UNIVERSIDADE DA BEIRA INTERIOR
Ciências

Bioanalytical tools for unraveling part of the puzzle of the biochemical phenomena in epilepsy: neuromediators and antiepileptic drugs

Beatriz Marques da Fonseca

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Orientador: Professor Doutor Gilberto Lourenço Alves

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RESUMO ALARGADO

As primeiras descrições de epilepsia remontam a 3000 anos A.C., a qual, ao longo da história, esteve ligada a fatores divinos e, somente em meados do século XIX, foi amplamente aceite como uma patologia com origem no cérebro. Assim, sumariamente, a epilepsia é uma doença do cérebro que envolve crises não provocadas recorrentes, as quais surgem devido a uma atividade neuronal anormal excessiva ou sincronizada. Embora o conhecimento científico tenha evoluído tremendamente nas últimas décadas, particularmente no campo das neurociências, acredita-se que ainda estamos longe de conhecer detalhadamente os fenómenos neurobioquímicos e moleculares intrínsecos à complexidade dos processos subjacentes a esta doença. De fato, uma compreensão mais profunda dos mecanismos que levam ao desenvolvimento da epilepsia (epileptogénese) e daqueles subjacentes à ictogénese será certamente favorável para encontrar novas abordagens terapêuticas, capazes de melhorar a supressão das crises epiléticas, particularmente em doentes que desenvolvem fenótipos de epilepsia farmacorresistente, mas também capazes de modificar, e se possível interromper, a cascata de alterações fisiopatológicas moleculares, estruturais e funcionais que ocorrem no cérebro durante a epileptogénese.

Dada a escassez de informação sobre os fenómenos neurobiológicos subjacentes à resposta farmacológica, bem como sobre os processos bioquímicos e moleculares responsáveis pela resistência a fármacos e pela epileptogénese, novos estudos são imperativos para contribuírem para o aumento do conhecimento acerca destes tópicos. O envolvimento de neuromediadores na iniciação e propagação das crises epiléticas é uma realidade cada vez mais reconhecida e a determinação quantitativa dessas substâncias endógenas será fundamental para melhorar o nosso conhecimento ao nível neurobioquímico, possibilitando uma melhor compreensão da etiopatogénese da epilepsia e também dos mecanismos de ação de fármacos antiepiléticos. Portanto, neste contexto, a bioanálise terá um papel primordial para ajudar a interpretar os resultados obtidos em muitos estudos de investigação desenvolvidos neste campo. Consequentemente, o objetivo global deste projeto de doutoramento foi desenvolver ferramentas bioanalíticas simples e fiáveis para a determinação de uma série de neuromediadores chave envolvidos na neurotransmissão e na excitabilidade neuronal. Além disso, o desenvolvimento de uma nova ferramenta bioanalítica para quantificar simultaneamente fármacos antiepiléticos importantes e alguns agentes quimioconvulsivantes amplamente utilizados foi também considerado.

Assim, o trabalho de investigação que sustenta esta tese de doutoramento começou com o desenvolvimento e validação de uma técnica analítica de cromatografia líquida de alta resolução acoplada à deteção por fluorescência (HPLC-FLD) para a quantificação simultânea de várias catecolaminas e compostos endógenos relacionados (dopamina, norepinefrina, epinefrina, ácido homovanílico, levodopa e 3-*O*-metildopa) em tecido cerebral de rato.

Posteriormente, outra metodologia utilizando o mesmo sistema analítico (HPLC-FLD) foi também desenvolvida para a determinação de cinco aminoácidos neuroativos (glutamato, aspartato, taurina, glutamina e ácido gama-aminobutírico) em tecido cerebral de rato; no entanto, neste caso, foi requerido um passo de derivatização pré-coluna porque estes aminoácidos não possuem fluorescência nativa.

Além disso, para realizar análises mais integradas dos efeitos anticonvulsivantes e convulsivantes em estudos não clínicos, será imprescindível a disponibilidade de ferramentas bioanalíticas que possibilitem a determinação simultânea dos fármacos antiepiléticos-alvo e de agentes convulsivantes. Portanto, no âmbito desta tese, foi também desenvolvido um método de cromatografia líquida acoplada à deteção por um sistema de díodos para a determinação simultânea, em amostras de plasma e cérebro de rato, três antiepiléticos estabelecidos (levetiracetam, zonisamida e lamotrigina) e dois importantes agentes quimioconvulsivantes (pentilenotetrazol e pilocarpina) frequentemente usados para induzir experimentalmente crises agudas e/ou epilepsia crónica em modelos animais.

Este conjunto de ferramentas bioanalíticas pode permitir o estudo de alterações neuroquímicas que ocorrem em várias regiões cerebrais associadas à epilepsia e/ou crises epiléticas, podendo assim ajudar a compreender múltiplos aspetos ainda pouco claros no campo das neurociências. Portanto, os resultados obtidos com os trabalhos apresentados nesta tese de doutoramento podem fornecer suporte quantitativo para futuros estudos não clínicos que visem aprofundar o conhecimento sobre os mecanismos neurobioquímicos subjacentes aos fenómenos de epileptogénese e de ictogénese, bem como à ação de fármacos antiepiléticos e de agentes quimioconvulsivantes. Finalmente, este trabalho representa uma contribuição pequena, mas útil, para desvendar parte do *puzzle* dos mecanismos neurobioquímicos complexos envolvidos na epilepsia.

Palavras-chave:

Aminoácidos neuroativos, antiepiléticos, bioanálise, catecolaminas, convulsivantes, cromatografia líquida, epilepsia, neuromediadores, rato.

ABSTRACT

The earliest descriptions of epilepsy date back to 3000 years B.C., which was, throughout history, linked to divine factors, and only in the mid-nineteenth century it was widely accepted as a pathology originating in the brain. Thus, summarily, epilepsy is a disease of the brain involving recurrent unprovoked seizures, which arise due to an abnormal excessive or synchronous neuronal activity. Although the scientific knowledge has evolved tremendously over the last decades, particularly in the field of neurosciences, it is believed that we are still far from knowing in detail the neurobiochemical and molecular phenomena intrinsic to the complexity of the processes underlying this disease. In fact, a deeper understanding of the mechanisms that lead to the development of epilepsy (epileptogenesis) and those underlying the ictogenesis will be certainly favorable to find novel therapeutic approaches, capable of improving the suppression of seizures, particularly in patients who develop phenotypes of drug-resistant epilepsy, but also capable of modifying, and if possible interrupting, the cascade of molecular, structural and functional pathophysiologic changes that occur in brain during epileptogenesis.

Given the scarcity of information on the neurobiological phenomena underlying the pharmacological response, as well as on the biochemical and molecular processes responsible for drug resistance and epileptogenesis, new studies are imperative to contribute to increase knowledge about these topics. The involvement of neuromediators in the initiation and spread of epileptic seizures is an increasingly recognized reality and the quantitative determination of these endogenous substances will be fundamental to improve our knowledge at neurobiochemical level, thus enabling a better understanding of the etiopathogenesis of epilepsy and the mechanisms of action of antiepileptic drugs as well. Therefore, in this context, bioanalysis will play a vital role in supporting and helping to interpret the results obtained from many research studies developed in this field. Consequently, the global aim of this doctoral project was to developed simple and reliable bioanalytical tools for the determination of a series of key neuromediators involved in the neurotransmission and neuronal excitability. In addition, the development of a novel bioanalytical tool to simultaneously quantify important antiepileptic drugs and some widely used chemoconvulsant agents was also considered.

