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Development of a method based on HPLC-DAD for the detection and quantification of glutamate and gamma-aminobutyric acid

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Escola superior de Saúde do Porto
Instituto Politécnico do Porto

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”In the long history of humankind (and animal kind, too) those who learned to collaborate and improvise most effectively have prevailed.”

Charles Darwin

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Abstract

Introduction: With the increasing incidence of neurodegenerative diseases, the need arose to study several molecules that may be useful to the study of new therapies and to treat the symptoms that come with these diseases, as is the case of depression. GABA and glutamate have a very important role in the homeostasis of the organism and molecules of interest and potential from the point of view of diagnosis are revealed. HPLC is a method that allows the detection and quantification of various analytes in different biological matrices, allowing rapid results with high precision, sensitivity and specificity.

Objectives: Perform a meta-analysis to understand the role of depression in neurodegenerative diseases and develop a simple, fast, non-derivatizing method based on HPLC-DAD for the detection and quantification of GABA and glutamate. To test its applicability in several biological matrices, so that it can be used as an additional tool for the study of neurodegenerative pathologies.

Materials and methods: The validation of the developed method was performed according to ICH guidelines, which was tested in standard solutions of GABA and glutamate and in samples of serum, urine and yeast extract. All assays were performed using a Hitachi LaChrom Elite® HPLC system, with separation on a Lichrospher LiChroCART® 250-4 100 (5 μm) RP-18 column and with DAD detection.

Results: At the end of several assays, the chosen method has as mobile phase H_2O and acetonitrile, eluted in gradient, at a flow rate of 1 mL/min for 10 minutes. The column temperature was 25°and the detection was performed at 210 nm. This method not only allows a rapid analysis but also a quantification in the order of μg/mL. Although the quantification was possible in the standard solutions, the same did not occur in the biological matrices tested.

Conclusion: Although it still needs to be optimized for biological matrices, the method developed allows an easy, fast and economically sustainable analysis of GABA and glutamate. It is, to date, the only method with DAD detection that allows the simultaneous detection of GABA and glutamate without recourse to derivatization of the sample.

Key words: Neurodegenerative diseases, depression, GABA, glutamate, HPLC-DAD

Resumo

Introdução: Com o aumento da incidência de doenças neurodegenerativas, surgiu a necessidade de estudar várias moléculas que possam ser úteis no estudo de novas terapias e tratar os sintomas que advêm dessas doenças, como é o caso da depressão. O GABA e o glutamato têm um papel muito importante na homeostase do organismo e revelam-se moléculas de interesse e com potencial do ponto de vista do diagnóstico. O HPLC é um método que permite a deteção e quantificação de vários analitos em diferentes matrizes biológicas, permitindo resultados rápidos com elevada precisão, sensibilidade e especificidade.

Objetivo: Realizar uma meta-análise para entender o papel da depressão nas doenças neurodegenerativas e desenvolver um método simples, rápido, sem recorrer a processos de derivatização, baseado em HPLC-DAD para a deteção e quantificação de GABA e glutamato. Testar a sua aplicabilidade em diversas matrizes biológicas, de forma a que possa ser utilizado como uma ferramenta adicional para o estudo de patologias neurodegenerativas.

Materiais e métodos: A validação do método desenvolvido ocorreu de acordo com as diretrizes da ICH, sendo que este foi testado em soluções padrão de GABA e glutamato e em amostras de soro, urina e extrato de levedura. Todos os ensaios foram realizados recorrendo a um sistema HPLC Hitachi LaChrom Elite®, com separação numa coluna RP-18 Lichrospher LiChroCART® 250-4 100 (5 μm) e com deteção por DAD.

Resultados: No final de vários ensaios, o método escolhido tem como fase móvel H_2O e acetonitrilo, eluido em gradiente, a um fluxo de 1 mL/min, durante 10 minutos. A temperatura da coluna era de 25°e a deteção realizada aos 210 nm. Este método não só permite uma análise rápida mas também uma quantificação na ordem dos μg/mL. Apesar de a quantificação ter sido possível em soluções padrão, o mesmo não se verificou nas matrizes biológicas testadas.

Conclusão: Apesar de ainda necessitar de ser otimizado para matrizes biológicas, o método desenvolvido permite uma análise fácil, rápida e economicamente sustentável de GABA e glutamato. É, até à data, o único método com deteção DAD que permite a deteção simultânea de GABA e glutamato sem recorrer à derivatização da amostra.

Palavras-chave: Doenças neurodegenerativas, depressão, GABA, glutamato, HPLC-DAD

Abbreviations and acronyms

ACN - Acetonitrile
AD - Alzheimer Disease
ALS - Amyotrophic Lateral Sclerosis
CNS - Central Nervous System
CSF - Cerebrospinal Fluid
DAD - Diode Array Detector
ECD - Electrochemical Detector
ESI LC/MS/MS - Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry
FDA - Food and Drug Administration
FLD - Fluorescent Detector
GABA - Gamma Aminobutyric Acid
HFBA - Heptafluorobutyric Acid
HPLC - High Performance Liquid Chromatography
ICH - International Conference on Harmonization
LOD - Limit of Detection
LOQ - Limit of Quantification
MAO-B - Monoamino Oxidase-B
NDA - Naphthalene-2,3-Dicarboxaldehyde
NMDA - N-Methyl-d-Aspartate
NP - Normal phase
NT - Neurotransmitter
OPA - O-phthalaldehyde
PCR - Polymerase Chain Reaction
PD - Parkinson Disease
RP-HPLC - Reverse Phase HPLC
THF - Tetrahydrofuran
UP - Ultrapure
USP - United States Pharmacopeia

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Chapter I

General Introduction

1.1 Neurodegenerative Diseases

Neurodegenerative diseases are becoming increasingly more frequent as the years pass, not only because of the demographic changes worldwide but also because of their higher incidence and prevalence with the increase of the average life expectancy. Globally, the most prevalent diseases are Alzheimer (33.9 million people (Barnes and Yaffe, 2011)), Parkinson (7 to 10 million people (Cacabelos, 2017)), Huntington (10.6 to 13.7 persons in 100.000 (McColgan and Tabrizi, 2017)); Amyotrophic Lateral Sclerosis (ALS) (1.9 persons in 100.000 (Chio et al., 2013)). The main symptoms and side effects vary according to the disease and are a consequence of the neuronal degeneration.

1.1.1 Alzheimer

Alzheimer's is an uncured disease in which amyloid plaques are deposited in the hippocampus - a brain structure with an important role in memory coding - and in other areas of the cerebral cortex involved in thinking and decision making. At the beginning of development, symptoms are unlikely to occur since certain brain regions are already affected years before the first symptoms appear (Figure 1.1). (Jannis, 2006)

Symptoms involve loss of memory, confusion, loss of spontaneity, and mood swings. In moderate cases, problems in the recognition of family and friends, difficulty with logical thinking, short-term memory loss and motor problems are already beginning to be seen. In advanced cases patients lose the hability to recognize people and comunicate, lose their sense of self and become dependent on the care of others. (Salawu and Olokoba, 2011) In cases of moderate Alzheimer, the symptoms are recognized by the physician. Even though it is not recommended to do this as a routine procedure, sometimes it is necessary to do a lumbar puncture to measure the levels of tau and phosphorylated tau in cerebrospinal fluid (CSF). In the case of Alzheimer's the tau levels are always elevated and the amyloid levels are diminished. Neuroimaging and consequent volumetric

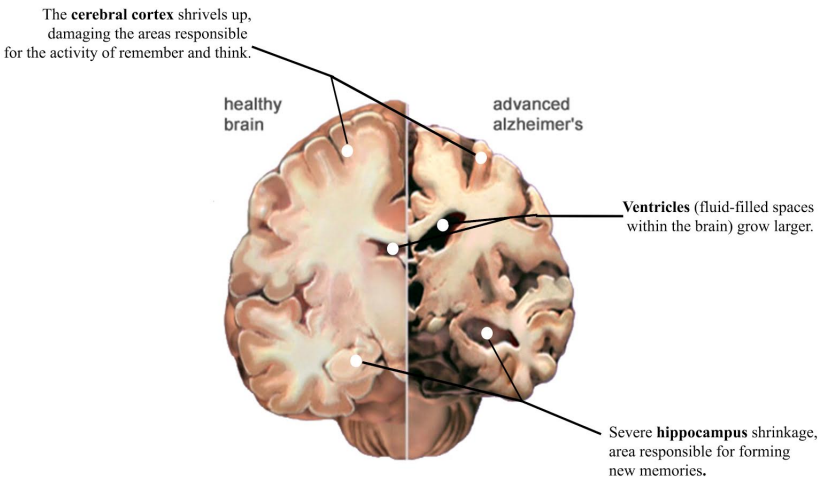


Figure 1.1: Structural differences between a normal brain and a brain with Alzheimer. (Adapted from (Jannis, 2006))

studies of the hippocampus can be used to exclude curable pathology's (chronic subdural hematoma or normal pressure hydrocephalus) that can cause a cognitive decline. (Klöppel et al., 2012; Sunderland et al., 2003)

The treatment of Alzheimer's does not cure or alter the progression of the disease, but rather treat secondary symptoms such as depression, agitation, delusions, etc. For this we use drugs that modulate neurotransmitters (NT) such as: antidepressants, anxiolytics, antiparkinsonian agents, beta blockers, among others. (Kumar et al., 2015)

1.1.2 Parkinson

Parkinson's is the second most common neurodegenerative disease, whose prevalence tends to increase. (Lebouvier et al., 2009) It is characterized by motor and non-motor manifestations, however there are other neurodegenerative diseases that share many symptoms with parkison such as dementia with Lewy Bodies and progressive supranuclear palsy. Genetic mutations can lead to the production of defective α-synuclein that agglomerates in an insoluble aggregate and deposits in neurons. In addition, systems such as the ubiquitin-proteosome, designed to degrade abnormal proteins, are also compromised. This leads to loss or degeneration of the dopaminergic neurons of the nigra substance and development of the Lewy bodies (cell inclusions and physiological hallmark whose major constituent is α-synuclein) (Figure 1.2).

This process starts years before the first physical symptoms appears and when these start already 60-70% of the neurons of the substantia nigra pars compacta have been degenerated. Another process that causes neuronal degeneration is the overproduction of reactive

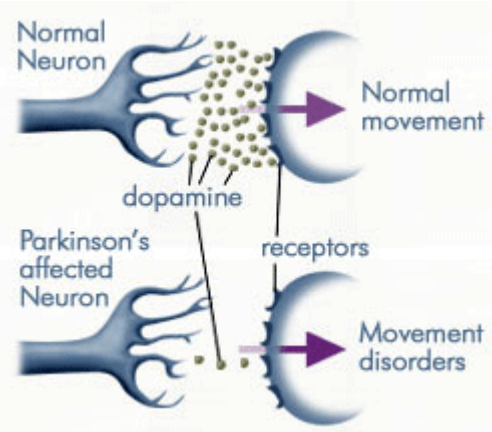


Figure 1.2: Normal vs affected neuron dopamine production in Parkinson Disease.
Patients that suffer from Parkinson's have an impaired production of dopamine, leading to the appearance of movement disorders. (Adapted from (Robertson, n.d.))

oxygen species (ROS) caused by mitochondrial dysfunction. (Beitz, 2014)
The diagnosis is based on the detection of the most common symptoms and the response to the treatment with levodopa. Therapeutic approaches depend on the age of the patient, stage of the disease, symptoms (Table I.1) and the benefits of the treatment. (Jankovic, 2008)

Table I.1: Classic and non classic motor and non motor sypmtoms in Parkinson disease. (Jankovic J, 2008)

	Motor	**Non Motor**
Classic	Tremors, bradykinesia (abnormal movements slow), stiffness, posture instability.	Cognitive / behavioral / neuropsychiatric disorders, autonomic nervous system failure, dementia, psychoses (often induced by treatment), sleep disturbance, olfactory dysfunction (hyposmia), and depression
Non classic	Decreased blink rate, scoliosis, parkinsonian gait, speech impairment, blurred vision	

The pharmacological approach revolves around the dopaminergic and/or other NT deficit. Drug therapy is recommended with levodopa, dopamine agonists, monoamine oxidase-B (MAO-B) inhibitors, among others as soon as signs of early motor symptoms appear. Medication can lead to problems of impulse control and behavioral disorders such as psychosis and hallucinations. A surgical approach, deep brain stimulation (DBS), can be used and is more effective in patients with low disease time and with a good preoperative response to levodopa. An electrode is implanted into the subthalamic nucleus, globus thalamus or ventral intermediate nucleus, and constant electrical currents stimulate these zones of the brain. This option, if effective, helps reduce motor symptoms and allows the drug doses to be decreased. DBS does not involve the destruction of brain tissue, it is reversible and can be adjusted to the progression of the disease. (Dhall and Kreitzman, 2016)

1.1.3 Amyotrophic lateral sclerosis

Progressive disease, of late onset, associated with the degeneration of cortical and spinal motor neurons. It can be divided into two categories: sporadic (90-95% of cases) and familial (5-10% of cases). More than half of the patients diagnosed with ALS do not survive and the prevalence tends to increase. Only 5% of the cases reach patients under the age of 30 and the first symptoms of the disease occur between 50 and 65 years of age. As the disease only develops at a later stage it is difficult to identify the main environmental factor that acted as a risk factor for the development of ALS. Smoking, diet and exposure to chemicals, heavy metals and radiation/electromagnetic fields are the most common risk factors. The most common cause is a mutation in the gene that encodes the expression of the antioxidant enzyme superoxide dismutase (SOD1). The mutant enzyme exhibits structural instability which can cause its aggregation in central nervous system (CNS) motor neurons. There are several hypotheses that justify the occurring neurodegeneration and the relationship with SOD1: (Zarei et al., 2015)

- Glutamate excitotoxicity: in a normal cell, glutamate is synthesized at the presynaptic terminal and released into the synaptic cleft during neurotransmission. Once released, the glutamate is removed through various protein transporters from the glial and neuronal cells. This process balances the glutamate concentration in the synaptic cleft and avoids excitotoxic neuronal damage. The motor cortex and spinal cord of patients with ALS present a reduction in the glutamate transporters, which leads to an increased concentration of extracellular glutamate, overstimulation of the receptors and excitotoxic neuronal degeneration (Figure 1.3). This leads to an influx of calcium that overstimulates the neurons and initiates in the cell various processes of biochemical destruction. (Turner et al., 2009)

- Structural and functional abnormalities of mitochondria: Mutant SOD1 is deposited in the electron transport chain in the mitochondrial membrane which leads to a defective prodution of adenosine triphosphate (ATP). (Zarei et al., 2015)

- Oxidative stress mediated by free radicals: Oxidative stress is a disturbance of the balance between the production of ROS and its elimination by antioxidant defense mechanisms. Accumulation of ROS causes various cell damage and the absence of SOD1, the major antioxidant enzyme, in the case of ALS is a factor for cells to enter into oxidative stress. (Barber et al., 2006)

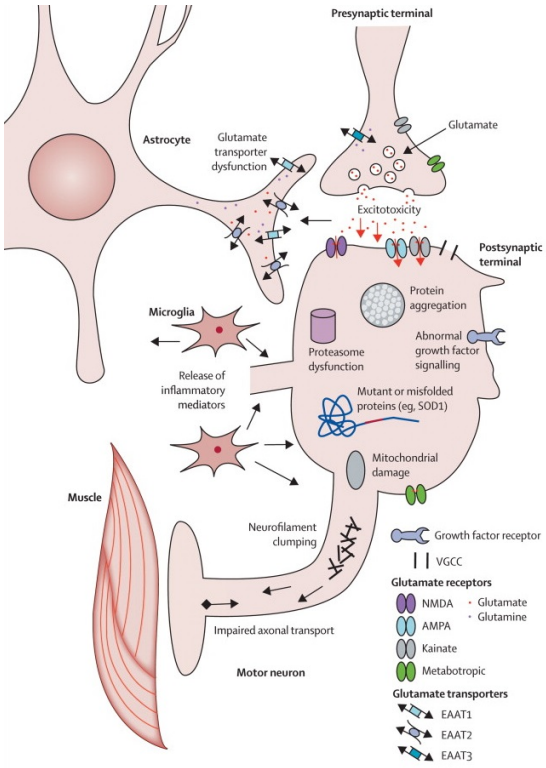


Figure 1.3: Representation of the excitotoxicity of glutamate (In (Turner et al., 2009))

Although it is known that in ALS respiratory failure is the major cause of death, there is a whole set of symptoms that appear with the worsening of the disease (Figure 1.4) (Chio et al., 2009).

