe typically consists of a cylindrical semipermeable membrane at its tip, connected with in- and outlet tubes (Figure 1.12 and Figure 1.13). Semipermeable membrane permits free transport of some but not all solutes. A variety of probe types with more than 20 different membrane and shaft length combinations are available (Chefer, 2009; Anonymous 7, 2013).

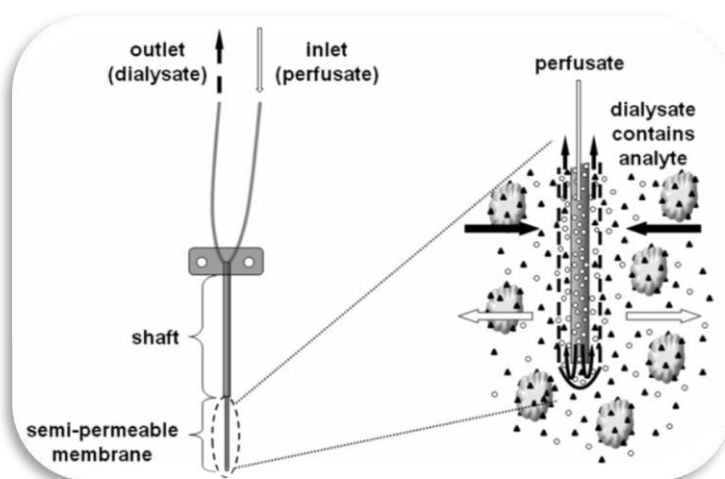


Figure 1.12. Schematic illustration of a microdialysis probe (Anonymous 8, 2013)

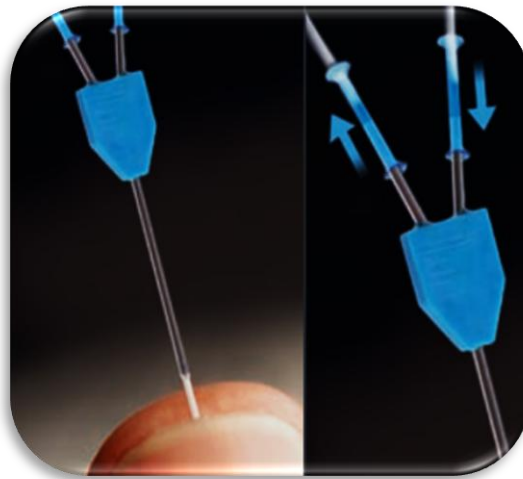


Figure 1.13. A microdialysis probe
(Anonymous 7, 2013)

Initially, the probe is implanted into the tissue. The probe is constantly perfused with a perfusion fluid (physiological salt solution) at a low flow rate (Anonymous 7, 2013). Perfusion rates of 0.3 to $3 \mu\text{l min}^{-1}$ are typically used depending upon the volume, sample collection time and analytical sensitivity (Chefer, 2009). The perfusion fluid should be isotonic to blood and should contain the relevant physiological ions consistently with cerebrospinal fluid or brain extracellular electrolyte composition (Orłowska-Madjak, 2004). Endogenous substances, such as neurotransmitters, are pass through pores in the membrane by diffusion out of the extracellular fluid into the perfusion medium. After a given amount of time, the perfusion solution inside the probe contains a representation of the chemicals found in the extracellular space. Samples are collected and then analyzed (Zhang and Beyer, 2006; Anonymous 7, 2013). The probe can be used to locally infuse exogenous compounds, nutrients and drugs by reversing the process (Anonymous 7, 2013).

The microdialysis technique has several important advantages. Concentration versus time profiles can be obtained from freely-moving animals. Information related to various compounds can be obtained simultaneously. Another distinct advantage of microdialysis is that the probe can be used for local delivery of drugs to specific brain regions. Moreover, simultaneous sampling from multiple sites is possible by implanting more than one probe in the same animal. Additionally, the dialysis principle provides protein free samples. So, samples can be analyzed directly avoiding sample clean up procedures with current analytical methods (Zeyden, 2008).

1.7. Chromatography

Chromatography is an analytical technique that is most widely used for the separation of the chemical components in complex mixtures. In addition, it is employed for the qualitative identification and quantitative determination of separated species. In all chromatographic methods the sample is dissolved in a mobile phase (which may be a gas, a liquid or a supercritical fluid) and then the mobile phase is moved through an immiscible stationary phase. The stationary phase remains fixed in place in a column or on a solid surface. All chromatographic separations are based upon differences in partitioning behavior of analyte species between a flowing mobile phase and a stationary phase. Chromatographic methods are generally classified according to the nature of the mobile phase: liquid, gas and supercritical fluid (Skoog, 1992; Skoog, 1996).

1.7.1. High-Performance Liquid Chromatography

High-performance liquid chromatography, HPLC, (formerly referred to as high-pressure liquid chromatography) is a type of liquid chromatography used to separate, identify and quantify compounds that have been dissolved in solution (Kupiec, 2004). HPLC was developed in the late 1960s and early 1970s. By reason of its versatility and wide applicability, HPLC is currently one of the most powerful tools in Analytical Chemistry. HPLC is optimum for the separation of chemical and biological compounds that are nonvolatile. HPLC is most often used to perform a target compound analysis.

There are four main separation modes that are used to separate most compounds. (1) Reversed-phase chromatography: the column packing is nonpolar (e.g. C18, C8, C3, phenyl, etc.) and the mobile phase is polar. This technique is the most common mode and can be used for nonpolar, polar, ionic molecules. (2) Normal-phase or adsorption chromatography: the column packing is polar (e.g. silica gel, cyanopropyl-bonded, amino-bonded, etc.) and the mobile phase is nonpolar. This technique is useful for water-sensitive compounds, geometric and cis-trans isomers and chiral compounds. (3) Ion exchange chromatography: the column packing contains ionic groups (e.g. sulfonic, tetraalkylammonium) and the mobile phase is an aqueous buffer. This technique is well convenient for the separation of inorganic and organic anions and cations in aqueous solution. Ionic dyes, amino acids and proteins can be separated because such compounds are salt in brine water. (4) Size exclusion chromatography: There is no interaction between the sample compounds and the column packing material. Molecules diffuse into pores of a porous medium. Molecules smaller than the pore opening enter the particle and are separated. But molecules larger than the pore opening do not diffuse into the particles. This

technique is mainly used for polymer characterization and for proteins. There are two modes as gel permeation and gel filtration chromatography (Anonymous 4, 2013).

The basic components of a HPLC system are shown in the schematic diagram in Figure 1.14. HPLC apparatus is equipped with a pump, an injector, a separation column and a detector. The chromatographic process begins by injection of a small volume of liquid sample into the moving mobile phase that passes through a column packed with particles of stationary phase. The injector can be a single injection or an automated injection system. An automated injection system providing reproducible injection volumes is extremely beneficial.



Figure 1.14. Flow scheme for HPLC (Anonymous 4, 2013)

The role of the pump is to force the mobile phase through the packed stationary phase at a specific flow rate, expressed in milliliters per minute. Normal flow rates in HPLC are in the 1 to 2 mL min⁻¹ range. Typical pumps can reach pressures in the range of 6000-9000 psi. High pressures are required because the stationary phase column packing consists of very small, tightly packed particles. A steady high pressure with no pulsating is needed to ensure reproducibility and accuracy.

Columns are commercially available in different lengths, pore sizes and packing materials. The correct selection of the column packing and the mobile phase are the most important factors for successful separation.

Eventually, an appropriate detector senses the presence of components different from the mobile phase and converts that information to an electrical signal. Since sample compound characteristics can be very different, many types of detectors have been developed to detect the compounds eluting from the column. The detectors based upon absorption of ultraviolet or visible radiation are the most widely used in HPLC. Another detector is based upon the changes in the refractive index of the solvent that is caused by analyte molecules. This detector is general rather than selective and responds to the presence of all solutes. Limited sensitivity is its disadvantage. Several types of electrochemical detectors are available that are based upon four electroanalytical methods including amperometry, potentiometry, voltammetry, coulometry and conductometry (Skoog, 1992).

Fluorescence detectors are probably the most sensitive among the HPLC detectors. It is possible to detect even a presence of a single analyte molecule in the flow cell. Typically, fluorescence sensitivity is 10-1000 times higher than that of the UV detectors analyzing strong UV absorbing compounds (Anonymous 2, 2013). Selectivity is also excellent because of the choice of excitation and emission wavelengths and the fact that only measurement of specific fluorescent species in samples. Non-fluorescent species can be detected if they are first converted to fluorescent species by pre- or postcolumn derivatization (Anonymous 9, 2014). Fluorescence detection is suitable for trace level analysis in complex matrices because of higher sensitivity and selectivity.

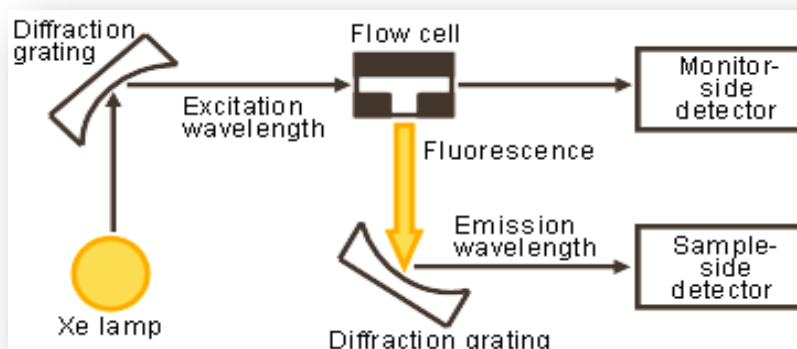


Figure 1.15. Illustration of a fluorescence detector optical system: while a UV/UV-VIS detector detects light that has passed through the flow cell, an FL detector detects fluorescence emitted in the direction orthogonal to the exciting light (Anonymous 3, 2013).

1.7.2. Fluorescence and Structure

Compounds containing aromatic functional groups with low-energy $\pi \rightarrow \pi^*$ transition levels give the most intense and most useful molecular fluorescence emission. Also, certain aliphatic and alicyclic carbonyl compounds or highly conjugated double-bonded structures may exhibit fluorescence.

Most unsubstituted aromatic hydrocarbons fluoresce in solution, with the quantum efficiency increasing with the number of rings and their degree of condensation. While the simplest heterocyclics (such as pyridine, furan, thiophene and pyrrole) do not fluoresce, fused-ring structures containing these rings often do.

Substitution on an aromatic ring generates shifts in the wavelength of absorption maxima and corresponding changes in the fluorescence peaks (Skoog, 1992).

1.8. Aims of the Study

The objectives of this study were:

- to develop simple and efficient method for simultaneous detection of GABA and glutamate in CSF by using HPLC-FLD,
- to develop simple and efficient method for simultaneous detection of melatonin, serotonin and noradrenaline in CSF by using HPLC-FLD,
- to validate these methods,
- to calculate measurement uncertainties for each compounds by applying 'bottom up' method.

CHAPTER 2

EXPERIMENTAL STUDY

2.1. Sampling

Samples were collected by using microdialysis technique. Microdialysis sampling on awake, freely-moving rats was conducted by Assoc. Prof. Dr. Alper KARAKAŞ in Department of Biology, Abant İzzet Baysal University. Adult wistar albino rats were used in this study. Rats were anesthetized and placed in stereotaxic apparatus. The stereotaxic coordinates of three brain regions in rats (amygdala, hippocampus and raphe nuclei) were determined according to the atlas for rat brain and literatures. Then guide cannulas were fixed to these coordinates. The animals were allowed to recover and the microdialysis experiment were performed one week later following cannulation. On the experimental day, the microdialysis probe (CMA 12 elite microdialysis probe; CMA Microdialysis AB, Sweden) was inserted into the guide cannula of the operated animals. Probe was perfused with perfusion fluid CNS (CMA Microdialysis AB, Sweden) (NaCl : 147 mmol L^{-1} , KCl : 2.7 mmol L^{-1} , CaCl_2 : 1.2 mmol L^{-1} , MgCl_2 : 0.85 mmol L^{-1} ; total chloride contents: $153.8 \text{ mmol L}^{-1}$). The flow rate of perfusion fluid was $2 \mu\text{L min}^{-1}$. The CSF was flowed out for initial one hour, then samples were started to collect. All microdialysis experiments was performed between 14:00 and 18:30 h. $5 \mu\text{L}$ EDTA and $5 \mu\text{L}$ perchloric acid were added into the plastic vials. The sample was pumped into these plastic vials. Each

sample was taken automatically at thirty minutes intervals. The samples were frozen immediately and stored -80°C until required. After the microdialysis study, rats were sacrificed by decapitation. A photograph which was taken during the experiment (Figure 2.1) shows a freely moving rat with a microdialysis probe and an injection cannula implanted surgically into a specific area within the brain. Also, each probe was individually tested to assess the recovery of the analytes.



Figure 2.1. A freely moving rat during the microdialysis experiment

2.2. Experiments of GABA and Glutamate

2.2.1. Overview of Derivatization Reaction

Amino acids in the CNS can be classified as neurotransmitter and non-neurotransmitter depending on their function. Aspartate, glycine, taurine, glutamate

and γ -aminobutyric acid (GABA) are neurotransmitter amino acids (Shah et al., 1999). The chemical structures of GABA and glutamate are shown in Figure 2.2.

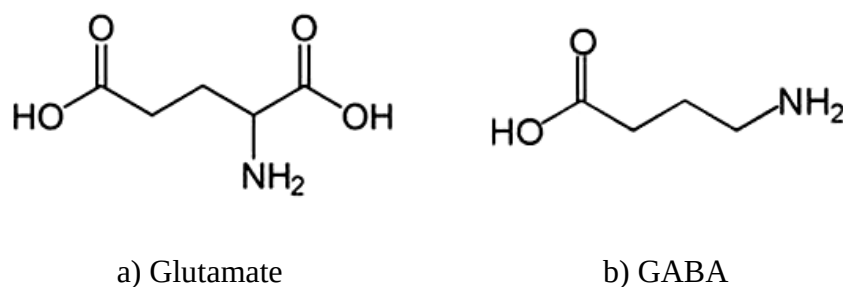


Figure 2.2. The chemical structures of glutamate (a) and GABA (b)

GABA and glutamate such as other amino acid neurotransmitters neither possess electroactive or fluorescent character nor show strong absorption in the ultraviolet/visible region. A derivatization step is performed in order to improve both the selectivity and sensitivity of detection of these molecules in biological samples. Different precolumn derivatising reagents have been used for this purpose including 1-dimethyl aminonaphthalenesulfonyl chloride (DANSYL), phenylisothiocyanate (PITC), 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC), ortho-phthalaldehyde (OPA) and naphthalene-2,3-dicarboxaldehyde (NDA) (Shah et al., 2002).

The well-known derivatization agent is OPA which reacts with primary amines rapidly in an aqueous basic medium and in presence of a thiol to form an isoindole derivative. The most important advantage of using OPA over other reagents is that it is not fluorescent or electroactive but its derivatives have suitable fluorescent and electrochemical characteristics. However, the thiols have a pungent odour and the resulting isoindole derivatives are usually found to be unstable

(Coppex, 2000; Shah et al., 2002; Clarke et al., 2007). The reaction of OPA with a primary amine is shown in Figure 2.3.

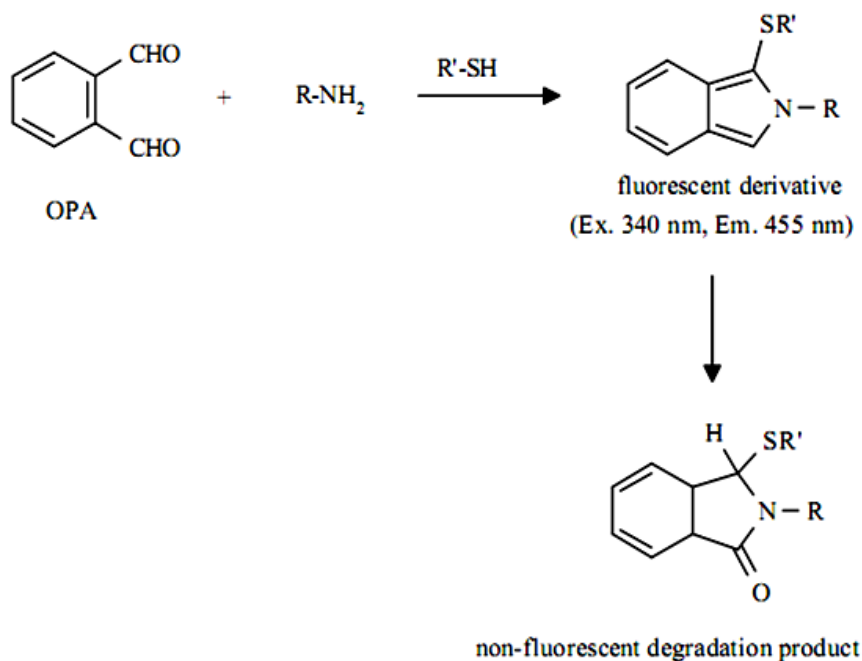


Figure 2.3. The reaction of OPA with a primary amine

To overcome the problems associated with OPA, naphthalene-2,3-dicarboxaldehyde (NDA) was designed and developed by de Montigny et al. (1987). NDA reacts with primary amines in the presence of cyanide ions (CN^-), to produce 1-cyanobenz[f]isoindole (CBI) derivatives which possess fluorescent and electroactive properties (de Montigny et al., 1987; Nussbaum et al., 1992). Derivatization reaction time with NDA is much longer than with OPA but the derivatives are more stable and are suitable for pre-column derivatization (Coppex, 2000). Also, NDA derivatives show excitation maxima (420 and 440 nm) in the visible region, efficient chemiluminescence by the peroxyoxalate method and improved fluorescence quantum efficiency. NDA has been used for the derivatization of amino acids and

peptides, catecholamines, aliphatic amines, heterocyclic amines, aliphatic diamines, drugs and lipids (Zotou and Notou, 2012).

The reaction of NDA with a primary amine is shown in Figure 2.4. In a multistep sequence, the primary amines react with one aldehyde moiety of NDA to form an imine. The addition of CN^- to imine results in intramolecular cyclization and finally leads to complete CBI derivative formation through a dehydration step (Zotou and Notou, 2012).

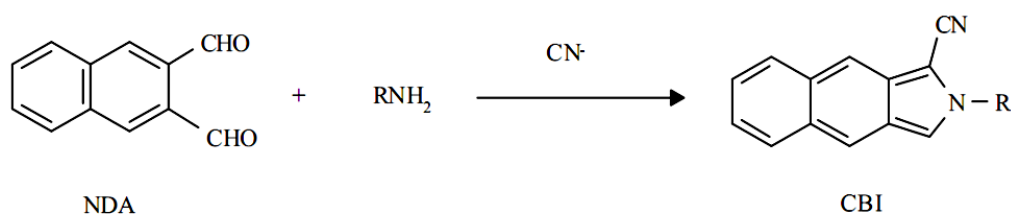


Figure 2.4. The reaction of NDA with a primary amine (Nussbaum et al., 1992)

The addition of a nucleophile, such as cyanide, to reaction is most important for the formation of stable CBI derivatives. The nucleophiles other than cyanide, such as hydrogen sulphite, isothiocyanate and 2-mercaptoethanol have been used. Montigny et al. (1987) found that CBI derivatives having a substituent other than cyano group on the indole ring both reduced stability and showed reduced fluorescence quantum efficiency (Shah et al. 1999).

McGill et al. (2005) reports the reaction of NDA with cyanide in the absence of amino acid. NDA self-condenses in the presence of cyanide ion and methanol at room temperature to yield a unique crystalline product, 15-hydroxybenzo[*g*]benzo[6,7]isochromeno[4,3-*c*]isochromen-7(15H)-one. The reaction

is given in Figure 2.5. The product is proposed to result from facile air oxidation of NDA to a methyl ester in combination with benzoin condensation.

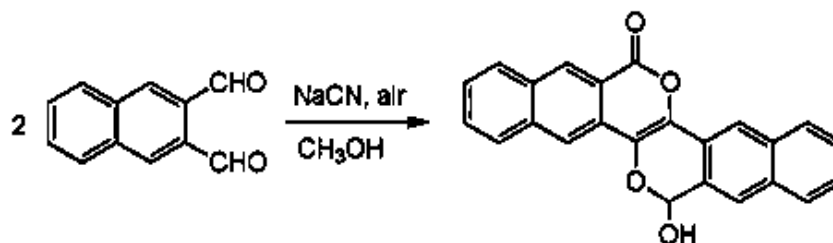


Figure 2.5. Condensation of NDA in the presence of air and cyanide in methanol (McGill et al., 2005)

2.2.2. Reagents, Materials and Equipment

4-aminobutyric acid (>98.0 %) and L-glutamic acid (>99.0 %) were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Boric acid (99.9 %), sodium hydroxide (99.3 %), potassium cyanide (99.5 %), ethylenedinitrilotetraacetic acid disodium salt dihydrate (99.8 %), hydrochloric acid (37.6 %) and methanol (≥ 99.9 %, HPLC grade) were supplied by Merck (Germany). 2,3-naphthalenedicarboxaldehyde (NDA) was obtained from Sigma-Aldrich (USA). Disodium hydrogen orthophosphate anhydrous was purchased from VWR International Ltd (England). Deionized water was obtained from SG LaboStar ultrapure water system (Germany) with a resistivity of 18.2 M Ω -cm. It was used throughout the experiments.

The Agilent 1100 series HPLC-FLD system (Germany) was used. The analytes were separated on a reversed phase Inertsil ODS-2 (150 x 4.6 mm i.d., 5 μ m) analytical column (Hichrom, England), which was protected by Inertsil ODS-2

(10 x 3.2 mm) guard cartridges (Hichrom, England). The analytical balance (0.1 mg) (And, GR 200, Japan) was used for all weighing processes. A pH-meter (WTW, pH 3310 SET2, Germany) and an ultrasonic bath (Bandelin Sonorex, Germany) were used in order to prepare mobile phase. Vortex mixer (Velp Scientifica, Italy) was used for homogenizing. Filtrations were performed with Durapore membrane filters (Merck, PVDF, pore size: 0.22 μ m, diameter: 47 mm) by using filtration apparatus (Jiteng, Japan). Automatic pipettes with different volumes (Rainin, USA) (100-1000 μ L, 10-100 μ L, 2-20 μ L) were used. Samples and solutions were light sensitive, so volumetric flasks (5 mL, 10 mL) and vials (2 mL) were chosen as amber glass.

2.2.3. Preparation of Chemicals

2.2.3.1. Preparation of Standards

998 ppm stock solution of glutamate was prepared by dissolving 0.0252 g of the glutamate standard in 25 mL of HPLC grade water. 996 ppm stock solution of GABA was prepared by dissolving 0.0254 g of the GABA standard in 25 mL of HPLC grade water.

The 0.5 mL of these stock solutions was diluted to 5 mL to obtain 100 ppm intermediate solutions. The standard solutions were prepared from the intermediate solutions with appropriate dilutions with HPLC grade water. All dilutions were done using automatic pipettes and A class volumetric flasks. All of the stock, intermediate and standard solutions were stored in a refrigerator. The intermediate and standard solutions were prepared daily.

2.2.3.2. Preparation of Mobile Phase

14.1960 g di-sodium hydrogen orthophosphate was dissolved in 1000 mL of HPLC grade water (0.1 M). 0.0093 g ethylenedinitrilotetraacetic acid disodium salt dihydrate was dissolved in 500 mL of HPLC grade water (50 μ M). The mobile phase was prepared by mixing 1000 mL of 0.1 M di-sodium hydrogen orthophosphate with 200 mL of 50 μ M ethylenedinitrilotetraacetic acid disodium salt dihydrate and the pH was adjusted to 5.3 with 1 M hydrochloric acid. The resulting solution was filtered through a 0.22 μ m membrane filter and degassed by ultrasonic shaking.