Thus, the research work supporting this doctoral thesis began with the development and full validation of an analytical technique of high-performance liquid chromatography coupled to fluorescence detection (HPLC-FLD) for the simultaneous quantification of several catecholamines and related endogenous compounds (i.e., dopamine, norepinephrine, epinephrine, homovanillic acid, levodopa and 3-*O*-methyldopa) in rat brain tissue. Posteriorly, another methodology using the same analytical system (HPLC-FLD) was also developed for the determination of five neuroactive amino acids (i.e., glutamate, aspartate, taurine, glutamine

and gamma-aminobutyric acid) in rat brain tissue; however, in this case, a precolumn derivatization step was required because these amino acids do not have native fluorescence.
Moreover, to perform more integrated analyses of the anticonvulsant and convulsant effects in nonclinical studies it will be essential the availability of bioanalytical tools that enable the simultaneous determination of the target antiepileptic drugs and convulsant agents. Therefore, within the scope of this thesis, it was also developed a liquid chromatography method coupled to diode array detection for the simultaneous determination, in plasma and rat brain samples, three established antiepileptics (levetiracetam, zonisamide and lamotrigine) and two important chemoconvulsant agents (pentylenetetrazole and pilocarpine) frequently used to experimentally induce acute seizures and/or chronic epilepsy in whole-animal models.
This set of bioanalytical tools may allow the study of neurochemical changes that occur in several brain regions associated with epilepsy and/or epileptic seizures, and thus can help to understand multiple aspects still unclear in the field of neurosciences. Hence, the achievements obtained with the work presented in this doctoral thesis may provide quantitative support for future nonclinical studies aimed at deepening the knowledge about the neurobiochemical mechanisms underlying the epileptogenesis and ictogenesis phenomena, as well as the action of antiepileptic drugs and chemoconvulsant agents. Finally, this work represents a small but useful contribution to unravel part of the puzzle of the complex neurobiochemical mechanisms involved in epilepsy.

Keywords:

Antiepileptic drugs, bioanalysis, catecholamines, convulsant agents, epilepsy, liquid chromatography, neuroactive amino acids, neuromediators, rat.

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LIST OF ABBREVIATIONS

A

Ach	Acetylcholine
AED	Antiepileptic drug
AMPA	α-Amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid
Asp	Aspartate (or aspartic acid)
ATP	Adenosine triphosphate

B

BBB	Blood-brain barrier
BH4	Tetrahydrobiopterin
BME	ß-mercaptoethanol
BRV	Brivaracetam
BZD	Benzodiazepine

C

CBZ	Carbamazepine
CLB	Clobazam
CLZ	Clonazepam
CNS	Central nervous system
COMT	Catechol-*O*-methyltransferase
CV	Coefficient of variation

D

DA	Dopamine
DAD	Diode array detection
DGAV	Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (*Direção Geral de Alimentação e Veterinária*)
DHBA	3,4-Dihydroxybenzylamine
DOPAC	3,4-Dihydroxyphenylacetic acid
DOPEG	3,4-Dihydroxyphenylglycol
DZP	Diazepam

E

E	Epinephrine
ECD	Electrochemical detection
EDTA	Ethylenediaminetetraacetic acid

EEG	Electroencephalogram
EMA	European Medicines Agency
EPSP	Excitatory postsynaptic potential
ESI	Electrospray ionization
ESL	Eslicarbazepine acetate
ESM	Ethosuximide

F

FBM	Felbamate
FDA	Food and Drug Administration
FLD	Fluorescence detection

G

GABA	γ-Aminobutyric acid
GAD	Glutamic acid decarboxylase
GAERS	Genetic absence epilepsy rat from Strasbourg
GBP	Gabapentin
GC	Gas chromatography
Gln	Glutamine
Glu	Glutamate (or glutamic acid)
GPCR	G-protein-coupled receptor

H

HFBA	Heptafluorobutyric acid
HILIC	Hydrophilic interaction liquid chromatography
5-HIAA	5-Hydroxyindoleacetic acid
5-HT	5-Hydroxytryptamine (or serotonin)
HPLC	High-performance liquid chromatography
HVA	Homovanillic acid

I

ILAE	International League Against Epilepsy
IPSP	Inhibitory postsynaptic potential
IS	Internal standard

K

KA	Kainic acid (or kainate)

L

LC	Liquid chromatography
LCM	Lacosamide

L-DOPA	L-3,4-Dihydroxyphenylalanine (or levodopa)
LEV	Levetiracetam
LLE	Liquid–liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LTG	Lamotrigine

M

m/*z*	Mass-to-charge ratio
MAO	Monoamine oxidase
MES	Maximal electroshock
MHPG	3-Methoxy-4-hydroxyphenylglycol
3-*O*-MD	3-*O*-Methyldopa
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

N

NBD-F	4-Fluoro-7-nitrobenzofurazan
NE	Norepinephrine
NMDA	*N*-Methyl-D-aspartic acid

O

ODS	Octadecylsilane
OPA	*O*-Phtalaldehyde
OSA	Octanesulfonic acid
OXC	Oxcarbazepine

P

PB	Phenobarbital
PER	Perampanel
PHT	Phenytoin
PIL	Pilocarpine
PPT	Protein precipitation
PRM	Primidone
PTZ	Pentylenetetrazole

Q

QC	Quality control

R

RFM	Rufinamide
RTG	Retigabine

S

scPTZ	Subcutaneous pentylenetetrazole
SE	*Status epilepticus*
SPE	Solid-phase extraction
STP	Stiripentol
SV2A	Glycoprotein 2A

T

Tau	Taurine
TBI	Traumatic brain injury
TGB	Tiagabine
TLE	Temporal lobe epilepsy
TMD	Trimethadione
TPM	Topiramate
Trp	Tryptophan
Tyr	Tyrosine

U

UFLC	Ultra-fast liquid chromatography
UV	Ultraviolet

V

VGB	Vigabatrin
VMA	Vanillylmandelic acid
VNS	Vagus nerve stimulation
VPA	Valproic acid (or valproate)

W

WHO	World Health Organization

Z

ZNS	Zonisamide

LIST OF PUBLICATIONS

Fonseca, BM., Rodrigues, M., Cristóvão, AC., Gonçalves, D., Fortuna, A., Bernardino, L., Falcão, A., and Alves, G. (2017). Determination of catecholamines and endogenous related compounds in rat brain tissue exploring their native fluorescence and liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci *1049-1050*, 51-59. doi: 10.1016/j.jchromb.2017.02.028

Fonseca, BM., Cristóvão, AC., and Alves, G. (2018). An easy-to-use liquid chromatography method with fluorescence detection for the simultaneous determination of five neuroactive amino acids in different regions of rat brain. J Pharmacol Toxicol Methods. *91,* 72-79. doi: 10.1016/j.vascn.2018.02.002

Fonseca, BM., Rodrigues, M., and Alves, G. (2018). First HPLC method for the simultaneous quantification of levetiracetam, zonisamide, lamotrigine, pentylenetetrazole and pilocarpine in rat plasma and brain samples. Anal Methods. *10*, 515-525. doi: 10.1039/C7AY02602A

LIST OF COMMUNICATIONS

Oral communications

Authors: da Fonseca B, Cristóvão AC, Alves G.
Title: A new HPLC-FLD method for the simultaneous determination of aspartate, glutamate, glutamine, taurine and gamma-aminobutyric acid in rat brain.
Local and date: XII Annual CICS-UBI Symposium; Covilhã, 6 e 7 julho de 2017.

Authors: da Fonseca B, Rodrigues M, Alves G.
Title: Simultaneous determination of three antiepileptics and two convulsant compounds in rat plasma and brain samples using HPLC-DAD.
Local and date: XII Annual CICS-UBI Symposium; Covilhã, 6 e 7 julho de 2017.

Authors: Beatriz Fonseca, Márcio Rodrigues, Ana Cristóvão, Ana Fortuna, Liliana Bernardino, Amílcar Falcão, Gilberto Alves.
Title: HPLC-FLD method for the simultaneous determination of catecholamines and related-compounds in rat brain tissue.
Local and date: SPF 2016 - XLVI Reunião Anual da Sociedade Portuguesa de Farmacologia, XXXIV Reunião de Farmacologia Clínica, XV Reunião de Toxicologia; Porto, 4 fevereiro de 2016.