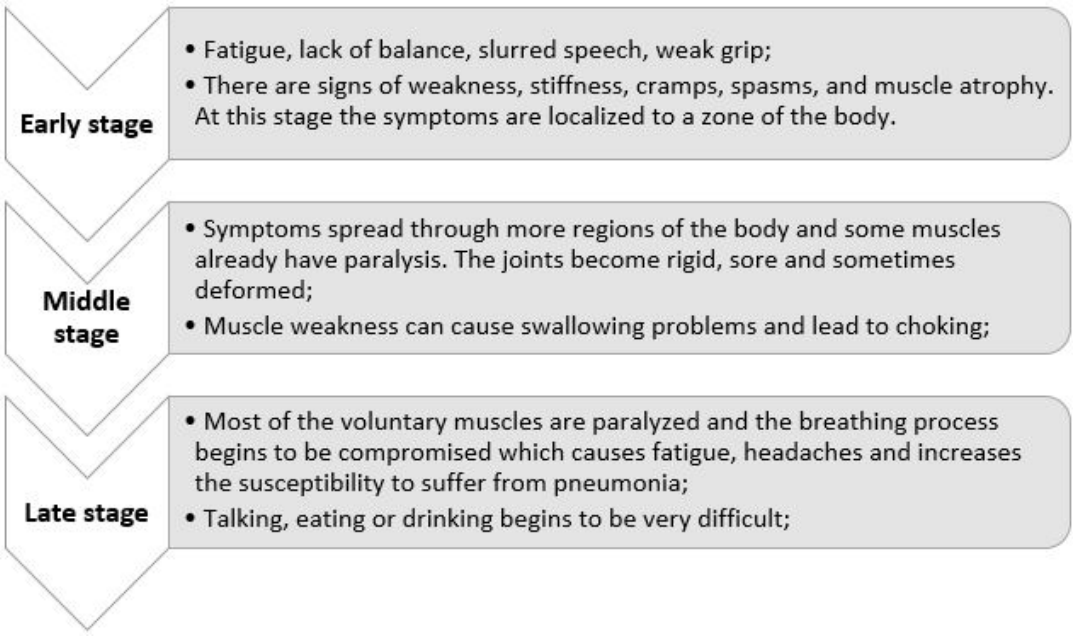


Figure 1.4: Development of symptoms according to the progression of the disease. ((Chio et al., 2009))

The complex and heterogeneous nature of ALS makes diagnosis difficult. There is an average of 13-18 months of delay between the first symptoms and the diagnosis. There are criteria and tests that help to make the differential diagnosis and exclude other pathologies that cause progressive dysfunction of upper and lower motor neurons. The diagnosis consists in obtaining a complete history of the patient, physical examination, electrodiagnostic, laboratorial, neuroimaging and genetic studies. (Zarei et al., 2015)

- Electrodiagnostic studies: EMG (electromyography) and nerve conduction are the most sensitive studies to detect ALS and quantify the degeneration of lower motor neurons. (de Carvalho et al., 2008)
- Laboratorial Studies: Laboratory studies include quantification of erythrocyte sedimentation rate, urine and serum electrophoresis, thyroid function tests, measurements of serum calcium and phosphate and cerebral spinal fluid (CSF) analysis. (Zarei et al., 2015)
- Neuroimaging studies: Neuroimaging involves the study of the brain and spinal cord and is useful for the exclusion of syndromes that mimic ALS. (Zarei et al., 2015)

Riluzole is the only Food and Drug Administration (FDA) approved treatment that has an increased survival rate of patients with ALS. This drug is known to trigger presynaptic inhibition and subsequent release of glutamate. (Turner et al., 2009)

1.1.4 Huntington Disease

Huntington's disease (HD) is a rare autosomal dominant and progressive disease with a distinct phenotype that includes symptoms such as chorea (involuntary movements of the face and extremities) and dystomia (repetitive muscle contractions), incoordination, cognitive decline, etc. (Walker, 2007) On average, the disease affects patients between the ages of 30 and 50, being the most common cause of death pneumonia, followed by suicide. (Roos, 2010)
The degenerative process begins by involving the medium spiny striatal neurons and, to a lesser extent, the cortical neurons. The GABAergic and enkephalin neurons of the basal ganglia are the most vulnerable to undergo degeneration and their dysfunction leads to the development of chorea. HD is caused by a mutation that leads to the expansion of a repeat of the CAG trinucleotide, encoding a polyglutamine tract within the huntingtin

(htt) protein. The mechanisms responsible for the development of the disease are still being discovered, however the non-mutated htt is responsible for the normal embryonic development and has apoptotic functions. (Kumar et al., 2010) The neurophysiological changes that occur are greater in the caudate and putamen regions. In these there is significant loss and atrophy of neurons (Figure 1.5). In brain tissue it is also possible to verify the appearance of nuclear and cytoplasmic inclusions containing mutante htt and polyglutamine. (Bates et al., 2015)

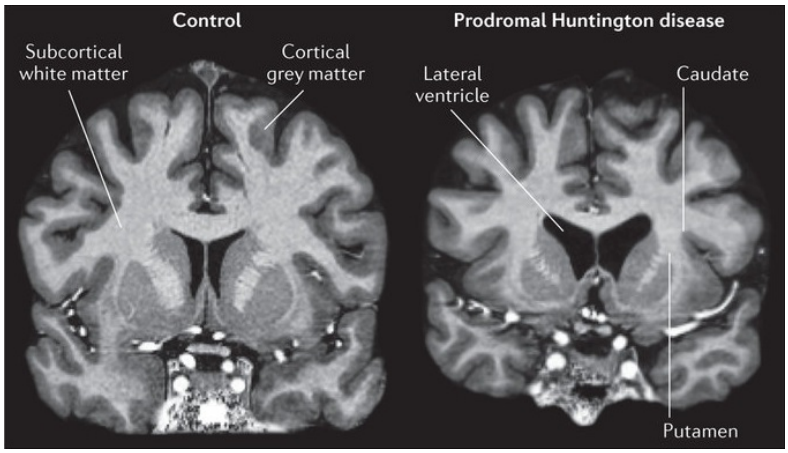


Figure 1.5: Differences in a brain without an with Huntington disease. (In (Bates et al., 2015))

Aside from the most common motor symptoms, patients begin to experience problems with speech and swallowing, developing bradykinesia (sluggish movements) and akinesia (difficulty in starting movements). Psychiatric symptoms appear at an early stage of the disease and often precede motor symptoms, with depression and dementia being the most frequent. (Roos, 2010)

The differential diagnosis is very important to distinguish HD from pathologies such as Huntington Disease-like 2, Spinocerebellar ataxia, Wilson disease, Benign hereditary chorea, etc. The diagnosis is based on genetic analysis to investigate the presence of the mutant allele through polymerase chain reaction (PCR)-based assays. This procedure is done after the patient showed signs of HD. (Margolis and Ross, 2003)

The treatment involves the prescription of drugs to treat chorea, depression and aggressive symptoms. In the case of Huntington's disease, surgical intervention is not effective and, to date, there are still no drugs with neuropathic effect or with the ability to delay the progression of the disease. (Roos, 2010)

1.2 Neurotransmitters

NT's are molecules released by the synaptic vesicles of neurons in response to nerve stimulation, being able to act in another neuron, muscle cells or even in glands. They are synthesized in only a few biosynthetic steps from precursors, such as amino acids obtained from the diet. Figure 1.6 is an example of the biosynthesis of some NT. (**?**Daubner et al., 2011)

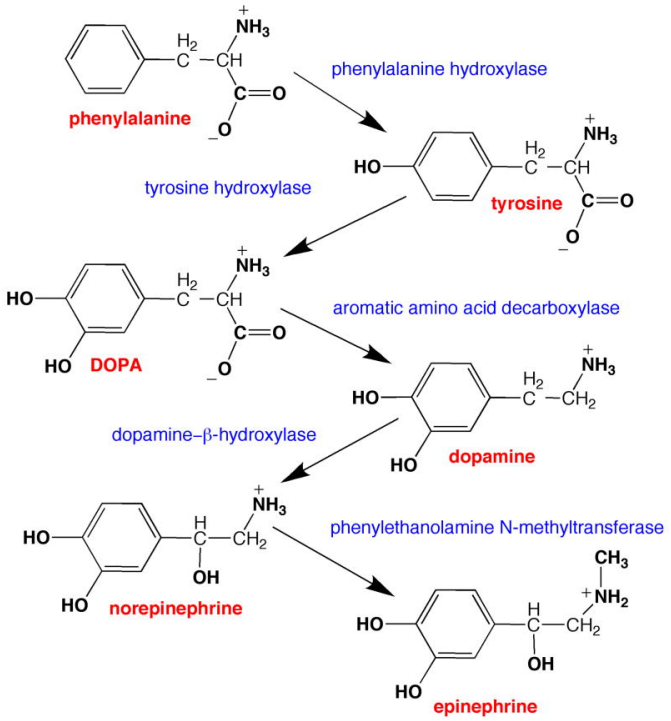


Figure 1.6: Example of a NT biosynthesis. (In (Daubner et al., 2011)

Even though for some classifications the division between peptides, monoamines and amino acids is enough, NT can be classified according to the following groups:

- Peptides: One exemple is somatostatin (GHIH – growth hormone inhibiting hormone), a peptide hormone produced not only in the hypothalamus but also throughout the CNS, in peripheral neurons, in the gastrointestinal (GI) tract and in the pancreatic islets of Langerhans. It acts by regulating the endocrine system, affecting neurotransmission and cell proliferation and inhibiting the production of other hormones like glucagon or insuline. (Patel, 1992)

- Purines: Adenosine, in the brain, acts as a inhibitory NT that promotes sleep and suppresses arousal and can also regulate the release of other NT. (Sebastiao and Ribeiro, 2009)

- Monoamines: Amino groups attached to an aromatic ring by a chain of two carbon. NTs such as serotonin, dopamine and epinephrine belong to this category. They are involved not only in cognitive, emotional and memory processes (Berton and Nestler, 2006; Kurian et al., 2011) but also in maintaining the integrity of neurons (Mele et al., 2010).

- Amino acids: Glutamate – major excitatory NT in the brain (Meldrum, 2000) - and GABA (γ-aminobutyric acid) - the major inhibitory NT in the central nervous system - are some examples (Bak et al., 2006) of amino acidic NTs.

- Gaseous NT: These were more recently discovered and include molecules like nitric oxide, carbon monoxide and hydrogen sulfide, responsible for promoting vasodilation, long-term potentiation, modulation of immune response, cardiovascular protection, among others. (Gadalla and Snyder, 2010)

- Others: Acetylcholine, synthesized from acetyl coenzime A and choline, is a NT at synapses in the ganglia of visceral motor system, neuromuscular junctions and other locations in the central nervous system. (Purves et al., 2001)

Synapses, that are essential for neuronal function, may be excitatory or inhibitory depending on NT function. They can promote – by opening the Na^+/K^+ channels causing a depolarization of the membrane - or inhibit – by opening the K^+ or C^+ channels, causing hyperpolarization of the membrane - the formation of an action potential in the postsynaptic neuron. The recycling of the synaptic vesicle is very rapid and occurs by endocytosis, a process that is very important considering that some neurons can synapse up to 50 times per second. With the exception of acetylcholine that is hydrolyzed to acetate and choline, all NTs from the synaptic cleft suffer re-uptake by the axon terminals. (Lodish et al., 2000) Given the important role of the different classes of NTs it is to be expected that any imbalance could affect the homeostasis of the organism. (Gao and Hong, 2008)

1.2.1 GABA and Glutamate

GABA is the main inhibitory NT of CNS, found naturally in the most varied species. It binds to transmembrane receptors of the plasma membrane or to pre and post synaptic neurons, leading to the opening of the chlorine and potassium channels, causing hyperpolarization. GABA receptors are divided into three: GABA A and C - ionotropic (the NT binds directly to the ion channel) and GABA B - metabotropic (the NT promotes ion channel opening through a coupled G protein). (Huang et al., 2015)
Its synthesis occurs from glutamate, in a chemical reaction catalyzed by the enzyme L-glutamic decarboxylase and with pyridoxal phosphate as cofactor. (Rowley et al., 2012) (Figure 1.7)

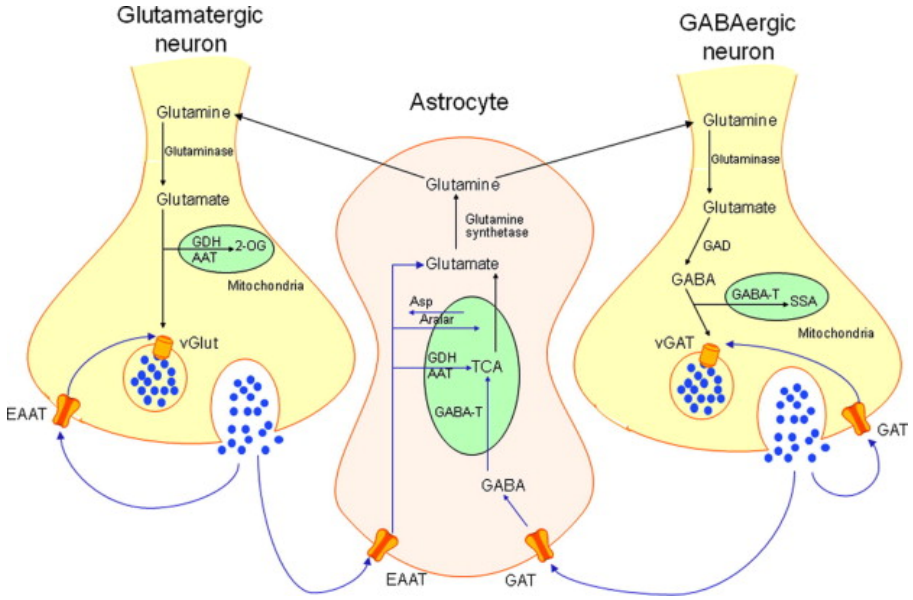


Figure 1.7: Schematic representation of the synthesis reaction of glutamate and GABA. **GDH** - Glutamate dehydrogenase; **EAAT** - Excitatory amino acid transporter; **GAT** - Glutamate aspartate transporter; **Glut** - Glutamate; **GAD** - Glutamic acid decarboxylase; **TCA** - Tricarboxilic acid; **AAT** - Aspartate aminotransferase; **SSA** - Synchronized electrical activity; (Adapted from ((Rowley et al., 2012))

Low concentrations of GABA are related to anxiety, epilepsy and depression, as well as to spasticity, involuntary movements, hypertension and hypoglycemia. Measurement of GABA concentration in CSF may be an important tool for the diagnosis of various diseases. (Chiu et al., 2005; Soltani et al., 2011; Kalueff and Nutt, 2007)
Glutamic acid or glutamate is the most abundant non-essential amino acid in mammals and the main excitatory NT of the CNS. Glutamate is stored in vesicles and, upon a nerve impulse, is released from the presynaptic cell into the synaptic cleft. Glutamate binds to receptors in the postsynaptic cell, such as N-Methyl-D-aspartate (NMDA), activates it

and is quickly removed by being transported through its receptors in glial/neuronal cells. (Figure 1.8) (Yelamanchi et al., 2016)

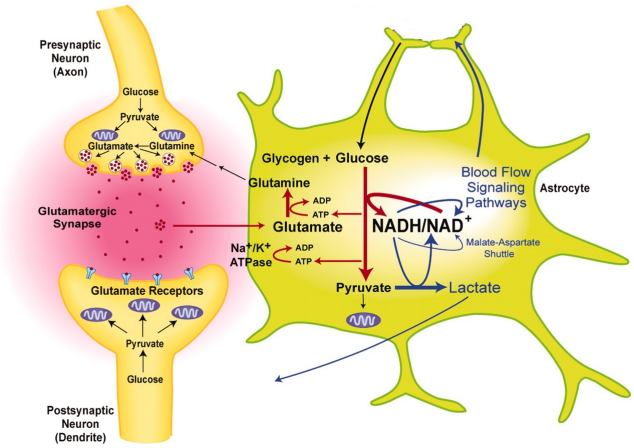


Figure 1.8: Metabolic pathway of the glutamic acid. After its production, glutamate is quickly removed from the synaptic cleft, by the glial cells, so it won't cause neuronal damages.
ATP - Adenosine Triphosphate; **ADP** - Adenosine Diphosphate; **NADH** - Nicotinamide adenine dinucleotide (reduced form); **NAD**$^+$ - Nicotinamide adenine dinucleotide (oxidized form); (Adapted from ((of Sciences of the United States of America, n.d.))

In case of damage/brain injury, glutaminergic neurons can release glutamate continuously without its removal, leading to its accumulation in the synaptic cleft and consequent neuronal damage. (Guerriero et al., 2015) The excitotoxicity of glutamate occurs as part of the ischemic cascade, and is thus associated with the appearance of strokes, (Chamorro et al., 2016) but it also occurs in diseases such as Alzheimer's, ALS and in cases of epileptic seizures. (Vishnoi et al., 2016)

1.2.2 GABA and glutamate in neurodegenerative diseases

NTs play an important role not only in the development of symptoms associated with ND, but also in the process of neurodegeneration. For instance, it is known that dopamine is involved in the development of PD since there is a decrease in its production in the brain. However, there are other NTs such as acetylcholine, GABA and glutamate that also play an important role in PD pathophysiology. There is an imbalance of NT in the extrapyramidal system which, in anatomy, is part of the motor system responsible to produce involuntary movements. There is a reduction in the concentration of dopamine and GABA and an excess production of acetylcholine and glutamate. GABA acts via GABAA receptors and exerts a presynaptic inhibitory function. Its hypofunction enhances the dopamine deficiency, and therefore a possible therapy is to use drugs that increase the neurotrans-

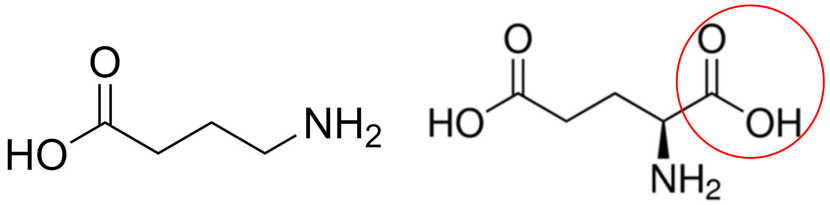
mission of GABAergic neurons by having an agonist effect on the GABAA receptors. Glutamate has an excitotoxic effect, which contributes to the neurodegenerative process. (Rinne et al., 1984; Werner and Coveñas, 2014)
In the case of AD, the amyloid β-peptide, in addition to toxic effects, also interacts with the enzyme glutamine synthetase, inactivating it and leading to an increase in the extracellular concentration of glutamate. (Campos-Peña and Meraz-Ríos, 2014) The activation of NMDA receptors is related to the processes of memory formation and learning, however, the over-activation of these receptors may lead to neuronal damage and justifies why Alzheimer's patients suffer a cognitive decline. (Danysz and Parsons, 2012) A mechanism of compensation for excess glutamate is the direct crosstalk process between the NMDA receptors and the GABAA receptors. Although the increased inhibitory function mediated by GABAergic neurons is an attempt to counter cell overexcitation, this process may interfere with other important processes such as long-term potentiation - main cellular mechanism responsible for learning and producing memories. (Li et al., 2016)
The role of glutamate and glutaminergic theory is already known in the case of ALS and has been extensively covered in this document but the role of GABA has yet to be consolidated. Although there is evidence that the loss of GABAergic neurons may contribute to the death of motor neurons, in animal models with ALS in which GABA was administered, it was found that there was an increase in the influx of Cl^{-}, which resulted in an increase in Ca^{2+} influx and, consequently, neuronal death. This suggests that the activation of GABAergic neurons may increase the excitotoxic effect of glutamate and contribute indirectly to the death of motor neurons, the main cause of ALS symptoms. (Van Den Bosch et al., 2006)
In Huntington's disease, the role of glutamate in the pathogenic process is quite similar to other neurodegenerative diseases and relies on excitotoxicity. In this case there is a substantial reduction of glutamate uptake, which leads to neuronal overexcitation, thus contributing to neurodegeneration. On the other hand, the concentration of GABA decreases, which has a great impact on the appearance of symptoms and progression of the disease. (André et al., 2010)