2.2.3.3. Preparation of Derivatization Reagents

0.1 M borate buffer (pH 9.5) was prepared by dissolving 0.3092 g boric acid in 50 mL of dilute aqueous sodium hydroxide and the pH was adjusted with 4 M sodium hydroxide. 0.0651 g potassium cyanide was dissolved in 100 mL of HPLC grade water (10 mM). Borate buffer and potassium cyanide solutions were filtered through a 0.22 μ m membrane filter. 0.0055 g NDA was dissolved in 5 mL of HPLC grade methanol (6 mM), protected from light. It was filtered through a 0.22 μ m syringe filter and stored in a refrigerator.

2.2.3.4. Preparation of Samples

The microdialysate samples are relatively clean. Hence, samples were analyzed directly without further sample clean up. All of the frozen samples (-20 $^{\circ}$ C) were thawed just before the experiment.

2.2.4. Derivatization Procedure

The derivatization procedure used in this study was based on the method described by Clarke et al., 2007. The derivatization reagents were added in a particular order to avoid benzoin condensation (Zotou and Notou, 2012). 100 μ L of sample or standard solution, 900 μ L of borate buffer, 200 μ L of potassium cyanide and 200 μ L NDA were added to an amber vial and mixed vigorously. The reaction mixture was left in the dark, at room temperature, for 1.5 hours. A 20 μ L of the derivative was injected to the HPLC system. The chromatographic conditions were summarized in Table 2.1.

Table 2.1. Chromatographic conditions for HPLC

Chromatographic Conditions	
Column	Inertsil ODS-2 (C18, 150 x 4.6 mm i.d., 5 μ m)
Mobile Phase	0.1 M Na ₂ HPO ₄ /50 mM EDTA (pH 5.3, 1 M HCl) and HPLC grade methanol (35:65, v/v); isocratic elution
Flow Rate	0.1 mL min ⁻¹
PMT-Gain	10
Injection Volume	20 μ L
Fluorescence Detector	Excitation wavelength : 420 nm Emission wavelength : 480 nm
Total Run Time	Stop Time: 60 min and Post Time: 15 min
Column Temperature	30 ⁰ C

2.2.5. Derivative Stability

The stability of the NDA derivatives was examined by using mixture of GABA and glutamate standards at a concentration of 50 ppb. After addition of reagents, it was waited from 1 to 4 hours at room temperature. This study was repeated for two days and average results were shown in Figure 2.6.

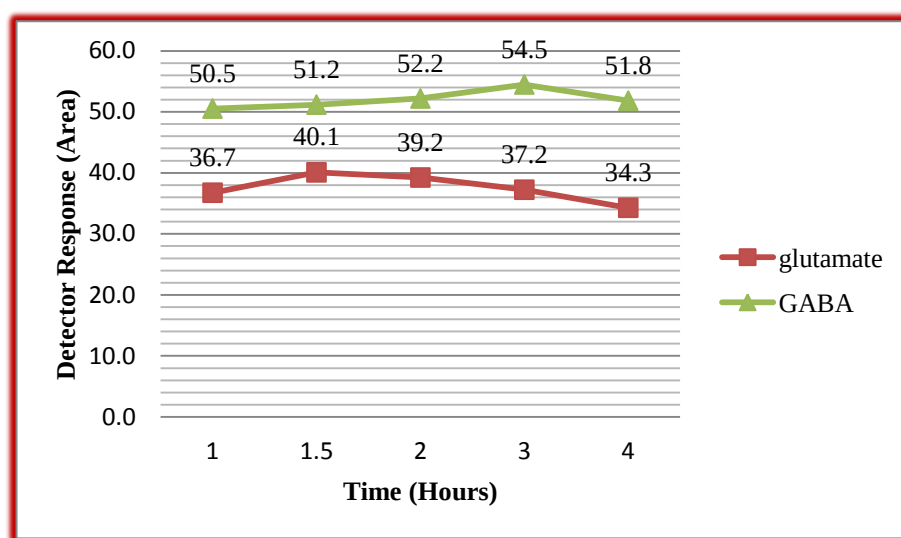


Figure 2.6. The responses of the GABA and glutamate standards (50 ppb) as a function of reaction time

2.2.6. Validation Parameters

The method performance was evaluated in terms of linearity, limit of detection and quantitation, recovery and precision.

2.2.6.1. Linearity

Standard addition method was used for eliminate the matrix effect. Intermediate solutions were prepared from the stock solutions of GABA and glutamate. Then, the standard solutions were prepared from the intermediate

solutions with appropriate dilutions with HPLC grade water. The samples which were taken from the amygdala, the hippocampus and the raphe nuclei regions of the rat brain were combined. The following derivatization procedures were used: 40 μ L of sample, 10 μ L of appropriate standard solution, 450 μ L of borate buffer, 100 μ L of potassium cyanide and 100 μ L of NDA were added to an amber vial and mixed vigorously. The reaction was allowed to proceed at room temperature in the absence of light for 1.5 hours. A 20 μ L of the derivative was injected to the HPLC system.

The nine matrix-matching calibration solutions were measured. Calibration curves were constructed by plotting peak areas versus concentrations (Figure 2.7 and Figure 2.8). Linear least squares regression was used to calculate the slopes, intercepts and squared correlation coefficients (R^2). Linearity was evaluated by using R^2 which was found to be 0.9997 for both GABA and glutamate.

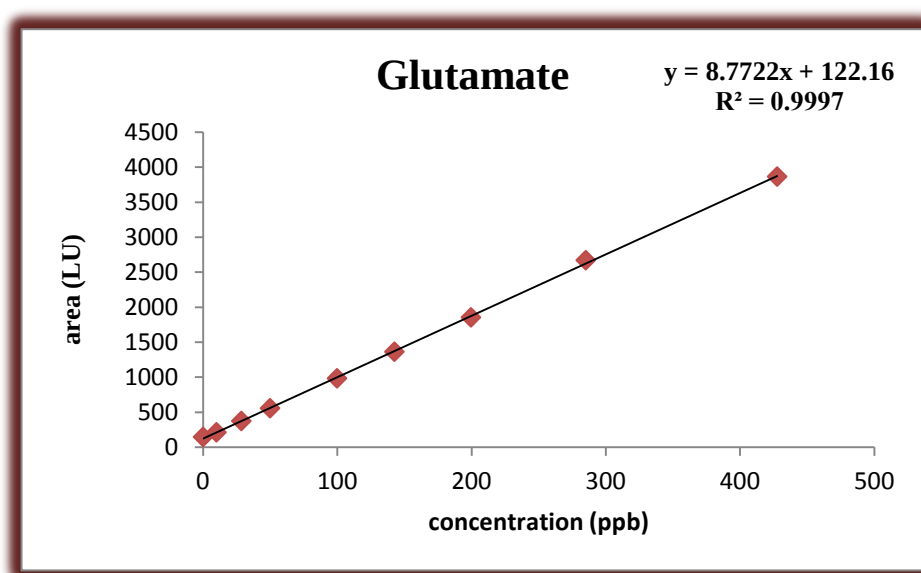


Figure 2.7. Calibration curve for glutamate

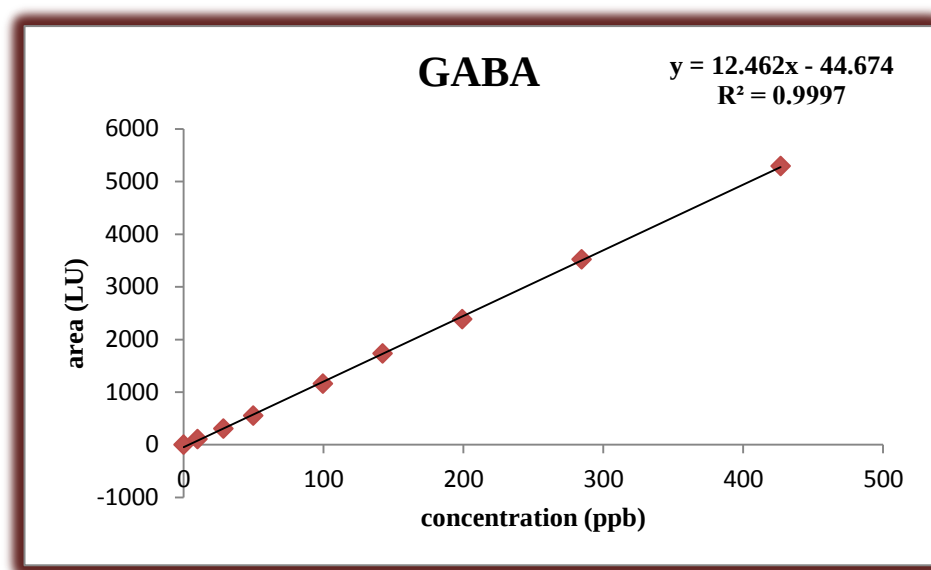


Figure 2.8. Calibration curve for GABA

2.2.6.2. Method Detection Limit (MDL) and Limit of Quantitation (LOQ)

The sample matrix was spiked with the mixture of the standard solutions. After the derivatization reaction, the spiked sample was analyzed with HPLC.

The peak height and peak background in the analyte peak (10 ppb) was measured. The method detection limits were calculated using S/N ratio of 3 and were found as 9 ppb for glutamate and 28 ppb for GABA. The limits of quantitation with the S/N ratio of 10 were found as 30 ppb for glutamate and 92 ppb for GABA.

2.2.6.3. Repeatability and Intermediate Precision

At the same day, five aliquots of cerebrospinal fluid sample were spiked with GABA/glutamate standard mixture and then derivatization was performed waited 1.5 hours and the injection to HPLC was realized. For the intermediate precision, these procedures were repeated daily in the consecutive five days. The peak areas were

measured and compared with calibration curves in order to quantify GABA/glutamate concentrations. The obtained results were used for precision and also recovery calculations.

Repeatability and intermediate precision values were expressed as relative standard deviation (RSD). RSD is also known as coefficient of variation (CV) and also frequently stated as a percentage. RSD was obtained by dividing the standard deviation to the mean concentration. The calculated results for GABA and glutamate are reported in Table 2.2.

Table 2.2. RSD values for GABA and glutamate

First Day Measurements for Glutamate			First Day Measurements for GABA		
Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
21.71	1214	138	46.06	1385	111
21.91	1222	139	46.67	1391	112
21.84	1223	139	46.46	1425	114
21.69	1174	134	45.39	1395	112
21.79	1216	139	46.12	1423	114
Mean Concentration (ppb)		138	Mean Concentration (ppb)		113
Standard Deviation		2.31	Standard Deviation		1.52
RSD %		1.67	RSD %		1.35
Second Day Measurements for Glutamate			Second Day Measurements for GABA		
Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
21.82	1187	135	46.31	1388	111
22.19	1209	138	47.48	1448	116
21.69	1190	136	45.65	1389	111
21.94	1213	138	46.56	1425	114
22.31	1193	136	47.17	1414	114
Mean Concentration (ppb)		137	Mean Concentration (ppb)		113
Standard Deviation		1.37	Standard Deviation		2.04
RSD %		1.00	RSD %		1.80

Table 2.2. RSD values for GABA and glutamate (continued)

Third Day Measurements for Glutamate			Third Day Measurements for GABA		
Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
22.33	1054	120	47.56	1251	100
21.82	1033	118	44.68	1283	103
22.06	1056	120	46.73	1293	104
22.13	1044	119	46.92	1290	103
22.17	1032	118	46.97	1271	102
Mean Concentration (ppb)		119	Mean Concentration (ppb)		103
Standard Deviation		1.28	Standard Deviation		1.36
RSD %		1.07	RSD %		1.33

Fourth Day Measurements for Glutamate			Fourth Day Measurements for GABA		
Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
22.24	1058	121	47.14	1366	110
22.30	1035	118	47.41	1345	108
21.89	1039	118	45.74	1366	110
22.10	1062	121	47.14	1366	110
Mean Concentration (ppb)		120	Mean Concentration (ppb)		109
Standard Deviation		1.50	Standard Deviation		0.85
RSD %		1.25	RSD %		0.78

Fifth Day Measurements for Glutamate			Fifth Day Measurements for GABA		
Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
22.17	991	113	47.32	1451	116
22.16	990	113	47.17	1478	119
22.25	985	112	47.68	1475	118
22.22	996	114	47.65	1469	118
22.06	975	111	46.96	1450	116
Mean Concentration (ppb)		113	Mean Concentration (ppb)		118
Standard Deviation		0.90	Standard Deviation		1.07
RSD %		0.80	RSD %		0.91

2.2.6.4. Recovery

Recovery was investigated by the analysis of spiked samples. This is because neither suitable certified reference materials nor reference materials are available. The spiking was performed at the 100 ppb level which is the middle part of the linear range of the calibration curve. The percent recovery values are reported in Table 2.3.

Table 2.3. Mean recovery values for GABA and glutamate

1 st Day	GLUTAMATE ($C_{\text{native}} = 49$ ppb; $C_{\text{spike}} = 99.792$ ppb)				GABA (C_{native} is below detection limit; $C_{\text{spike}} = 99.568$ ppb)			
	retention time (min)	area	C_{observed} , ppb	Recovery %	retention time (min)	area	C_{observed} , ppb	Recovery %
	21.71	1214	138	89	46.06	1385	111	112
	21.91	1222	139	90	46.67	1391	112	112
	21.84	1223	139	90	46.46	1425	114	115
	21.69	1174	134	85	45.39	1395	112	112
	21.79	1216	139	89	46.12	1423	114	115
	Mean Recovery %			89	Mean Recovery %			113
2 nd Day	GLUTAMATE ($C_{\text{native}} = 49$ ppb; $C_{\text{spike}} = 99.792$ ppb)				GABA (C_{native} is below detection limit; $C_{\text{spike}} = 99.568$ ppb)			
	retention time (min)	area	C_{observed} , ppb	Recovery %	retention time (min)	area	C_{observed} , ppb	Recovery %
	21.82	1187	135	86	46.31	1388	111	112
	22.19	1209	138	89	47.48	1448	116	117
	21.69	1190	136	87	45.65	1389	111	112
	21.94	1213	138	89	46.56	1425	114	115
	22.31	1193	136	87	47.17	1414	114	114
	Mean Recovery %			87	Mean Recovery %			114
3 rd Day	GLUTAMATE ($C_{\text{native}} = 30$ ppb; $C_{\text{spike}} = 99.792$ ppb)				GABA (C_{native} is below detection limit; $C_{\text{spike}} = 99.568$ ppb)			
	retention time (min)	area	C_{observed} , ppb	Recovery %	retention time (min)	area	C_{observed} , ppb	Recovery %
	22.33	1054	120	90	47.56	1251	100	101
	21.82	1033	118	88	44.68	1283	103	103
	22.06	1056	120	91	46.73	1293	104	104
	22.13	1044	119	89	46.92	1290	103	104
	22.17	1032	118	88	46.97	1271	102	102
	Mean Recovery %			89	Mean Recovery %			103

Table 2.3. Mean recovery values for GABA and glutamate (continued)

4 th Day	GLUTAMATE ($C_{\text{native}} = 29$ ppb; $C_{\text{spike}} = 99.792$ ppb)				GABA (C_{native} is below detection limit; $C_{\text{spike}} = 99.568$ ppb)			
	retention time (min)	area	C_{observed} , ppb	Recovery %	retention time (min)	area	C_{observed} , ppb	Recovery %
	22.24	1058	121	92	47.14	1366	110	110
	22.3	1035	118	89	47.41	1345	108	108
	21.89	1039	118	90	45.74	1366	110	110
	22.1	1062	121	92	47.14	1366	110	110
	Mean Recovery %			91	Mean Recovery %			110

5 th Day	GLUTAMATE ($C_{\text{native}} = 14$ ppb; $C_{\text{spike}} = 99.792$ ppb)				GABA (C_{native} is below detection limit; $C_{\text{spike}} = 99.568$ ppb)			
	retention time (min)	area	C_{observed} , ppb	Recovery %	retention time (min)	area	C_{observed} , ppb	Recovery %
	22.17	991	113	99	47.32	1451	116	117
	22.16	990	113	99	47.17	1478	119	119
	22.25	985	112	99	47.68	1475	118	119
	22.22	996	114	100	47.65	1469	118	118
	22.06	975	111	97	46.96	1450	116	117
	Mean Recovery %			99	Mean Recovery %			118

2.3. Experiments of Melatonin, Serotonin and Noradrenaline

2.3.1. Reagents, Materials and Equipment

Serotonin hydrochloride (98.5 %) and L-noradrenaline (98.7 %) were purchased from Alfa Aesar (Germany). Melatonin (≥ 99 %) was purchased from Fluka (China). Sodium acetate (anhydrous), acetonitrile (≥ 99.9 %, HPLC grade), methanol (≥ 99.9 %, HPLC grade) and hydrochloric acid (37.6 %) ($d = 1.19$ g/cm³ at 20 °C) were supplied by Merck (Germany). Glacial acetic acid (100.0 %) was purchased from Sigma-Aldrich (Germany). Deionized water was obtained from SG LaboStar ultrapure water system (Germany) with a resistivity of 18.2 M Ω -cm. It was used throughout the experiments.

The Agilent 1100 series HPLC-FLD system (Germany) was used. The analytes were separated on a reversed phase Inertsil ODS-2 (150 x 4.6 mm i.d., 5 μ m) analytical column (Hichrom, England), which was protected by Inertsil ODS-2 (10 x 3.2 mm) guard cartridges (Hichrom, England). The analytical balance (0.1 mg) (And, GR 200, Japan) was used for all weighing processes. A pH-meter (WTW, pH 3310 SET2, Germany) and an ultrasonic bath (Bandelin Sonorex, Germany) were used in order to prepare mobile phase. Vortex mixer (Velp Scientifica, Italy) was used for homogenizing. Filtrations were performed with Isopore membrane filters (Merck, polycarbonate, pore size: 0.22 μ m, diameter: 47 mm) by using filtration apparatus (Jiteng, Japan). Automatic pipettes with different volumes (Rainin, USA) (100-1000 μ L, 10-100 μ L, 2-20 μ L) were used. Samples and solutions were light sensitive, so volumetric flasks (5 mL, 10 mL) and vials (2 mL) were chosen as amber glass. Also, insert vials were used inside the vials because of small sample volumes.

2.3.2. Preparation of Chemicals

2.3.2.1. Preparation of Standards

Stock solutions were prepared. The stock solutions were stored in a freezer. The exact calculations concerning to stock solutions are as shown below and they are used in all calculations related to experiments.

- 823 ppm serotonin was prepared by dissolving 0.0252 g serotonin hydrochloride in 25 mL of methanol/HPLC grade water mixture (1/9, v/v).
- 1007 ppm noradrenaline was prepared by dissolving 0.0255 g L-noradrenaline in 25 mL of hydrochloric acid (0.1 M).

- 990 ppm melatonin was prepared by dissolving 0.0250 g melatonin in 25 mL of methanol/HPLC grade water mixture (1/9, v/v).

Intermediate solutions were prepared:

- The 50 μ L of serotonin (823 ppm) was diluted with HPLC grade water to 5 mL to obtain 8.23 ppm serotonin,
- The 50 μ L of noradrenaline (1007 ppm) was diluted with HPLC grade water to 5 mL to obtain 10.07 ppm noradrenaline,
- The 50 μ L of melatonin (990 ppm) was diluted with HPLC grade water to 5 mL to obtain 9.90 ppm melatonin.

The standard solutions were prepared from the intermediate solutions with appropriate dilutions with HPLC grade water. All dilutions were done using automatic pipettes and A class volumetric flasks. All of the intermediate and standard solutions were stored in a refrigerator. The intermediate and standard solutions were prepared daily.

2.3.2.2. Preparation of Mobile Phase

75 mM sodium acetate was prepared: 6.1500 g sodium acetate was dissolved in 1000 mL of HPLC grade water.

The mobile phase was prepared by mixing 720 mL of 75 mM sodium acetate with 280 mL of acetonitrile and the pH was adjusted to 5.0 with glacial acetic acid. The resulting solution was filtered through a 0.22 μ m membrane filter and degassed by ultrasonic shaking.

2.3.2.3. Preparation of Samples

The microdialysate samples are relatively clean. Hence, samples were analyzed directly without further sample clean up. All of the frozen samples (-20 °C) were thawed just before the experiment.

2.3.3. Chromatographic Conditions

A fluorescence HPLC method for the simultaneous detection of melatonin, serotonin and noradrenaline in microdialysates from rat brain was used. Separation was performed with a C18 reverse-phase column using isocratic elution. The chromatographic conditions were summarized in Table 2.4.

Table 2.4. Chromatographic conditions for HPLC

Chromatographic Conditions	
Column	Inertsil ODS-2 (C18, 150 x 4.6 mm i.d., 5 µm)
Mobile Phase	75 mM sodium acetate-acetonitrile (pH 5.0, acetic acid) (72:28, v/v); isocratic elution
Flow Rate	1 mL min ⁻¹
PMT-Gain	15
Injection Volume	20 µL
Fluorescence Detector	Excitation wavelength : 280 nm
	Emission wavelength : 340 nm
Total Run Time	Stop Time: 10 min and Post Time: 5 min

2.3.4. Validation Parameters

The method performance was evaluated in terms of linearity, limit of detection and quantitation, recovery and precision.

2.3.4.1. Linearity

Standard addition method was used for eliminate the matrix effect. Intermediate solutions were prepared from the stock solutions. Then, the standard solutions were prepared from the intermediate solutions with appropriate dilutions with HPLC grade water. The samples which were taken from the amygdala, the hippocampus and the raphe nuclei regions of the rat brain were combined. 40 μ L of sample and 10 μ L of appropriate standard solution were added to an inserts vial and mixed vigorously. For the initial concentration which was set at 0, 10 μ L of HPLC grade water was added instead of standard. A 20 μ L of the mixture was injected to the HPLC system.

The five matrix-matching calibration solutions were measured with two replicates. Calibration curves were constructed by plotting peak areas versus concentrations (Figure 2.9, Figure 2.10 and Figure 2.11). Linear least squares regression was used to calculate the slopes, intercepts and squared correlation coefficients (R^2). Linearity was evaluated by using R^2 which was found to be 0.9971 for noradrenaline, 0.9947 for serotonin and 0.9991 for melatonin.