Authors: Beatriz M. Fonseca, Márcio Rodrigues, Ana C. Cristóvão, Ana Fortuna, Liliana Bernardino, Amílcar Falcão, Gilberto Alves.
Title: Quantitative analysis of several catecholamines and related-compounds in rat brain samples using their native fluorescence and liquid chromatography.
Local and date: I Congress in Health Sciences Research: Towards Innovation and Entrepreneurship; Covilhã, 26 a 28 novembro de 2015.

Poster communications

Authors: Beatriz da Fonseca, Sandra Ventura, Márcio Rodrigues, Gilberto Alves.
Title: A novel HPLC-DAD method for simultaneous quantification of levetiracetam, zonisamide, lamotrigine, pentylenetetrazole and pilocarpine in rat plasma and brain samples.
Local and date: 32nd International Epilepsy Congress; Barcelona, 2 a 6 setembro de 2017.

Authors: Beatriz da Fonseca, Sandra Ventura, Ana C. Cristóvão, Gilberto Alves.

Title: Simultaneous quantification of five neuroactive amino acids in different regions of rat brain by HPLC-FLD.

Local and date: 32nd International Epilepsy Congress; Barcelona, 2 a 6 setembro de 2017.

CHAPTER I – General introduction

I.1. Epilepsy

I.1.1. Historical perspective

Epilepsy was one of the first brain disorders to be described. More than 3000 years ago, in ancient Babylon, epilepsy was described as a condition representing an evil state of mind or possession **(Eadie, 1995; Goldenberg, 2010; Wahab, 2010)**. The term "epilepsy" is derived from the Greek word *epilambanein*, meaning to attack or seizure **(Baloyannis, 2013; Goldenberg, 2010)**. People once thought that epileptic individuals were being invaded by evil spirits, demons or gods.

Later, in 400 B.C., Hippocrates suggested that epilepsy was a disorder of the brain and epileptic seizures were generated there as a consequence of various natural etiologies **(Eadie, 1995; Goldenberg, 2010)**. Although Hippocrates and his followers regarded the epilepsy as a physical disorder due to natural causes, a multitude of supposed supernatural causes of epilepsy strongly prevailed until the nineteenth century, period from which natural philosophers and physicians initiated to undertake studies of brain function **(Pitkänen and Engel, 2014)**. Indeed, an important contribution was made by Hughlings Jackson in 1875, who recognized epilepsy as the result of abnormal brain discharges **(Eadie, 1995; Wahab, 2010)**. Since then, there have been notable advances in genetics, molecular biology, neurophysiology, and functional imaging and neurochemical techniques, which have expanded the Jackson's concepts.

I.1.2. Epidemiology

Approximately 50 million people are currently living with epilepsy worldwide. The estimated proportion of the general population with active epilepsy (i.e. continuing seizures or with the need for treatment) is from 4 to 10 per 1,000 population. Some studies suggest that this proportion is much higher in low- and middle-income countries, with a value of 7 to 14 per 1,000 people **(WHO, 2018)**.

According to the World Health Organization (WHO), it is estimated that around 2.4 million people are diagnosed with epilepsy each year. In high-income countries, annual new cases are between 30 and 50 per 100,000 people in the general population, whereas in low- and middle-income countries this number can be up to two times higher. This higher incidence of epilepsy in low- and middle-income countries is likely to be associated with the increased risk of endemic conditions, such as malaria or neurocysticercosis; the higher incidence of road traffic injuries

and birth-related injuries; and worse conditions in terms of medical infrastructures, lower availability of preventive health programs and accessible care. Indeed, nearly 80% of the patients with epilepsy live in low- and middle-income countries **(WHO, 2018)**.

As regards epilepsy in Europe, it is estimated that it affects between 2.6 and 6 million people **(Abad et al., 2011; Gustavsson et al., 2011)**. The disparity of these estimates highlights the lack of sufficiently accurate epidemiological data on the prevalence of epilepsy. Indeed, population-based studies are missing from the majority of European countries. However, taking into consideration the available data, the prevalence of active epilepsy in Europe varies across different countries from 3.3 to 7.8 per 1,000 inhabitants in the general population and from 3.2 to 5.1 per 1,000 inhabitants in pediatric studies **(Forsgren et al., 2005)**. Actually, the estimation of epilepsy prevalence and incidence in Europe has been derived by extrapolation of the results obtained from studies carried out in different countries **(Behr et al., 2016)**. Depending on the study, the annual incidence of epilepsy in Europe ranges from 24 to 82 per 100,000 population. It is also worthy of note that age-related incidence follows a bimodal distribution, with an increased incidence in children and elderly patients. In fact, the age-specific incidence curve for epilepsy has a characteristic U-shaped profile with the highest rates in the youngest children and in the elderly **(Forsgren et al., 2005; Kotsopoulos et al., 2002)**. Epidemiologic studies in epilepsy aim to assess the risk factors for developing the disorder, evaluate its burden, comorbidities and outcomes, and identify opportunities for preventing epilepsy and its consequences. Improved epilepsy data collection and measurement are thus necessary for better epidemiologic research, along with well-designed and targeted studies to characterize the significant trends and inform health care providers, policy-makers, and the society as a whole. Hence, further research is needed to improve knowledge about epilepsy incidence, prevalence, risk factors, comorbidities and outcomes, which will determine future prevention efforts **(England, 2012)**.

I.1.3. Definitions and classification of epileptic seizures and epilepsy

In 2005, the International League Against Epilepsy (ILAE) and International Bureau for Epilepsy proposed consensus definitions for “epilepsy” and “seizure”. Epilepsy was defined as “a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiological, cognitive, psychological, and social consequences of this condition”, which required the occurrence of at least one epileptic seizure. In turn, an epileptic seizure was defined as “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain” **(Fisher, 2005)**. However, the above definition of epilepsy is currently considered theoretical and not adequately detailed to provide a guidance on how enduring predisposition should be defined, particularly for those individuals presenting a single unprovoked seizure. Thus, after years of discussion new recommendations

have been published and adopted as a position of the ILAE. According to the revised definition, “epilepsy” is a disease of the brain defined by any of the following conditions: (1) at least two unprovoked (or reflex) seizures occurring greater than 24 h apart; (2) one unprovoked (or reflex) seizure and a probability of further seizures similar to the general recurrence risk (at least 60%) after two unprovoked seizures, occurring over the next 10 years; (3) diagnosis of an epilepsy syndrome. Moreover, epilepsy is considered to be resolved for individuals who had an age-dependent epilepsy syndrome but are now past the applicable age or those who have remained seizure-free for the last 10 years, with no antiseizure medicines for the last 5 years **(Fisher et al., 2017a)**.

In terms of seizure types classification, as current knowledge is still insufficient to form a scientifically based classification, the ILAE through the Commission for Classification and Terminology published in March 2017 a revised operational (practical) classification system **(Fisher et al., 2017b)**.

Although the establishment of a classification system is a long and complicated process, the classification of seizure types is important for several reasons. Among others, the classification becomes a worldwide shorthand form of communication among clinicians caring for epilepsy patients, allows grouping of patients for therapies, allows researchers to better focus their studies on mechanisms of different seizure types, and some regulatory agencies approve drugs or devices indicated for specific seizure types. Actually, the ILAE 2017 operational classification of seizure types (Table I.1) had as primary goal to provide a communication framework for clinical use, because seizure types are relevant to clinical practice in humans; additionally, it aimed to make the classification understandable by patients and families and broadly applicable to all ages **(Fisher et al., 2017b)**.