1.2.3 Detection and quantification methods

There are already different established methods developed over the years for the detection of GABA and/or glutamate, all of which have positive and negative sides. Depending on the type of sample, there are different methods that can be used for the detection and

quantification of these NT's. In this case, glutamate and GABA are amino acidic NTs, and their analysis is useful not only in the medical-pharmaceutical industry, but also in the food industry. The most commonly used method for *in vivo* sampling of glutamate is microdialysis where the dialysates are further analyzed by High Performance Liquid Chromatography - Fluorescence Detector (HPLC-FLD). Although it is a method that allows sample collection in live/anesthetized animals and also allows drug administration locally for studies of neurotransmission mechanisms, it has a long analysis time (5-15 min) and the probe used, depending on the size, may cause nerve/tissue damage. (Hugo Cifuentes Castro et al., 2014) Another way of detecting glutamate *in vivo* would be the use of small probes that detect enzyme-catalyzed reaction products. For example, glutamate oxidase is responsible for the oxidative deamination of glutamate, producing α-ketoglutarate, ammonia (NH_3) and hydrogen peroxide (H_2O_2) (electrochemically detectable). Electrochemical methods are extremely selective and have the ability to detect quantities in the order of μM, however some probes are slightly smaller than those used in microdialysis, presenting the same problem when it comes to possible brain damage. (Qin et al., 2008) Smaller sensors are more difficult to manufacture with reproducibility and, depending on the material, to adapt to freely moving animals. (Qin et al., 2008)
Any analytical method has advantages and disadvantages, depending on the analyte and type of analysis in question. HPLC is a fast and efficient technique that provides accurate results with high resolution and reproducibility. Although it is a versatile technique in component identification and quantification, an HPLC analysis can be quite expensive as it spends large quantities of more expensive organic reagents. It is an automatic technique and relatively easy to use from the user's point of view however there are a wide variety of reagents, columns, etc, that can be used which makes it difficult to develop new methods and troubleshoot problems. On the other hand, gas chromatography (GC) is a technique that not only allows rapid sample analysis with high sensitivity and resolution but is also cheaper than HPLC (which has a high cost of maintenance and operation). Although the GC has advantages over HPLC, the samples used in GC must be in the gaseous state for which they are vaporized using high temperatures. Biological samples exist in the solid and liquid state, and are more stable in their natural form. (Kaspar et al., 2008) The heating process may alter the stability of the molecules of interest and induce their decomposition. Liquid chromatography allows a non-destructive analysis of less stable and larger molecules, such as large polymers/biomolecules, and the use of the sample in its natural physical state. (Services, 2017) Given this, the analysis using gas-chromatography (GC) is not impossible, however, it is necessary to modify the sample by adding different functional groups, so it becomes less reactive and more volatile.

The ideal method for the detection of GABA would be microdialysis followed by HPLC analysis with fluorescence or electrochemical detection, however this process requires derivatization of the sample since GABA is neither fluorescent nor electroactive. (Hugo Cifuentes Castro et al., 2014) Derivatization is a process common to almost all chromatographic methods described for the simultaneous detection of GABA and glutamate, since their structure (Figure 1.9) and chemical properties are similar (both are extremely polar). Although the derivatization of the sample facilitates the detection process, it has disadvantages like: the chromatographic conditions have to be optimized according to the separation of the derivatised product and not of the analyte of interest; it produces artifact peaks resulting from the excess of the derivatizing reagent; the derivatised product must exhibit stability under the chosen chromatographic conditions;
(Parriott, 2012)



a) GABA b) Glutamate

Figure 1.9: Structural similarities between GABA and Glutamate. Glutamate has an extra carboxyl group than GABA.

Table I.2 has described a few examples of different chromatographic methods developed for the detection of GABA and glutamate in biological samples. 1 - (de Freitas Silva et al., 2009) 2 - (Clarke et al., 2007) 3 - (Eckstein et al., 2008) 4 - (Zhang et al., 2005)

1.3 Chromatography and RP-HPLC

Chromatography is a commonly used technique for the separation of compounds that not only can be applied in clinical practice (Pollock et al., 2013), but also has great importance in food (Wahed et al., 2016) and environmental industries. (Patrolecco et al., 2013) There are several distinct types - TLC (thin layer chromatography), liquid and gaseous chromatography - which vary depending on the type of sample being analyzed, the mobile and stationary phases and also the separation method. (Zweig et al., 1972)

Table I.2: Description of some existing methods for the detection of GABA and glutamate in biological samples.

	Method	Flow	Mobile phase	Derivatization	Time of analysis	Year of publication	Detector
1	Reverse phase (RP)-HPLC	Isocratic (1.00 mL/min)	0,05 M sodium acetate +THF+methanol (50:1:49 v/v)	O-phthalaldehyde (OPA)	9 min	2008	FLD
		Isocratic (0.65 mL/min)	0.1M di-sodium hydrogen orthophosphate/50 M EDTA (pH=5.6) + HPLC grade methanol (35:65)		20 min		ECD
2	RP-HPLC	Isocratic (0.1 mL/min)	0.1M di-sodium hydrogen orthophosphate/50 M EDTA (pH=5.3) + HPLC grade methanol (35:65)	Naphthalene-2,3-Dicarboxaldehyde (NDA) in the presence of cyanide ions	15 min	2006	FLD
3	Normal phase (NP)-HPLC	Gradient	Mobile phase A: 1% formic acid and 0.5% Heptafluorobutyric Acid (HFBA) in HPLC grade water Mobile phase B: 1% formic acid and 0.5% HFBA in ACN	Ion pairing with HBFA	5 min	2008	ESI LC /MS/MS
4	RP-HPLC	Isocratic (200μL/min)	GABA: 0.1 M sodium acetate (pH 6.0) and 100 mg/L EDTA sodium in 25% ACN	instead of derivatization	40 min	2005	ECD
		Isocratic (150μL/min)	Glutamate: 0.1 M sodium acetate (pH 6.0) and 100 mg/l EDTA sodium in 15% ACN	OPA	30 min		

Although gas chromatography is cheaper and produces less waste, most compounds are not volatile enough to utilize GC (Schultz et al., 2015). HPLC (high-performance liquid chromatography) - a chromatographic technique widely used in biochemistry - not only allows the separation of miscible, non-volatile/semi volatile compounds but also their identification and quantification. The HPLC system can be divided into the following items: (Figure 1.10)

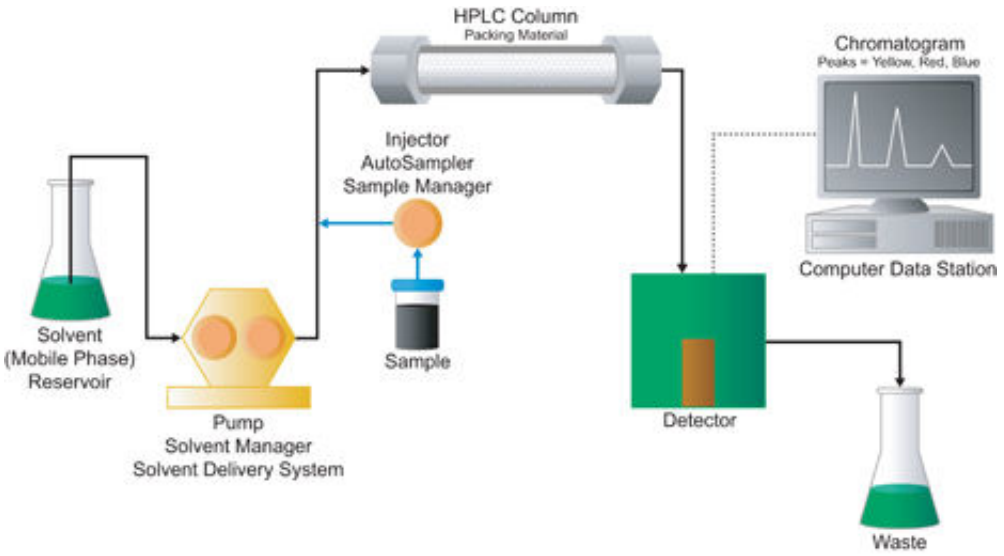


Figure 1.10: Schematic representation of a modular HPLC instrument. (Adapted from ((Waters, n.d.))

- *Pump:* Moves the mobile phase at high pressure through the column. It contains two pistons: one that pumps and the other that makes the refill. The pumped eluent

may have a fixed (isocratic elution) or variable composition (gradient elution). The pressure depends on the flow, viscosity of the mobile phase and the particle size of the stationary phase. (Rouessac and Rouessac, 2013)

- *Injetor:* Since the mobile phase flow must be stable from the column to the detector, the injection of a precise volume of sample must be done as quickly as possible. This process can be performed by a high pressure manual valve or automatically. (Harris, 2010)

- *Column:* C18 columns are the most common in HPLC analysis. They are filled with a non-polar stationary phase therefore this type of column is useful for the analysis of compounds with high polarity. High carbon loads are advantageous to use when analyzing complex samples that require a high degree of separation. The higher the carbon content, the more hydrophobic the stationary phase is. It is also important to consider the column size, with smaller columns (30-50 mm) resulting in shorter run times, fast balance, low backpressure and high sensitivity. In contrast, longer columns (250-300 mm) provide greater resolution power despite using more solvent. The inner walls are coated with an inert material and within the column is the stationary phase whose particle size ranges from 1.7 to 5 μm. The stationary phase in RP-HPLC is hydrophobic and chemically bound to the surface of a silica sphere. They can vary from hydrocarbon chains to amine groups. (Gupta et al., 2012)

  The efficiency of column packing increases with the decreasing of the particle size. This allows you to increase the resolution of the peaks or maintain the resolution in case the running time decreases. Samples and solvent must always be filtered to avoid irreversible adsorption of unwanted particles. (Harris, 2010) Temperature plays an important role in the retention, selectivity and efficiency of the column when analyzing small molecules. In addition, there is also a higher resolution of the method when high temperatures are utilized since the temperature of the column affects the viscosity of the mobile phase, which, in turn, affects the resolution.

- *Detector:* The separation itself is not visible and results are not readable unless a detection method is used such as fluorescence detectors or other techniques like mass spectroscopy. (Zweig et al., 1972) In order for the results to be interpreted, the retention time - time that the sample takes to be detected since it was injected in the column – needs to be calculated and this value is influenced by the affinity of the sample to both phases. (Snyder et al., 2012) Regarding the different detection

methods, DAD (diode array detector) is considered the method of choice for protocol optimization taking into account that enables the selection of the optimal range of wavelengths to be analyzed resulting in a more complete and objective analysis. (Parriott, 2012; Skoog et al., 2017) The DAD consists of a light source from a deuterium or tungsten lamp. Unlike UV-VIS detection, light passes directly through the flow cell and the amount of scattered light is quantized for each wavelength in the photodiode arrays. (Swadesh, 2000)

In reverse-phase HPLC (RP-HPLC) the stationary phase is less polar than the mobile phase and efficient separation will depend not only on the polarity of the sample but also the ratio between polar and non-polar components of the mobile phase. (Parriott, 2012) The RP-HPLC is now one of the most widely used methods because of its effectiveness in separating compounds such as simple or aromatic hydrocarbons, amines, lipids and even metabolically active compounds. However it proves to be inefficient in the separation of inorganic ions, polysaccharides, polynucleotides and compounds too hydrophobic. There are some critical parameters in reversed phase chromatography that are important to consider when developing a method:

Flow rate: The increase / decrease of the flow rate can affect both the pressure and the resolution of the peaks, since it affects the efficiency of the column. There are advantages in increasing the flow rate, such as reducing the time of analysis, but there are also disadvantages. Increased flow rates lead to increased pressure which, over time, can decrease the half-life of the column and wear the HPLC system. If the flow rate is above the optimum value the column efficiency and peak resolution decreases. The same results happen when the flow rate value is lower than the optimal value. The advantage of the decrease in flow rate is the consumption of less mobile phase and decrease in pressure. The change in flow rate may also change the order of elution (selectivity). (Witt, 2017)

Column length: The length of the column is directly proportional to the efficiency of the separation, ie, increasing the length of the column translates into an increase in efficiency. Small columns (30-50 mm) are useful for gradient analysis. In addition, they allow a quick analysis and low backpressures. For higher resolution, bigger columns (250-300 mm) are recommended, however the analysis takes more time and at a higher cost. (Jones et al., 2015)

Temperature of the column: The temperature of the column is a factor which can be easily changed to optimize the resolution of the chromatogram peaks, since the increase in temperature leads to a decrease in the viscosity of the mobile phase. In RP-HPLC it plays an important role in controlling both the retention time and the selectivity. (Dolan,

2002)

Mobile phase: This is a very important parameter in RP-HPLC since it can have an effect on the retention of the molecule. The variation of the composition of the mobile phase is very important and, since reverse phase chromatography assumes that the stationary phase is non-polar, the mobile phase must have some polarity. Depending on the analyte in question, the mobile phase may have a greater or lesser polarity. For example, a very polar analyte will exhibit a higher affinity for a polar mobile phase, being less retained in the stationary phase and being eluted earlier. The choice of mobile phase is critical for HPLC separation. The most common constituents are water and ACN, and these are a good starting point for the development of a method. Other frequently used organic solventes that are miscible in water are tetrahydrofuran (THF) and methanol. (Snyder et al., 2012) The composition of the mobile phase may either remain constant (isocratic elution) or vary (gradient elution). In the case of an isocratic separation, not only the composition of the mobile phase but also the flow rate are constant. The gradient separation implies that during the analysis the percentages of the components of the mobile phase change, and the approach may be linear, segmented, convex or concave. A correct selection of the most appropriate gradient not only decreases the analysis time but also increases the resolution of the peaks. (Joshi et al., 2015; Schellinger and Carr, 2006)

1.4 Objectives

Neurodegenerative diseases are increasingly more frequent and hard to diagnose, so new researches should be made towards the development of optimized analytical techniques that could help not only with prevention but also with diagnosis and therapeutic follow-up, leading to a possible improvement of the disease condition and its symptoms. Knowing that there is a lack of studies regarding the role of GABA and glutamate in both depression and neurodegenerative diseases and that the core goal of our work is to unravel the triad depression, neurodegenerative diseases and NTs, the following objectives were defined:

- Perform a meta analysis not only to do a small revision on depression in neurodegenerative diseases context but also to understand if the prescription of antidepressants is as frequent as expected.
- Develop a HPLC-DAD based method to detect and calculate the concentrations of glutamate and GABA, given that there are no current methods available that simultaneously use DAD as a detector, don't use derivatization of the sample and are of fast analysis.
- Validate the method by applying it to different samples of biological origin.

Chapter II

Antidepressants in neurodegenerative diseases: a meta-analysis

This manuscript is in accordance with a submission made to Neuroscience & Biobehavioral Reviews and is awaiting feedback.

2.1 Abstract

Neurotransmitters are chemical messengers that enable communication between cells, specially between neurons. Any imbalance in their production may be associated to the development of several pathologies, including neurodegenerative diseases. Overtime research efforts were directed towards the discovery of new therapeutics or ways to mitigate some side effects that come with the most frequent neurodegenerative diseases, such as Alzheimer's or Parkinson's.

Objective: Understand if the prescription of antidepressants is frequent among patients with neurodegenerative diseases.

Materials and methods: A meta-analysis was carried out based on one database (PubMed) results for a search that included the Mesh Terms: neurotransmitters, neurodegenerative diseases and antidepressants. Inclusion and exclusion criteria were applied and the Cochrane guidelines were followed to improve the quality of the reported meta-analysis. The results obtained were the effect size, presented as a value of proportion, and the heterogeneity value. Both of these were obtained using the Comprehensive Meta-analysis software.

Results: After the application of the exclusion/inclusion criteria, thirteen studies were included in the meta-analysis. To evaluate the effect size and heterogeneity we used a random effects model. The value of the effect size obtained for the studies was 0.355 or 35,5%, being this the percentage of patients diagnosed with neurodegenerative diseases that were prescribed with antidepressants. Since the p-value is lower than 0.05, we reject the null hypothesis and rely on the confidence interval to state, with 95% confidence, that the value of the effect size for the general population is within 0.263 and 0.459. The I^2

value was 35.830, indicating a low to moderate existence of heterogeneity among studies. This was already foreseen when choosing the regression model and so we conclude that the degree of the existence of heterogeneity is low and may be due to the variation of methods utilised in the different studies analysed.
Conclusion: There are, at least, a minimum of 26.3 to a maximum of 45.9% of patients with neurodegenerative diseases prescribed with antidepressants. In spite of this there are still different opinions regarding the benefits of this treatment. Further studies are necessary to determine whether the advantages of this kind of therapy outdo the disadvantages.