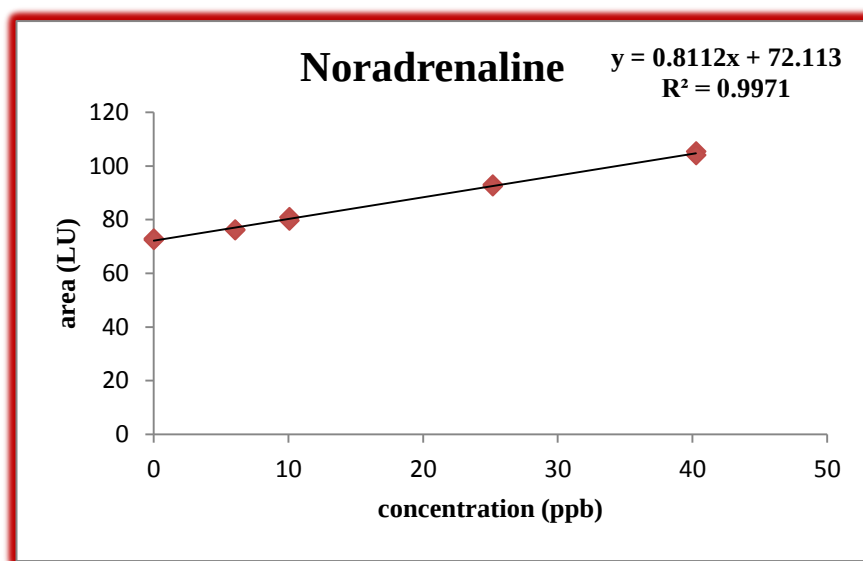


Figure 2.9. Calibration curve for noradrenaline

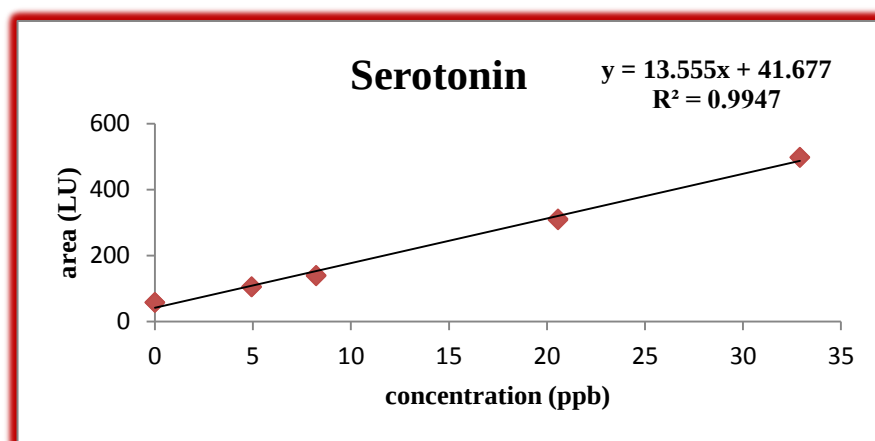


Figure 2.10. Calibration curve for serotonin

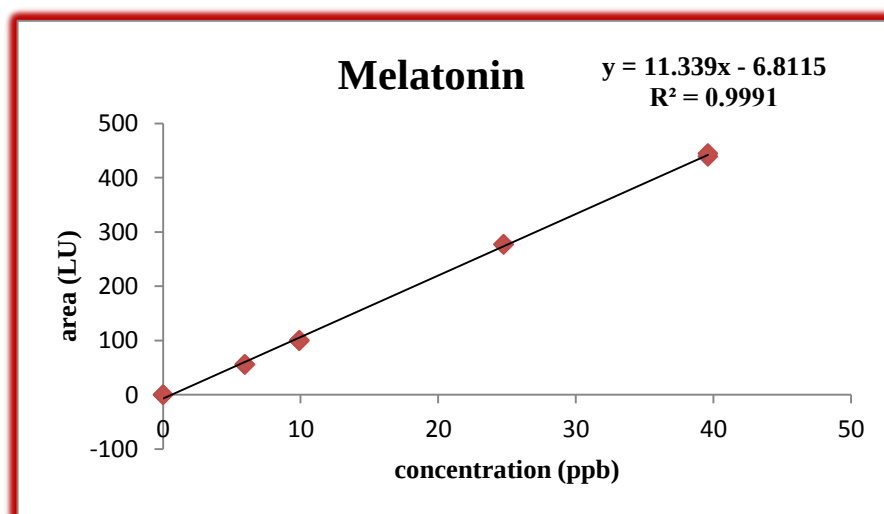


Figure 2.11. Calibration curve for melatonin

2.3.4.2. Method Detection Limit (MDL) and Limit of Quantitation (LOQ)

MDL and LOQ for each compound were estimated by analysing ten replicate spiked samples at the final concentration of 5 ppb. MDL values were calculated as three times the standard deviation of ten replicates and were found as 0.5 ppb for melatonin, as 1.2 ppb for serotonin and as 2.7 ppb for noradrenaline. LOQ values were calculated as ten times the standard deviation of ten replicates and were found as 1.7 ppb for melatonin, as 3.8 ppb for serotonin and as 8.8 ppb for noradrenaline. The calculated results are reported in Table 2.5.

Table 2.5. MDL and LOQ values for noradrenaline, serotonin and melatonin

NORADRENALINE			SEROTONIN			MELATONIN		
Retention time (min)	Area	Conc. (ppb)	Retention time (min)	Area	Conc. (ppb)	Retention time (min)	Area	Conc. (ppb)
1.58	6	5	1.97	66	5	5.58	28	5
1.58	5	4	1.97	67	5	5.58	28	5
1.58	4	3	1.97	67	5	5.58	29	5
1.58	4	3	1.97	69	5	5.58	30	5
1.59	4	3	1.97	72	5	5.57	29	5
1.58	4	3	1.98	73	5	5.58	28	5
1.59	3	3	1.98	73	5	5.59	28	5
1.59	3	2	1.98	76	6	5.59	28	5
1.59	3	2	1.99	77	6	5.58	26	4
1.59	2	2	1.99	82	6	5.60	27	4
Mean Concentration (ppb)		3	Mean Concentration (ppb)		5	Mean Concentration (ppb)		5
Standard Deviation		0.88	Standard Deviation		0.38	Standard Deviation		0.17
MDL		2.7	MDL		1.2	MDL		0.5
LOQ		8.8	LOQ		3.8	LOQ		1.7

2.3.4.3. Repeatability and Intermediate Precision

The spiking was performed at two fortification levels representing initial and final parts of the linear range. At the same day, six aliquots of cerebrospinal fluid sample were spiked with melatonin-serotonin-noradrenaline standard mixture at each fortification level and then analyzed. For the intermediate precision, these procedures were repeated daily in the course of the consecutive five days. The peak areas were measured and compared with calibration curves in order to quantify concentrations of these neurotransmitters. The obtained results were used for precision and also recovery calculations.

Repeatability and intermediate precision values were expressed as RSD. The calculated results with related to compounds are reported in Table 2.6, Table 2.7 and Table 2.8.

2.3.4.4. Recovery

Recovery was investigated by the analysis of spiked samples. This is because neither suitable certified reference materials nor reference materials are available. The spiking was performed at two fortification levels. The calculated percent recovery values are reported in Table 2.9, Table 2.10 and Table 2.11.

Table 2.6. RSD values for noradrenaline

First Day Measurements			Second Day Measurements			Third Day Measurements			Fourth Day Measurements			Fifth Day Measurements		
Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
1.59	77.9	96.0	1.58	75.3	92.8	1.60	30.8	38.0	1.61	24.2	29.8	1.56	102.7	126.6
1.58	78.4	96.6	1.59	76.7	94.5	1.60	32.4	39.9	1.60	22.9	28.3	1.56	101.5	125.2
1.59	78.5	96.8	1.58	75.6	93.2	1.60	31.8	39.2	1.60	23.0	28.3	1.56	101.9	125.7
1.58	77.6	95.7	1.58	77.7	95.8	1.60	32.8	40.4	1.60	24.1	29.7	1.56	99.2	122.3
1.58	78.1	96.3	1.69	77.8	95.9	1.60	32.4	39.9	1.60	24.4	30.1	1.56	99.1	122.1
1.58	78.0	96.1	1.58	75.4	93.0	1.60	31.5	38.9	1.60	23.0	28.3	Mean Conc. (ppb)		124.4
Mean Conc. (ppb)		96.2	Mean Conc. (ppb)		94.2	Mean Conc. (ppb)		39.4	Mean Conc. (ppb)		29.1	Standard Deviation		2.05
Standard Deviation		0.42	Standard Deviation		1.42	Standard Deviation		0.89	Standard Deviation		0.87	RSD %		1.65
RSD %		0.44	RSD %		1.51	RSD %		2.25	RSD %		2.99			

Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
1.55	53.5	65.9	1.65	51.9	64.0	1.47	7.9	9.7	1.48	7.3	9.0
1.55	52.4	64.6	1.56	51.5	63.5	1.47	7.3	9.0	1.48	7.5	9.2
1.56	52.8	65.1	1.66	52.0	64.1	1.48	8.2	10.1	1.48	7.4	9.1
1.55	52.6	64.9	1.56	50.0	61.7	1.47	8.9	11.0	1.48	7.4	9.2
1.55	53.1	65.5	1.66	51.5	63.5	1.48	7.7	9.5	Mean Conc. (ppb)		9.1
1.57	52.7	65.0	1.56	51.2	63.1	1.48	9.0	11.1	Standard Deviation		0.10
Mean Conc. (ppb)		65.2	Mean Conc. (ppb)		63.3	Mean Conc. (ppb)		10.1	RSD %		1.07
Standard Deviation		0.46	Standard Deviation		0.89	Standard Deviation		0.83			
RSD %		0.70	RSD %		1.41	RSD %		8.24			

Table 2.7. RSD values for serotonin

First Day Measurements			Second Day Measurements			Third Day Measurements			Fourth Day Measurements			Fifth Day Measurements		
Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
1.99	490	36.2	1.99	554	40.9	1.96	481	35.5	1.98	546	40.3	1.97	480	35.4
1.98	476	35.1	2.00	530	39.1	1.96	545	40.2	1.97	542	40.0	1.97	481	35.5
1.99	480	35.4	1.99	534	39.4	1.96	545	40.2	1.97	532	39.3	1.97	472	34.8
1.99	480	35.4	1.99	531	39.2	1.97	549	40.5	1.97	529	39.0	1.96	464	34.2
1.99	474	35.0	2.12	555	41.0	1.97	548	40.4	1.97	523	38.6	1.97	453	33.5
1.99	465	34.3	1.99	526	38.8	1.97	541	39.9	1.97	529	39.0	Mean Conc. (ppb)		34.7
Mean Conc. (ppb)		35.2	Mean Conc. (ppb)		39.7	Mean Conc. (ppb)		39.4	Mean Conc. (ppb)		39.4	Standard Deviation		0.86
Standard Deviation		0.61	Standard Deviation		0.96	Standard Deviation		1.96	Standard Deviation		0.64	RSD %		2.48
RSD %		1.72	RSD %		2.41	RSD %		4.98	RSD %		1.64			

Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
1.99	131	9.7	2.10	153	11.3	1.97	189	14.0	1.97	216	15.9
1.99	132	9.7	1.99	144	10.6	1.97	188	13.9	1.97	217	16.0
1.99	133	9.8	2.12	152	11.2	1.97	188	13.9	1.97	213	15.7
1.99	131	9.6	2.00	144	10.6	1.97	182	13.4	1.98	191	14.1
1.99	130	9.6	2.13	152	11.2	1.97	172	12.7	1.97	213	15.7
1.99	132	9.7	1.99	141	10.4	1.97	178	13.1	Mean Conc. (ppb)		15.5
Mean Conc. (ppb)		9.7	Mean Conc. (ppb)		10.9	Mean Conc. (ppb)		13.5	Standard Deviation		0.78
Standard Deviation		0.08	Standard Deviation		0.39	Standard Deviation		0.50	RSD %		5.05
RSD %		0.84	RSD %		3.61	RSD %		3.68			

Table 2.8. RSD values for melatonin

First Day Measurements			Second Day Measurements			Third Day Measurements			Fourth Day Measurements			Fifth Day Measurements		
Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
5.53	436	38.4	5.61	508	44.8	5.33	544	48.0	5.43	442	39.0	5.44	455	40.1
5.54	437	38.5	5.62	483	42.6	5.33	544	48.0	5.42	444	39.1	5.46	451	39.8
5.55	430	37.9	5.61	502	44.2	5.33	544	47.9	5.41	432	38.1	5.46	449	39.6
5.55	429	37.9	5.88	501	44.2	5.33	540	47.6	5.42	429	37.8	5.45	440	38.8
5.56	436	38.5	Mean Conc. (ppb)		43.9	5.33	539	47.6	5.42	427	37.7	5.46	432	38.1
5.57	434	38.3	Standard Deviation		0.96	Mean Conc. (ppb)		47.8	5.41	437	38.5	Mean Conc. (ppb)		39.3
Mean Conc. (ppb)		38.2	RSD %		2.18	Standard Deviation		0.20	Mean Conc. (ppb)		38.4	Standard Deviation		0.83
Standard Deviation		0.28				RSD %		0.43	Standard Deviation		0.60	RSD %		2.12
RSD %		0.73							RSD %		1.58			

Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)	Retention Time (min)	Area	Conc. (ppb)
5.56	99	8.7	5.86	116	10.3	5.37	134	11.9	5.42	127	11.2
5.56	99	8.7	5.89	116	10.3	5.37	135	11.9	5.42	127	11.2
5.58	98	8.7	5.63	115	10.1	5.37	135	11.9	5.42	126	11.2
5.56	100	8.8	5.91	117	10.3	5.38	131	11.6	5.42	126	11.1
5.56	100	8.8	Mean Conc. (ppb)		10.2	5.40	134	11.8	Mean Conc. (ppb)		11.2
5.57	98	8.7	Standard Deviation		0.08	5.37	131	11.6	Standard Deviation		0.04
Mean Conc. (ppb)		8.7	RSD %		0.79	Mean Conc. (ppb)		11.8	RSD %		0.31
Standard Deviation		0.05				Standard Deviation		0.16			
RSD %		0.62				RSD %		1.38			

Table 2.9. Mean recovery values for noradrenaline

First Day Measurements (C _{native} =57 ppb; C _{spike} =40 ppb)				Second Day Measurements (C _{native} =53 ppb; C _{spike} =40 ppb)				Third Day Measurements (C _{native} is below detection limit; C _{spike} =40 ppb)				Fourth Day Measurements (C _{native} is below detection limit; C _{spike} =40 ppb)				Fifth Day Measurements (C _{native} =92 ppb; C _{spike} =40 ppb)			
Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %
1.59	77.9	96.0	98	1.58	75.3	92.8	99	1.60	30.8	38.0	94	1.61	24.2	29.8	74	1.56	102.7	126.6	86
1.58	78.4	96.6	99	1.59	76.7	94.5	104	1.60	32.4	39.9	99	1.60	22.9	28.3	70	1.56	101.5	125.2	83
1.59	78.5	96.8	100	1.58	75.6	93.2	100	1.60	31.8	39.2	97	1.60	23.0	28.3	70	1.56	101.9	125.7	84
1.58	77.6	95.7	97	1.58	77.7	95.8	107	1.60	32.8	40.4	100	1.60	24.1	29.7	74	1.56	99.2	122.3	75
1.58	78.1	96.3	98	1.69	77.8	95.9	107	1.60	32.4	39.9	99	1.60	24.4	30.1	75	1.56	99.1	122.1	75
1.58	78.0	96.1	98	1.58	75.4	93.0	100	1.60	31.5	38.9	97	1.60	23.0	28.3	70	Mean Recovery %			81
Mean Recovery %			98	Mean Recovery %			103	Mean Recovery %			98	Mean Recovery %			72				

First Day Measurements (C _{native} =57 ppb; C _{spike} =10 ppb)				Second Day Measurements (C _{native} =53 ppb; C _{spike} =10 ppb)				Third Day Measurements (C _{native} is below detection limit; C _{spike} =10 ppb)				Fourth Day Measurements (C _{native} is below detection limit; C _{spike} =10 ppb)			
Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %
1.55	53.5	65.9	92	1.65	51.9	64.0	112	1.47	7.9	9.7	97	1.48	7.3	9.0	89
1.55	52.4	64.6	79	1.56	51.5	63.5	107	1.47	7.3	9.0	90	1.48	7.5	9.2	92
1.56	52.8	65.1	84	1.66	52.0	64.1	113	1.48	8.2	10.1	100	1.48	7.4	9.1	90
1.55	52.6	64.9	82	1.56	50.0	61.7	89	1.47	8.9	11.0	109	1.48	7.4	9.2	91
1.55	53.1	65.5	88	1.66	51.5	63.5	107	1.48	7.7	9.5	94	Mean Recovery %			90
1.57	52.7	65.0	83	1.56	51.2	63.1	103	1.48	9.0	11.1	110				
Mean Recovery %			85	Mean Recovery %			105	Mean Recovery %			100				

Table 2.10. Mean recovery values for serotonin

First Day Measurements (C _{native} =3 ppb; C _{spike} =33 ppb)				Second Day Measurements (C _{native} =4 ppb; C _{spike} =33 ppb)				Third Day Measurements (C _{native} =4 ppb; C _{spike} =33 ppb)				Fourth Day Measurements (C _{native} =6 ppb; C _{spike} =33 ppb)				Fifth Day Measurements (C _{native} =3 ppb; C _{spike} =33 ppb)			
Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %
1.99	490	36.2	99	1.99	554	40.9	113	1.96	481	35.5	94	1.98	546	40.3	104	1.97	480	35.4	108
1.98	476	35.1	96	2.00	530	39.1	107	1.96	545	40.2	109	1.97	542	40.0	104	1.97	481	35.5	108
1.99	480	35.4	97	1.99	534	39.4	108	1.96	545	40.2	109	1.97	532	39.3	101	1.97	472	34.8	106
1.99	480	35.4	97	1.99	531	39.2	107	1.97	549	40.5	110	1.97	529	39.0	101	1.96	464	34.2	104
1.99	474	35.0	96	2.12	555	41.0	113	1.97	548	40.4	109	1.97	523	38.6	99	1.97	453	33.5	102
1.99	465	34.3	94	1.99	526	38.8	106	1.97	541	39.9	108	1.97	529	39.0	101	Mean Recovery %			105
Mean Recovery %			97	Mean Recovery %			109	Mean Recovery %			106	Mean Recovery %			102				

First Day Measurements (C _{native} =3 ppb; C _{spike} =8 ppb)				Second Day Measurements (C _{native} =4 ppb; C _{spike} =8 ppb)				Third Day Measurements (C _{native} =4 ppb; C _{spike} =8 ppb)				Fourth Day Measurements (C _{native} =6 ppb; C _{spike} =8 ppb)			
Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %
1.99	131	9.7	76	2.10	153	11.3	90	1.97	189	14.0	116	1.97	216	15.9	122
1.99	132	9.7	77	1.99	144	10.6	82	1.97	188	13.9	114	1.97	217	16.0	123
1.99	133	9.8	77	2.12	152	11.2	90	1.97	188	13.9	114	1.97	213	15.7	119
1.99	131	9.6	76	2.00	144	10.6	82	1.97	182	13.4	109	1.98	191	14.1	100
1.99	130	9.6	75	2.13	152	11.2	90	1.97	172	12.7	100	1.97	213	15.7	119
1.99	132	9.7	77	1.99	141	10.4	80	1.97	178	13.1	106	Mean Recovery %			117
Mean Recovery %			76	Mean Recovery %			86	Mean Recovery %			110				

Table 2.11. Mean recovery values for melatonin

First Day Measurements (C _{native} is below detection limit; C _{spike} =40 ppb)				Second Day Measurements (C _{native} is below detection limit; C _{spike} =40 ppb)				Third Day Measurements (C _{native} is below detection limit; C _{spike} =40 ppb)				Fourth Day Measurements (C _{native} is below detection limit; C _{spike} =40 ppb)				Fifth Day Measurements (C _{native} is below detection limit; C _{spike} =40 ppb)			
Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %
5.53	436	38	97	5.61	508	45	113	5.33	544	48	121	5.43	442	39	98	5.44	455	40	101
5.54	437	38	97	5.62	483	43	107	5.33	544	48	121	5.42	444	39	99	5.46	451	40	100
5.55	430	38	96	5.61	502	44	112	5.33	544	48	121	5.41	432	38	96	5.46	449	40	100
5.55	429	38	96	5.88	501	44	112	5.33	540	48	120	5.42	429	38	96	5.45	440	39	98
5.56	436	38	97	Mean Recovery %			111	5.33	539	48	120	5.42	427	38	95	5.46	432	38	96
5.57	434	38	97					Mean Recovery %		121		5.41	437	39	97	Mean Recovery %			99
Mean Recovery %			97									Mean Recovery %		97					

First Day Measurements (C _{native} is below detection limit; C _{spike} =10 ppb)				Second Day Measurements (C _{native} is below detection limit; C _{spike} =10 ppb)				Third Day Measurements (C _{native} is below detection limit; C _{spike} =10 ppb)				Fourth Day Measurements (C _{native} is below detection limit; C _{spike} =10 ppb)			
Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time (min)	Area	C _{observed} (ppb)	Recovery %	Ret. Time	Area	C _{observed} (ppb)	Recovery %
5.56	99	9	88	5.86	116	10	104	5.37	134	12	120	5.42	127	11	113
5.56	99	9	88	5.89	116	10	104	5.37	135	12	121	5.42	127	11	113
5.58	98	9	88	5.63	115	10	102	5.37	135	12	121	5.42	126	11	113
5.56	100	9	89	5.91	117	10	104	5.38	131	12	117	5.42	126	11	112
5.56	100	9	89	Mean Recovery %			103	5.40	134	12	119	Mean Recovery %			113
5.57	98	9	88					5.37	131	12	117				
Mean Recovery %			88					Mean Recovery %		119					

CHAPTER 3

RESULT AND DISCUSSION

In recent decades, neurochemical measurements have led to many improvements in connection with the relationship between chemistry in the CNS and the behavioral, cognitive and emotional state of an organism (Perry et al., 2009). In vivo measurement of the neurotransmitters in the extracellular space of the CNS is an important tool for these studies. Microdialysis technique enables the continuous measurement of small molecular weight substances like most neurotransmitters from the extracellular space in living tissue systems. In this study, CSF samples from three different regions (the amygdala, the hippocampus and the raphe nuclei) of the rat brain were collected by using microdialysis technique.

The concentration of neurotransmitters in the extracellular space is very low. Shah et al. (1999) report that the levels of amino acids in microdialysates were found to be significantly lower than in brain tissue samples. In addition to this, samples are obtained by microdialysis technique at the small volumes. Hence, the analytical methods should provide low detection limits and should require less sample volume. Moreover, microdialysis samples are complex mixtures and the perfusion fluid contains inorganic salts. Finally, it is said that sensitive analytical methods are needed for the separation and quantification of neurotransmitters in microdialysis

samples. In this context, HPLC methods are more sensitive and accurate methods than other analytical techniques.

Due to the small sample volume, simultaneous determination can be performed for different neurotransmitters in microdialysates. But on the other hand, different methods can be required for the measurement of neurotransmitters depended on their chemical structure.

In this study, two methods were developed for the neurotransmitters (GABA, glutamate, serotonin and noradrenaline) and melatonin hormone in cerebrospinal fluid samples from the rat brain by using HPLC with fluorescence detection and a C18 reversed-phase column. The methods were validated. Moreover, measurement uncertainties were calculated by applying ‘bottom up’ approach.

3.1. Determination of GABA and Glutamate

3.1.1. Method Development of GABA and Glutamate

Abnormalities in the levels of GABA and glutamate are known to be associated with neurological and psychiatric disorders like Alzheimer’s disease, schizophrenia and epilepsy. Therefore, the measurement of the levels of these neurotransmitters in animal models may provide an additional tool for drug design. Furthermore, identifying changes in the concentration of selected amino acids in different regions of the brain can be useful to monitor drug efficiency (Shah et al., 1999; de Freitas Silva et al., 2009). For this reason, sensitive and accurate methods are needed for determination of neurotransmitters in microdialysis samples.