The purpose of the ILAE 2017 operational classification of seizure types was to recognize that some seizures can have either a focal or generalized onset, allow classification when the onset is unobserved, include some missing seizure types, and adopt more transparent designations. Accordingly, in relation to previous classification, the main changes introduced by this new operational classification of seizure types included the following: (1) “partial” became “focal”; (2) awareness was used as a classifier of focal seizures; (3) the terms dyscognitive, simple partial, complex partial, psychic, and secondarily generalized were eliminated; (4) new focal seizure types included automatisms, behavior arrest, hyperkinetic, autonomic, cognitive, and emotional; (5) atonic, clonic, epileptic spasms, myoclonic, and tonic seizures can be of either focal or generalized onset; (6) focal to bilateral tonic–clonic seizure replaced secondarily generalized seizure; (7) new generalized seizure types are absence with eyelid myoclonia, myoclonic absence, myoclonic–atonic, myoclonic–tonic–clonic; and (8) seizures of unknown onset may have features that can still be classified. Therefore, this new classification enables a greater flexibility and transparency in naming seizure types. In fact, the clinician’s first task is to determine if an event has the characteristics of a seizure and the next step is to make its classification into a specific seizure type **(Fisher et al., 2017b)**.

Table I.1. The International League Against Epilepsy (ILAE) 2017 classification of seizure types **(Fisher et al., 2017b)**.

FOCAL ONSET[a]	GENERALIZED ONSET	UNKNOWN ONSET
Motor Onset	**Motor**	**Motor**
Automatisms	Tonic-clonic	Tonic-clonic
Atonic[1]	Clonic	Epileptic spasms
Clonic	Tonic	**Non-Motor**
Epileptic spasms[1]	Myoclonic	Behavior arrest
Hyperkinetic	Myoclonic-tonic-clonic	
Myoclonic	Myoclonic-atonic	**Unclassified[2]**
Tonic	Atonic	
	Epileptic spasms	
Non-Motor Onset	**Non-Motor (absence)**	
Autonomic	Typical	
Behavior arrest	Atypical	
Cognitive	Myoclonic	
Emotional	Eyelid myoclonia	
Sensory		
Focal to bilateral tonic-clonic		

[a]Focal onset seizures are separated into focal aware, and focal impaired awareness.
[1]Degree of awareness usually is not specified.
[2]Due to inadequate information or inability to place in other categories.

In addition to the classification of seizure types, the ILAE also presented recently an updated classification of the epilepsies (Figure I.1), which reflects the current understanding of the epilepsies and their underlying mechanisms. This new classification of the epilepsies is a multilevel classification of diagnosis, that is, where possible, a diagnosis at all three levels should be sought (seizure type, epilepsy type and epilepsy syndrome) as well as the etiology of the individual's epilepsy; furthermore, it was designed to allow the classification of epilepsy in different clinical environments. Indeed, its primary purpose is for diagnosis of patients, but it is also essential for epilepsy research, development of antiepileptic drugs (AEDs), and communication around the world **(Scheffer et al., 2017)**.

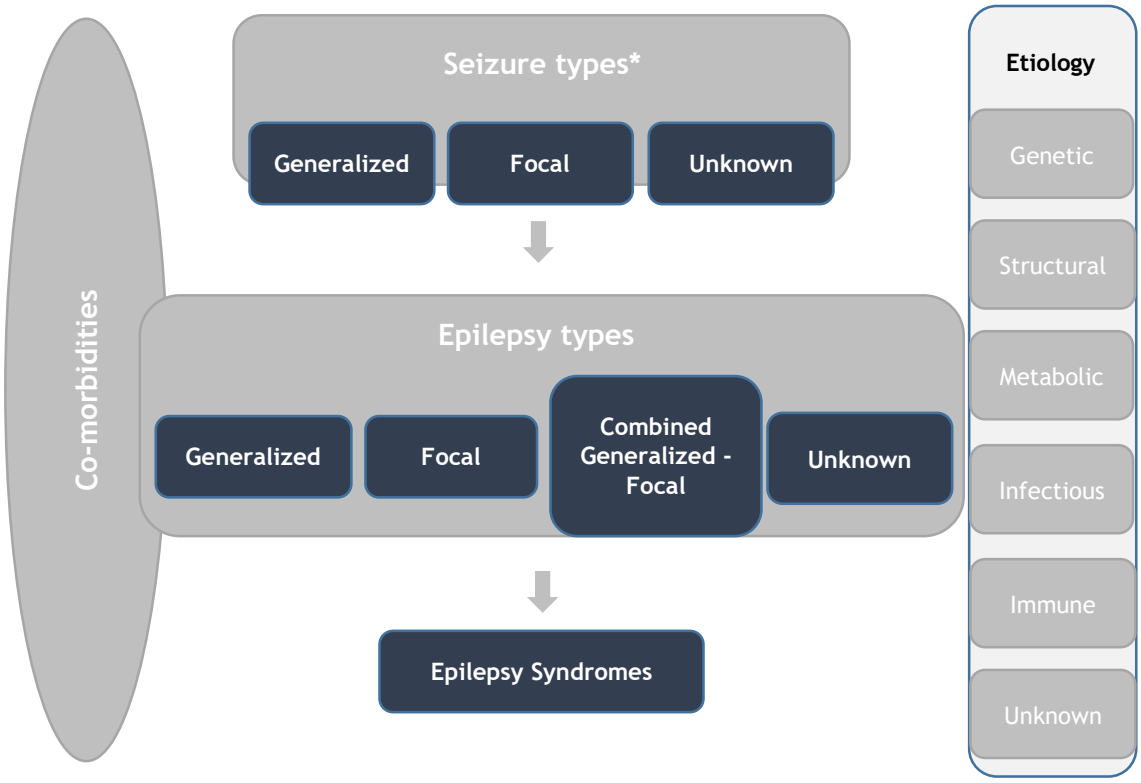

Figure I.1. Framework for classification of the epilepsies **(Scheffer et al., 2017)**. *Denotes onset of seizure.

Briefly, the ILAE 2017 classification of the epilepsies presents three levels, starting with seizure type, where it assumes that the patient is having epileptic seizures as defined by the new ILAE operational classification of seizure types. Then, the second level involves the diagnosis of epilepsy type, including focal epilepsy, generalized epilepsy, combined generalized and focal epilepsy, and also an unknown epilepsy group. The third level is that of epilepsy syndrome, where a specific syndromic diagnosis can be made. The new classification integrates etiology along each stage, highlighting the need to consider etiology at each step of diagnosis, as it often has important treatment implications. Etiology is classified into six subgroups, selected because of their potential therapeutic consequences. New terminology was introduced such as developmental and epileptic encephalopathy, and the term "benign" was substituted by the terms "self-limited" and "pharmacoresponsive" to be used where appropriate **(Scheffer et al., 2017)**.

I.1.4. Pathophysiology of epilepsy: A brief overview

Despite the advances that have been achieved over the last decades, the pathophysiologic mechanisms involved in the initiation and propagation of seizures (ictogenesis), as well as those involved in transforming the normal brain tissue into a seizure-prone tissue (epileptogenesis) still remain to be fully elucidated **(Pitkänen and Engel, 2014)**.

The term epileptogenesis frequently refers to the set of brain/neuronal changes that occur gradually over a period of time from the occurrence of a precipitating brain insult or injury to the onset of recurrent seizures. Hence, the suggested definition for epileptogenesis involves the development of an epileptic disorder and/or progression of the epilepsy after it is established **(Łukawski et al., 2018; Pitkänen, 2010)**. In fact, experimental evidence indicates that epileptogenesis is a dynamic phenomenon comprising a set of continuous molecular, structural and functional pathophysiologic modifications in seizure-prone brain areas **(Vezzani, 2015)**.