2.2 Introduction

Neurodegenerative diseases are becoming increasingly more frequent as the years pass, not only because of the demographic changes worldwide but also because of their higher incidence and prevalence with the increase of the average life expectancy. Two of the most common are Alzheimer, that affects nearly 44 million people worldwide, and Parkinson disease that affects around 10 million people. (Dorsey et al., 2007; Martin et al., 2006) The main symptoms and side effects vary according to the disease and are a consequence of the neuronal degeneration. Depression has been described as one of the neurodegenerative diseases most common side effect that may appear as an early or main manifestation. (Baquero and Martín, 2015)
Alzheimer is a neurodegenerative disease characterized by cognitive impairment and dementia due to the deposition of amyloid plaques as a consequence of increased Aβ peptide concentration in cerebrospinal fluid (CSF). (Martin et al., 2006; Sheline et al., 2014) Once formed, the plaque may acquire neurotoxic conformations that are dependent on Aβ peptide concentration. 25-40% of patients with Alzheimer (Mizukami et al., 2009) have significant depression symptoms. There are authors that defend that the prescription of high doses of citalopram (selective serotonin reuptake inhibitor) decreases the concentration of Aβ peptide in healthy youngsters. (Sheline et al., 2014) Also in animal models (rat) it was found that the treatment with serotonin or agonists that activate their receptors, in cases of Alzheimer disease, leads to a reduction in the concentration of Aβ peptide in the interstitial fluid of the brain and, in the case of prolonged intake, leads to blockage of growth, reduction of plaque size and also prevents the formation of new amyloid plaque.(Sheline et al., 2014)
Parkinson disease is an idiopathic disease in which there is degeneration and cell death of the dopaminergic neurons present at the base of the midbrain. (Van Den Eeden et al.,

2003) At least 20-40% of patients with Parkinson's disease also present significant depression symptoms. (Krøigård et al., 2014) There are also authors that support the use of antidepressants for the treatment of Parkinson disease, given that depression is one the major non-motor symptoms and it is associated with a more rapid progression of the physical symptoms and a greater decline in cognitive abilities. (Menza et al., 2009)
ALS (Amyotrophic lateral sclerosis) is a neurodegenerative motor disease characterized by the formation of protein inclusions in the cellular and axonal bodies of the motor cortex, leading to neuronal death, not only by accumulation of free radicals, but also by cytoplasmic hypercalcemia caused by excessive activity of excitatory neurotransmitters. Inspite of being a disease without a cure it is known that prolonged exposure to serotonin increases the excitability of spinal motor neurons so antidepressants, such as selective serotonin reuptake inhibitors (SSRI's) can be prescribed as one of the treatments for ALS. (Koschnitzky et al., 2014; Kiernan et al., 2011; Chio et al., 2013)
Huntington's disease is caused by an autosomal dominant genetic mutation that results in abnormal production of the huntintin protein (HTT), gradually damaging the brain. The first symptoms to be detected are physical (abnormal dance-like movements) since the cognitive and behavioral changes are not serious enough to be detected at a very early stage. Inspite of this, major depressive disorder (MDD) is the most common symptom among pre-symptomatic patients. The chronic intake of antidepressants activates different neuroprotective mechanisms hence slowing down the progression of the disease. (Renoir et al., 2012; Jamwal and Kumar, 2015; Van Duijn et al., 2007)
Although it is logical to treat the depressive symptons, depression is hard to diagnose, fact that can decrease the quality of life of the patient and increase the risk of mortality. (Caballero et al., 2006) Besides that, the use of antidepressants can be quite controversial and the opinions regarding its prescription are divergent. There are authors that argue that not all categories of antidepressants are recommended. Tricyclic antidepressants improve the symptoms of depression but they are associated to a few side effects such as arrhythmia's, hypotension and anticholinergic effect that can cause tachycardia's. SSRI's are the most commonly used because patients experience fewer side effects but paired with MAO inhibitors may lead to the appearance of serotonergic syndrome (STS), although rarely. (Hauser RA, 1997) This syndrome causes changes in mental status, tremors and incoordination. However, in a large patient study, it was concluded that the risk of developing STS is not greater than the benefit of the therapy in question. (Panisset et al., 2014)
There are differrent classes of antidepressants being the most commom: (d'Souza and Jago, 2014)

- Mono amine oxidase inhibitors (MAOIs): Inhibitors of the activity of MAO (mono amine oxidase), an enzyme that metabolizes biogenic amines such as serotonin, noradrenaline and dopamine via oxidative deamination, thus increasing their concentration in the synaptic cleft and at respective postsynaptic receptor sites. (Riederer and Laux, 2011)

- Tricyclic: Cyclic antidepressants can block the absorption (reuptake) of the neurotransmitters serotonin and norepinephrine, increasing the levels of these two neurotransmitters in the brain. Despite being effective they have been replaced due to the side effects associated. (Morgan et al., 1987)

- SSRI: Selective serotonin reuptake inhibitors (SSRI's) increase extracellular mono amine levels by blocking serotonin reuptake transporters (SERTs) on presynaptic terminals. (Koschnitzky et al., 2014)

Knowing that theoretically the use of antidepressants is something recommended for the therapy of several neurodegenerative diseases, it is our goal with this meta-analysis to understand if the clinical practice reflects the theoretical recommendation and quantify how frequent antidepressants really are prescribed to patients suffering from neurodegenerative diseases.

2.3 Materials and Methods

Following the Cochrane Guidelines, we performed a meta-analysis of studies already published where the numerical data extracted from the studies were statistically analyzed. The main goal was to assess if the prescription of antidepressants was frequent among patients diagnosed with neurodegenerative diseases.

2.3.1 Search Strategy

Systematic search of the literature were conducted on the PubMed database (National Library of Medicine, National Institutes of Health, Bethesda, MD, USA; http://www.ncbi.nih.gov/PubMed) on the 24^{th} of April of 2016. The mesh terms "neurodegenerative diseases", "antidepressants" and "neurotransmitters" were used and a total of 199 articles were obtained.

2.3.2 Selection Criteria

In order to carry out a primary selection of the studies, inclusion criteria were applied at a specific order:

- 1st inclusion criteria: Be written only in English; Not be a meta-analysis or a review.
- 2nd inclusion criteria: Have all the mesh terms included in the abstract.

After analysing the articles obtained, exclusion criteria were also applied:

- Exclusion criteria: Using non-human experimental models; Not provide the needed measures/parameters to be included in the meta analysis.

2.3.3 Statistical analysis

The data extraction process was performed individually and quality assessments were completed independently by the remaining authors. We used the Comprehensive Meta-Analysis (CMA V3) software to conduct the meta-analysis and calculate the effect size, with a confidence interval (CI) at 95%, and measure the heterogeneity between studies. By default of the software and for the use of this statistical tool the following hypotheses were considered:

$$\text{H0: } \pi = 0.5 \text{ versus } H1 : \pi \neq 0.5.$$

2.3.4 Heterogeneity test

The evaluation of heterogeneity is of great importance in a meta-analysis given that it is what defines the most appropriate statistical treatment to use. It is generally evaluated by the Q test, proposed by Cochran, or quantified by the I^2 statistic defined by Higgins and Thompson. In meta-analyzes with a low number of studies the power of the Cochran test may be low and may not detect heterogeneity. In the case of a high number of studies the power of the Cochran test is high but despite presenting statistical significance, it may not be clinically significant. The statistic of I^2, which unlike the Q test does not depend on the value of degrees of freedom, reveals the value of heterogeneity between studies. (Higgins et al., 2003) Given that our meta-analysis includes a total of 13 studies it seems important that we present not only the Q test but also the I^2 test.

2.3.5 Effect size and forest plot

Since the studies included in the meta-analysis are not cases-control or cohort we can not use the most frequently used effect sizes such as the odds ratio or the relative risk. For the purpose outlined it seemed appropriate to use as the effect size a proportion calculated by dividing the number of patients diagnosed with neurodegenerative diseases that were prescribed with antidepressants by the total number of patients suffering from neurodegenerative diseases. The results of the individual studies were integrated and each received a weight, according to its precision, obtained by the inverse of the variance of the effect size. To calculate this estimate, regression models can be used and they can be of two types: fixed - in which the studies are assumed to be homogeneous - or random - in which some variation between studies is due to differences between the respective populations and protocols employed. (Loureiro and Gameiro, 2011)
The forest plot is a graphical model designed to compare results from multiple scientific studies. Although it can have several forms, it is presented in the form of two columns: one with the authors name and the year of publication and another with the measure of the effect and the confidence interval for each of the studies. The measure of the effect is represented by a square (the diamond represents the measure of the general effect for the fixed or random effects model) and the larger the design the greater the weight assigned to the study. The confidence interval is represented by a horizontal line.

2.4 Results

2.4.1 Search results

The PubMed search resulted in 199 records. Out of these, 105 were excluded from full review because they were either meta-analysis/reviews or they were not written in English.
Out of the 94 articles remaining, only 22 included all the mesh terms in the abstract, which means that 72 records were excluded. Out of the 22, 9 articles were also left out because the experimental model used was non human or because they were lacking data regarding the amount of patients included in the study. In the end, 13 articles were submitted to the meta-analysis (Fig.2.1).

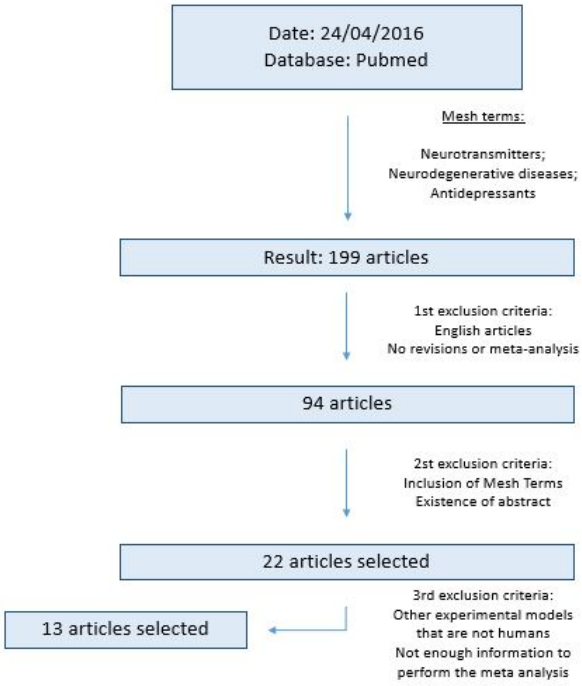


Figure 2.1: Flow diagram of inclusion/exclusion criteria results

2.4.2 Heterogeneity

Figure 2.2 shows the results of the heterogeneity test for the fixed and random models, including the Q-value which depends on the degrees of freedom, the p-value and the I^2-value. Since we assume that there is no homogeneity between the articles and giving the low number of studies included (n=13), we used as reference the values obtained by the I^2 test, instead of the Q test, for the random model. The I^2 statistic can range from negative values up to 100% and according to Higgins and Thompson (Higgins et al., 2003) values from 25% to 50% might indicate low to moderate heterogeneity. In this case we obtained a value of 35.830%. Knowing in advance that clinical variability (heterogeneity) exists among the studies, that is, variation in the type of participants, interventions used, exposures or expected results, the value obtained may be considered moderate and justified by it. The p-value of I^2 is equivalent to the p-value of the Q test, being this statistically significant ($< 0{,}001$), reinforcing the presence of heterogeneity.

2.4.3 Effect size and forest plot

The effect size is a value of proportion that allows us to assess to what degree antidepressant treatment is a reality within patients diagnosed with neurodegenerative diseases,

Model	Number of studies	Heterogeneity			
		Q-value	df (Q)	P-value	I-squared
Fixed	13	1884,5	12	< 0,001	99,363
Random	13	18,700	12	< 0,001	35,830

Figure 2.2: Heterogeneity test results

taking into account the values provided by each of the 13 studies included in the meta-analysis. In figure 2.3, it is shown not only the effect size results (named as event rate) for each individual study, but also the overall effect size for the fixed and random models. The decision process between using a fixed or random model should be very well considered but, in this case, since the results show that there is no homogeneity, we use the random model that distributes the weight of the studies in a more uniform way, valuing the contribution of smaller studies. In addition to the effect size value, the confidence interval at 95% and the *p*-value are also described. According to the random effects model, the value of the effect size is 0.355 which translates into a percentage of 35.5% of patients diagnosed with neurodegenerative diseases who are prescribed with antidepressants. This value refers to the sample of the 13 studies included in the meta-analysis. The hypotheses (H0 and H1) formulated refer to the general population and their validity depends on the *p*-value. The *p*-value is the probability of obtaining the observed effect or larger under a null hypothesis. (Higgins and Green, 2011) For the random model, we obtained a *p*-value $= 0.007 < 0.05$, rejecting the null hypothesis and being able to state that the proportion value for prescribing antidepressants to patients with neurodegenerative diseases in the general population is different than 50%. As the test is bilateral, we can't verify if it is higher or lower, but with the confidence interval given by the software, we are 95% confident that the value of the effect size is within the range: $[0.263 - 0.459]$. Clinically, it means that the percentage of patients diagnosed with neurodegenerative diseases and prescribed with antidepressants can be as low as 26.3% or as high as 45.9%.

Figure 2.4 contains the names of the main authors of each study, the Z-value (measures the difference between an observed statistic and the mean of the population in units of the standard deviation), p-value and the forest plot that summarizes the weight assigned to each study, effect size and its confidence interval.

Model	Study Name	Statistics for each study			
		Event rate	Lower limit	Upper limit	p-Value
	Mazzucchi 2014	0,363	0,286	0,447	0,002
	Krøigård 2014	0,225	0,206	0,245	0,000
	Mizukami 2009	0,389	0,246	0,554	0,186
	Sharp 2008	0,824	0,573	0,942	0,015
	Kulisevsky 2008	0,300	0,252	0,353	0,000
	Chen 2007	0,193	0,189	0,197	0,000
	Maurits 2007	0,315	0,302	0,329	0,000
	Caballero 2006	0,586	0,487	0,678	0,089
	Martin 2006	0,500	0,415	0,585	1,000
	Bellelli 2005	0,167	0,143	0,194	0,000
	Panisset 2014	0,662	0,638	0,686	0,000
	Joutsa 2012	0,089	0,060	0,129	0,000
	Kompoliti 1998	0,500	0,244	0,756	1,000
Fixed		0,223	0,220	0,227	0,000
Random		0,355	0,263	0,459	0,007

Figure 2.3: Evaluation of the effect size for each article included in the meta-analysis and overall effect size for the random and fixed models

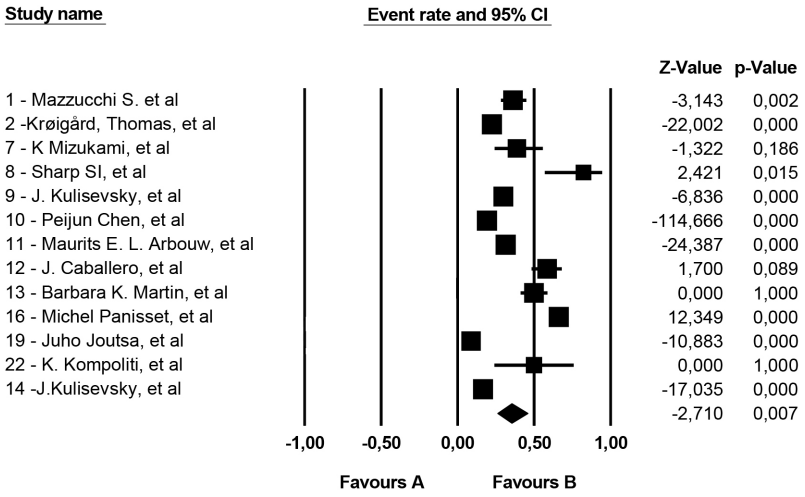


Figure 2.4: Forest plot showing the weight attributed to each study and the event rate value with the confidence interval at 95%

2.5 Discussion

This meta-analysis includes data from 13 studies assessing a total of 50.894 patients with different neurodegenerative diseases out of which 11.248 were prescribed with antidepressants. Given this we used meta-analysis to evaluate the extent of prescription of antidepressants in patients with neurodegenerative diseases. The main results were the event rate and the heterogeneity value.

In meta-analyzes that evaluate medical interventions, the effect size is referred to as relative risk, odds ratio, or risk difference. However this applies to case-control studies,

and in this case we are interested in obtaining an overall value of the percentage of individuals diagnosed with neurodegenerative diseases and prescribed with antidepressants, regardless of the type of study in question. For this we choose as the effect size a proportion, referred to as event rate. The fact that the data extracted from the articles are independent of the research type assures us that there is no omission of data/bias and that our results are as reliable as possible. Knowing this, it is anticipated that clinical heterogeneity exists, something that justifies the moderate value (35,830%) of heterogeneity that was obtained in the I^2 test. The main result, and the one that answers to the question that originated this meta-analysis, is the event rate. For the random model, assuming the non-homogeneity of the studies, we obtained a value of 0.355 or 35,5% for the thirteen study sample. Since the p-value was less than 0.05 we reject the previously defined hypothesis for the general population that said that the summary value of the effect size was 0.5, however we are 95% confident that this value is between 0.263 and 0.459. The advantage of this meta-analysis is that we do not specify a type of disease and so in this interval are included diseases such as Alzheimer's, Parkinson's, Huntington's and Amyotrophic Lateral Sclerosis. We are interpretively concluding that between 26,3 and 45,9% patients with neurodegenerative diseases are prescribed with antidepressants. This values can be considered small since according to the studies included in this meta-analysis the prescription of antidepressants, especially SSRIs, is highly recommended. Nonetheless these results reflect the clinical practice and can be applied to the global population. There are cases where this kind of treatment can cause a delay or even reverse the progression of the disease, but these occur occasionally or in non-human models. (Sheline et al., 2014) There are also studies showing that treatment with antidepressants combined, for example, with other drugs may cause worsening of the main symptoms. (Panisset et al., 2014)

These results confirm the need to clarify the impact of antidepressant therapy in the progression of neurodegenerative diseases. In order to define exactly how harmful/beneficial this type of treatment can be, new methods have to be developed for the study of neurodegenerative diseases and unravel the exact role of antidepressants in the ongoing pathogenic process.

Chapter III

Development of a chromatographic method for the detection of GABA and Glutamate

3.1 HPLC method development

The development and validation of HPLC-based methods is very important not only for the food, chemical and pharmaceutical industries, but also for research. The developed method must be validated in standards to demonstrate that the analytical procedure is suitable for the intended use. For this, it is important to know the physical and chemical properties of the compounds to be analyzed, such as chemical structure, solubility, pKa value, polarity, etc. In order to save time, is also relevant to know already validated methods to use as a starting point. (Sarma Krishna pathy, 2013) In the development of a chromatographic method there are some key steps that have to be followed:

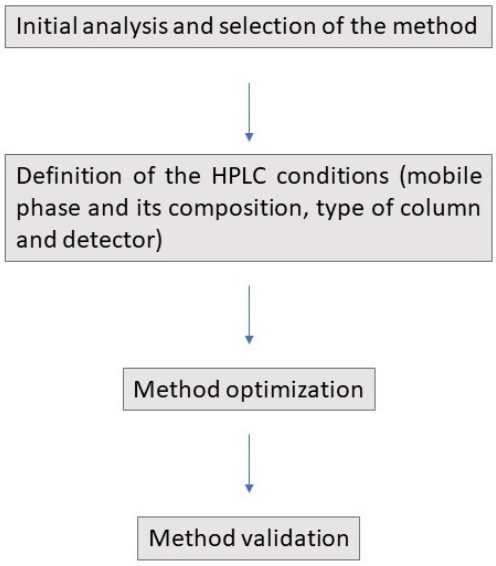


Figure 3.1: Important steps for the development of a HPLC method.