GABA and glutamate neither possess electroactive or fluorescent character nor show strong absorption in the ultraviolet/visible region (Shah et al., 2002; Clarke

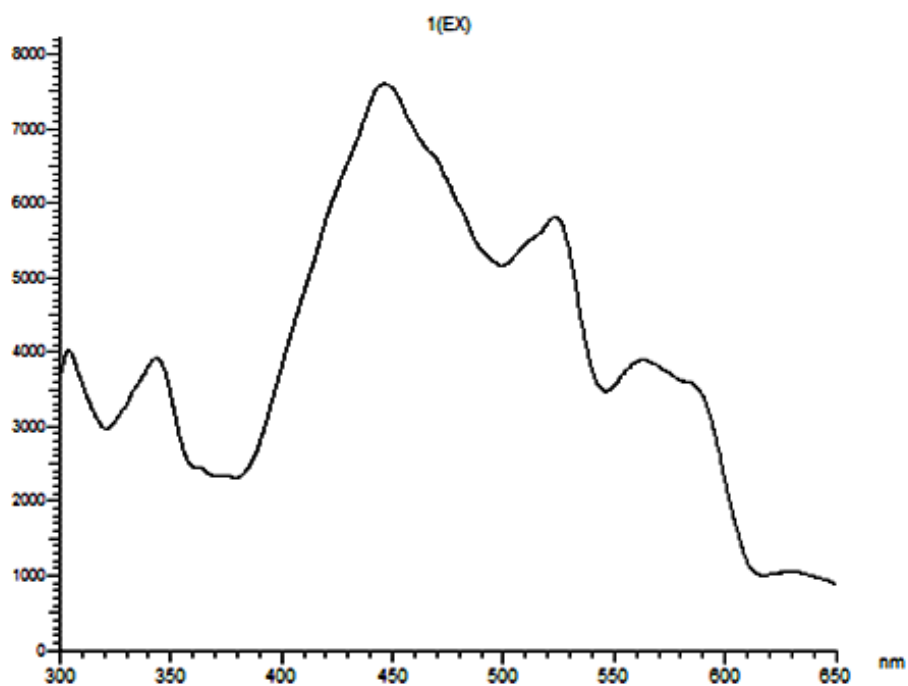
et al., 2007; de Freitas Silva et al., 2009). Therefore, a derivatization step was performed before the elution with HPLC.

OPA as a derivatization agent has a widespread usage in literature. But in this study, derivatization was performed with NDA. Many researchers have commented on the instability of the OPA derivatives. de Freitas Silva et al. (2009) studied with OPA and they reported the low stability of the OPA derivatives at room temperature. Derivatization reaction and elution is much longer with NDA. But, NDA derivatives are more stable and NDA is more suitable for pre-column derivatization.

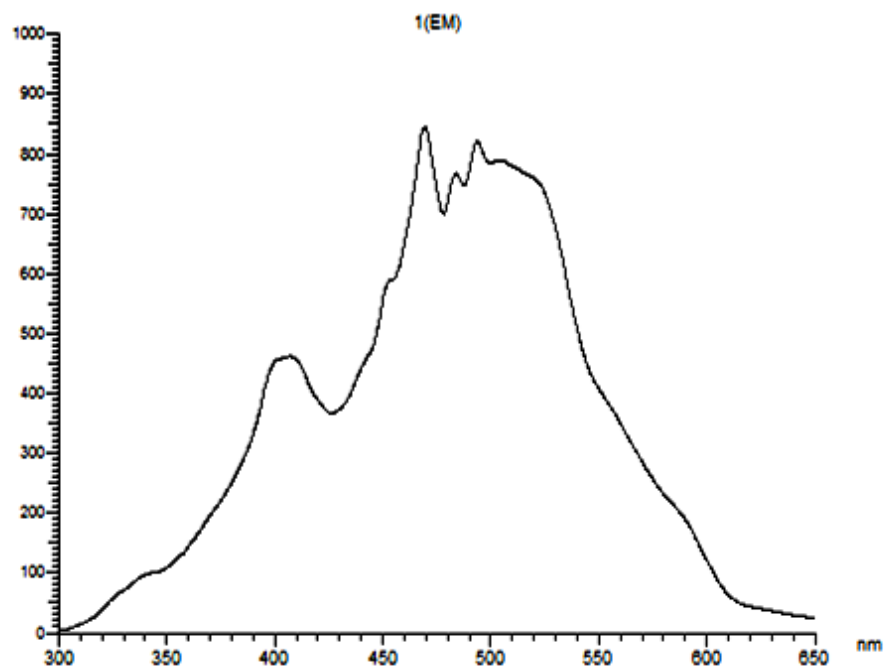
In this study, a fluorescence HPLC method for the simultaneous detection of GABA and glutamate in microdialysates from rat brain was used. The procedure used in this study was adapted from the method described by Clarke et al. (2007). Clarke et al. studied with rat brain tissue samples. But, it was studied with microdialysate samples in this study. GABA/glutamate were pre-column derivatized with NDA in the presence of cyanide ion as the nucleophile. The derivatives were separated on a C18 reverse-phase column using isocratic elution.

Excitation and emission wavelengths were optimized for the simultaneous detection of derivatives of GABA and glutamate. 5 ppb of the GABA and glutamate standards was derivatized and carried out wavelength scan with fluorescence spectrophotometer (Hitachi F-4500, Japan). The excitation spectrums of derivatives of GABA and glutamate were found maximum at 450 nm and 440 nm, respectively. The maximum emission spectrums of derivatives of GABA and glutamate were found separately at 470 nm. Figure 3.1 and Figure 3.2 illustrate spectrums obtained using fluorescence spectrophotometer. Also, the excitation and emission wavelengths

were found from the literature. Finally, the fluorescence detector was set at an excitation wavelength of 420 nm, an emission wavelength of 480 nm.

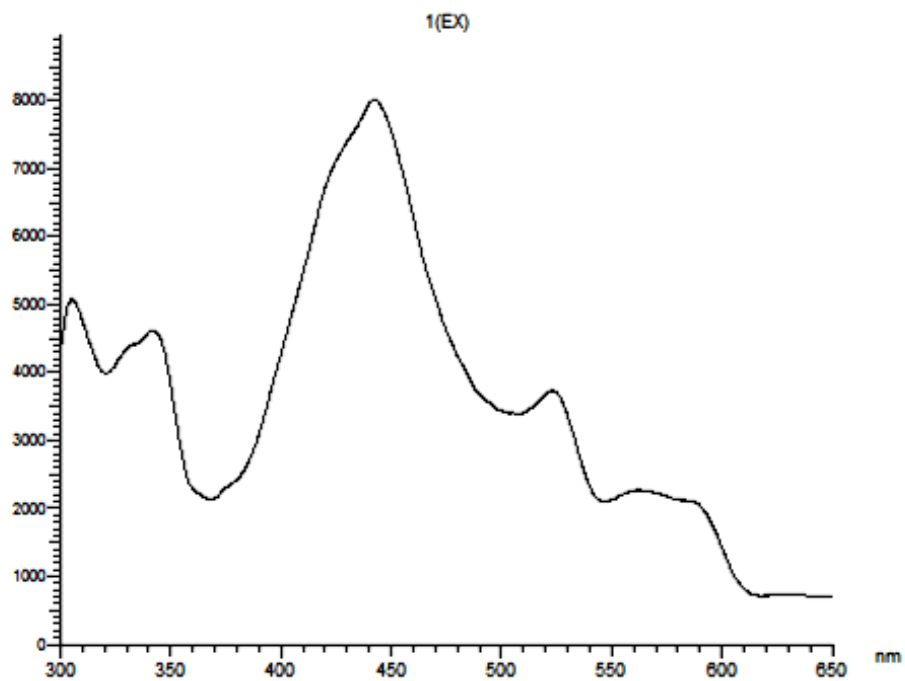


a) Excitation spectrum of GABA

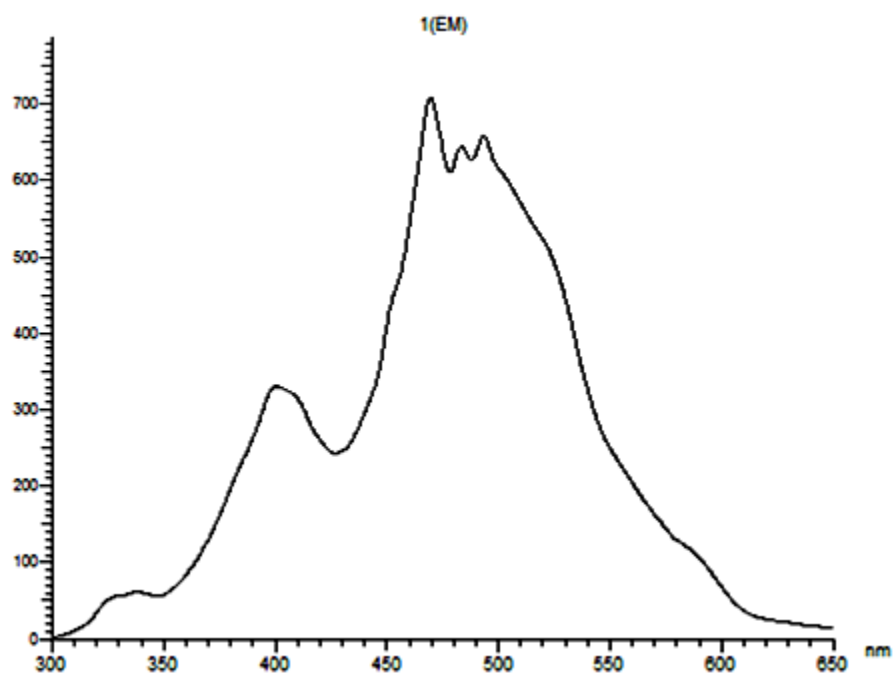


b) Emission spectrum of GABA

Figure 3.1. Wavelength scans for the derivatives of GABA (5 ppb)



a) Excitation spectrum of glutamate



b) Emission spectrum of glutamate

Figure 3.2. Wavelength scans for the derivatives of glutamate (5 ppb)

The flow rate was increased from 0.1 to 0.25 mL min⁻¹ in order to decrease the measurement time with HPLC. But, the sufficient separation was not obtained by using the flow rate of 0.25 mL min⁻¹ on the chromatogram. So, the lower flow rate (0.1 mL min⁻¹) was preferred.

The stability of the NDA derivatives was examined. It was observed that the derivatization reaction was not occurred in the refrigerator. After addition of derivatization reagents, it was waited from 1 to 4 hours at room temperature. Optimum reaction yield was obtained in 3 hours for GABA. On the other hand, optimum reaction yield for glutamate was obtained in one and a half hour and an approximately 7% degradation was observed at the third hour. Hence, the reaction mixture was left in the dark, at room temperature, for 1.5 hours.

To identify GABA and glutamate peaks, the derivatives of the standards at the increasing concentrations were injected to HPLC. Also, the derivatization reagents without sample or standard were analyzed since many peaks were observed on the chromatogram. Then, the increasing area at the definite retention time was determined on the chromatogram. GABA and glutamate standards were studied at the concentrations of 10, 20, 50 and 100 ppb. The chromatograms obtained from mixture of GABA and glutamate standards at the concentrations of 50 and 100 ppb are given in Figure 3.3.

Elution was achieved in 60 minutes. GABA/glutamate were identified by their characteristic retention times. As expected, GABA derivative with a larger hydrophobic (non-polar) character was retained longer. The retention time of glutamate was ranged between 21.50 min. and 22.33 min. However, the retention time of GABA was ranged between 44.68 min. and 47.56 min. Clarke et al. (2007) report total run time as 15 min. This might be explained with differences between the analytical columns.

Microdialysis samples were analyzed: 25 μ L of sample, 225 μ L of borate buffer, 50 μ L of potassium cyanide and 50 μ L of NDA were added to an amber vial and mixed vigorously. The reaction was allowed to proceed at room temperature in the absence of light for the 1.5 hours. A 20 μ L of the derivative was injected to the HPLC system. Also, standard mixture was spiked into microdialysis samples to confirm the existence of GABA and glutamate. 25 μ L of sample and 25 μ L of 1400 ppb standard mixture, 450 μ L of borate buffer, 100 μ L of potassium cyanide and 100 μ L of NDA were added to an amber vial and mixed vigorously. In this way, it was provided that the standard concentration of the resulting solution was at 50 ppb. After the derivatization reaction, a 20 μ L of the derivative was injected to the HPLC system. As a result, proportional rise were observed at the areas of the peaks of GABA/glutamate. The chromatograms obtained from the sample (from the hippocampus region) and the spiked sample (spiking was performed on the same sample) are given in Figure 3.4 as an example.

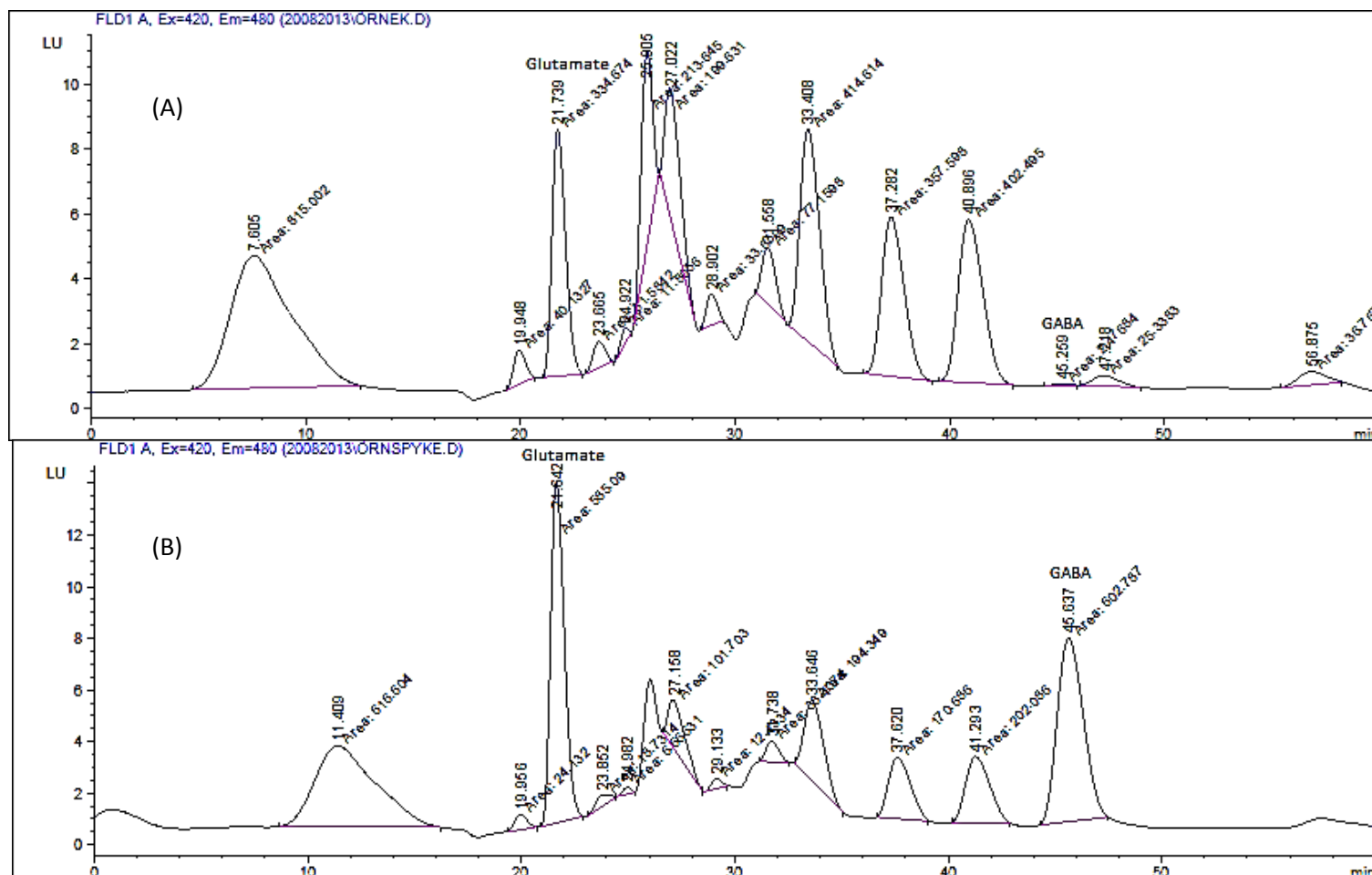


Figure 3.4. The chromatograms of the sample (A) and the spiked sample (B) (Chromatographic conditions: flow rate:0.1 mL min⁻¹, mobile phase: 0.1 M Na₂HPO₄/50 mM EDTA (pH 5.3, 1 M HCl) and HPLC grade methanol (35:65, v/v), isocratic, 20 µL injection, column: inertsil ODS-2 (C18, 150 x 4.6 mm i.d., 5 µm))

3.1.2. Method Validation of GABA and Glutamate

ISO/IEC 17025:2005 defines validation as “the **confirmation** by examination and the provision of **objective evidence** that the particular requirements for a **specific intended use** are fulfilled”. Validation has three important parts: (1) the specific intended use means the analytical requirement. (2) the objective evidence is usually provided from the calculated appropriate method performance parameters (3) the confirmation is taken as a satisfactory comparison of the performance data with what is required, i.e., demonstrating that the method is fit-for-purpose (Eurachem Workshop, 2006). Method validation is an essential requirement of laboratory accreditation according to ISO/IEC 17025.

Possible matrix effects coming from the sample material must be checked. The range of concentrations by the standard addition should be the same as that of the matrix-free calibration. In other words, the slopes of both calibration plots can be compared for significant difference. If the slopes are not significantly different, there is no need to compensate for matrix effects (NATA, Technical Note 17, 2009). The slopes of the plots for GABA and glutamate were found as 1.1955 and 0.6545 by using external calibration method (Figure 3.5), respectively. But, when the standard addition method was used, the slopes of the plots for GABA and glutamate were found as 12.462 (Figure 2.8) and 8.7722 (Figure 2.7), respectively.

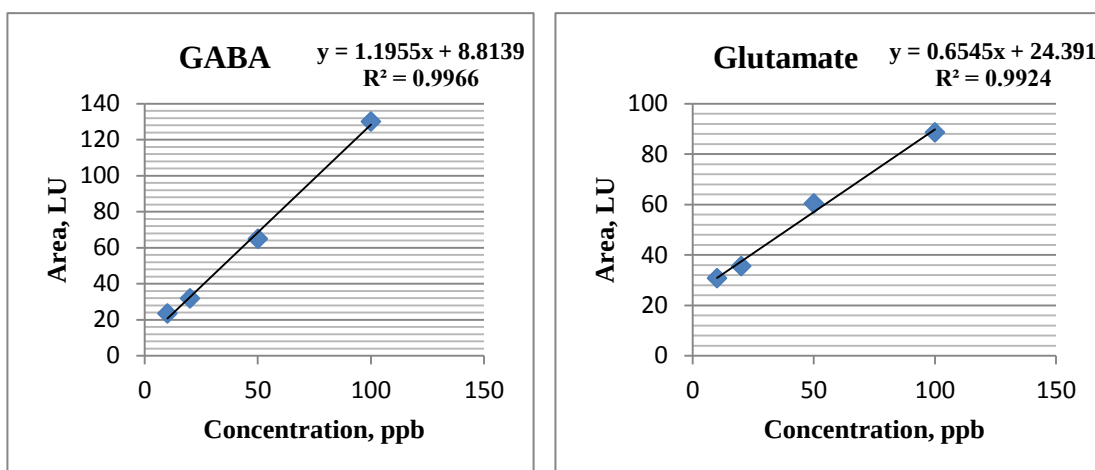


Figure 3.5. External calibration curve for GABA and glutamate

Accordingly, the standard addition method was used for eliminate the matrix effect. For this purpose; equal volumes of the sample were taken, all but one were separately spiked with known amounts of the different standard solutions and all were then diluted to the same volume. Consequently, nine matrix-matching calibration solutions were analyzed according to the procedure which was mentioned in the Section 2.2.6.1.

The matrix-matching calibration solutions were measured with two injections in the instrument. But, due to degradation of both GABA and glutamate derivatives, lower peak areas were observed in the second measurements. Hence, only the results of first measurements were used.

The calibration curves were constructed by plotting the peak areas against the concentrations with the initial unknown concentration set at 0. Linear regression equation was found for GABA as $y = 12.462x - 44.674$ and for glutamate as $y = 8.7722x + 122.16$. Linearity was evaluated by using R^2 which was found to be 0.9997 for both GABA and glutamate.

The limit of detection is defined as the lowest concentration of analyte in a sample that can be detected but not necessarily quantitated as an exact value under the stated conditions of the method. The limit of quantitation is strictly the lowest concentration of analyte in a sample which can be quantitatively determined with an acceptable level of repeatability precision and trueness (ICH Harmonised Tripartite Guideline, 1994).

Several approaches for determining the detection limit and the quantitation limit are possible. For the determination of GABA and glutamate, signal-to-noise ratio (S/N) method was used since it is not possible to run sample many times due to the degradation problem. Determination of the S/N was performed by measuring the peak height and peak background in the analyte peak.

A S/N ratio between 3:1 or 2:1 is generally considered acceptable for estimating the detection limit. A typical S/N ratio is 10:1 for estimating the quantitation limit (ICH Harmonised Tripartite Guideline, 1994). In this study, the limits of detections were calculated using S/N ratio of 3 and were found as 9 ppb for glutamate and 28 ppb for GABA. The limits of quantitation with the S/N ratio of 10 were found as 30 ppb for glutamate and 92 ppb for GABA.

Low values were obtained for the detection limits of GABA and glutamate when a comparison was made with previously reported data (Clarke et al., 2007).

Precision is related to random measurement error and is a measure of how close results are to one another. The random error can be reduced by making replicate measurements and calculating the mean value (Eurachem Guide, 2011).

Repeatability conditions refer to measurements being made on portions of the same material by the same analyst using the same procedure and the same equipment

in a same laboratory over a short time period. Additionally, under intermediate measurement conditions, measurements are made on portions of the same material using the same procedure, but over an extended time period. Different analyst who may be using different equipment is also possible (Eurachem Guide, 1998; Eurachem Guide, 2011).

Due to the shortage of microdialysis samples and the long HPLC measurement time, five independent repeats were performed at the repeatability conditions. Experiments were repeated daily in the consecutive five days. The standard deviation and RSD of the results were calculated. The percentage RSD values ranged from 0.78 to 1.80 for GABA and from 0.80 to 1.67 for glutamate.

To determine intermediate precisions of GABA and glutamate, the pooled relative standard deviations (RSD_{pool}) were calculated. RSD_{pool} was obtained using the following equation (Barwick and Ellison, 2000):

$$RSD_{pool} = \sqrt{\frac{[(RSD_1^2 \times (n_1 - 1))] + [(RSD_2^2 \times (n_2 - 1))] + \dots}{(n_1 - 1) + (n_2 - 1) + \dots}} \quad (1)$$

where RSD_1 was the RSD calculated for the first day results, n_1 was the number of replicates for that day, etc. RSD_{pool} was found for GABA as 1.31 % and for glutamate as 1.19 %.

Because of the absence of a suitable reference material, recovery was investigated through spiking studies (IUPAC Technical Report, 1999). The recovery for each sample, $R_{(i)}$, was calculated by the Eq. (2) (Eurachem Guide, 1998; Barwick and Ellison, 2000; Yenisoy-Karakaş, 2012):

$$R_{(i)} = \frac{C_{\text{obs}(i)} - C_{\text{native}}}{C_{\text{spike}}} \quad (2)$$

where $C_{\text{obs}(i)}$ was the concentration of the GABA/glutamate in the spiked sample, C_{native} was the concentration of the GABA/glutamate in the unspiked (native) sample and C_{spike} was the concentration of the spike (pure standard) added to the CSF sample.

Recovery is often tested to determine whether it is significantly different from 1.0 (100 %) (Barwick and Ellison, 1999). The closer R is to 1, the smaller the bias in the procedure (TrainMIC, 2003).

The average recovery of the five days for glutamate was found 91% (the recoveries changed between 87 % and 99 %). Also, the average recovery of the five days for GABA was found 112 % (the recoveries changed between 103 % and 118 %).

3.1.3. Estimation of Uncertainty for GABA and Glutamate

Eurachem/Citac guide (2012) defines the measurement uncertainty as “a parameter associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand”. The estimation of the measurement uncertainty is mandatory for laboratories accredited under ISO 17025.