Overall, three basic phases can be distinguished in the process of epileptogenesis: (1) acute brain damage (initial insult), (2) latent period with "maturation" of the epileptic focus, and (3) actual epilepsy **(Maguire, 2016; Pitkänen and Lukasiuk, 2011).** The consequence of the initial insult starting the epileptogenesis is an immediate and progressive process of varying course over time; immediate response involves neuronal activation with intracellular calcium ion accumulation and further stages of excitotoxicity, starting the system of secondary messengers, activation of gene expression and protein synthesis. The latent phase may take years or even decades, and this fact might explain that the progression of modifications in response to the initial insult is critical to the expansion of the epileptic condition **(Maguire, 2016; Pitkänen and Lukasiuk, 2009)**.

Nowadays, it is recognized that epileptogenesis can be originated by a variety of initial brain insults such as traumatic brain injury (TBI), tumors, stroke, infections, craniotomy, cerebral palsy, intrapartum hypoxia, neurodegenerative diseases and prolonged acute symptomatic seizures including complex febrile seizures and *status epilepticus* (SE) **(Löscher and Brandt, 2010; Mani et al., 2011)**. These several types of brain injuries are causes of acquired epilepsy, and brain trauma is probably one of the most common causes of idiopathic epilepsy **(Maio, 2014)**. However, the genetic influence is supposed to be more strongly associated with idiopathic epilepsies, whereas mechanisms of circuitry reorganization after a brain insult are most extensive in the acquired symptomatic ones. It should be noted that these mechanisms are not separate realities but, in some cases, functional consequences of brain injury can depend on genetic background **(Pitkänen and Lukasiuk, 2009)**.

For instance, mutated DNA sequences in genes encoding for ion channels or neurotransmitter receptors have been identified in hereditary focal or generalized epilepsies **(Kobow and Blümcke, 2018)**. About 25% of genes identified in epilepsy encode for ion channels **(Oyrer et al., 2018)**. Indeed, channelopathies are presumed to have a major contributing role in the development of seizures **(Pitkänen and Lukasiuk, 2009)**. Molecular analysis of ion channels after epileptogenic insults has particularly revealed that brain injury can result in changes in both ligand- and receptor-gated ion channels that are associated with the altered function when investigated using single-cell electrophysiology assays **(Brooks-Kayal et al., 1998; Chen et al., 2012; Shah et al., 2004; Su et al., 2002)**. This phenomenon, called acquired channelopathy, has been described in the dendritic, somatic, and axonal channels. Some of the subunit and functional changes can last even weeks and, thus, this can contribute to the

establishment of a lowered seizure threshold in parallel with other plastic neuronal alterations. For example, several research groups have described changes in the expression of genes encoding various subunits of ligand- or voltage-gated ion channels after SE by analyzing the RNA in single neurons, including changes in $GABA_A$ receptors and sodium, potassium and calcium channels **(Pitkänen and Lukasiuk, 2009)**. There is evidence that, for example, changes in $GABA_A$ receptor subunit composition can affect the efficacy of AEDs **(Goodkin et al., 2007)**.

In spite of its complex etiology, a common feature of the epileptic disorders is a paroxysmal excitatory activity, which is capable to produce the same pathological features that are ultimately recognized clinically as epileptic disease **(Maio, 2014)**. Abnormal electrophysiological activity underlying seizures in people with epilepsy results from neurobiochemical processes initiated at the cellular level. Neuronal hyperexcitability and hypersynchrony underlie an altered seizure threshold. The expression of ictogenesis and epileptogenesis requires pooled neuronal involvement that intermittently results when large networks of neurons are recruited **(Tatum et al., 2018)**. In this context, electroencephalography is an essential diagnostic tool, enabling the record at the scalp of complex electrophysiological signals generated by the brain composed of summated pools of neuronal excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) **(Merricks et al., 2015)**.

Neurobiochemical phenomena involving voltage-gated ion channels and currents give rise to electrophysiological manifestations of epileptogenic activity. While inward current flow of calcium or sodium ions causes depolarization resulting in an EPSP, activation of calcium mediated outward flow of potassium ions or γ-aminobutyric acid (GABA) mediates chloride influx and results in hyperpolarization that generates an IPSP at the dendritic synapses. Astrocytes contribute to the spread of current flow by enhancing calcium influx into the neurons **(Tatum et al., 2018)**.

Neuronal pools also continuously interact with other neurons in local neural networks and other networks in more distant regions of the brain. Actually, seizure generation involves an imbalance between excessive excitatory neurotransmitters and a relative reduction of inhibitory neurotransmission. Thus, ictogenesis results from augmented synchronization of neuronal epileptiform discharges due to increased neuronal activity. However, the specific mechanisms involved may differ among different seizure types. In absence epilepsy, it depends on synchronization of transient low-threshold T-type calcium channels in the thalamus via rhythmic activity of inhibitory neuronal networks. On the other hand, in focal seizures, mechanism for initiating a seizure involves excessive firing and synchronization of neurons; with a seizure, potassium concentration in the extracellular space is increased at the cellular level and it results in breakdown of the local inhibitory mechanisms, excitation and depolarization of neurons, and thus in seizure initiation **(Tatum et al., 2018)**.

The best understanding of the epileptogenic process comes largely from animal studies that have investigated SE-induced epileptogenesis. This model produces alterations similar to those

found in resected temporal lobe tissue of patients who have undergone surgery for drug-refractory epilepsy. Nevertheless, it has been recognized that SE may not be the condition most representative of human epileptogenesis because it is a relatively rare cause of epilepsy in humans, particularly in adults, and is often associated with other epileptogenic insults as stroke and TBI. Furthermore, typically, the damage induced in animals exceeds that found in humans **(Bosse et al., 2013; Pitkänen and Lukasiuk, 2009)**. Also, human data available from surgically operated epileptic tissue represent a relatively small population of patients and, thus, it may distort our view of the severity of pathology in most patients with acquired temporal lobe epilepsy (TLE). Despite these caveats, it is, however, important to acknowledge that these conditions have already provided a database of information about molecular and cellular changes in epilepsy that can be used to create testable hypotheses for understanding and discontinuing the epileptogenic process **(Pitkänen and Lukasiuk, 2009)**.

A number of theories have been proposed regarding mechanisms contributing to epilepsy progression, including the progressive primary etiologies, such as expanding lesions and progressive neurodegeneration, development of pharmacologic resistance, and seizure-induced plasticity/kindling **(Maguire, 2016; Pitkänen and Lukasiuk, 2011)**. Among the many proposed mechanisms of epileptogenesis, excluding the molecular aspects, the most essential are the following: oxidative stress, short- and long-term adaptive changes in sensitivity to GABAergic neurotransmission by means of $GABA_A$ receptors, impairment of the fine tuning mediated by dopamine (DA) receptors activity, inflammation and inflammatory processes affecting the extracellular neuronal matrix integrity, neuronal cell death, the impact of hormones or even mechanisms of circadian rhythmicity **(Łukawski et al., 2018)**.

Nevertheless, little is still known about the exact mechanisms underlying epilepsy progression. The most common changes observed during the developing seizures include increased neurogenesis in the dentate gyrus, hilar ectopic dentate granule cells, loss of hilar cells (interneurons and mossy cells), and formation of mossy fiber sprouting **(Dudek and Sutula, 2007; Łukawski et al., 2018; Parent, 2007)**. These changes are proposed to be induced by brain insults, which may result in the rewiring of the hippocampal network to establish a possible epileptic circuitry **(Coulter, 2001; Kokaia, 2011; Łukawski et al., 2018; McNamara, 1994; Rakhadehor and Jensen, 2010)**. Brain regions that are very sensitive to insults and can cause epileptogenesis include temporal lobe structures such as hippocampus, amygdala and piriform cortex **(Łukawski et al., 2018)**. In fact, TLE is the most common form of refractory epileptic disorder, often related to childhood seizures. The symptomatic manifestations of TLE appear only after a widespread irreversible damage of entorhinal cortex **(Bartolomei et al., 2005)**, hippocampus **(Mathern et al., 2002)** and perirhinal cortex, which has a major role in the spread of limbic seizures **(Biagini et al., 2013; Maio, 2014)**. These pathological features of TLE decrease the possibility of successful therapeutic approaches, often rendering the disease refractory. The complex clinical management of chronic TLE and the limited success rate of surgical approaches, amplify the debilitating nature of this specific epileptic disorder **(Maio, 2014)**.