3.1.1 Method optimization

Once defined the initial conditions for method development, there is need to optimize it so that it is more efficient and has a better resolution. First of all, it is important to decide which chromatographic variables we are willing to change and how the remaining ones can be optimized. The flow rate, and consequently the pressure, temperature, column size and percentages of the different compounds of the mobile phase are some examples of variables that can be optimized. (Majors et al., 2010)

3.1.2 Method validation

When developing a method, it must be ensured that the analysis of the compounds for which this method has been developed generates significant results and can be applied in general practice. Each year, the FDA and United States Pharmacopeia (USP) update the guidelines and add new criteria for the validation of methods. Another possible source for finding validation criteria is the International Conference on Harmonization (ICH) which still adds more requirements to those proposed by the other entities. (Shabir, 2003) (Food et al., 2017) According to ICH the following parameters are essential for the validation of a method:

3.1.2.1 Specifity:

ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc." This definition implies the identification of an analyte and a purity test, to ensure that all the analytical procedures performed allow an accurate statement of the content of impurities of an analyte. (ICH et al., 2005)

3.1.2.2 Accuracy:

Expresses the proximity of the value obtained to the reference/accepted value. There are several methods available to determine accuracy: (i) Analysis of a sample of known concentration (reference material) and comparison to the accepted value; (ii) Comparison of the results of a well-characterized procedure (where the accuracy of it is stated and/or defined) with the results obtained with the proposed analytical procedure. (iii) based on the recovery of known amounts of analyte (minimum to prepare in triplicate at three levels) and (iv) based on the recovery of spiked analyte with standard additions. (ICH et al., 2005)

3.1.2.3 Precision:

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. (ICH et al., 2005) Precision may be considered at three levels:

- Repeatability: It can also be reffered as intra-assay precision and it is defined as the precision under the same operating conditions over a short interval of time. It should be assessed using a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each), or from a minimum of six determinations at 100% of the test concentration.
- Intermediate precision: Express variations within the same laboratory (different days, equipment, analysts, etc).
- Reproducibility: It expresses the precision between different laboratories and is used for the standardization of methodologies.

3.1.2.4 Detection limit:

The detection limit of an individual analytical procedure is the lowest value detected, not necessarily quantified, in a sample. (ICH et al., 2005)

- Based on Visual Evaluation: The detection limit is determined by establishing the minimum level at which the analyte can be reliably detected and by the analysis of samples with known concentrations of analyte.
- Based on Signal-to-Noise: Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.
- Based on the Standard Deviation of the Response and the Slope: The limit of detection (LoD) may be expressed as:

$$LoD = \frac{3.3\sigma}{S}$$

σ - the standard deviation of the response.
S - the slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte and the σ value can be estimated either from the (i) *Standard Deviation of the Blank:* or the (ii) *Calibration Curve*.
(i) - Analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.
(ii) - Using samples containing an analyte in the range of LoD. The standard deviation may be calculated by the residual standard deviation of a regression line or by the standard deviation of y-intercepts of regression lines.

3.1.2.5 Quantification limit:

Lowest quantity of analyte that can quantified in a sample with precision and accuracy. (ICH et al., 2005)

- Based on Visual Evaluation: The quantification limit is generally determined by establishing the minimum level at which the analyte can be quantified with acceptable accuracy and precision and the analysis of samples with known concentrations of analyte.

- Based on Signal-to-Noise: Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

- Based on the Standard Deviation of the Response and the Slope: The limit of quantification (LoQ) may be expressed as:

$$LoQ = \frac{10\sigma}{S}$$

σ - the standard deviation of the response.
S - the slope of the calibration curve.

The slope S may be estimated from the calibration curve of the analyte and the σ value can be estimated either from the (i) *Standard Deviation of the Blank:* or the (ii) *Calibration Curve*.
(i) - Analyzing an appropriate number of blank samples and calculating the standard deviation of these responses.

(ii) - Using samples containing an analyte in the range of LoQ. The standard deviation may be calculated by the residual standard deviation of a regression line or by the standard deviation of y-intercepts of regression lines.

3.1.2.6 Linearity:

Capacity (within a known range) to obtain results directly proportional to the analyte concentration in the sample. The linearity should be determined at least with a series of five standard solutions for which the expected concentrations should cover 80%-120% of the desired concentration. The result should be proportional to the concentrations of the analytes and it should be evaluated by appropriate statistical methods, for example, by calculation of a regression line by the method of least squares. (ICH et al., 2005)

3.1.2.7 Range:

The range of a procedure is the interval between the lowest and highest concentrations of analyte in the sample. It is calculated knowing that the method has adequate values of accuracy, precision and linearity. (ICH et al., 2005)

3.1.2.8 Robustness:

Robustness is a measure that exists to evaluate variations in the results obtained in response to small intentional changes in the parameters of the method to be evaluated, being an indicator of reliability during a normal use. The pH and composition of the mobile phase, flow rate, column type and temperature are parameters that can be changed. (ICH et al., 2005)

3.2 Material and methods

3.2.1 Instrument and software

All experiments were performed on a Hitachi LaChrom Elite® HPLC system (Hitachi High - Technologies Corporation, Tokyo, Japan) composed by HTA L-2130 LaChrom Elite quaternary pumps (Hitachi High-Technologies Corporation), L-2200 LaChrom Elite autosampler (Hitachi High-Technologies Corporation), L-2300 LaChrom Elite column heater (Hitachi High-Technologies Corporation), L-2455 LaChrom Elite photo DAD (Hitachi High-Technologies Corporation). EZChrom Elite Compact Software Version 3.3.2.

(Agilent Technologies, Inc., Santa Clara, CA, United States) was used for data collection and treatment.

3.2.2 Reagents and consumables

L-glutamic acid and sodium acetate were purchased from Merck S.A. (Algés, Portugal). 4-Aminobutyric acid was purchased from Thermo Fisher (Waltham, Massachusetts, USA). Trifluoroacetic acid was purchased from Biochem Chemopharma (Ligne, Cosne sur Loire, France). ACN and methanol (HPLC GOLD Ultra Gradient) were purchased from Carlo Erba Reagents (Chaussée du Vexin, Val de Reuil, France). Ultrapure water (up) was obtained from the Water Purification System TKA Barnstead™ GenPure™ capsule 0.2 μm (Thermo Fisher Scientific, Wilmington, DE, EUA). LiChrospher® 100 RP-18 (5 μm) LiChroCART® 250-4 was purchased from Merck S.A. (Algés, Portugal). Membrane filters 0.45 μm, 47mm, were purchased from Advantec®, Toyo Roshi Kaisha, Ltd. (Tokyo, Japan). PuradiscTM, 0.2 μm, 25mm sterile and endotoxin free filters were purchased from WhatmanTM (GE Healthcare UK Limited, Buckinghamshire, UK).

3.2.3 Analytical procedure

3.2.3.1 Mobile phase and chromatographic conditions

Different mobile phases were tested so that specific peaks of GABA and glutamate could be detected in a sample in which the two were present.

Mobile phase - 0.5% CH_3COOH:MeOH:H_2O (15:15:70)

The 0.5% CH_3COOH:MeOH:H_2O up solutions were prepared in a proportion of 15:15:70 and eluted firstly at a flow rate of 0.5 and then 1 mL/min during 20 minutes, with the temperature of the column set at 25°C.

Mobile phase - ACN:0.1% CH_3COOH:H_2O up (15:15:70)

The ACN:0.1% CH_3COOH:H_2O up solutions were prepared in a proportion of 15:15:70 and eluted at a flow rate of 1 mL/min during 20 minutes, with the temperature of the column set at 25°C.

Mobile phase - ACN:0.05% CH_3COOH:H_2O up (15:15:70)

The ACN:0.05% CH_3COOH:H_2O up solutions were prepared in a proportion of 15:15:70 and eluted at a flow rate of 1 mL/min during 20 minutes, with the temperature of the column set at 25°C.

Mobile phase - ACN:TFA 0.1%

The ACN:TFA 0.1% solutions were prepared in a proportion of 50:50, 70:30 and 80:20, then eluted at a flow rate of 1 mL/min during 20 minutes, with the temperature of the column set at 25°C.

Mobile phase - MeOH:Acetate buffer (0.1 M, pH=5.5/6)

The acetate buffer was prepared using sodium acetate and acetic acid, to adjust the pH value. Two pH values were tested, being those 5.5 and 6. The MeOH:Acetate buffer mobile phase was tested for two proportions (50:50 and 30:70), each eluted at a flow rate of 1 mL/min during 20 minutes. The column was set at 25°C and after this, the assay was repeated with the column at 40°C.

Mobile phase - ACN:H_2O

The gradient eluition of ACN and H_2O is described in more detail in table III.1.

Table III.1: Composition of the mobile phase during the whole run.

Time (min)	Composition of the mobile phase
0 - 2.5	100% H_2O
2.5 - 7	30% H_2O + 70% ACN
7 - 9	100% H_2O
9 - 10	100% H_2O

This mobile phase was pumped into the column - at a temperature of 25°C - at a flow rate of 1.00 mL/min and the injection volume of the standards was 30 μL. The detection was made at 195-400 nm for 10 minutes (total run time). All mobile phases were filtered through a 0.45μm membrane.

3.2.3.2 Calibration standards

4.3 mg/mL Glut and GABA stock solutions were made using up water a solvent. All stock solutions were filtered through a 0.45μm filter membrane device. The first assays were performed using standard solutions containing either Glut or GABA in the following concentrations: 0.014, 0.070, 0.350, 1.750 and 8.750 mg/mL. In the subsequent assays, standard solutions containing both Glut and GABA were prepared in the following concentrations: 0.0675, 0.1351, 0.2703, 0.5406, 1.0812 and 2.162 mg/mL.

3.2.4 Method optimization

3.2.4.1 Optimization of the gradient

For the method to be optimized, there was the need of perfecting the gradient by choosing the right time to increase the percentage of organic phase over the aqueous phase. Three different gradients were tested where the composition of the mobile phase varied at 2.5, 3 and 4 minutes (Table III.2):

Table III.2: Different gradients tested to optimize the time for the variation of the composition of the mobile phase.

Time (minutes)			Composition of the mobile phase
Gradient 1	Gradient 2	Gradient 3	
0 - 2.5	0 - 3	0 - 4	100% H_2O
2.5 - 7	3 - 7	4 - 7	30% H_2O + 70% ACN
7 - 9	7 - 9	7 - 9	100% H_2O
9 - 10	9 - 10	9 - 10	100% H_2O

3.2.4.2 Optimization of the temperature

In order to find the best temperature for the method, different assays were performed using two standard solutions of glutamate (2.81; 5.62 mg/mL), GABA (3.22; 6.45 mg/mL) and MIX [glut (1.40; 2.81 mg/mL); GABA(1.61; 3.22 mg/mL)]. The mobile phase, composed of a gradient of ACN and H_2O, was pumped at a 1 mL/min flow rate through a column set at different temperatures (15, 25, 35 and 40°C).

3.2.5 Method validation

The method tested was validated according to the ICH guidelines for procedure validations.

3.2.5.1 Specifity

The specificity of the methods was determined by comparing chromatograms obtained from GABA and glutamate spiked-samples.

3.2.5.2 Linearity

Six standard solution concentrations comprising the range between 0.0675 - 2.162 mg/mL were assayed. Calibration curves were constructed by plotting average peak area versus

concentrations. The linearity was evaluated using regression analysis.

3.2.5.3 Accuracy

Accuracy was determined by measuring recovery in standard solutions: 0.0675, 0.1351, 0.2703, 0.5406, 1.0812 and 2.162 mg/mL.

3.2.5.4 Precision

Precision was determined by means of the repeatability (intraday precision). The repeatability was evaluated by analysing the standard solutions: 0.0675, 0.1351, 0.2703, 0.5406, 1.0812 and 2.162 mg/mL.

3.2.5.5 LoD and LoQ

The LoD and LoQ were calculated using the following equation, where σ is the standard deviation of the blank sample and S is the slope of the calibration curve:

$$LoD = \frac{3.3\sigma}{S} \text{ and } LoQ = \frac{10\sigma}{S}$$

3.2.6 Statistical analysis

Statistical analysis was performed using SPSS v.24.0 (IBM Corp., Armonk, NY, USA). Calibration curves equations were obtained using linear regression analysis.

3.3 Results and Discussion

Considering the important role that both glutamate and GABA have in the neurodegenerative process, it is fundamental that there are well established methods for their detection and quantification both *in vivo* and in *vitro*, thus contributing to a better understanding of how these NT work under normal and pathological conditions. Due to GABA and glutamate structural similarities, their separation and detection requires some work. For the analysis of proteins and amino acids, the use of reverse phase chromatography is the most appropriate and the most frequently used method is HPLC coupled to fluorometric, spectrophotometric or electrochemical detection systems. (Hugo Cifuentes Castro et al., 2014) . Any HPLC-based method has to be developed and validated according to the previously defined ICH guidelines. (ICH et al., 2005) All the following chromatographic assays were performed using a C18 column and for the detection of glutamate and GABA to be possible it was necessary to optimize some important parameters.

3.3.1 DAD and wavelength of detection

In order to determine the optimal wavelength for the detection of GABA and glutamate, we performed an HPLC run using standard solutions within the range of UV (190-400 nm) and the absorption spectrum of the two substances was obtained. Both GABA and glutamate are detected between 190 and 220 nm (figure 3.2). With this in mind, we defined the values of 200, 210 and 220 nm as optimal wavelengths for the detection of these NT in all assays.

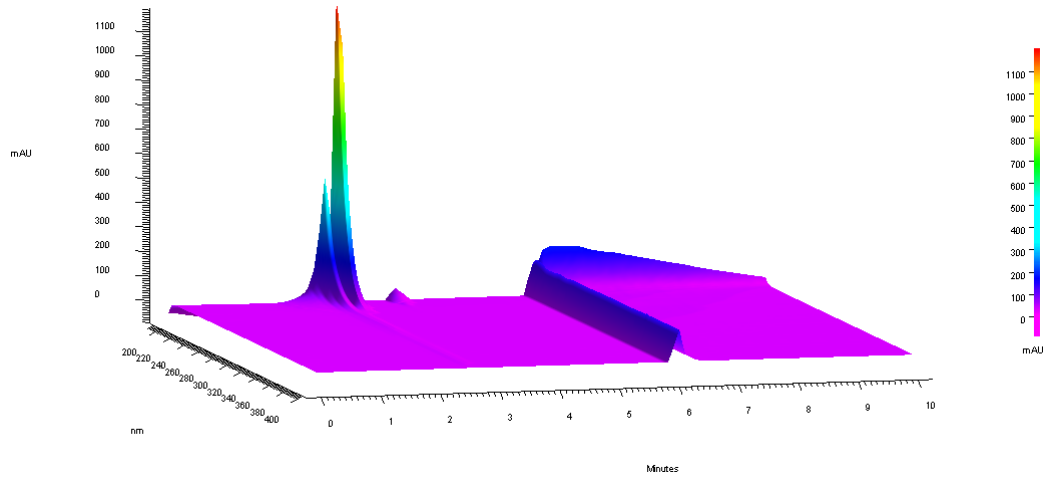


Figure 3.2: Glutamate and GABA absorption spectra, obtained by DAD.

3.3.2 Preparation of the standard solutions

The first step in the preparation of the standard solutions was to find the water solubility value at room temperature of GABA and glutamate. After this step, calculations were performed in order to verify the minimum concentration of NT soluble in aqueous solution without the need to resort to heat sources. Another important value to take into consideration was the value of these naturally occurring NT in human plasma samples, whereas for glutamate it is 248.04 μmol/L (Fadel et al., 2014) and for GABA is 100 nmol/L (Petty, 1994). Considering these values, standard solutions were made using successive dilutions of 1:500, starting from a more concentrated to a less concentrated solution (2.1625 to 0.0676 mg/ml).

3.3.3 Development of the mobile phase

Three standard solutions of glutamate (0.75, 0.075 and 0.0075 mg/mL), three of GABA (10.31, 1.031 and 0.1013 mg/mL) and five of a solution called MIX (GLUT: 0.375 mg/mL to 0.0375 μg/mL; GABA: 5.155 mg/mL to 0.5155 μg/mL) were prepared and these were the standard solutions utilized for all the following methods tested. All the solutions were prepared according to the different mobile phases, being this both the solvent used to dissolve the NT and the blank solution. In order to save time, the first mobile phase was prepared considering others already existent that were used for the detection of NT. As a first approach, we used a mobile phase that had an aqueous component (70% H_2O UP), an acidic component (15% of a 0.5% acetic acid solution) and an organic component (15% methanol). The assay was performed at 25 °C and at a flow rate of 1.00 mL/min. After obtaining the chromatograms we proceed to the analysis of the results and compare the retention times of the GABA and glutamate solutions with those of the MIX. Ideally, if the peaks appear in the individual chromatograms they should also appear in the MIX solution. In order to confirm if any of the peaks is not an artifact of the mobile phase, we also compare the results with the blank sample chromatogram. In our case, all the blank solutions assays were negative for the presence of GABA and glutamate. In figure 3.3 we observe a chromatogram of the most concentrated glutamate solution at an absorbance of 210 nm, the glutamate peak having a retention time of approximately 5.7 min. On the other hand, the most concentrated standard solution of GABA has a retention time of 7.1 min. (Figure 3.4)

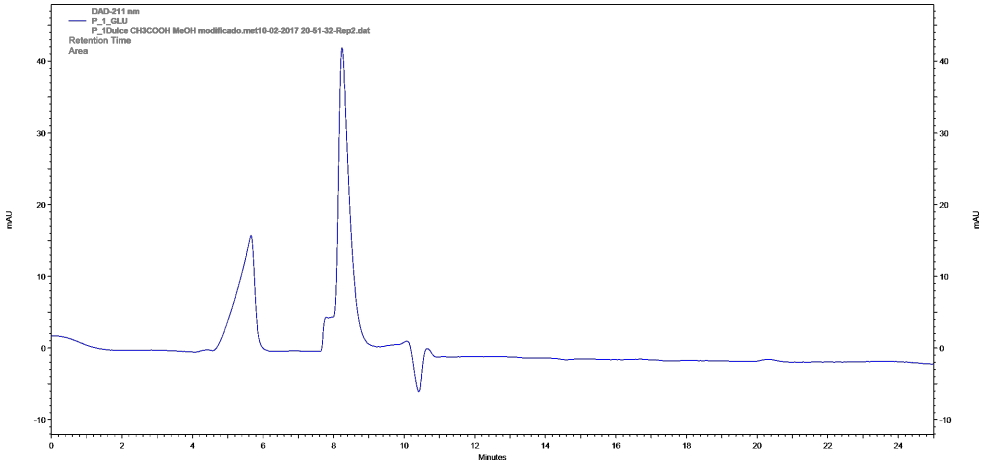


Figure 3.3: Chromatogram of the glutamate assay using as mobile phase CH_3COOH 0.5% : MeOH : H_2O up (15:15:70).