There are two different approaches to evaluating measurement uncertainty: ‘bottom up’ and ‘top down’. The ‘bottom up’ approach involves the quantification of

each source of uncertainty separately. In contrast, the ‘top down’ approach uses the method performance data (such as recovery and precision) which account for a number of sources of uncertainty (Eurachem Workshop, 2006).

When the measurement uncertainty is evaluated correctly, it gives more realistic estimation for the true spread of results. It provides confidence to the analyst and the customer that decisions based on the results (Eurachem Workshop, 2006). Also, it is unavoidable to calculate the measurement uncertainty for the trace and ultra trace analysis (Yenisoy-Karakaş, 2009). Furthermore, thanks to the detailed analysis of uncertainty sources, an Analytical Chemist evaluate the critical stages of the method where uncertainty should be reduced (Yenisoy-Karakaş, 2006).

For these reasons, in this study, the measurement uncertainty was evaluated for the neurotransmitters which are trace levels in the CSF. The ‘bottom up’ approach was applied.

The measurement uncertainty for the determination of glutamate was shown by explaining each step. Since the similar calculations were performed for GABA and glutamate, the uncertainty result for GABA was reported.

3.1.3.1. Specification of the Measurand and Measurement Procedure

The glutamate concentration in the microdialysate sample, C_{GLU} , expressed in ppb, is calculated by using the following model equation:

$$C_{GLU} = C_0 \times DF \quad (3)$$

where C_0 is the concentration of the glutamate obtained from the calibration curve (ppb) and DF is the dilution factor ($V_{initial} = 20 \mu L$, $V_{final} = 280 \mu L$).

The procedure is illustrated schematically in Figure 3.6.

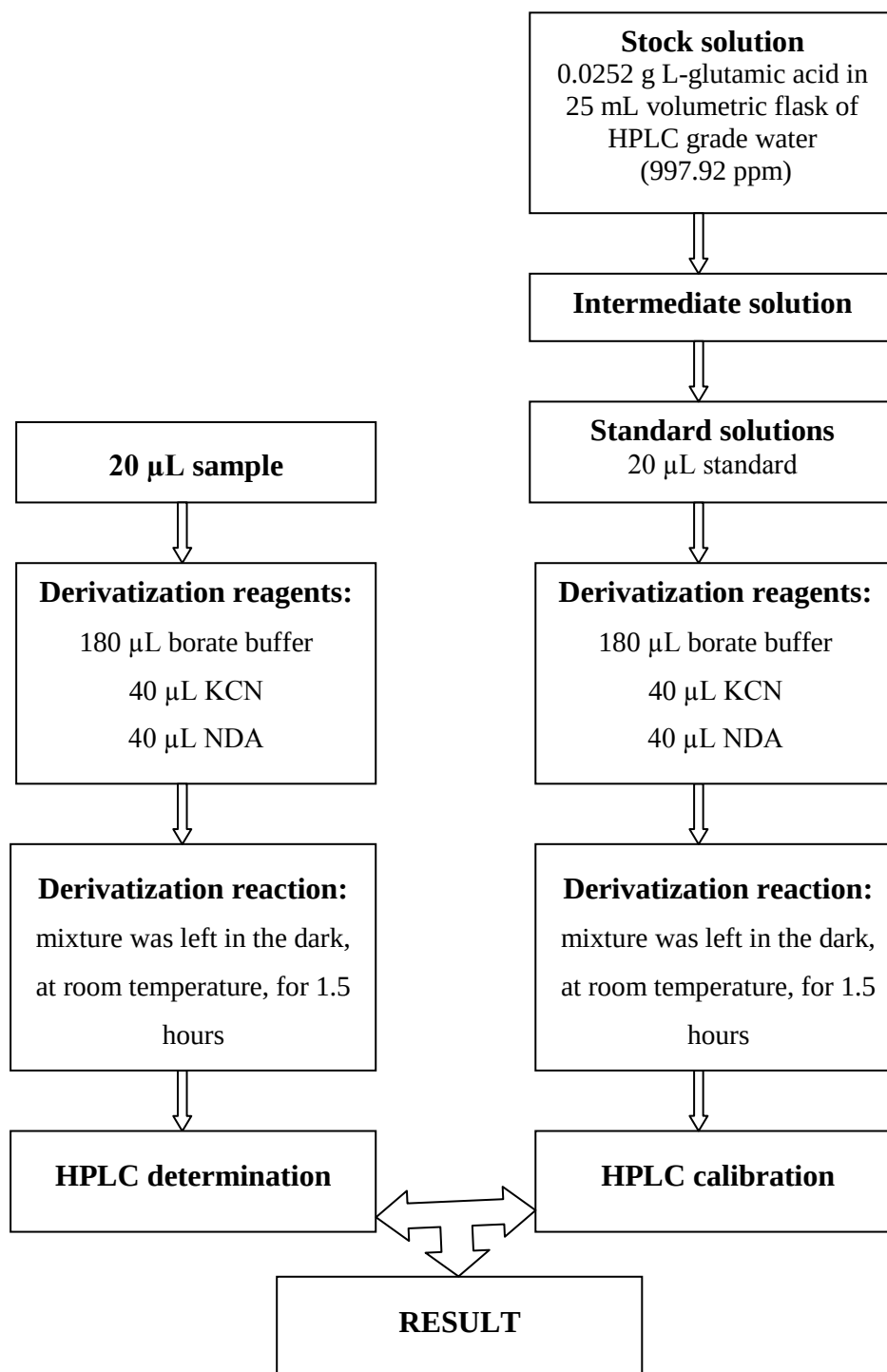


Figure 3.6. Measurement procedure for glutamate

3.1.3.2. Identification of the Uncertainty Components

For the identification of all relevant uncertainty sources, a cause and effect diagram (also known as the Ishikawa or fishbone diagram) was used (Figure 3.7). The parameters in the model equation are represented by the main branches of the diagram. Also, other parameters are added to the diagram considering each step in the procedure.

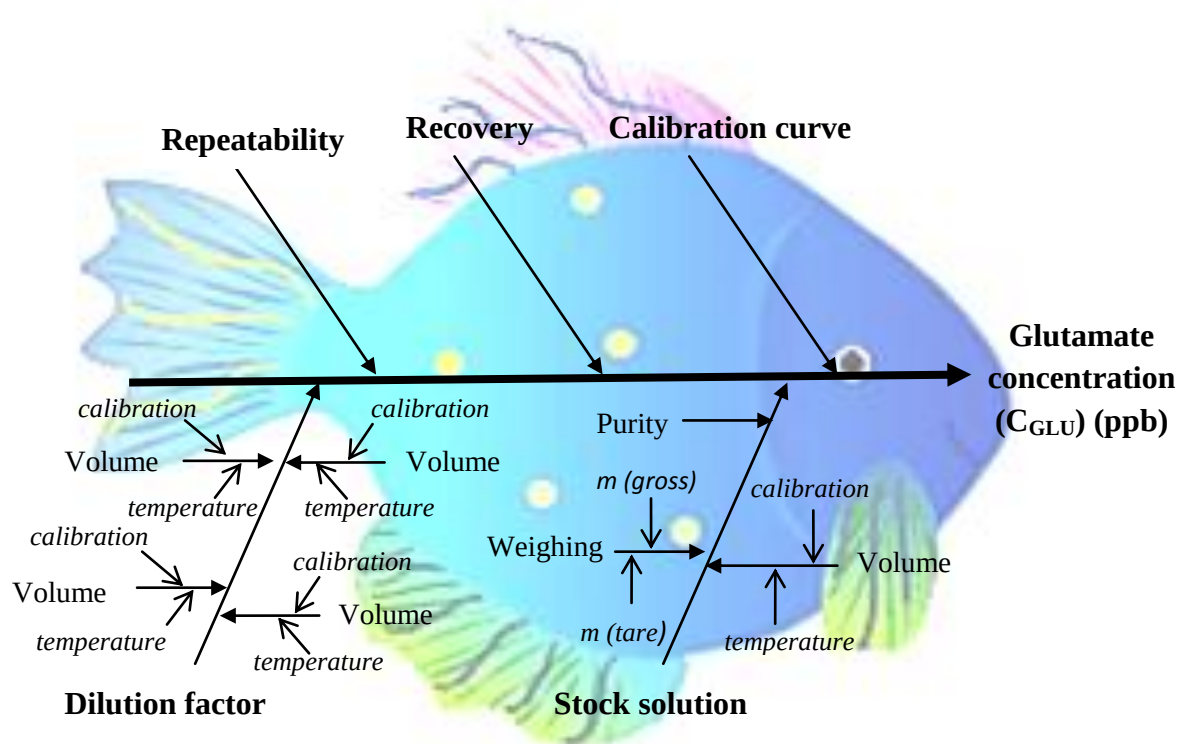


Figure 3.7. Cause and effect diagram for the determination of glutamate

The uncertainty components that contribute to the glutamate concentration in the CSF are as follows:

- 1) Calibration curve (C_0)
- 2) Stock solution of the glutamate (C_{stock})
- 3) Dilution factor (DF)

4) Repeatability (Rep)

5) Recovery ($\bar{R}$).

3.1.3.3. Quantification of the Uncertainty Components

All uncertainty contributions was expressed as standard uncertainties (as standard deviation) before the combination.

3.1.3.3.1. Estimation of the Uncertainty Derived from the Linear Calibration Curve

The uncertainty associated with the calibration curve, $u(C_0)$, was calculated with the Eq. (4) defined by Eurachem/Citac guide (2012).

$$u(c_0) = \frac{S}{B_1} \sqrt{\frac{1}{p} + \frac{1}{n} + \frac{(c_0 - \bar{c})^2}{S_{xx}}} \quad S_{xx} = \sum_{j=1}^n (c_j - \bar{c})^2 \quad (4)$$

where

S : residual standard deviation

B_1 : slope

p : number of measurements to determine C_0

n : number of measurements for the calibration

C_0 : determined glutamate concentration in CSF

$\bar{c}$: mean value of the different calibration standards (n number of measurements)

j : index for the number of measurements to obtain the calibration curve

C_j : individual calibration standard value obtained from the calibration equation.

As mentioned in Section 2.2.6.1, the nine matrix-matching calibration solutions were measured. Because of the degradation problem, one measurement was performed for each calibration solution. So, n is equal to 9 and p is equal to 1. Calibration curve was constructed by plotting peak areas versus concentrations. The slope of the calibration curve (B_1) was found as 8.7722 (Calibration equation: $y = 8.7722x + 122.16$). Residual standard deviation (S) was calculated from CurveExpert version 1.3 and it was found as 22.482 (Figure 3.8).

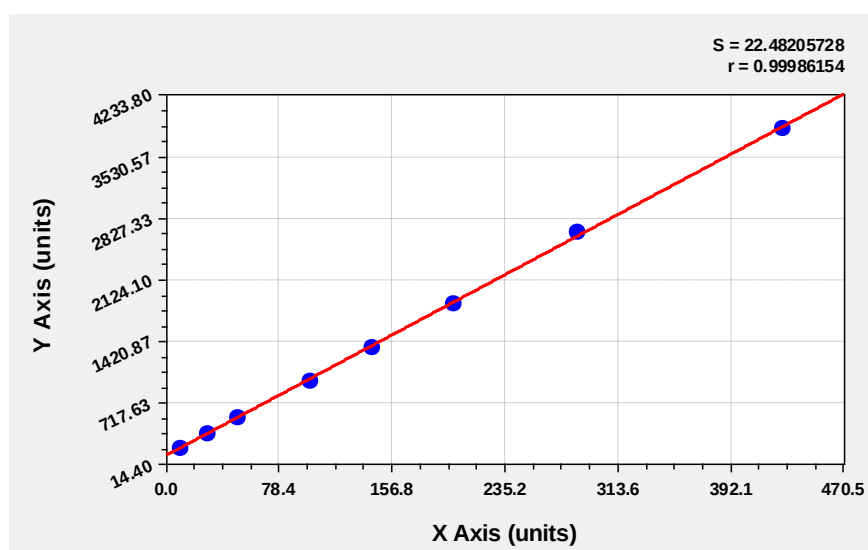


Figure 3.8. Calibration curve for the determination of glutamate

S_{xx} and $\bar{C}$ was calculated as 166082 and as 138.1, respectively. The calculations were shown in Table 3.1.

Table 3.1. Calculation of the S_{xx} and $\bar{c}$

Conc. (ppb)	Area	C_j	$C_j - C_{ort}$	$(C_j - C_{ort})^2$
0.0	144	2.5	-135.6	18395
10.0	210	10.0	-128.1	16409
28.5	372	28.5	-109.6	12023
49.9	554	49.3	-88.9	7899
99.8	981	97.9	-40.2	1616
142.6	1361	141.2	3.1	10
199.6	1852	197.2	59.1	3489
285.1	2668	290.2	152.1	23142
427.7	3862	426.4	288.3	83100

$$\bar{c} = 138.1$$

$$S_{xx} = 166082$$

Finally, when C_0 was assumed as 100 ppb;

$$u(c_0) = \frac{22.482}{8.7722} \sqrt{\frac{1}{1} + \frac{1}{9} + \frac{(100 - 138.1)^2}{166082}} \quad (5)$$

$$u(c_0) = 2.71 \text{ ppb}$$

3.1.3.3.2. Estimation of the Uncertainty Derived from Stock Solution

A stock solution of glutamate at 997.92 ppm was prepared for the calibration curve. 0.0252 g glutamate standard was weighed on the analytical balance. It was dissolved within 25 mL (volumetric flask) of HPLC grade water. The concentration of stock solution depends on the weighing of the glutamate standard, its purity and the volume of the HPLC grade water in which it is dissolved. Therefore, the uncertainties of three parameters were combined in order to estimate the uncertainty derived from stock solution (Eq. (6)).

$$\frac{u(C_{\text{stock}})}{C_{\text{stock}}} = \sqrt{\left(\frac{u(P)}{P}\right)^2 + \left(\frac{u(W)}{W}\right)^2 + \left(\frac{u(V)}{V}\right)^2} \quad (6)$$

Purity

The purity of the glutamate standard is given on the supplier's certificate as >99.0 % (>0.99). Because there was no additional information about the uncertainty value, a rectangular distribution was assumed. To obtain the standard uncertainty $u(P)$, the value of 0.01 was divided by $\sqrt{3}$.

$$u(P) = \frac{0.01}{\sqrt{3}} = 0.0058 \quad (7)$$

Weighing

Solid standard (25.2 mg) was weighed on the analytical balance. The uncertainty with the mass of the glutamate was estimated using the data from the calibration certificate of the analytical balance. The expanded uncertainty ($k=2$) of the balance is given as 0.6 mg. The standard uncertainty was calculated by using normal distribution and it was found as 0.3 mg ($0.6 \text{ mg}/2 = 0.3 \text{ mg}$).

This contribution was considered twice since the weighing process involves a difference.

$$u(W) = \sqrt{2 \times (0.3)^2} = 0.424 \text{ mg} \quad (8)$$

Volume

The volume of the stock solution contained in the volumetric flask (25 mL) has two major sources of uncertainty: temperature effects and calibration.

➤ Calibration : The manufacturer's specification is ± 0.040 mL for the volumetric flask (25 mL) at 20°C . This value is given without any confidence level or distribution information. A triangular distribution was assumed. To obtain the standard uncertainty, the value of 0.040 was divided by $\sqrt{6}$.

$$\frac{0.040 \text{ mL}}{\sqrt{6}} = 0.016 \text{ mL} \quad (9)$$

➤ Temperature : The volumetric flask was calibrated at a temperature of 20°C according to the manufacturer. The uncertainty of the temperature effect was calculated from the variation in the laboratory temperature and the coefficient of the volume expansion of the solvent.

Temperature variation in the laboratory was assumed as $\pm 3^{\circ}\text{C}$. Since the volume expansion of the liquid is significantly larger than that of the glass, the volume expansion of the flask was neglected. The coefficient of volume expansion for water is $2.1 \times 10^{-4}^{\circ}\text{C}^{-1}$ at 20°C , which leads to a volume variation of

$$\pm (25\text{mL} \times 3^{\circ}\text{C} \times 2.1 \times 10^{-4}^{\circ}\text{C}^{-1}) = \pm 0.0157 \text{ mL} \quad (10)$$

The standard uncertainty was calculated by using rectangular distribution and found as 0.009 mL.

$$\frac{0.0157 \text{ mL}}{\sqrt{3}} = 0.009 \text{ mL} \quad (11)$$

The two contributions were combined to give the standard uncertainty $u(V)$.

$$u(V) = \sqrt{0.016^2 + 0.009^2} = 0.019 \text{ mL} \quad (12)$$

The uncertainties associated with each parameter were combined as follows:

$$\frac{u(C_{\text{stock}})}{C_{\text{stock}}} = \sqrt{\left(\frac{0.0058}{1}\right)^2 + \left(\frac{0.424}{25.2}\right)^2 + \left(\frac{0.019}{25}\right)^2} = 0.0178 \quad (13)$$

$$u(C_{\text{stock}}) = 997.92 \text{ ppm} \times 0.0178 = 17.80 \text{ ppm} \quad (14)$$

3.1.3.3.3. Estimation of the Uncertainty Derived from the Dilution Factor

Dilution factor was calculated as 14 ($V_{\text{final}}/V_{\text{initial}} = 280 \text{ } \mu\text{L}/20 \text{ } \mu\text{L} = 14$). 20 μL of the sample (V_{initial}) was taken into a vial by using 20 μL capacity automatic pipette. 180 μL of the borate buffer was taken by using 1 mL capacity automatic pipette. 40 μL of the KCN and 40 μL of the NDA were taken separately by using 100 μL capacity automatic pipette. In this way (by adding derivatization reagents), the sample was diluted to 280 μL (V_{final}). The related uncertainty data and calculated standard uncertainty results are given in Table 3.2.

Table 3.2. Standard uncertainties for dilution factor

			Type of distribution	Standard uncertainty	Standard uncertainty $u(V)$ of volume V
Volume of the borate buffer, μL	Temperature change in the lab. $^{\circ}\text{C}$	3	rectangular	0.065	0.133
	The coefficient of volume expansion for water $^{\circ}\text{C}^{-1}$	0.00021			
	Volume of the volumetric pipette, μL	180			
	Uncertainty of volumetric pipette, μL	0.20	rectangular	0.115	
Volume of the KCN, μL	Temperature change in the lab. $^{\circ}\text{C}$	3	rectangular	0.015	0.038
	The coefficient of volume expansion for water $^{\circ}\text{C}^{-1}$	0.00021			
	Volume of the volumetric pipette, μL	40			
	Uncertainty of volumetric pipette, μL	0.06	rectangular	0.035	
Volume of the NDA, μL	Temperature change in the lab. $^{\circ}\text{C}$	3	rectangular	0.103	0.109
	The coefficient of volume expansion for methanol $^{\circ}\text{C}^{-1}$	0.00149			
	Volume of the volumetric pipette, μL	40			
	Uncertainty of volumetric pipette, μL	0.06	rectangular	0.035	
Volume of the sample, μL	Temperature change in the lab. $^{\circ}\text{C}$	3	rectangular	0.007	0.030
	The coefficient of volume expansion for water $^{\circ}\text{C}^{-1}$	0.00021			
	Volume of the volumetric pipette, μL	20			
	Uncertainty of volumetric pipette, μL	0.05	rectangular	0.029	

The standard uncertainty for V_{initial} was calculated as 0.030 (Table 3.2). The total uncertainty for V_{final} was calculated as 0.178.

$$u(V_{\text{final}}) = \sqrt{(0.133)^2 + (0.038)^2 + (0.109)^2 + (0.030)^2} = 0.178 \quad (15)$$

In order to obtain the uncertainty comes from dilution factor, these values were combined as follows:

$$u(\text{DF}) = \sqrt{\left(\frac{0.178}{280}\right)^2 + \left(\frac{0.030}{20}\right)^2} = 0.0016 \quad (16)$$

3.1.3.3.4. Estimation of the Uncertainty Derived from Repeatability

All possible repeatability uncertainties in the procedure were collected under this part. The five independent experiments for glutamate analysis were repeated daily. Also, the repetition of the analysis was performed in the consecutive five days. The mean RSD of the five days was found as 0.0116.

The standard uncertainty derived from the repeatability was calculated by using the following equation (standard deviation of the mean):

$$u(\text{Rep}) = \frac{\text{RSD}}{\sqrt{n}} = \frac{0.0116}{\sqrt{5}} = 0.0052 \quad (17)$$

3.1.3.3.5. Estimation of the Uncertainty Derived from Recovery

For glutamate, the spiked samples were prepared from the sample matrix which contained the analyte. The mean recovery, $\bar{R}$, was calculated by the following formula (Barwick and Ellison, 2000; Yenisoy-Karakaş, 2012):

$$\bar{R} = \frac{1}{n} \sum_{i=1}^n \frac{C_{\text{obs}(i)} - C_{\text{native}}}{C_{\text{spike}(i)}} \quad (18)$$

The mean recovery for glutamate was found as 0.91.

The standard uncertainty comes from the recovery, $u(\bar{R})$, for glutamate was calculated by using Eq. (19) (Barwick and Ellison, 2000; Yenisoy-Karakaş, 2012).

$$u(\bar{R}) = \frac{1}{\bar{C}_{\text{spike}}} \sqrt{\frac{\bar{u}(C_{\text{obs}(i)})^2}{n} + u(C_{\text{native}})^2} \quad (19)$$

$$u(\bar{R}) = \frac{1}{99.792} \sqrt{\frac{(0.017)^2}{24} + (0.197)^2} = 0.0020 \quad (20)$$

For GABA, the spiked samples were prepared by using the blank sample matrix. 10 µL volume of the 100 ppb GABA standard was added to each blank sample matrix (40 µL). Therefore, $\bar{R}$ and $u(\bar{R})$ were calculated by using Eq. (21) and (22) (Barwick and Ellison, 2000; Pecorelli et al., 2005).

$$\bar{R} = \frac{\bar{m}_{\text{obs}}}{m_{\text{spike}}} \quad (21)$$

$$u(\bar{R}) = \bar{R} \times \sqrt{\left(\frac{S_{m_{\text{obs}}}^2}{n \times \bar{m}_{\text{obs}}^2} \right) + \left(\frac{u(m_{\text{spike}})}{m_{\text{spike}}} \right)^2} \quad (22)$$

To decide whether the mean recovery was significantly different from 1.0, a statistical test was used. The significance test statistic t was calculated.

$$t = \frac{|1 - \bar{R}|}{u(\bar{R})} = \frac{|1 - 0.91|}{0.0020} = 45 \quad (23)$$

This t value was compared with two-tailed critical value t_{critical} , for $(n-1)$ degrees of freedom at 95 % confidence. Since the calculated t value was greater than t_{critical} , $\bar{R}$ is significantly different from 1.0.

$$t = 45 \geq t_{\text{critical};23} = 2.069$$

In the normal application of the method no recovery correction in the model equation was applied (i.e., $\bar{R}$ was assumed to equal 1). But, the uncertainty associated with method recovery was increased (Barwick and Ellison, 2000; Pecorelli et al., 2005; Yenisoy-Karakaş, 2012; Aslan-Sungur et al., 2014) and the following equation was used:

$$u(\bar{R})' = \sqrt{\left(\frac{1 - \bar{R}}{k}\right)^2 + u(\bar{R})^2} \quad (24)$$

where k was the coverage factor. $u(\bar{R})'$ was calculated as 0.045 applying Eq. (24).

$$u(\bar{R})' = \sqrt{\left(\frac{1 - 0.91}{2}\right)^2 + (0.0020)^2} = 0.045 \quad (25)$$

3.1.3.4. Calculating of the Combined Uncertainty

The values and their uncertainties associated with each component were summarized in Table 3.3.