Taking into consideration the time dependency and the various processes that occur simultaneously during the development and progression of seizures, an instant response includes neuronal activation with intracellular calcium ion accumulation and additional stages of excitotoxicity, beginning the system of secondary messengers, activation of gene expression and protein expression. In the subsequent days, at the site of injury, inflammatory processes succeed and mediators of inflammation, glial and endothelial cell responses are activated **(Łukawski et al., 2018; Sendrowski and Sobaniec, 2013)**.

Neuronal cell death has been implicated as a causal factor leading to the development of the epileptic disorder. The findings support the idea that repeated seizures mediate neuronal necrosis and apoptosis prevalently associated with the activation of certain distinct anti/pro-apoptotic B-cell lymphoma 2 family factors. In hippocampus, executioner caspase-3 and 6 are activated and actively expressed **(Narkilahti and Pitkänen, 2005; Narkilahti et al., 2003; Weise et al., 2005)**. Moreover, B-cell lymphoma 2 family proteins, such as bcl-2-like protein 4 and B-cell lymphoma 2, are also involved in pathogenesis of human TLE models **(Engel and Henshall, 2009; Henshall and Simon, 2005; Liou et al., 2003)**.

Studies have shown that during the epileptogenesis process the number of newly generated neurons augment in the subgranular zone at early stages after brain insults **(Parent, 2002; Zhong et al., 2016)**. Acute seizures are also associated with augmentation of neurogenesis and migration of newly born neurons into ectopic regions such as the hilus and the molecular layer of the dentate gyrus. Thus, abnormally migrated newly born neurons play a role in the occurrence of epileptogenic hippocampal circuitry characteristically seen after acute seizures, SE or head injury **(Kuruba et al., 2009; Łukawski et al., 2018)**. Among the main factors responsible for increased cellular proliferation have been enumerated, (1) the release of mitogenic factors from dying neurons, differenced granule cells and reactive glia; (2) the increased levels of GABA in the dentate gyrus during the early post-seizure period; (3) the influence of increased levels of neuropeptide Y after acute seizures which enhances the proliferation of neural stem cells in the dentate gyrus; (4) the modulation of neuron-restrictive silencing factor activity; (5) the influence of increased neuronal activity during and after seizures on neural stem cells and the production of new neurons **(Banerjee et al., 2005; Botterill et al., 2015; Croll et al., 2003; Deisseroth et al., 2004; Ge et al., 2006; Howell et al., 2010; Jessberger et al., 2007; Łukawski et al., 2018; Scharfman and Gray, 2007, 2015; Shetty et al., 2004)**.

Besides acute changes, chronic changes stimulating and leading to epileptogenesis have also been discussed. The last ones are changes that follow over weeks to months and include anatomical changes, such as neurogenesis, gliosis, mossy fiber sprouting and network reorganization **(Łukawski et al., 2018)**.

Overall, four major glial cell types are involved in epileptogenesis, which include astrocytes, microglia, oligodendrocytes, and NG2 cells (also known as polydendrocytes). Glial cells can contribute to the epileptogenic process in several ways, including structural support, water and

ionic homeostasis, regulation of neurotransmission, inflammatory responses, and neurogenic potential. However, most of the available data on the role of glial cells in the epileptogenic process come from astrocytes and microglia **(Pitkänen and Lukasiuk, 2009)**. Astrocytes may influence the pathogenesis and pathophysiology of epilepsy by the homeostatic control of synaptic neurotransmission via release of gliotransmitters, such as glutamate (Glu), adenosine triphosphate (ATP) and D-serine **(Clasadonte et al., 2013; Haydon and Carmignoto, 2006)**.
Disruption of the glial component of the blood-brain barrier (BBB), as well as glia-induced inflammation have been implicated not only in ictogenesis but most importantly also in the epileptogenesis. Astrocytes play an important 'upstream' homeostatic role in controlling the uptake, degradation, and recycling of neurotransmitters. Impaired reuptake of neurotransmitters such as Glu **(Coulter and Eid, 2012)**, or dysregulated metabolism of neuromodulators such as adenosine **(Boison et al., 2013)** influence the development of epileptiform activity. Since glucose is considered to be a key transmitter of bi-directional communication between astrocytes and neurons, a decrease in glucose is a very sensitive measure for the onset of epileptogenesis, whereas reduction of *N*-acetyl aspartate is detectable later. This decrease in glucose levels can be an indicator of neuronal death and/or mitochondrial impairment and may indicate beginning of gliosis. In the chronic phases of the hippocampal formation, astrocyte metabolism becomes more activated because the number of neurons is reduced **(Hadera et al., 2015; Łukawski et al., 2018)**.
Astrocytes form large intercellular networks and perturbations of glial metabolism can, therefore, affect the entire neuronal circuitry. These network effects of astrocytes may, indeed, be a reason why the neuronal networks in epilepsy synchronize; similarly, fluctuations in metabolic functions of glia may explain why seizures are sporadic **(Boison and Steinhäuser, 2017)**.
Microgliosis can manifest itself as cell proliferation, migration, or secretion of various compounds into the extracellular space. All this is accompanied by characteristic morphological changes. Microglial cells are extremely sensitive to disturbances in brain homeostasis and respond quickly to detected pathology such as damage to neighboring neurons **(Hailer, 2008)**. Microglia and astrocytes activated by epileptogenic insults increase the synthesis and release of inflammatory molecules, thus contributing to the generation of neuroinflammation **(Vezzani, 2015)**. Activated microglia become secretory and release a number of compounds with harmful effects on neurons, such as pro-inflammatory cytokines [as interleukin (IL)-1ß, IL-6 and tumor necrosis factor-alpha] and their receptors, proteases and nitric oxide **(Allan et al., 2005; Hailer, 2008; Kreutzberg, 1995; Pitkänen and Lukasiuk, 2009)**. On the other hand, activated microglia can also secrete transforming growth factor-beta, brain-derived neurotrophic factor, neurotrophin-3, or nerve growth factor, which have a neuroprotective effect and can promote regeneration **(Elkabes and Black, 1996; Miwa et al., 1997)**. Nowadays, it is widely recognized that inflammatory processes in brain can affect the extracellular neuronal matrix integrity, which plays a critical role in the modulation of α-amino-3-hydroxy-5-methyl-isoxazole-4-

propionic acid (AMPA) glutamate receptor mobility, paired-pulse depression, L-type voltage-dependent calcium channel activity and long-term potentiation processes **(Maio, 2014)**.

As expected, changes taking place in the brain impair the neuronal plasticity. For instance, brain injury from seizures results in reorganization of a variety of excitatory and inhibitory neurotransmitter/neuromodulator systems **(Hamed, 2008)**. Moreover, altered expression of AMPA and *N*-methyl-D-aspartic acid (NMDA) glutamate receptor subunits has been reported throughout various phases of the epileptogenic process **(Mathern et al., 1998)**. Also, GABA receptors and GABAergic neuronal pathways play a key role in neuronal inhibition in the central nervous system (CNS). Recurrent seizures lead to a progressive internalization of postsynaptic $GABA_A$ receptors and, consequently, to an erosion of inhibitory mechanisms **(Kaila et al., 2014)**. Several classes of adhesion proteins contribute to activity-induced changes in synaptic efficacy and, in particular, to the potentiation of glutamatergic neurotransmission. It has been proved that seizures stimulate integrin and matrix protein expression, as well as extracellular proteolysis; these processes are in some instances known, and in other instances likely, to facilitate reactive axonal growth and circuit modification following seizures. Thus, integrins and their matrix targets are significantly involved in multiple processes that contribute to the development of epilepsy most particularly in adult brain **(Gall and Lynch, 2004; Łukawski et al., 2018)**.