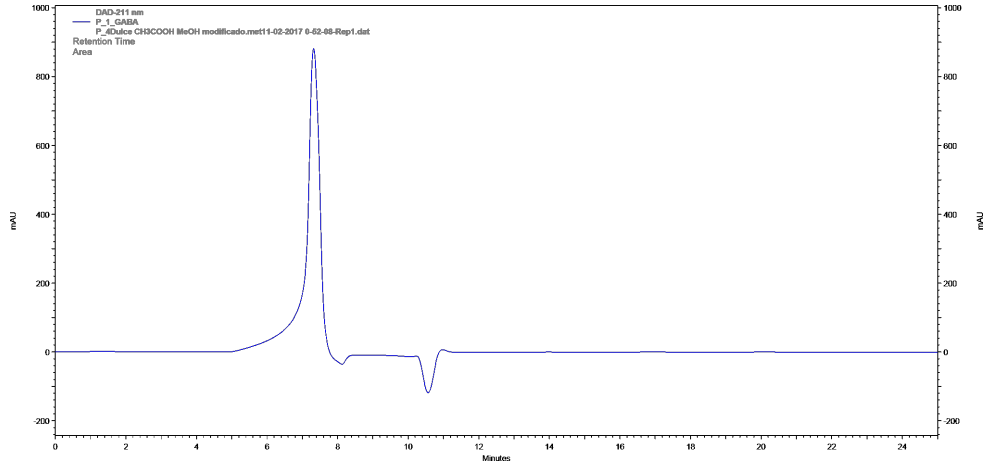


Figure 3.4: Chromatogram of the GABA assay using as mobile phase CH_3COOH 0.5% : MeOH : H_2O up (15:15:70).

It would be expected that in the MIX solution there would be a representation of the two peaks and even though this method has already worked with other amino acids (Teixeira, 2015), it seems to be no separation of glutamate and GABA in the MIX assay. Either glutamate is not detected or the peaks overlap (with peak from 5.2 to 7.5 min) (Figure 3.5)

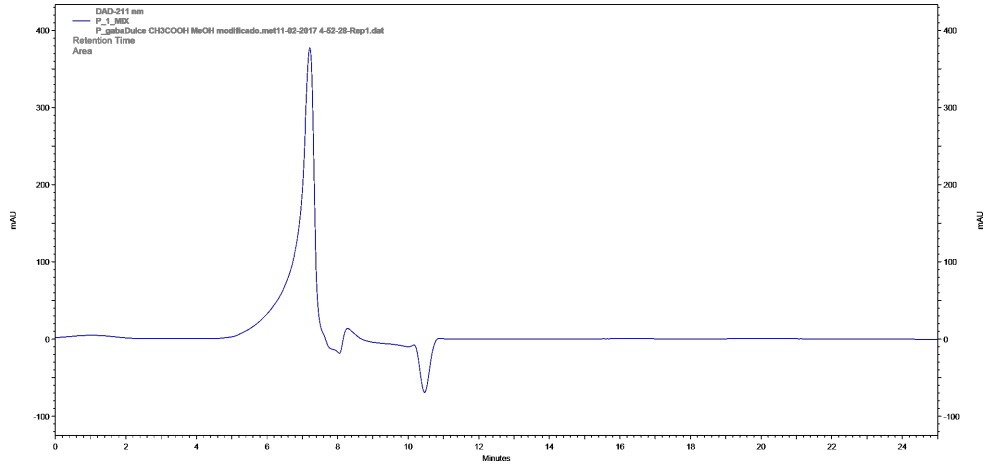


Figure 3.5: Chromatogram of the MIX assay using as mobile phase CH_3COOH 0.5% : MeOH : H_2O up (15:15:70).

Given the results obtained, we decided to decrease the percentage of acid in the aqueous solution and change to a different and stronger organic phase by experimenting with the following mobile phases: CH_3COOH 0.1%:ACN:H_2O up (15:15:70 %) and CH_3COOH 0.05%:ACN:H_2O up (15:15:70 %). Despite all, the retention time of GABA and glutamate in the individual assays did not vary significantly (2.7 for glutamate and 2.8 minutes for GABA) and the peaks again appeared to be overlapped in the MIX assay. (Figure 3.6 and 3.7) There were no significant differences between the two mobile phases tested

which indicates that lower percentages of acid do not have an influence on the separation of GABA and glutamate.

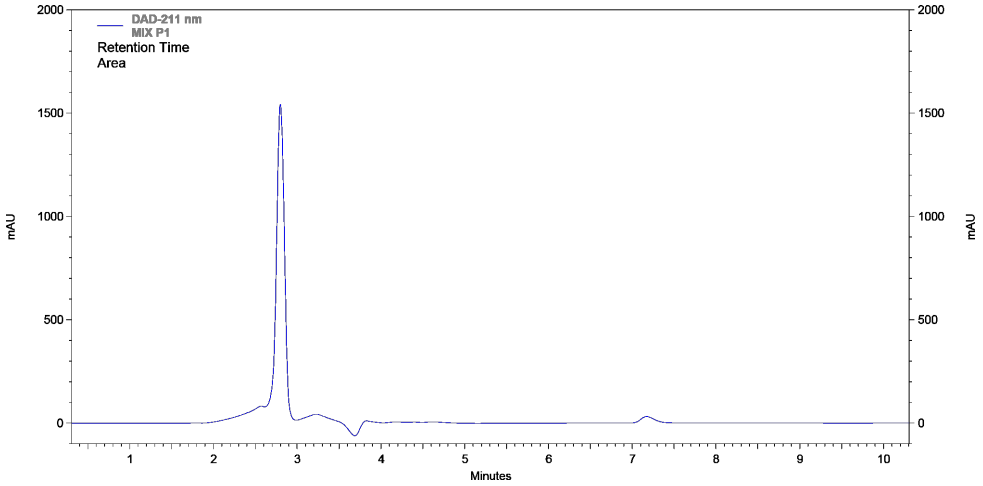


Figure 3.6: Chromatogram of the MIX assay using as mobile phase CH_3COOH 0.1% : MeOH : H_2O up (15:15:70).

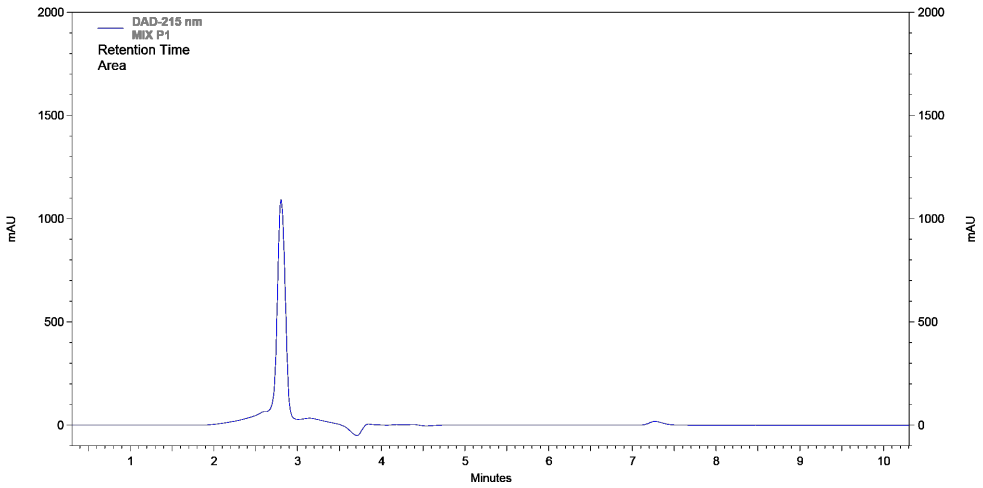


Figure 3.7: Chromatogram of the MIX assay using as mobile phase CH_3COOH 0.05% : MeOH : H_2O up (15:15:70).

Given this problem, we decided to reduce the flow of the mobile phase from 1.00 to 0.500 mL/min, this way the analytes could be pumped at a lower pressure and have more time to be in contact with the stationary phase. Even though there was a delay of the retention time of glutamate and GABA, the peaks were still overlapped. Since the previous mobile phases were not suitable for the separate detection of the two NT, we decided to opt for an acidified aqueous phase, this time with TFA 0.1% (a stronger acid) and a more apolar organic phase (ACN), in a ratio of 50:50. The low pH of the mobile phase leads to the ionization of the amino acids and can vary its structure enough that there is a difference between the retention times. Another important factor is the apolarity of the ACN. Since glutamate and GABA are extremely polar, a slightly less polar mobile phase could make

a difference, along with the low pH, in the retention time. The result obtained was the overlap of the peaks with individual detection of each of the NT. In Figures 3.8 and 3.9 we can see that glutamate is detected at approximately 2.10 minutes and GABA is detected at 2.3 minutes.

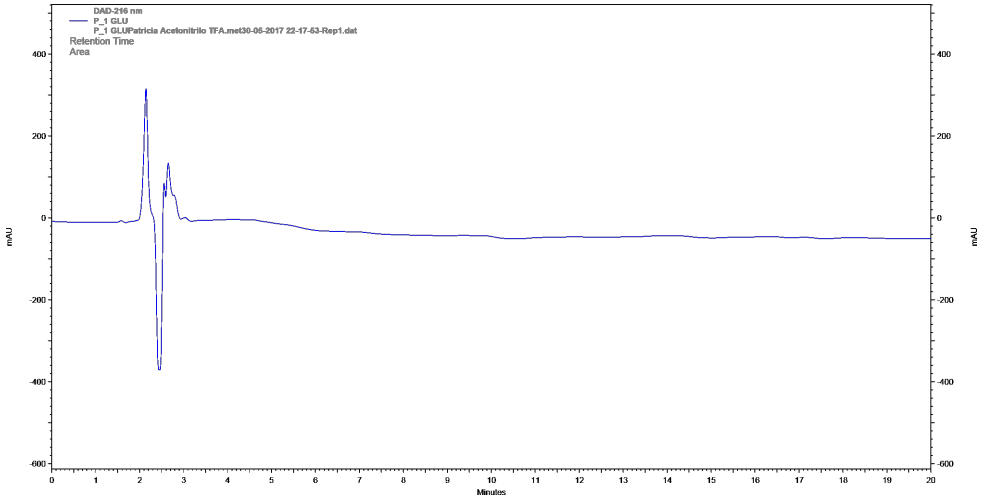


Figure 3.8: Chromatogram of the glutamate assay using as mobile phase TFA 0.1% : ACN (50:50).

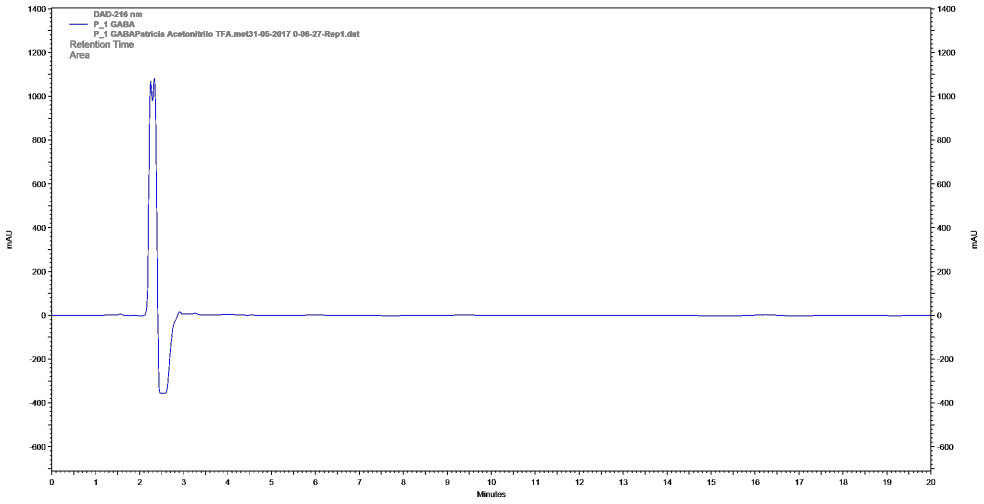


Figure 3.9: Chromatogram of the GABA assay using as mobile phase TFA 0.1% : ACN (50:50).

Since the two NT have similar retention times, there is no obvious separation in the MIX chromatogram. (Figure 3.10).

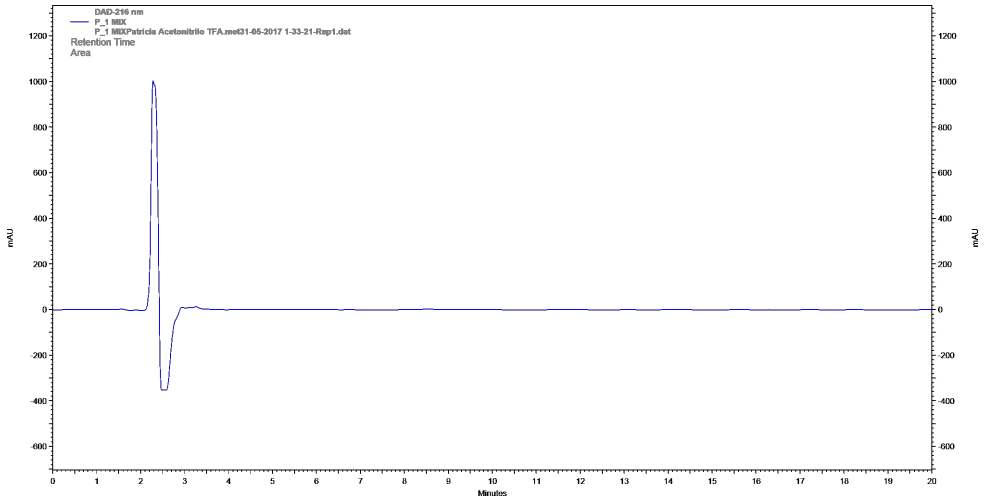


Figure 3.10: Chromatogram of the MIX assay using as mobile phase TFA 0.1% : ACN (50:50).

Thus, we attempt other variants of this method by increasing the percentage of the organic phase from 50 to 70% (figure 3.11) and then from 70 to 80% (figure 3.12). The goal was to see if there was an influence of the increase of the percentage of ACN in the separation of the NT's, nonetheless the peaks of glutamate and GABA continued to overlap and, given that the retention time for both assays was of 2.72 minutes, there were no significant changes with the increase of the organic phase.

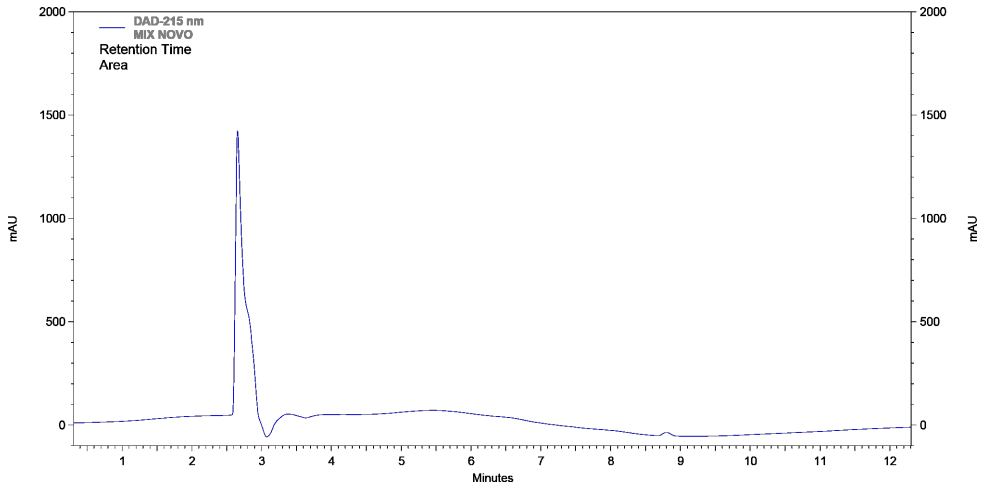


Figure 3.11: Chromatogram of the MIX assay using as mobile phase TFA 0.1% : ACN (50:70).

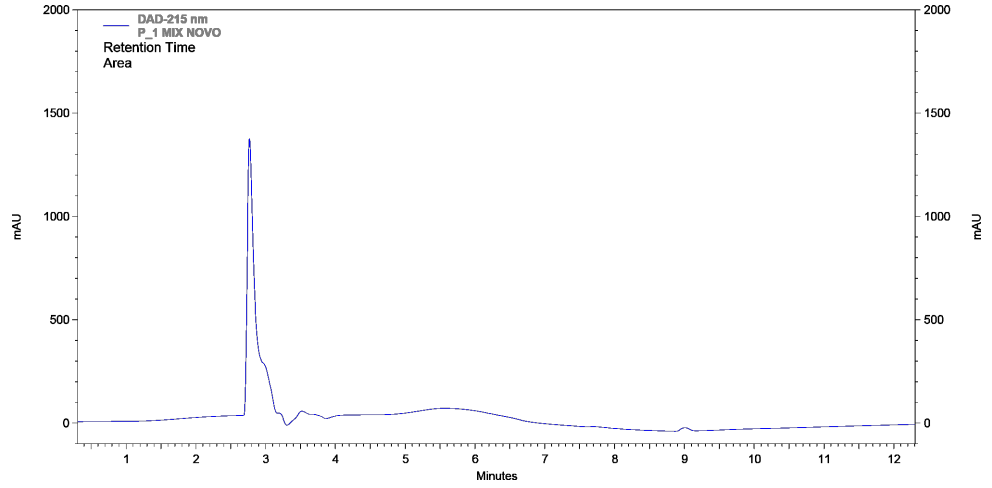


Figure 3.12: Chromatogram of the MIX assay using as mobile phase TFA 0.1% : ACN (50:80).