Table 3.3. The values and their uncertainties

Description	Value x	Standard uncertainty u(x)	Relative standard uncertainty u(x)/x
calibration curve	100	2.71	0.0271
stock solution	997.92	17.80	0.0178
repeatability	1	0.0052	0.0052
dilution factor	14	0.0015	0.0001
increased recovery	0.91	0.045	0.0492

Standard uncertainties associated with each component were combined accordingly:

$$\frac{u_C(C_{GLU})}{C_{GLU}} = \sqrt{\left(\frac{u(C_0)}{C_0}\right)^2 + \left(\frac{u(C_{stock})}{C_{stock}}\right)^2 + \left(\frac{u(DF)}{DF}\right)^2 + \left(\frac{u(Rep)}{Rep}\right)^2 + \left(\frac{u(\bar{R})}{\bar{R}}\right)^2} \quad (26)$$

$$\frac{u_C(C_{GLU})}{100} = \sqrt{\left(\frac{2.71}{100.0}\right)^2 + \left(\frac{17.80}{997.92}\right)^2 + \left(\frac{0.0015}{14}\right)^2 + \left(\frac{0.0052}{1}\right)^2 + \left(\frac{0.045}{0.91}\right)^2} = 0.0592 \quad (27)$$

$$u_C(C_{GLU}) = 5.92 \text{ ppb}$$

The combined uncertainty for determination of glutamate in CSF was found as 5.92 ppb.

The sizes of these uncertainty contributions can be compared using the histogram in Figure 3.9. The dilution factor has virtually no influence on the overall

uncertainty. Repeatability is an another component for the combined uncertainty and it is derived from the overall variability in the method. Standard preparation for the calibration curve is an important uncertainty contribution and also the calibration curve is the important step to be careful. Recovery has the major effect on the combined uncertainty.

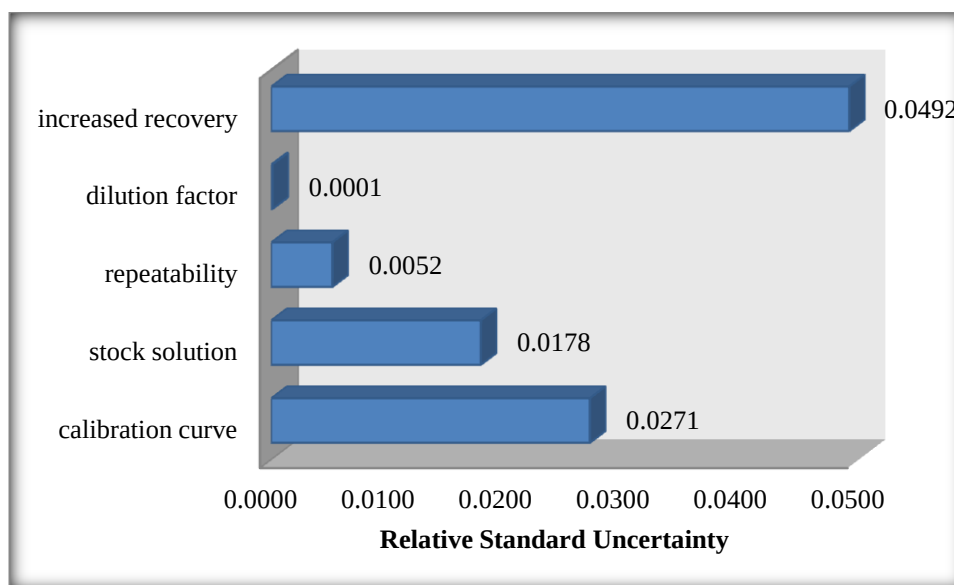


Figure 3.9. Uncertainty contributions for glutamate determination

3.1.3.5. Expanded Uncertainty

The expanded uncertainty $U(C_{GLU})$ was obtained by applying a coverage factor (k) of 2 (a level of confidence of approximately 95 %).

$$U(C_{GLU}) = 2 \times 5.92 = 11.8 \text{ ppb} \quad (28)$$

The result is reported as $(100 \pm 12) \text{ (ppb)}^*$.

*The reported uncertainty is an expanded uncertainty calculated using a coverage factor of 2 which gives a level of confidence of approximately 95 %.

3.2. Determination of Melatonin, Serotonin and Noradrenaline

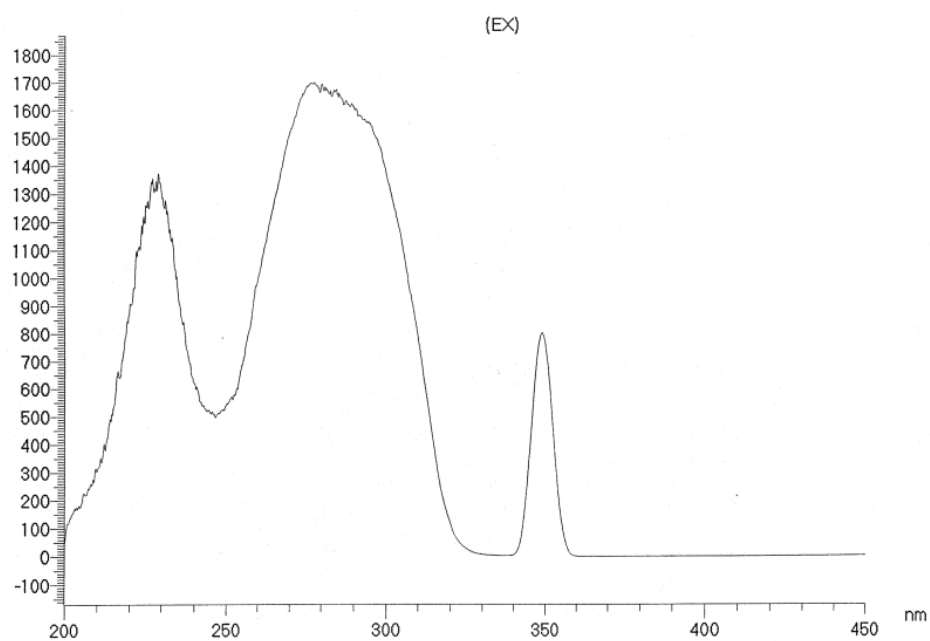
3.2.1. Method development of Melatonin, Serotonin and Noradrenaline

The biogenic amines have importance as markers of neuroblastoma, stress condition and other autonomic nervous system disorders (Mishra et al., 2009). Sensitive and accurate methods are needed for determination of neurotransmitters in microdialysis samples and HPLC methods meet this need.

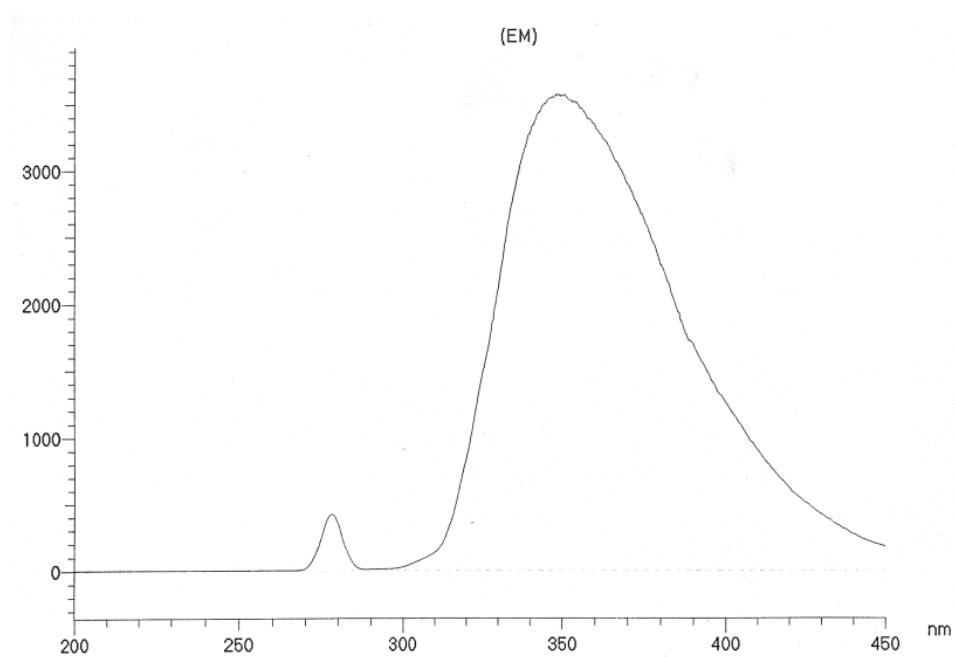
In this study, a fluorescence HPLC method for the detection of melatonin, serotonin and noradrenaline in microdialysates from rat brain was developed. The procedure used in this study is rapid and simple. Detection is performed by using native fluorescence character of these compounds without any derivatization reaction. The compounds were separated in one chromatographic run, on a C18 reverse-phase column using isocratic elution. Then, the method was validated.

Firstly, chromatographic conditions for the determination of melatonin, serotonin and noradrenaline were investigated and optimized.

The excitation and the emission wavelengths were optimized for the simultaneous detection of melatonin, serotonin and noradrenaline. At the result of the wavelength scanning with fluorescence spectrophotometer (Hitachi F-4500, Japan), the maximum excitation wavelengths of melatonin, serotonin and noradrenaline were found at 278 nm, 278 nm and 280 nm, respectively in the spectrums. The maximum emission wavelengths of these compounds were found as 350 nm, 337 nm and 312 nm, respectively. In light of the foregoing, the fluorescence detector was set at the excitation wavelength of 280 nm, the emission wavelength of 340 nm. Figure 3.10, Figure 3.11 and Figure 3.12 illustrate spectrums obtained using fluorescence spectrophotometer.

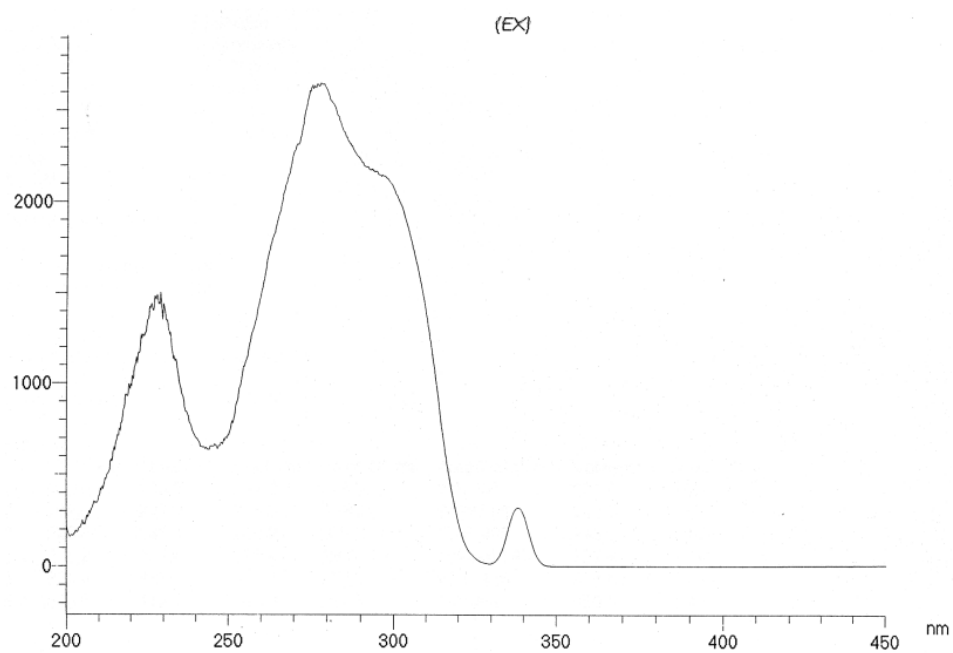


a) Excitation spectrum of melatonin (500 ppb)

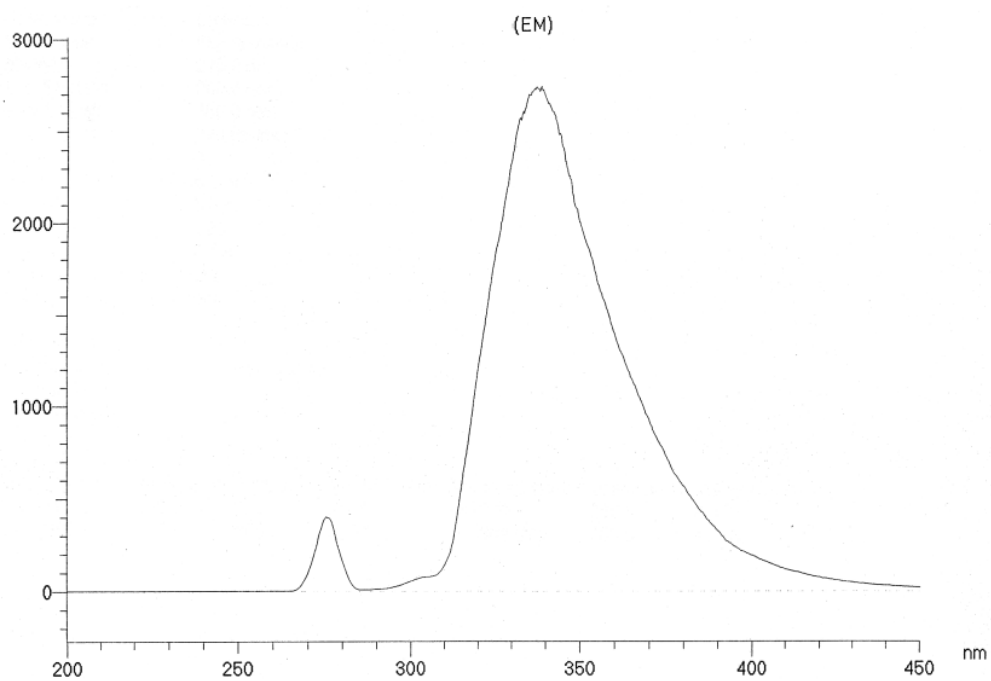


b) Emission spectrum of melatonin (1 ppm)

Figure 3.10. Wavelength scans for the melatonin

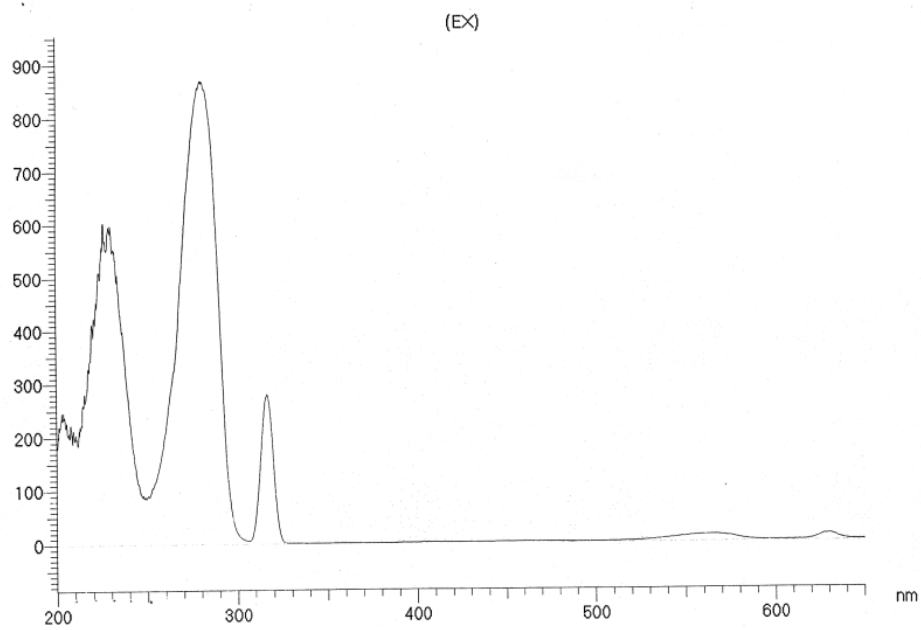


a) Excitation spectrum of serotonin (1 ppm)

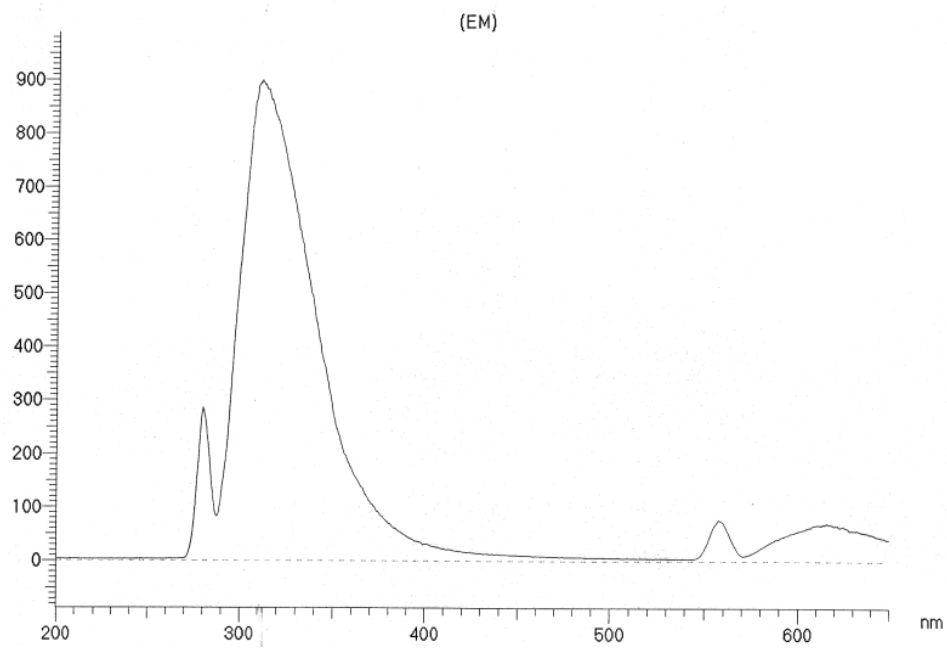


b) Emission spectrum of serotonin (1 ppm)

Figure 3.11. Wavelength scans for the serotonin



a) Excitation spectrum of noradrenaline



b) Emission spectrum of noradrenaline

Figure 3.12. Wavelength scans for the noradrenaline

Different mobile phases were studied. 50 mM sodium acetate buffer (pH 4.0, acetic acid) was used as a mobile phase. Serotonin and noradrenaline peaks were observed by using this mobile phase. But melatonin peaks were not observed. Also, 75 mM sodium acetate buffer (pH 5.0, acetic acid) was used as another mobile phase but results were not considered as satisfactory.

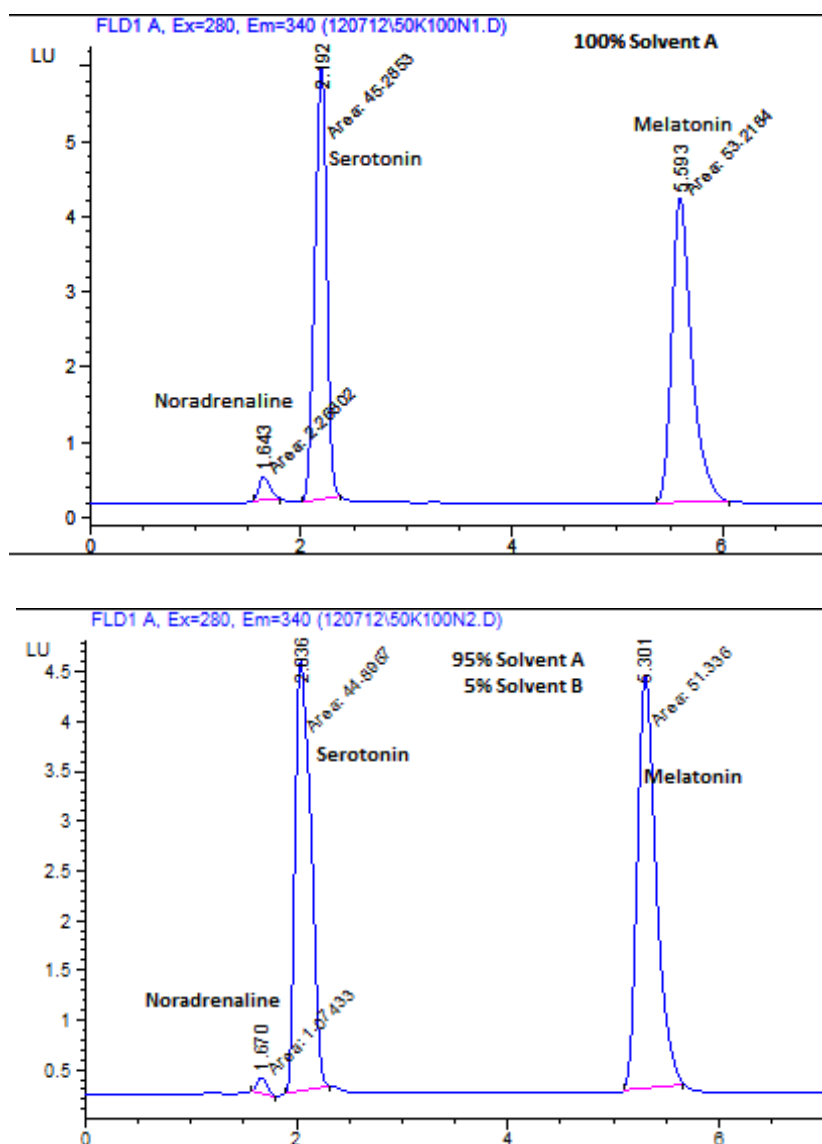
The mobile phase which was used in this study was composed of 75 mM sodium acetate and acetonitrile in a proportion of 72:28 (v/v) (pH 5.0, acetic acid). This mobile phase was taken from Rizzo et al. (2002) and Ms Thesis of Özkan (2009). Rizzo et al. proposed a HPLC method with fluorescence detection to measure the melatonin concentration in the human plasma and CSF. Also, Özkan studied with HPLC-FLD for the determination of melatonin in human plasma. But, in this study mobile phase was used for the simultaneous determination of melatonin, serotonin and noradrenaline in rat brain CSF.

Two solvents were tried (Solvent A: 75 mM sodium acetate-acetonitrile (pH 5.0, acetic acid) (72:28, v/v) and Solvent B: methanol) containing different percentages in the mobile phase. Compounds were separated with isocratic elution at a flow rate of 1 mL min⁻¹. Four different mobile phases were compared with each other. Solvent percentages in the mobile phase were

- 100% Solvent A,
- 95% Solvent A-5% Solvent B,
- 90% Solvent A-10% Solvent B,
- 85% Solvent A-15% Solvent B.

The chromatograms obtained from the mixture of the standards (50 ppb melatonin, 50 ppb serotonin and 100 ppb noradrenaline) by using four different mobile phases

are given in Figure 3.13. The best separation was achieved by using 100 % Solvent A. The decreasing of the percentages of methanol in the mobile phase resulted in an improvement in the separation of noradrenaline and serotonin peaks. Finally, it was decided to only Solvent A in the mobile phase.