Dendritic spines are subcellular structures that participate in synaptic transmission in the brain **(Wong, 2005)**. It is likely that alterations in these structures can affect the availability of various receptor types as well as their stoichiometry and, thus, compromise the information flow from afferent inputs **(Pitkänen and Lukasiuk, 2009)**. Seizure-related changes in dendritic spines may represent a mechanistic basis for cognitive deficits in epilepsy. Indeed, studies on human epilepsy revealed that a loss of dendritic spines occurs in hippocampal pyramidal neurons and dentate granule cells in patients with TLE **(Łukawski et al., 2018; Wong, 2005)**. Taken together, scientific studies **(Raijmakers et al., 2016; Wong and Guo, 2013)** carried out so far regarding the relationship between neuronal plasticity and epileptogenesis seem to be important not only to confirm that homeostasis is active in epilepsy, but also to understand the advantages and disadvantages of decreasing or enhancing these homeostatic mechanisms **(Łukawski et al., 2018)**.

I.1.5. Signaling pathways: Focus on neurotransmitters

The receptors and ion channels in neurons have a complex interacting network of membrane proteins linking the external influences to intracellular signaling systems and alterations in gene transcription; thus, theoretically, the epileptic cells can be affected by many different ways. However, all forms of epilepsy result from a shifting of the excitatory-inhibitory balance of nerve cells towards an excitatory state causing hyperexcitability, which can be spread in the CNS. In this context, the fast neurotransmitters as Glu and GABA are considered key

neuromediators involved in the genesis of epileptic seizures. Nevertheless, an optimal fine tuning of excitatory-inhibitory balance of neurons can be achieved by other neurotransmitters and substances that do not transmit fast information in synapses but rather modulate the efficacy of neurotransmission directly or indirectly using various signal transduction mechanisms **(Dobolyi et al., 2014)**.

A number of studies has revealed that neurotransmitters of central monoaminergic systems, such as norepinephrine (NE), DA, serotonin (also called as 5-hydroxytryptamine, 5-HT), histamine, as well as adenosine and acetylcholine (Ach), can play an inhibitory role in seizure activity, but it depends on the pathways and receptor subtypes involved **(Chen et al., 2016a; Morimoto et al., 2004; Strac et al., 2016; Tripathi and Bozzi, 2015; Werner and Coveñas, 2017)**. The proconvulsant and anticonvulsant actions of some of these neurotransmitters are also dose-dependent. For instance, NE at low doses in the hippocampus exerts proconvulsant effects and at high doses anticonvulsant effects. DA and 5-HT also seem to exert modulating effects on seizures; in fact, selective DA reuptake inhibitors and citalopram, a selective 5-HT reuptake inhibitor, at low doses had no protective effect on rats with limbic seizures, but at high doses, they had anticonvulsant effects **(Werner and Coveñas, 2017)**. Relatively to serotonergic neurotransmission, expression of various types of 5-HT receptors is evident in most of the networks associated with seizure onset **(Tripathi and Bozzi, 2015)**. 5-HT_{2A} receptors have been implicated in various psychiatric and neurological disorders, including epilepsy **(Crunelli et al., 2018)**. Moreover, a study showed that mutant mice lacking 5-HT_{2C} receptors were more prone to spontaneous death from seizures, suggesting that 5-HT_{2C} receptors mediate tonic inhibition of neuronal network excitability **(Tecott et al., 1995)**; in contrast, normally, neuronal excitability can be decreased during hyperpolarization of glutamatergic neurons by 5-HT_{1A} receptors, depolarization of GABAergic neurons by 5-HT_{2C} receptors and inhibition of 5-HT_3 and 5-HT_7 receptors **(Bagdy et al., 2007)**. In this scope, it has been suggested that the elevated levels of 5-HT and DA metabolites during epilepsy may represent an epiphenomenon, rather than a concerted strategy of local or distal neurons to contain an epileptogenic focus. Indeed, the rate of monoaminergic metabolism (i.e., synthesis, uptake, and clearance) does not significantly correlate with the epileptic condition in baboon **(Strac et al., 2016).** Regarding the cholinergic system, it is believed that cholinergic innervation plays a key role in the normal control of neuronal excitability, although the exact role of cholinergic dysfunction is not completely known. However, according to available experimental data, the *in vivo* application of pilocarpine (PIL) or other muscarinic agonists and of blockers of the ACh-hydrolyzing enzyme (acetylcholinesterase) readily induces seizures and may lead to SE, resulting in spontaneous seizures following a latent period **(Friedman et al., 2007)**. It is also well-known that Ach exerts its effects through activation of muscarinic and nicotinic cholinergic receptors, and it appears that the activation of muscarinic-1 (M1) cholinergic receptors has proconvulsant effects, whereas the activation of nicotinic α7 cholinergic receptors enhances GABAergic neurotransmission **(Werner and Coveñas, 2017)**. About adenosine, presynaptic and postsynaptic adenosine receptor stimulation generates an inhibitory effect on many excitatory

pathways such as those mediated by Glu. Presynaptic adenosine receptor stimulation may reduce the release of the excitatory neurotransmitter Glu and postsynaptic adenosine receptor stimulation may directly inhibit excitatory pathways. Indeed, adenosine type one (A_1) receptor antagonists like theophylline and caffeine can reduce seizure thresholds and prolong seizures by interfering with mechanisms of seizure termination **(Chen et al., 2016a)**.

In addition, different neuromodulatory systems have been suggested to affect the initiation and propagation of seizures. For instance, molecules involved in the modulation of synaptic neurotransmission, such neuropeptides, have been implicated in epilepsy and many of them are considered to have endogenous neuroprotective actions. The neuropeptide Y, somatostatin, ghrelin and galanin act as regulators of diverse synaptic functions and occur concomitantly with the classic neurotransmitters **(Brain and Cox, 2006; Oztas et al., 2017).** Most of the neuropeptide receptors are members of the G-protein–coupled receptors (GPCRs) family. Several neuropeptides and their receptors have been implicated in the pathogenesis of epilepsy, mostly by regulating the classic neurotransmitters systems, either by modifying the release of neurotransmitters or by regulating the effects of their activated receptors **(Casillas-espinosa et al., 2012)**. As example, some studies demonstrated that neuropeptide Y gene therapy in animal models reduced and delayed the progression of seizures **(Noé et al., 2009; Noè et al., 2008; Powell et al., 2018)**. Also, galanin, a 29- or 30-amino acid peptide, had a role in reducing the susceptibility to seizures during seizure induction **(Kanter-Schlifke et al., 2007)**.

In light of what has been previously discussed, changes in many classical neurotransmitters and neuromodulators may be responsible for dysfunctions in neural circuits, determining an imbalance between excitatory and inhibitory neurotransmission systems, thus contributing to the phenomena underlying epileptogenesis and/or ictogenesis. Hence, given the increasing interest in neuroactive amino acids and the consolidated observations on the relevance of catecholamines in epilepsy, these two main groups of neurotransmitters in the CNS are core subjects in the context of this thesis. Therefore, they will be focused in more detail in the following sections.

I.1.5.1. Neuroactive amino acids

Amino acids are the essential building blocks of proteins and peptides, and some of them have neuroactive functions, acting as neurotransmitters in the CNS. The two neuroactive amino acids that have been more studied as neurotransmitters involved in epilepsy are Glu and GABA.

Glu, or glutamic acid, is the predominant excitatory neurotransmitter in brain that is responsible for generating EPSP by depolarizing the neurons **(Meldrum et al., 1999; Voglis and Tavernarakis, 2006)**. Four Glu receptor types are identified: three ionotropic (ligand-gated cation channels) receptors, i.e. NMDA, AMPA and kainate (KA) receptors, and one metabotropic (G-protein-coupled) receptor **(Kew and Kemp, 2005; Meldrum et al., 1999; Voglis and Tavernarakis, 2006)**.