Given the inefficiency of these tested methods, we decided to create a controlled pH environment to ensure that both GABA and glutamate were in the optimal ionic form so that there would be some even more significant difference between them. The best pH value was between 5 and 6, because the charge of glutamate would be -1 and the charge of GABA would be 0. In order to test the effect of pH on the separation of the two NT, we decided to test different mobile phases using a Acetate buffer (acetic acid and sodium acetate) and an organic phase (methanol). The choice of buffer was made taking into account other methods already developed and the fact that the phosphate buffers may cause precipitates in the HPLC system. Given this, we decided to use methanol and an acetate buffer prepared from sodium acetate and acetic acid (pH 5.5, 0.1 M) in a ratio of 30:70%. Although there is a controlled environment of pH, the retention time of GABA and glutamate is 2.73 and 2.70 min, so in MIX assay the peaks continue to overlap (figure 3.13). In order to test the influence of temperature on the separation of the two NTs, we decided to repeat the same assay but increase the temperature of the column from 25 to 40°(figure 3.14), however there was no difference in the retention time of GABA and glutamate, just a change in the peak shape. It is possible to verify that at 3.75 min there is another peak with an area of approximately 50 mAU, nonetheless this peak is common to all chromatograms and is probably an impurity being detected.

MeOH:Acetate buffer (0.1 M, pH=5.5) - 30:70 % Column set at 25°C

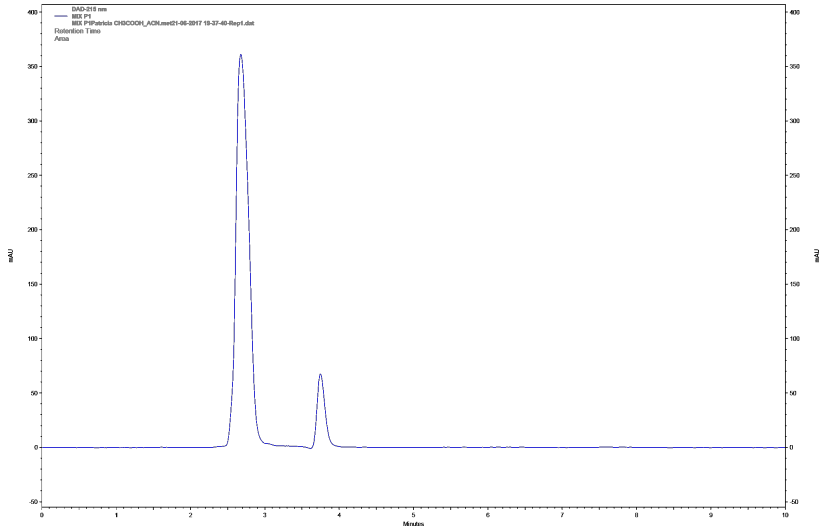


Figure 3.13: Chromatogram of the MIX assay using as mobile phase MeOH:Acetate buffer 0.1 M, pH=5.5 (30:70 %).

MeOH:Acetate buffer (0.1 M, pH=5.5) - 30:70 % Column set at 40°C

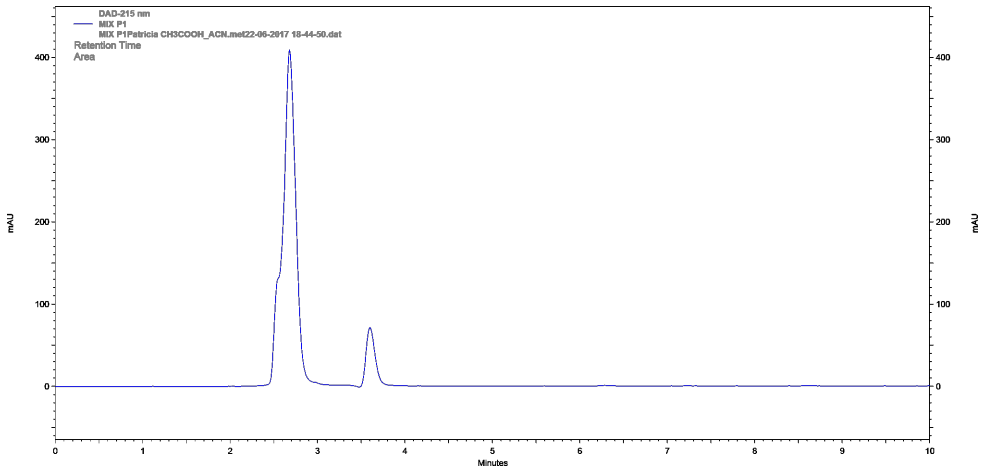


Figure 3.14: Chromatogram of the MIX assay using as mobile phase MeOH:Acetate buffer 0.1 M, pH=5.5 (30:70 %).

Given these results, we decided to test once more the effect of increasing the organic phase from 30% to 50% of methanol, however there was only a 0.06 second variation in the retention time of the NTs, and the peaks were still overlapped. The increase of the temperature to 40°did not provide significant variations and the retention time for both NTs decreased from 2.64 min to 2.61 min.

MeOH:Acetate acid buffer (0.1 M, pH=5.5) - 50:50 % Column set at 25°C

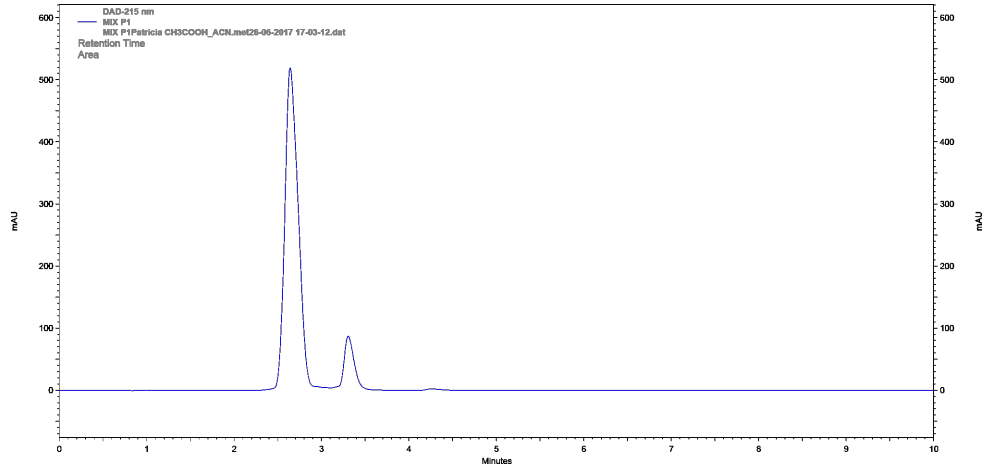


Figure 3.15: Chromatogram of the MIX assay using as mobile phase MeOH:Acetate buffer 0.1 M, pH=5.5 (50:50 %)

MeOH:Acetate acid buffer (0.1 M, pH=5.5) - 50:50 % Column set at 40°C

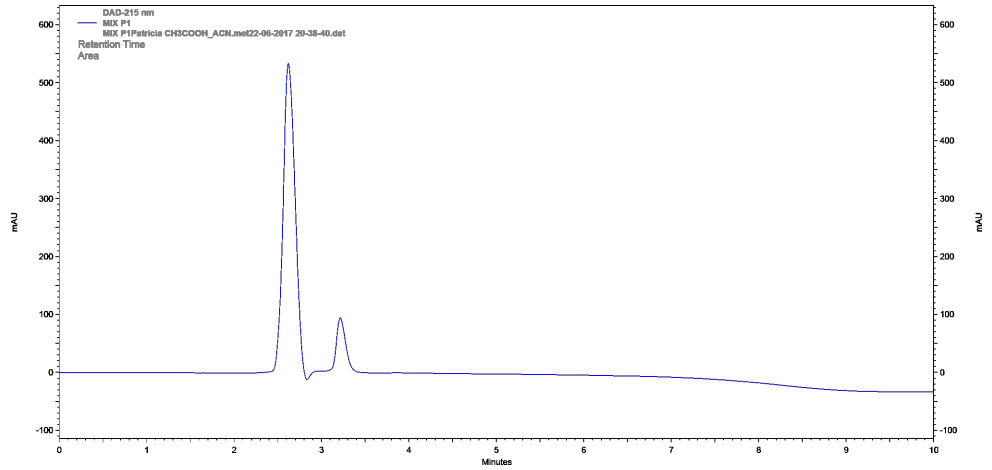


Figure 3.16: Chromatogram of the MIX assay using as mobile phase MeOH:Acetate buffer 0.1 M, pH=5.5 (50:50 %).

MeOH:Acetate buffer 0.1 M, 50:50 % - pH=6 Column set at 25°C

As we know that the optimal pH for GABA and glutamate to be in different ionic states is between 5.5 and 6, we decided to test if there was any change in the chromatograms with the increase of the buffers pH to 6 (figure 3.17), however there was no variation of the retention time of both NTs and the peaks appeared at 2.64 minutes.

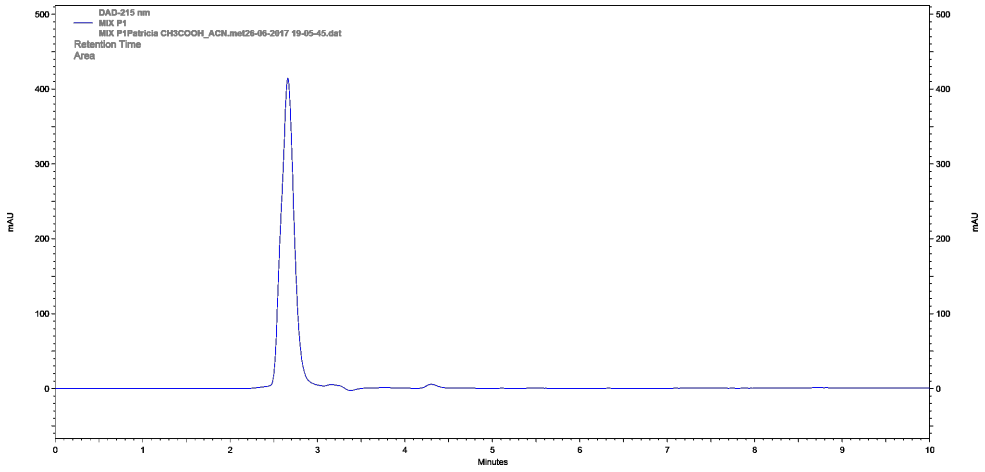


Figure 3.17: Chromatogram of the MIX assay using as mobile phase MeOH:Acetate buffer 0.1 M, pH=6 (50:50 %).

After the analysis of the results obtained, and since the separation of GABA and glutamate had not yet been achieved, we decided to dissolve the NT in UP H_2O (and thus create a solution with high polarity) and use as mobile phase ACN (less polar than methanol), hoping that this difference between polarities would be sufficient to vary the retention times of both NT. The pH value was measured to ensure that it was close to 5.5, that way the structural differences of GABA and glutamate were still enhanced.

Given that there was a small separation, we decided to opt for a gradient elution in order to separate completely the two peaks. The initial method started with 100% H_2O until 3 minutes. From 3 to 7 minutes the mobile phase was composed of 50% H_2O up and 50% ACN. At 7 minutes the mobile phase was again composed of 100% H_2O and the race ends at 10 minutes. The mobile phase was pumped at a flow rate of 1.00 mL/min through a 25°column.

Figure 3.18 shows a chromatogram of the MIX assay with two peaks, one at 2.56 min and the other at 2.76 min. The analysis of the chromatogram of the GABA assay shows a peak at 2.76 min, indicating that on the MIX assay, the second peak belongs to GABA. The same results happened for the glutamate assay, with it being detected at 2.53 min, corresponding to the first peak in the MIX assay.

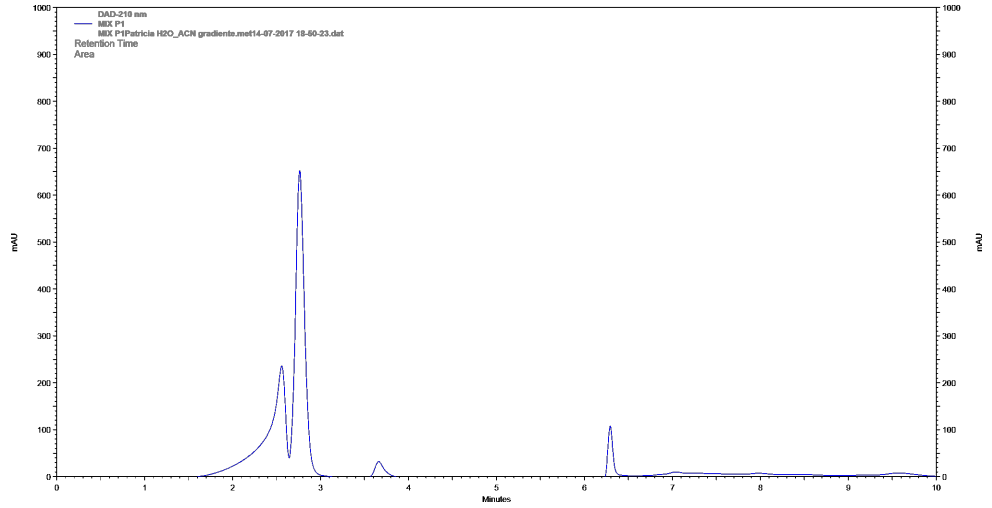


Figure 3.18: Chromatogram of the MIX assay using a gradient mobile phase of ACN:H_2O.

3.3.4 Optimization of the chosen method

Considering all the results obtained, the only mobile phase that allowed a clear separation of the peaks of GABA and glutamate was a gradient of H_2O and ACN (figure 3.18), with a flow rate of 1 mL/min and the temperature of the column at 25°C. Even though the separation of the two NT was achieved, there is no complete separation of the two peaks. The next step prior to the method validation is its optimization by adjusting the gradient and the column temperature.

3.3.4.1 Optimization of the gradient

A part of the optimization process is the choice of the most suitable gradient for the separation of GABA and glutamate. Knowing that the two NTs are detected between 2.5 and 3 minutes, we decided to test the best time for increasing the organic phase of the mobile phase. Considering the results obtained with the last method (gradient 1), two additional assays were performed where the composition of the mobile phase varied at 2.5 (gradient 3) and 4 minutes (gradient 2).

For the more concentrated solutions there are no significant variations between the different gradients and in all there is a difference of 20 seconds between the glutamate and GABA peaks. In assays 1 and 3 there is no change in the retention time of the two NTs (glutamate peak at 2.55 and GABA at 2.76 min), however in assay 2 there is an increase in the retention time (glutamate and GABA peaks at 3.21 and 3.41 minutes) as a consequence of the late entry of the organic phase into the mobile phase. In less concentrated solutions, there is a greater difference between the peaks of the two NTs in assay 3 (mean differences of 40 seconds) which makes this gradient the most appropriate. In

all assays, the retention time of glutamate decreases at each dilution while the retention time of GABA remains constant. This may be justified by the increase of the amount of water in the sample (since it is less concentrated), which leads to an increased affinity for the mobile phase and makes the elution happen faster, detecting glutamate progressively earlier.

3.3.4.2 Optimization of the temperature of the column

After the gradient was set to a temperature of 25°C we decided to repeat the same assay at 15, 20, 35 and 40°C to verify the optimal temperature for this method. Temperatures between 20 and 25°C originated peaks with higher resolution and more specific to both GABA and glutamate.
Considering these results, we defined as the mobile phase H_2O and ACN eluted in gradient (table III.2, gradient 2), at a flow rate of 1 mL/min, through a column set at 25°C for 10 minutes.

3.3.5 Method validation

Having defined the best temperature and gradient conditions, we proceeded to the preparation of the standard solutions. The concentration of the solutions was chosen so we could have in consideration both the hydrossolubility of glutamate/GABA and the normal values found in biological samples. A stock solution MIX was made with concentration of GABA and glutamate was of 4.325 mg/mL. From this solution, six successive dilutions of 1:500 were made. These samples were analyzed using the method defined above and different parameters were evaluated in order to validate the method.

3.3.5.1 Specificity

Specificity is defined by the ability of a method to detect a substance even in the presence of other structurally similar ones. Considering that GABA and glutamate have a very alike structure and that in the chromatogram both peaks are detected, we can conclude that the method allows for a specific detection of the two NT.

3.3.5.2 Linearity, LoD and LoQ

Table III.3 presents not only the LoD and LoQ values but also the linearity parameters of the calibration curves for GABA and glutamate.

Table III.3: Linearity, retention time, LoD and LoQ

	Glutamate ($\lambda = 210nm$)	GABA ($\lambda = 210nm$)
Retention time (min $\pm$ SD)	2.366 $\pm$ 0.215	2.773 $\pm$ 0.206
LoD ($\mu g/mL$)	0.166	0.271
LoQ ($\mu g/mL$)	0.503	0.822
Linearity		
Regression equation	y = 3x10^6x + 155179	y = 3x10^6x + 272759
Correlation coefficient (r^2)	0.973	0.995

The linearity of the method was studied using linear regression calculations. A regression equation was obtained and from this the LoD and LoQ values were calculated. It was also possible to calculate the correlation coefficient value (r^2), which allows to evaluate the linear relation between two variables (the closer to 1, the better the linearity). (Mukaka, 2012) Both r^2 values were close to 1, so the method developed presents good linearity. It also allows for a quantification and detection of GABA and glutamate in the order of the μg/mL, relatively low compared with other similar methods.