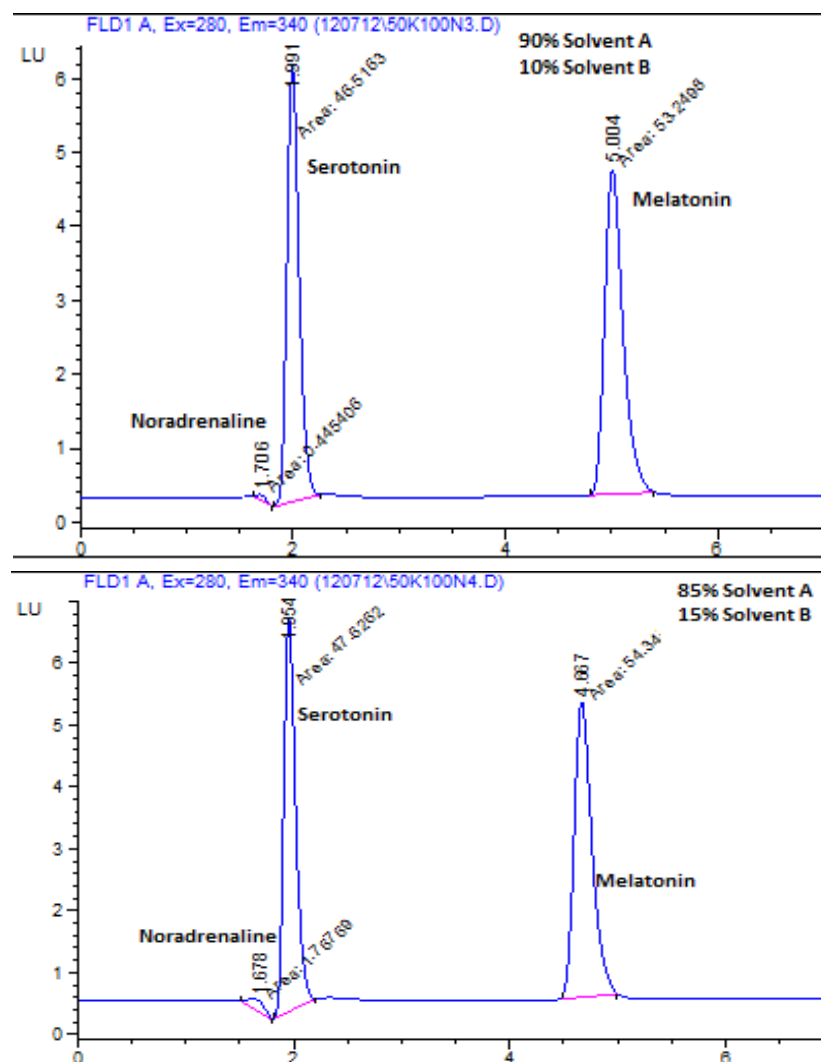


Figure 3.13. The chromatograms of four different mobile phases (Chromatographic conditions: flow rate: 1 mL min⁻¹, 20 µL injection, column: inertsil ODS-2 (C18, 150 x 4.6 mm i.d., 5 µm))

The main problem of these studies was the high detection limit of noradrenaline. Noradrenaline was not observed at small concentrations. Noradrenaline was also studied at the excitation wavelength of 240 nm and the emission wavelength of 315 nm. But, the signal of noradrenaline was not changed.

The fluorescence detector was set first at a PMT gain of 10. But then, the signals of the compounds were amplified using PMT gain of 15. When the PMT gain

was increased from 10 to 15, the area of the serotonin (50 ppb) increased from 12 to 442 and the area of the melatonin (50 ppb) increased from 7 to 220. Also, the area of the noradrenaline (50 ppb) increased from 2 to 65 by increasing PMT gain. In this way, the small areas of the noradrenaline peak in the microdialysate samples were observed.

Elution was achieved in 10 minutes. Melatonin, serotonin and noradrenaline were identified by their characteristic retention times. No interfering peaks with the separation and identification of these compounds were observed. The retention times of the noradrenaline, serotonin and melatonin were found as 1.6, 2.0 and 5.5, respectively. The chromatograms obtained from noradrenaline, serotonin and melatonin standards at the concentration of 50 ppb are given in Figure 3.14, Figure 3.15 and Figure 3.16, respectively. Also, the chromatogram obtained from the mixture of the standards at the concentration of 30 ppb is given in Figure 3.17.

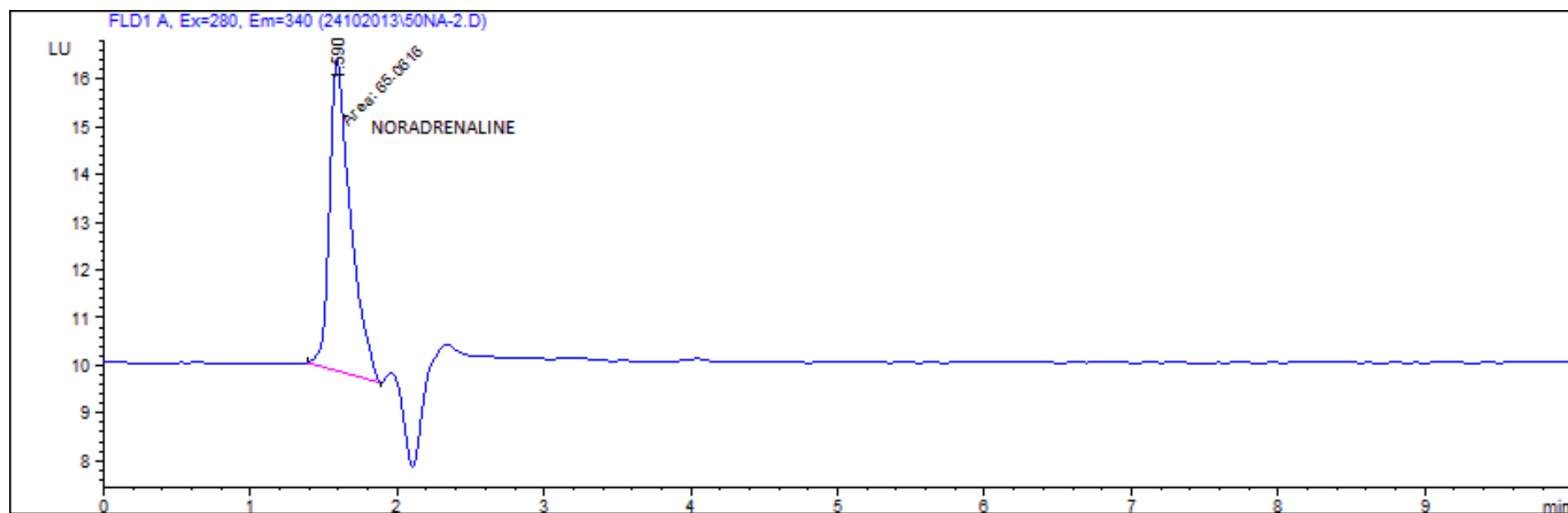


Figure 3.14. The chromatogram of noradrenaline standard at the concentration of 50 ppb

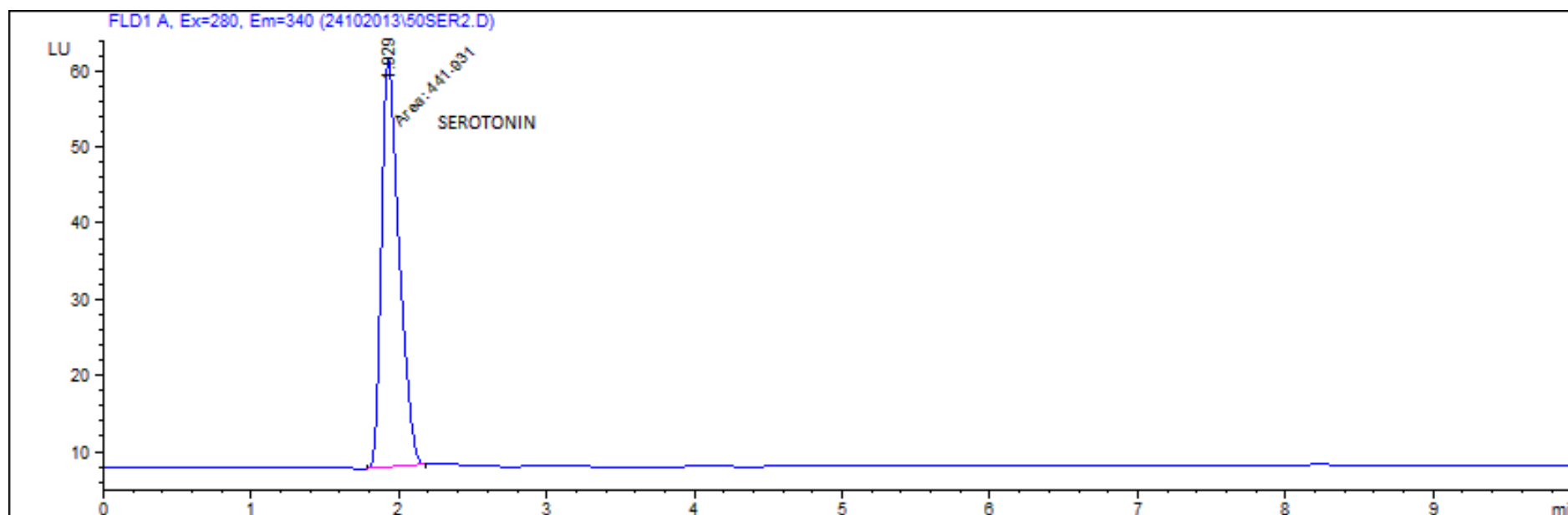


Figure 3.15. The chromatogram of serotonin standard at the concentration of 50 ppb

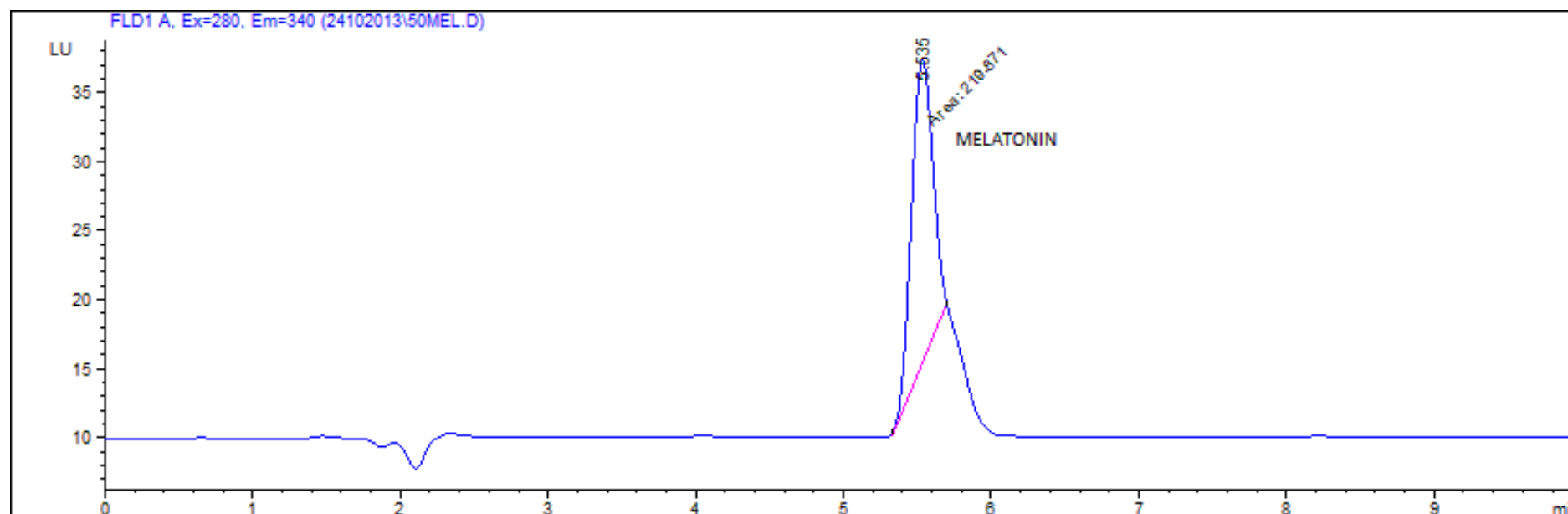


Figure 3.16. The chromatogram of melatonin standard at the concentration of 50 ppb

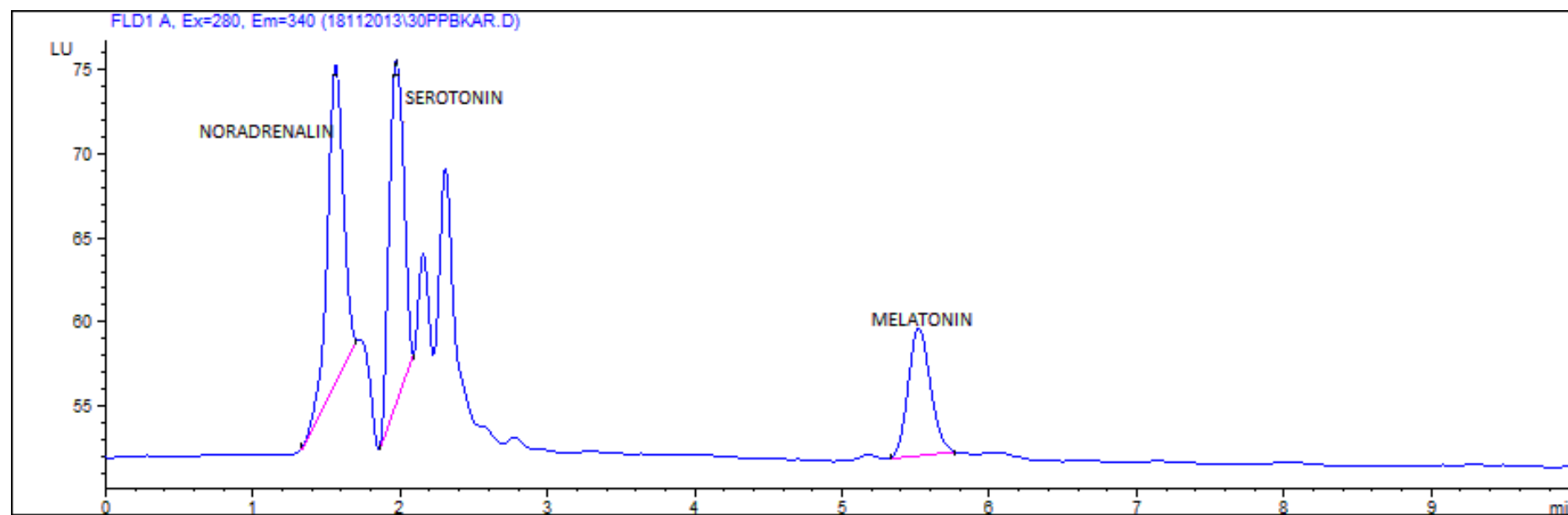


Figure 3.17. The chromatogram of the standard mixture at the concentration of 30 ppb

Before the analysis of the microdialysis samples, the perfusion fluid was analyzed with HPLC to see its effects on the chromatogram. The chromatogram obtained from the perfusion fluid is given in Figure 3.18.

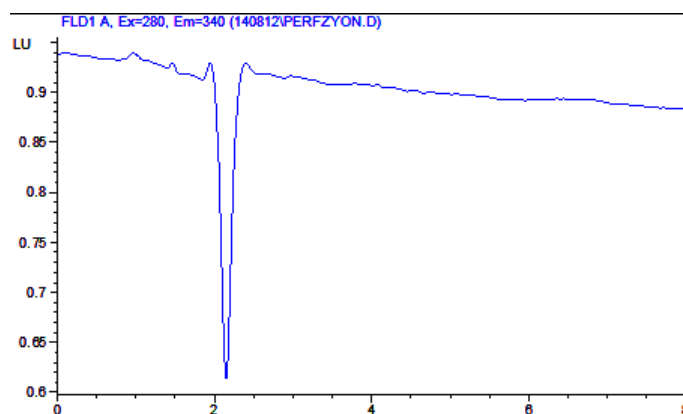


Figure 3.18. The chromatogram of the perfusion fluid

Microdialysis samples were analyzed by the injection of 20 μ L of sample to the HPLC system. A sample chromatogram obtained from raphe nuclei is given in Figure 3.19. In this sample, the peaks of noradrenaline and serotonin were observed. But here, there were no melatonin peak. Also, the same sample (20 μ L) was spiked with 10 μ L of standard mixture (100 ppb). The chromatogram obtained from the spiked sample is given in Figure 3.20.

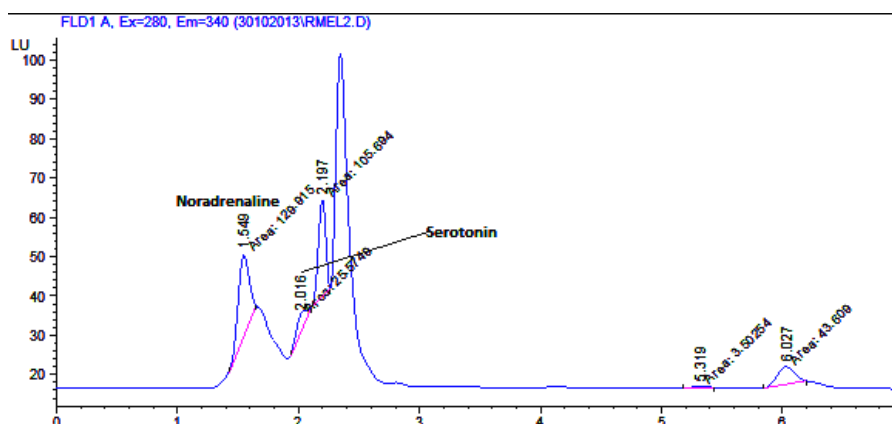


Figure 3.19. The chromatogram of the sample

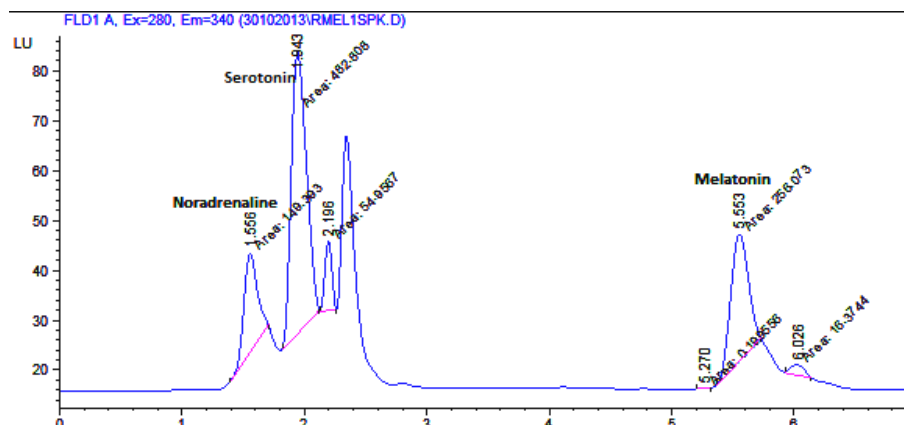


Figure 3.20. The chromatogram of the spiked sample

3.2.2. Method Validation of Melatonin, Serotonin and Noradrenaline

Firstly, it was decided that the standard addition and the internal standard methods were used together to obtain the calibration curve. For this purpose, 5-fluorotryptamine hydrochloride was used (its retention time was 3.4 min.). Calibration curves were constructed using matrix-matching calibration solutions which included 25 ppb of the internal standard. But, better results were observed without internal standards. Since, there was an interference problem in the internal standard peak.

Therefore, the standard addition method was used only to obtain calibration curves. Matrix-matching calibration solutions were analyzed according to procedure which was mentioned in the Section 2.3.4.1. The five matrix-matching calibration solutions were measured with two replicates.

Calibration curves were constructed by plotting the peak areas against the concentrations with the initial unknown concentration set at 0. The results are summarized in Table 3.4.

Table 3.4. The results of linear least squares regression for noradrenaline, serotonin and melatonin

Analyte	Linear regression equation	R ²
Noradrenaline	$y = 0.811x + 72.113$	0.9971
Serotonin	$y = 13.555x + 41.677$	0.9947
Melatonin	$y = 11.339x - 6.811$	0.9991

The limit of detection and quantitation were calculated according to Eurachem Guide (1998). For each analyte, ten replicates of the spiked samples at the final concentration of 5 ppb were measured and the standard deviation from measured signals was calculated. As a result, limit of detection values were found as 0.5 ppb for melatonin, 1.2 ppb for serotonin and 2.7 ppb for noradrenaline. The limit of quantitation values were found as 1.7 ppb for melatonin, 3.8 ppb for serotonin and 8.8 ppb for noradrenaline.

Six independent repeats were performed at repeatability conditions and at two concentration levels. Experiments were repeated daily in the consecutive five days. The standard deviation and RSD values of the results were calculated. The percentage RSD values changed between 0.31 and 2.18 for melatonin, 0.84 and 5.05 for serotonin and 0.44 and 8.24 for noradrenaline. To determine intermediate precisions of compounds, RSD_{pool} values were calculated using Eq. (1). RSD_{pool} was found for noradrenaline as 3.27 %, for serotonin as 3.21 % and for melatonin as 1.29 %.

Because of the absence of a suitable reference material, recovery was investigated through spiking studies (IUPAC Technical Report, 1999). The recovery for each sample, $R_{(i)}$, was calculated by the Eq. (2).

The average recovery of the five days at two spiking levels for noradrenaline was found 92 % (the recoveries changed between 72 % and 105 %). The average recovery of the five days at two spiking levels for serotonin was found as 101 % (the recoveries changed between 76 % and 117 %). Also, the average recovery of the five days at two spiking levels for melatonin was found as 105 % (the recoveries changed between 88 % and 121 %).

3.2.3. Estimation of Uncertainty for Melatonin, Serotonin and Noradrenaline

The measurement uncertainty for the determination of serotonin was shown by explaining each step. Since the similar calculations were performed for melatonin, serotonin and noradrenaline, the uncertainty result for melatonin and noradrenaline was just reported.

3.2.3.1. Specification of the Measurand and Measurement Procedure

The measurand in this study is the serotonin concentration in the microdialysate sample (C_{SER} , expressed in ppb) and it is calculated by using the calibration curve.

$$C_{SER} = C_0 \quad (29)$$

C_0 : the concentration of the serotonin obtained from the calibration curve (ppb)

The stages in the procedure are shown in Figure 3.21.

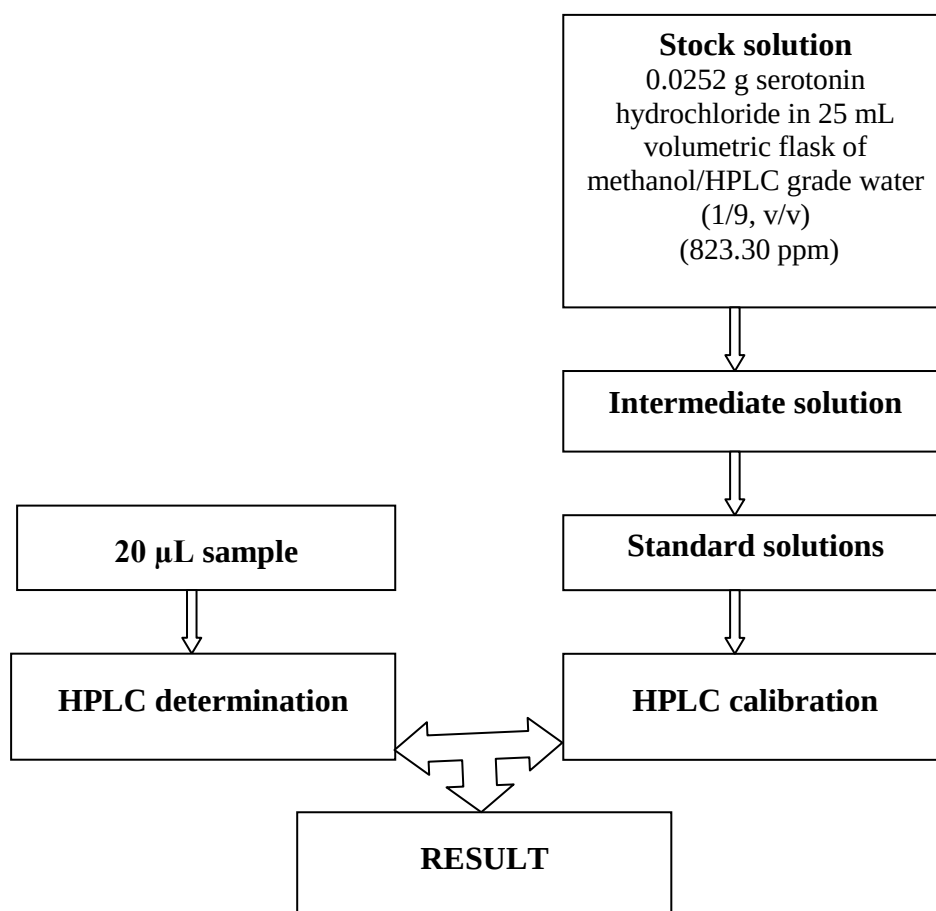


Figure 3.21. Measurement procedure for serotonin

3.2.3.2. Identification of the Uncertainty Components

The relevant uncertainty components are shown using the cause and effect diagram in Figure 3.22.