3.3.5.3 Accuracy - recovery rate

Tables III.4 and III.5 present the accuracy results of the method that is defined by the approximation of the concentration measured experimentally to its nominal/actual value. These results were obtained using the standard solutions described above and are presented in the form of a relative error (RE %).

Table III.4: Analytical results of accuracy test by standard solutions of glutamate.

True concentration	Mean concentration calculated	RE (%)	Number of samples
2,162	1,779	-17,72	6
1,0813	1,0859	0,42	6
0,5406	0,6454	19,39	6
0,2703	0,2233	-17,37	6
0,1352	0,0827	-38,86	6
0,0676	-0,0092	-113,54	6

RE% is calculated using the equation: RE % = ((Mean cal. conc. - True conc.) / True conc.) $\times$ 100%

Table III.5: Analytical results of accuracy test by standard solutions of GABA.

True concentration	Mean concentration calculated	RE (%)	Number of samples
2,15	2,3537	9,48	6
1,075	1,3069	21,57	6
0,5375	0,6489	20,73	6
0,2688	0,3185	18,49	6
0,1344	0,1366	1,64	6
0,0672	0,04130	-38,55	6

RE% is calculated using the equation: RE % = ((Mean cal. conc. - True conc.) / True conc.) $\times$ 100%

According to theory, the mean value obtained must be within 15% of the actual value. (Sonawane et al., 2014) The inaccuracy of the method for glutamate ranges from -113.54 to 19.39, with only one value within the 15% range. For GABA, the inaccuracy ranges from -38.55 to 21.57, with two values with the 15% range. Knowing this, we conclude that the accuracy parameter it is not validated for this method.

3.3.5.4 Precision

Tables III.6 and III.7 present the value of precision for this method in the form of the relative standard deviation (RSD %).

The value of precision for each concentration level of the GABA and glutamate standard solutions (2.1625 to 0.0676 mg/mL) must be inferior to 15% (Sonawane et al., 2014). The RSD (%) value for the standard solution of glutamate ranged from 5.43 to 21.74%, with two concentrations having results greater that 15%, whilst the RSD (%) value for the standard solution of GABA ranged from 1.40 to 4.19%. This means that the method shows better repeatability (intra-day precision) for the analysis of GABA than for the analysis of glutamate.

Table III.6: Analytical results for repeatability (intraday test) from standard solutions of glutamate

Concentration (mg/mL)	RSD (%)	No. of samples
2.1625	7.28	6
1.0813	15.86	6
0.5406	8.24	6
0.2703	5.43	6
0.1352	21.74	6
0.0676	7.97	6

RSD% is calculated using the equation: RSD % = (Standard deviation / Mean peak area) $\times$ 100%

Table III.7: Analytical results for repeatability (intraday test) from standard solutions of GABA

Concentration (mg/mL)	RSD (%)	No. of samples
2.15	1.91	6
1.075	2.46	6
0.5375	4.19	6
0.2688	1.40	6
0.1344	1.87	6
0.0672	1.51	6

RSD% is calculated using the equation: RSD % = (Standard deviation / Mean peak area) $\times$ 100%

3.3.6 Conclusions

After testing different mobile phases, we conclude that the best mobile phase for the quantification of glutamate and GABA is a gradient of H_2O:ACN, at a flow rate of 1 mL/min, at 25°and with detection at 210 nm. Although the method does not present good accuracy, it presents a good linearity, specificity and precision, especially for the quantification of GABA. This method provides a value of LoD and LoQ in the order of the μg/mL for both NT, obtained from a calibration curve with a correlation coefficient of 0.973 for glutamate and 0.995 for GABA. The analysis time is 10 minutes, allowing a rapid analysis of the two molecules.

Chapter IV

Application of the developed method for the quantification of GABA and glutamate

The objective of this chapter was to evaluate the performance of the method described and developed in Chapter III for the quantification of glutamate and GABA when applied to biological matrices such as yeast, urine and serum extract.

GABA and glutamate are two NTs that can be quantified predominantly in CSF, urine, serum/plasma of the blood, among others. In Table IV.1 are described some methods for the quantification of GABA and glutamate, presenting data regarding concentrations obtained, type of analysis and type of sample in both normal and pathological conditions. Basal concentrations of GABA and glutamate may vary depending on the type of sample and other factors that may increase/reduce the production of these NTs. Although protein content is lower than in serum, CSF is widely used for the development of several biomarkers for different diseases, since it is in direct contact with the extracellular space of the brain, better reflecting the pathophysiological changes. (Blennow et al., 2015) Blood serum and plasma are not only easier to obtain but also have a higher reproducibility of results. In addition, the detection of biomarkers in the serum shows a high sensitivity, explained by the high concentration of metabolites. (Yu et al., 2011) Urine collection requires non-invasive methods and although there is information that says it is a source of unreliable results (Ryberg et al., 2004), it is known that, unlike blood, urine is not subject to homeostatic mechanisms, better reflecting the changes that exist in the human body. There are also authors who consider that the biomarkers found in urine can be useful for the diagnosis and study of brain diseases. (An and Gao, 2015) Although yeast (*Saccharomyces cerevisiae*) has a very different cellular complexity when compared to human neurons, some basic mechanisms (oxidative stress, protein aggregation, apoptosis and mitochondrial damage) involved in the development of neurodegenerative diseases were well preserved, proving that yeast is a good model for the study of neurodegenerative diseases and that its extract can be a good source of biomarkers. (Miller-Fleming et al., 2008)

Table IV.1: GABA and glutamate concentrations in different biological matrices, quantified using different methodologies.

Reference	Sample	Concentration	Method
(de Freitas Silva et al., 2009)	Rodent brain	GABA - 0,22 μg/mL Glut - 4,86 μg/mL	HPLC-FL
(Huisman et al., 2010)	Rat urine	GABA - 10,5 mg/dL of creatinine	ELISA
(Defaix et al., 2018)	Mice frontal córtex	Glut - 1,25 ng/mL GABA - 0,63 ng/mL	HILIC-MS/MS
(Zhang et al., 2005)	Dialysates	Glut - 0,8 pmol GABA - 0,03 pmol	HPLC-ECD
(Kaul et al., 2011)	Dialysates	Glut - 6 nmol/L GABA - 10 nmol/L	Capillary electrophoresis-FLD
(Lee et al., 2010)	Serum	Glut - 12,76 mg/g tissue GABA - 66,85 mg/g tissue	HPLC-FLD
(Buck et al., 2009)	Dialysates	Glut - 100 nmol GABA - 48 nmol	LC-MS/MS
(Monge-Acuña and Fornaguera-Trías, 2009)	Rat brain homogenates	Glut - 0,88 μg/mg of tissue GABA - 1,06 μg/mg of tissue	HPLC-ECD
(González et al., 2011)	Rat brain homogenates	Glut - 1000 μg/g of tissue GABA - 30 μg/g of tissue	UHPLC–MS/MS
(O'Byrne et al., 2011)	Bacterial cells	GABA - 1,12 mM	Enzymatic microtiter plate assay

4.1 Materials and methods

4.1.1 Instrument and software

The instruments and software used for the development of the method are described in 3.2.1.

4.1.2 Reagents and consumables

The reagents and consumables used for the development of the method are described in 3.2.2.

4.1.3 Analytical procedure

4.1.3.1 Mobile phase

The mobile phase was composed by H_2O and ACN which were eluted in a gradient described in table III.1. Both H_2O and ACN were filtered through a 0.45 μm membrane.

4.1.3.2 Calibration standards

For calibration purposes we prepared a stock solution of glutamate and GABA diluted in water, with the concentration of 4.3 mg/mL. This solution was filtered through a mem-

brane filter device and from this six successive dilutions of 1:500 were made, originating six solutions with concentrations of 0.0675, 0.2703, 0.5406, 1.0812 and 2.162 mg/mL.

4.1.3.3 Sample preparation

The preparation of the urine (1) and serum (2) samples was the same, being the first step the addition of 1 ml of 15% TFA (freshly prepared) to 1 ml of each sample to promote protein precipitation. (Peixoto, 2012) After vortexing for 20 seconds, the samples were centrifuged at 14.5 rpm for 10 minutes. After this procedure, the supernatant was separated from the pellet, filtered and spiked with a MIX solution (equal concentrations of GABA and glutamate - 1,081 mg/ml). Simultaneously, non-spiked samples were tested. Yeast samples (*S. cerevisiae*) were inoculated into yeast extract peptone dextrose (YPD, VWR), incubated overnight at 37and adjusted to an optical density (OD) at 600 nm between 0.08 and 0.1 using sterile UP water. After this, the yeast grew at 30°C at 120 rpm to an optical density between 1.502 and 1.902. To promote cell lysis and the removal of their content, the cells passed through a freezing(-80°)/thawing (room temperature) cycle for 20 minutes and then sonicated for 10 minutes (Silent Crusher at 47-63 Hz). After this procedure, the samples were treated according to the same protocol applied to the serum and urine samples.

(1) Serum A - Randox - Human precision control level 2 (UE 1557); Serum B - Randox - Human precision control level 3 (UE 1558)
(2) Urine - Cornay - Urine control level 1 (5-161)

4.1.3.4 Chromatographic conditions

The chromatographic conditions used are described in 3.2.3.1.

4.2 Results

The purpose of this chapter is to verify if the method developed in the previous chapter allows the quantification of GABA and glutamate in different biological matrices where these two NT's are available. Urine, serum and yeast extract assays were performed, however, it was not possible to obtain any specific peak of GABA and glutamate for any of the assays, thus not allowing the validation of the method. In figure 4.2 we can see the chromatograms obtained for the non-spiked samples and in the figure 4.1 the chromatograms

obtained using samples spiked with solutions of known concentration of GABA and glutamate. In terms of the results, serum A and B present similar chromatograms, something to be expected since their constitution is the same. There are two peaks common to the four existing assays in both spiked and non-spiked samples, detected at 4.5 and 6.10 minutes. This leads us to believe that those are the GABA and glutamate peaks (as we verified in the absorption spectrum), however, the spiked samples have a lower peak area than the peak area of the non-spiked samples, which makes it doubtful if those two peaks belong to the two NT's. In addition, with the method developed in Chapter III, GABA and glutamate were detected before the 3 minutes mark so if there were two peaks for the two NT's they should be detected between 2 and 3 minutes, something that is not visible in none of the chromatograms of figures 4.1 and 4.2.

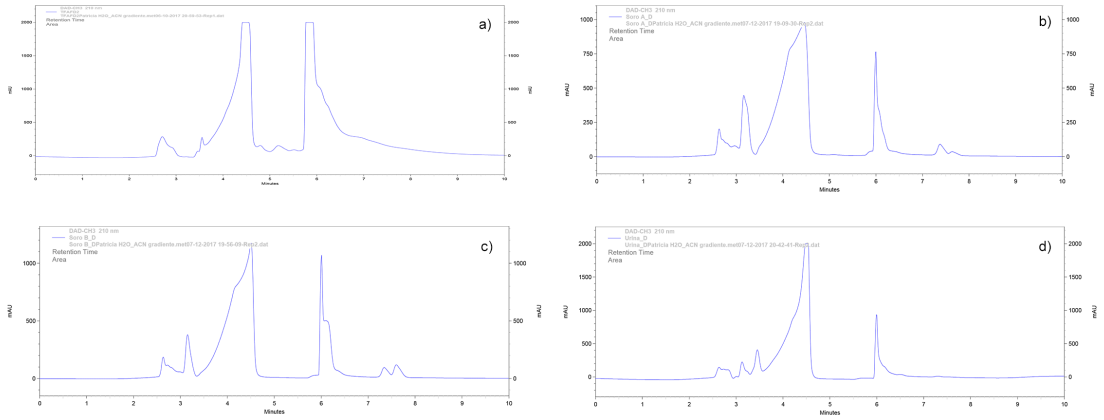


Figure 4.1: Chromatograms obtained by the analysis of a) yeast extract, b) serum A, c) serum B and d) urine samples, spiked with glutamate and GABA.

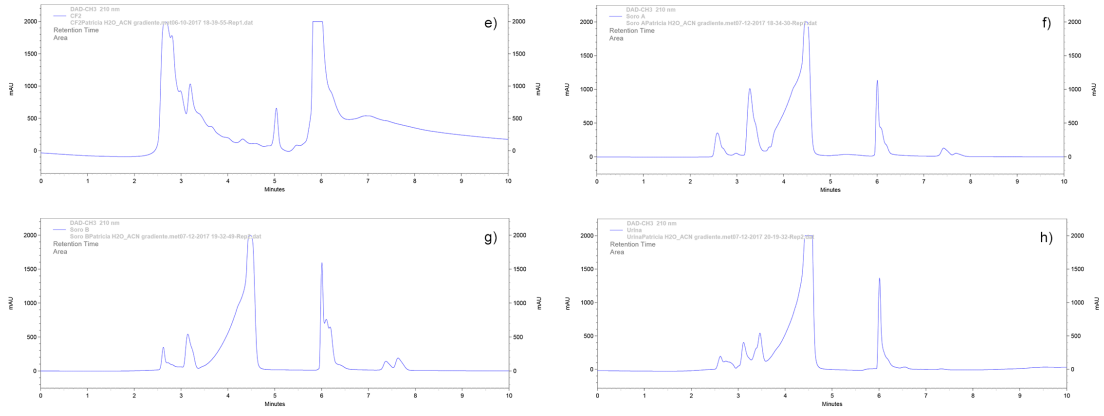


Figure 4.2: Chromatograms obtained by the analysis of e) yeast extract, f) serum A, g) serum B and h) urine samples.

These results can be justified by the difference of the pH value between the method developed for the standard solutions and the method used for biological samples. In

order to promote protein precipitation, 1 mL of a 15% TFA solution prepared on the same day (as older solutions may lead to erroneous results) was added to the samples. At the time of the development of the method it was concluded that the ideal pH of the mobile phase for the separation and quantification of the two NT's was between 5.5 and 6. The acidification of the samples with TFA (which is a very strong acid) lowers the pH of the samples to a value of approximately 2 and this difference may be sufficient to not allow the detection of GABA and glutamate in the different matrices tested.

Even though the parameters necessary for the validation of a method were validated (chapter III), it was not possible to apply the protocol developed to the biological matrices tested. In order to prove the applicability and the efficacy of the method, it is necessary to readjust the sample preparation protocol, replacing the acidification step with another alternative.

Chapter V

Discussion

5.1 Main conclusions

The major goal of this work is to study the triad depression - neurodegenerative diseases - NT. In a search using these key words, we have found that studies that focus on these three topics simultaneously, explore the role of the absence of serotonin (which causes symptoms of depression) and dopamine in the development of various neurodegenerative diseases. When we realized that depression was one of the main secondary symptoms of this type of illness, we decided to do a meta-analysis not only to better understand the effect of depression medication in the patogenic process, but also to quantify the percentage of patients with neurodegenerative diseases that are prescribed with antidepressants. The percentage value ranged from 26.3 to 45.9%, nonetheless there are mixed opinions regarding the benefits of the prescription of antidepressants to patients with neurodegeneration. These results, and the scarcity of more varied studies, justified the study of the role of other NT in the development of neurodegenerative diseases and the appearance of symptoms of depression.

We decided to focus on studying the main excitatory and inhibitory NT and develop a method that allows them to be quantified in a simple, fast and efficient way, facilitating future studies in the field of neurosciences. Since HPLC is a strong analytical technique used to separate, identify and quantify different components in a mixture, its use is increasingly more frequent and the equipment necessary for its application is nowadays found in the most different laboratories.

Knowing that GABA and glutamate may play a relevant role in the diagnosis and treatment of the most varied neurodegenerative diseases and their secondary symptoms such as depression, a gradient method based on HPLC-DAD was developed using as mobile phase H_2O and ACN. The method developed was based on another method for the detection of other amino acids and underwent changes varying parameters such as pH, constitution of the mobile phase and the type of elution, in order to allow the separation of GABA and glutamate. This method is not only providing a simple and fast analysis (10 minutes with detection of the two NTs between 2.5 and 3 minutes) but also the prac-

tice of a sustainable chemistry since in 10 mL of mobile phase only 3.15 mL are organic phase. After an optimization process, the method was validated using standard samples of known concentrations. For both NT's the method had a LoD and LoQ in the order of μg/mL and presents good linearity and specificity. As far as GABA detection is concerned, the method also shows good accuracy.

In order to test its applicability, the method was tested in different biological matrices such as samples of serum, urine and yeast extract, however, no specific peak of GABA or glutamate could be obtained. One of the reasons for lack of specific peaks might be the differences between the pH value of the standard samples (pH=5.5) and the pH value of the biological samples (pH=2) given that some of the mobile phases tested on chapter III had TFA and presented overlap of the peaks. Having said this, in the future, a protocol for treating samples without acidification or using a weaker acid has to be developed. The problem that arises from this process is that without acidification of the sample there is no protein precipitation and, consequently, it is not possible to quantify GABA or glutamate. A weaker acid could raise the pH value but it might not be enough since the optimal range is between 5.5 and 6.

Although the method still lacks optimization of some parameters, the continuity of this study is very important, since to this date there is no record of any rapid analysis method with DAD detection that does not resort to the process of derivatization of the sample. There are methods for detecting the two NTs separately however, considering that both GABA and glutamate have a very important role in the homeostasis of the organism, a method allowing the detection of the two NT's simultaneously is not only better but also allows for a more complete and economic analysis.

5.2 Future perspectives

In order to give applicability to the method, it is necessary to solve the problem of the pH differences between the standard samples and the biological samples and to optimize other parameters. The simplest solution would be to neutralize the sample by using a solution of sodium hydroxide to obtain the desired pH and to enable a test that guarantees that the conditions of the analysis of the biological samples are as similar as possible to the standard tests.

The next step may be to substitute ACN for methanol, which is cheaper and more eco-friendly. If the polarity variation affects the effectiveness of the method, another alterna-

tive is to reduce the percentage of organic phase used. If the method is still not validated for the biological matrices, one must return to the validation step for the standard samples and try to vary other parameters in order to be able to move forward again.
After the validation step is complete, it would be interesting to see if the method is also applicable to non-synthetic samples drawn from animal models.

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