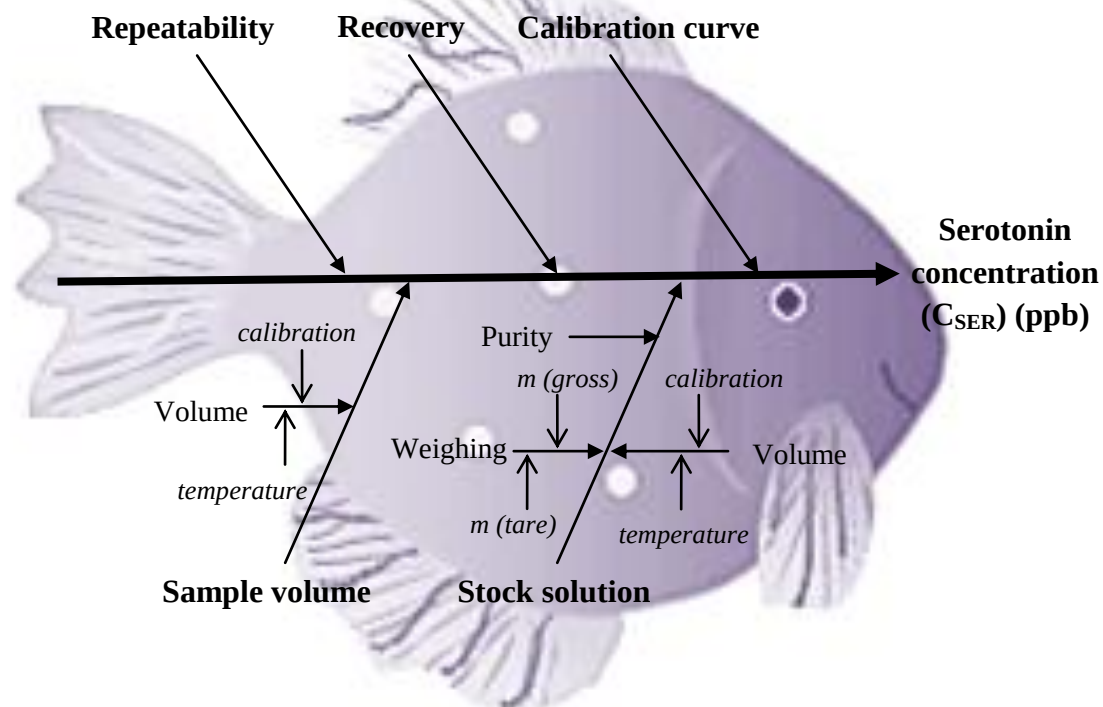


Figure 3.22. Cause and effect diagram for the determination of serotonin

The uncertainty components that contribute to the serotonin concentration in the CSF are as follows:

- 1) Calibration curve (C_0)
- 2) Stock solution of the serotonin (C_{stock})
- 3) Volume of sample (V_{sample})
- 4) Repeatability (Rep)
- 5) Recovery ($\bar{R}$).

3.2.3.3. Quantification of the Uncertainty Components

All uncertainty contributions was expressed as standard uncertainties (as standard deviation) before the combination.

3.2.3.3.1. Estimation of the Uncertainty Derived from the Linear Calibration Curve

The uncertainty associated with the calibration curve, $u(C_0)$, was calculated with the Eq. (4) defined by Eurachem/Citac guide (2012).

Two replicate measurements were performed for the five matrix-matching calibration solutions. So, n is equal to 10 and p is equal to 2. Calibration curve was constructed by plotting peak areas versus concentrations. The slope of the calibration curve (B_1) was found as 13.555 (Calibration equation: $y = 13.555x + 41.677$). Residual standard deviation (S) was calculated from CurveExpert version 1.3 and it was found as 13.612 (Figure 3.23).

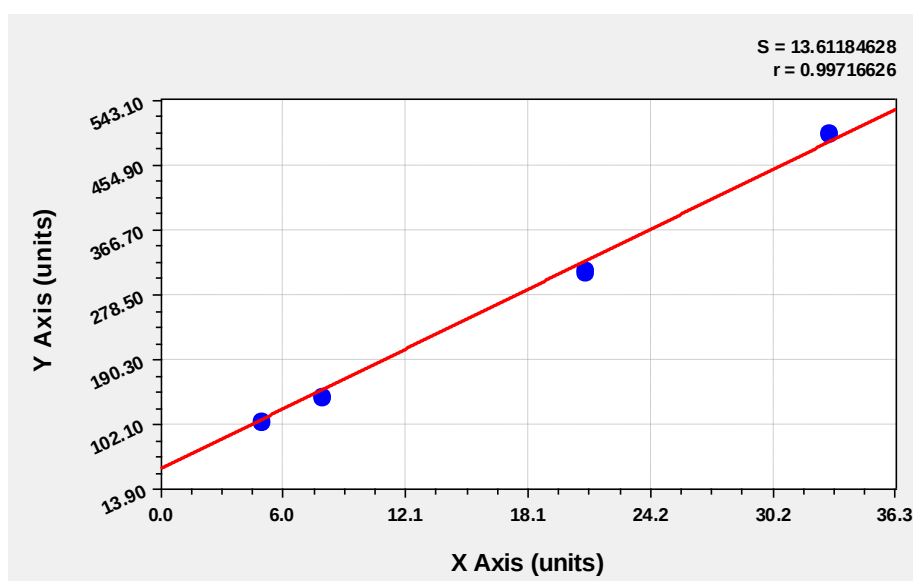


Figure 3.23. Calibration curve for the determination of serotonin

S_{xx} and $\bar{c}$ was calculated as 1427 and as 13.3, respectively. The calculations were shown in Table 3.5.

Table 3.5. Calculation of the S_{xx} and $\bar{c}$

Conc. (ppb)	Area	C_j	$C_j - C_{ort}$	$(C_j - C_{ort})^2$
0	59	1.2	-12.1	146
0	58	1.2	-12.1	147
5	106	4.8	-8.6	73
5	105	4.7	-8.7	75
8	141	7.3	-6.0	36
8	139	7.1	-6.2	38
21	312	19.9	6.6	43
21	308	19.7	6.3	40
33	499	33.7	20.4	416
33	497	33.6	20.3	412

$$\bar{c} = 13.3$$

$$S_{xx} = 1427$$

Finally, when C_0 was assumed as 10.0 ppb;

$$u(c_0) = \frac{13.612}{13.555} \sqrt{\frac{1}{2} + \frac{1}{10} + \frac{(10.0 - 13.3)^2}{1427}} \quad (30)$$

$$u(c_0) = 0.78 \text{ ppb}$$

3.2.3.3.2. Estimation of the Uncertainty Derived from Stock Solution

A stock solution of serotonin at 823.30 ppm was prepared for the calibration curve. 0.0252 g serotonin hydrochloride (purity: 98.5 %) standard was weighed on the analytical balance. It was dissolved within 25 mL (volumetric flask) of

methanol/HPLC grade water mixture (1/9, v/v). The concentration of stock solution depends on the weighing of the serotonin standard, its purity and the volume of the HPLC grade water in which it is dissolved.

The results were only reported here and the detailed calculations were shown in Section 3.1.3.3.2. Table 3.6 shows standard uncertainties comes from three parameters.

Table 3.6. Standard uncertainties

Description	Value x	Standard uncertainty u(x)
Volume of the flask, ml	25	0.019
Purity of the serotonin standard	1	0.009
Mass of the serotonin standard, mg	25.2	0.424

The uncertainties of three parameters were combined in order to estimate the uncertainty derived from stock solution.

$$\frac{u(C_{\text{stock}})}{C_{\text{stock}}} = \sqrt{\left(\frac{0.009}{1}\right)^2 + \left(\frac{0.424}{25.2}\right)^2 + \left(\frac{0.019}{25}\right)^2} = 0.019 \quad (31)$$

$$u(C_{\text{stock}}) = 823.30 \text{ ppm} \times 0.019 = 15.65 \text{ ppm} \quad (32)$$

3.2.3.3.3. Estimation of the Uncertainty Derived from Sample Volume

20 µL of the sample (V_{sample}) was taken into an insert vial by using 20 µL automatic pipette. $u(V_{\text{sample}})$ was calculated as 0.030 (Table 3.7).

Table 3.7. Standard uncertainty of the sample volume

			Type of distribution	Standard uncertainty	Standard uncertainty $u(V_{\text{sample}})$
Volume of the sample, μL	Temperature change in the lab. $^{\circ}\text{C}$	3	rectangular	0.007	0.030
	The coefficient of volume expansion for water $^{\circ}\text{C}^{-1}$	0.00021			
	Volume of the pipette, μL	20			
	Uncertainty of pipette, μL	0.05	rectangular	0.029	

3.2.3.3.4. Estimation of the Uncertainty Derived from Repeatability

All possible repeatability uncertainties in the procedure were collected under this part. The six independent experiments at two concentration levels for serotonin analysis were repeated daily. Also, the repetition of the analysis was performed in the consecutive five days. The mean RSD of the five days was found as 0.0297.

The standard uncertainty derived from the repeatability was calculated by using following equation (standard deviation of the mean):

$$u(\text{Rep}) = \frac{\text{RSD}}{\sqrt{n}} = \frac{0.0297}{\sqrt{9}} = 0.0099 \quad (33)$$

3.2.3.3.5. Estimation of the Uncertainty Derived from Recovery

The spiked samples were prepared from the sample matrix which contained the analyte. Using Eq. (18), the mean recovery was calculated from the analysis of the spiked samples and it was found as 1.038.

Also, the standard uncertainty comes from the recovery was calculated by using Eq. (19).

$$u(\bar{R}) = \frac{1}{32.906} \sqrt{\frac{(0.012)^2}{29} + (0.114)^2} = 0.0035 \quad (34)$$

For melatonin, the spiked samples were prepared by using the blank sample matrix. 10 μL volume of the 40 ppb melatonin standard was added to each blank sample matrix (40 μL). Therefore, $\bar{R}$ and $u(\bar{R})$ were calculated by using Eq. (21) and (22).

To determine whether the mean recovery was significantly different from 1.0, a statistical test was used. The significance test statistic t was calculated.

$$t = \frac{|1 - \bar{R}|}{u(\bar{R})} = \frac{|1 - 1.038|}{0.035} = 11 \quad (35)$$

This t value was compared with two-tailed critical value t_{critical} , for $(n-1)$ degrees of freedom at 95 % confidence. Since the calculated t value was greater than t_{critical} , $\bar{R}$ is significantly different from 1.0.

$$t = 11 \geq t_{\text{critical};28} = 2.048$$

In the normal application of the method no recovery correction in the model equation was applied (i.e., $\bar{R}$ was assumed to equal 1). The uncertainty associated with method recovery was increased using Eq. (24).

$$u(\bar{R})' = \sqrt{\left(\frac{1 - 1.038}{2}\right)^2 + (0.0035)^2} = 0.0194 \quad (36)$$

3.2.3.4. Calculating of the Combined Uncertainty

The values and their uncertainties associated with each component were summarized in Table 3.8.

Table 3.8. The values and their uncertainties

Description	Value x	Standard uncertainty u(x)	Relative standard uncertainty u(x)/x
calibration curve	10.0	0.78	0.0783
stock solution	823.30	15.65	0.0190
repeatability	1	0.0099	0.0099
sample volume	20	0.030	0.0015
increased recovery	1.04	0.0194	0.0187

Standard uncertainties associated with each component were combined accordingly:

$$\frac{u_C(C_{SER})}{C_{SER}} = \sqrt{\left(\frac{u(C_0)}{C_0}\right)^2 + \left(\frac{u(C_{stock})}{C_{stock}}\right)^2 + \left(\frac{u(V_{sample})}{V_{sample}}\right)^2 + \left(\frac{u(Rep)}{Rep}\right)^2 + \left(\frac{u(\bar{R})'}{\bar{R}}\right)^2} \quad (37)$$

$$\frac{u_C(C_{SER})}{10.0} = \sqrt{\left(\frac{0.78}{10.0}\right)^2 + \left(\frac{15.65}{823.30}\right)^2 + \left(\frac{0.0030}{20}\right)^2 + \left(\frac{0.0099}{1}\right)^2 + \left(\frac{0.0194}{1.04}\right)^2} = 0.0833 \quad (38)$$

$$u_C(C_{SER}) = 0.83 \text{ ppb}$$

The combined uncertainty for determination of serotonin in CSF was found as 0.83 ppb.

The sizes of these uncertainty contributions can be compared using the histogram in Figure 3.24. The volume of sample has virtually no influence on the

overall uncertainty. Repeatability is another component for the combined uncertainty and it is derived from the overall variability in the method. The standard preparation for the calibration curve, repeatability and recovery are the important uncertainty sources. The calibration curve is the important step to be careful. It has the major effect on the combined uncertainty.

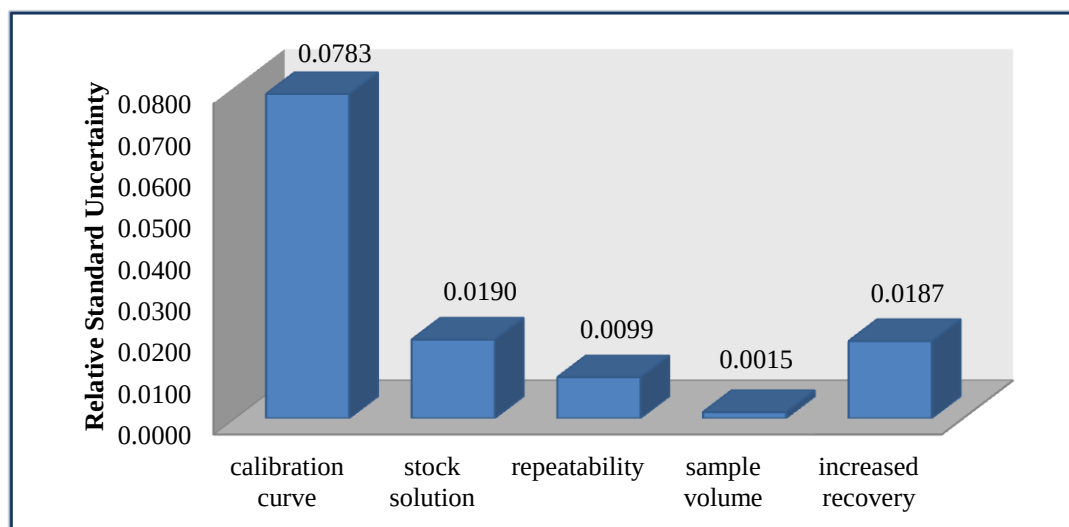


Figure 3.24. Uncertainty contributions for serotonin determination

3.2.3.5. Expanded Uncertainty

The expanded uncertainty $U(C_{SER})$ was obtained by applying a coverage factor (k) of 2 (a level of confidence of approximately 95 %).

$$U(C_{SER}) = 2 \times 0.83 = 1.7 \text{ ppb} \quad (39)$$

The result is reported as $(10.0 \pm 1.7) \text{ (ppb)}^*$

*The reported uncertainty is an expanded uncertainty calculated using a coverage factor of 2 which gives a level of confidence of approximately 95 %.

3.3. Results of Validation Parameters and Expanded Uncertainties

Table 3.9 shows the results obtained from the validation studies of the two developed methods.

Table 3.9. Results of the validation studies

Validation parameters	Melatonin	Serotonin	Noradrenaline	Glutamate	GABA
Retention time (min)	5.5	2.0	1.6	21.5-22.3	44.7-47.6
Linearity (R^2)	0.9991	0.9947	0.9971	0.9997	0.9997
Linear Range (ppb)	1.7-40	3.8-33	8.8-40	30-428	92-427
MDL (ppb)	0.5	1.2	2.7	9*	28*
LOQ (ppb)	1.7	3.8	8.8	30	92
Repeatability (RSD %)	0.31-2.18	0.84-5.05	0.44-8.24	0.80-1.67	0.78-1.80
Intermediate precision (RSD _{pool} %)	1.29	3.21	3.27	1.19	1.31
Mean recovery of the five days %	105	101	92	91	112
*S/N = 3					

Table 3.10 shows the expanded uncertainty results for the compounds.

Table 3.10. Expanded uncertainty results

Compound	Expanded Uncertainty Results
Glutamate	(100 ± 12) (ppb)*
GABA	(100 ± 13) (ppb)*
Serotonin	(10.0 ± 1.7) (ppb)*
Melatonin	(10.0 ± 1.0) (ppb)*
Noradrenaline	(10.0 ± 1.6) (ppb)*
*The reported uncertainty is an expanded uncertainty calculated using a coverage factor of 2 which gives a level of confidence of approximately 95 %.	

3.4. Comparison of This Study and Other Similar Methods from Literature

Table 3.11 shows a comparison of this study and other similar methods from the literature. In this study, MDL was calculated. MDL is different from LOD in terms of using the matrix. Lower values can be obtained with LOD by using only standards prepared by water or other solvents. The literature values in Table 3.11 used LOD values except for Santos-Fandila et al. (2013) who gave MDL values.

Clarke et al. (2007) studied with both electrochemical (ECD) and fluorescence detectors (FLD) and they reported lower detection limits with fluorescence detector. Also, in this study better sensitivity was obtained than the studies of Clarke et al. (2007) and de Freitas Silva et al. (2009) who used OPA as a derivatization agent for the determination of GABA and glutamate. Shah et al. (1999) calculated LOD using $S/N=2$ and they reported lower detection limits. Similarly Rizzo et al. (2002) calculated LOD and they reported lower detection limit for melatonin.

Santos-Fandila et al. (2013) developed a method for different families of neurotransmitters with ultrahigh performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). They calculated MDL in their study. Although UHPLC-MS/MS is more sensitive and selective technique, MDL values of glutamate and noradrenaline are lower in our study and also MDL values of GABA and serotonin are similar in two methods.

Table 3.11. Similar methods from literature

Reference	Analytes	Matrix	Detection method	Limit of detection
Shah et al., 1999	GABA, glutamate (NDA derivatization)	microdialysate and tissue from rat brain	HPLC-FLD	GABA: 0.1-0.4 ppb* glutamate: 0.1-0.6 ppb*
Rizzo et al., 2002	melatonin	human plasma and CSF	HPLC-FLD	melatonin: 0.5×10^{-3} ppb
Fotopoulou and Ioannou, 2002	noradrenaline	urine samples	HPLC-FLD	noradrenaline: 1.7 ppb**
Clarke et al., 2007	GABA, glutamate (NDA derivatization)	rat brain tissue	HPLC-FLD	GABA: 55 ppb* glutamate: 90 ppb*
Clarke et al., 2007	GABA, glutamate (NDA derivatization)	rat brain tissue	HPLC-ECD	GABA: 400 ppb* glutamate: 650 ppb*
de Freitas Silva et al., 2009	GABA, glutamate (OPA derivatization)	rodent brain tissue	HPLC-FLD	GABA: 100 ppb*** glutamate: 1000 ppb***
Zhen et al., 2011	serotonin	blood samples	HPLC-ultraviolet detection	serotonin: 0.4 ppb**
Santos-Fandila et al., 2013	serotonin, noradrenaline, GABA, glutamate	microdialysate from rat brain	UHPLC-MS/MS	GABA: 20 ppb** glutamate: 20 ppb** serotonin: 2 ppb** noradrenaline: 20 ppb**
This study, 2014	serotonin, melatonin, noradrenaline	microdialysate from rat brain	HPLC-FLD	serotonin: 1.2 ppb noradrenaline: 2.7 ppb melatonin: 0.5 ppb
	GABA, glutamate			GABA: 28 ppb** glutamate: 9 ppb**

* S/N = 2

** S/N = 3

***The smallest concentration in the calibration curve

CHAPTER 4

CONCLUSION

In this study, two methods were developed for the determination of the neurotransmitters (GABA, glutamate, serotonin and noradrenaline) and the melatonin hormone in cerebrospinal fluid samples by using HPLC with fluorescence detection and a C18 reversed-phase column. Samples were collected from three brain regions (amygdala, hippocampus and raphe nuclei) in rats by using microdialysis technique.

In the first method, pre-column derivatization with NDA in the presence of cyanide ion was performed for the simultaneous detection of GABA and glutamate before the analysis with HPLC. The stability of the NDA derivatives was examined. Results showed that good stability like previously reported data (Clarke et al., 2007; Zotou and Notou, 2012).

First method was adapted with slight modifications from the method described by Clarke et al. (2007). Clarke et al. studied with rat brain tissue samples. But, it was studied with microdialysis samples in this study. Our work was more difficult. Because the levels of neurotransmitters in microdialysates are significantly lower than in brain tissue samples according to the previously reported data. Accordingly, detection limit is an important parameter. Clarke et al. reported the

sensitivity problem of the method (they found LOD as 55 ppb for GABA and as 90 ppb for glutamate). But, in this study lower detection limits for GABA and glutamate were obtained. They said that it is unlikely that researchers seeking to analyse microdialysis samples will be able to use the methods described. So, we have managed to analyze microdialysis samples with better sensitivity. Elution was achieved in 60 minutes. The retention time of GABA and glutamate might be shortened by using different analytical column.

The second method involves the simultaneous detection of melatonin, serotonin and noradrenaline. The method does not require extraction or derivatization. Detection is performed by using native fluorescence character of these compounds. The procedure used in this method is rapid and simple. The method is suitable for microdialysis samples. Also, in some studies (Drijfhout et al., 1996; Moujir et al., 1997; Frese et al., 2009) different methods were used for the determination of these three compounds instead of single method. To our knowledge, there is no available method for the simultaneous determination of these compounds in the literature. Therefore, this is the first time that simultaneous determination was performed for these compounds.

The methods were validated. The performance of the methods was evaluated in terms of linearity, method detection limit, limit of quantitation, recovery and precision. Samples are obtained by microdialysis technique at the small volumes. Therefore, the replicates were limited during the validation studies. The standard addition method was used to obtain calibration curves. Although the external standard method is used commonly in the literature, the standard addition method is more suitable because of elimination of the matrix effect. There was no a suitable reference material of the compounds. Therefore, recovery was investigated by the

analysis of spiked samples over the matrix. Sufficient results were obtained from the validation studies.

Finally, measurement uncertainties of all compounds were calculated by applying 'bottom up' method. Uncertainty calculations of these compounds were performed for the first time in the literature. The critical stages of the method were evaluated. The major sources of the uncertainty budget were calibration curves, stock solution and recovery.

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