The NMDA receptors are integral membrane proteins incorporating four large subunits (tetramers) that form a central ion channel pore selective for cations, which are highly permeable to calcium ions. These receptors do not form a homogeneous population but rather exist as multiple subpopulations that differ in their functional and, presumably, physiopathological properties. More specifically, NMDA receptors are heteromeric assemblies composed of four homologous Glu receptor subunits within a large set of different subunits belonging to three families: GluN1 (occurs as eight distinct isoforms), GluN2 (occurs as four different subunits GluN2A to D) and GluN3 (occurs as two different subunits GluN3A and B). NMDA receptors form a unique family of Glu-gated ion channels central to excitatory neurotransmission, and the members of this family share common structural and functional features but also differ one from the other in many important aspects, such as channel kinetics, permeation or block by divalent cations or sensitivity to endogenous modulators **(Paoletti, 2011)**. The NMDA receptor activation requires the simultaneous binding of Glu and glycine/D-serine and AMPA receptor-mediated initial depolarization for removing magnesium ions which block NMDA receptors at resting membrane potentials. Glu binds with a higher affinity to NMDA receptors than to AMPA receptors. Also, the conductance of NMDA receptors for calcium ions is higher than that of other cation-conducting ionotropic receptors. As expected, the concentration of calcium ions entering the cells determines the frequency of synaptic stimulation, occurring the activation of protein kinases (when the intracellular calcium level is low) or phosphatases (when the intracellular calcium level is high). These phenomena may be responsible for processes of synaptic potentiation or depression *via* the regulation of AMPA receptor phosphorylation **(Lasoń et al., 2013)**.

The AMPA receptor, which is responsible for generation of fast EPSP in the CNS, forms channels permeable to sodium and potassium ions and, with some limitation, to calcium ions. In the presence of an agonist, the AMPA receptor desensitizes and this desensitization state is inhibited by positive allosteric modulators. The AMPA receptor is composed by four homologous proteins GluR1–4 (or GluRA–D) in various configurations **(Lasoń et al., 2013)**.

KA receptors are several times less abundant in the brain tissue than AMPA receptors, but their widespread distribution suggests that they are present in most neuronal cells. Cloning studies showed that KA receptors could be composed of GluR5, GluR6, GluR7 and KA1 and KA2 subunits. Long-lasting depolarization of neurons by postsynaptically localized KA receptors leads to intracellular flow of calcium ions *via* the voltage-dependent calcium channels, and this effect may be of significance in synaptic plasticity, but also in seizurogenic and neurotoxic effects of KA **(Bloss and Hunter, 2010; Vincent and Mulle, 2009)**.

The metabotropic Glu receptors comprise a family of 8 subtypes of receptors, mGluR1 to mGluR8, which are divided into 3 groups on the basis of differences in amino acid sequence, pharmacological characteristics and intracellular signaling pathways. The activation of mGluRs belonging to group I (mGluR1 and mGluR5) stimulates phosphatidylinositol synthesis and the release of intracellular calcium, whereas the stimulation of group II (mGluR2 and mGluR3) and group III (mGluR4, mGluR6-8) mGluRs inhibits adenylate cyclase **(Conn and Pin, 1997)**. The

group I mGluRs are localized postsynaptically on both hippocampal and cortical glutamatergic cells and GABAergic interneurons. Their activation leads to phosphorylation and inactivation of several types of potassium channels, which leads to depolarization of neuronal cells. The group III mGluRs are presynaptic inhibitory autoreceptors localized on glutamatergic cell endings and presynaptic inhibitory heteroreceptors on some GABAergic neuronal cells **(Lasoń et al., 2013)**. The process of Glu release and uptake in brain tissue engages several membrane transporters. There are 5 Glu transporters (GLAST, GLT-1, EAAC1, EA4 and EAAT5), which are localized in membranes of astrocytes and neurons. The synthesis and activity of Glu transporters is regulated by the concentration of Glu, as the main signal, whereas inactivation of these proteins results in seizures and neurotoxicity **(Ueda et al., 2001; Urbanska et al., 1998; Werner and Covenas, 2011)**.

Mechanisms such as upregulation of Glu receptors, elevation in extracellular Glu concentration, abnormalities in glutamatergic transporters and autoimmune mechanisms contribute to excessive glutamatergic activity, which in turn plays an important role in hyperexcitability and consequently in epilepsy disorders **(Albrecht and Zielińska, 2017; Aroniadou-Anderjaska et al., 2008; Aronica et al., 2000; Barker-haliski and White, 2015; Bien et al., 2005; Debanne et al., 2006; DeLorenzo et al., 2005; Seifert et al., 2002; Touret et al., 2007; Yi and Hazell, 2006)**.

In opposition to Glu, GABA is the main inhibitory neurotransmitter that generates inhibitory presynaptic potentials by hyperpolarizing the neurons **(Morimoto et al., 2004; Treiman, 2001)**. Thus, GABAergic system has an important role for counter-balancing the neuronal excitability and therefore in suppressing the epileptiform activity **(Morimoto et al., 2004)**. It has been firmly established that GABA is produced by intraneuronal decarboxylation of Glu, and after being released, its intrasynaptic action is quickly terminated by glia and presynaptic neuronal uptake involving high-affinity GABA transporters. There are four GABA transporters expressed in the human brain that are named as GAT-1, GAT-2, GAT-3 and betaine-GABA transporter BGT1. The glial and neuronal GAT-1 received particular attention, as besides the GAT-3, GAT-1 predominantly participates in GABA uptake **(Lasoń et al., 2013)**.

GABA mediates its inhibitory effects *via* GABA receptors, which are either ionotropic $GABA_A$ receptors or metabotropic $GABA_B$ receptors. The pentameric $GABA_A$ receptor complex is composed of two α and two ß subunits and one γ subunit, which form the ligand-gated chloride channel, and depending on the subunit composition of the receptor it mediates tonic or phasic inhibition **(Mula, 2009)**. The $GABA_A$ receptor configuration also determines the affinity of its agonists, antagonists and allosteric modulators. Activation of $GABA_A$ receptor in the brain generates fast IPSP by increasing influx of chloride and the key role of this receptor complex in the prevention and inhibition of seizures is commonly accepted, except for the absence non-convulsive seizures **(Depaulis et al., 1997)**. In contrast to the well-recognized role of ionotropic $GABA_A$ receptor in the pathomechanism of seizures, less consistent data have been obtained on engagement of metabotropic $GABA_B$ receptor in the regulation of brain excitability. $GABA_B$

receptors are localized presynaptically and mediate slow inhibition by increasing the potassium conductance and decreasing the calcium entry; thus, they can inhibit release of both excitatory and inhibitory neurotransmitters **(Meldrum, 1989; Treiman, 2001)**.
It has been conjectured that epileptogenesis can be induced by the reduction or loss of GABAergic inhibition, increasing the probability of generating EPSP and synchronizing neuronal burst discharges **(Aroniadou-Anderjaska et al., 2008)**. In this context, impairment of GABA release, changes in GABA receptors, decrease of GABA synthesis and neuronal loss are the main GABAergic dysfunction mechanisms implicated **(Brooks-Kayal et al., 1998; Butler et al., 1993; Buzzi et al., 2012; Dinkel et al., 1998; Knopp et al., 2008; Kobayashi and Buckmaster, 2003; Schwarzer et al., 1997)**.

Besides Glu and GABA neurotransmitters, other neuroactive amino acids are known to be involved in neurotransmission, such as glutamine (Gln), aspartate (Asp), glycine and taurine (Tau) **(Albrecht et al., 2010; Baran, 2006; Meldrum et al., 1999; Morimoto et al., 2005; Perry et al., 2009)**. Gln is also found abundantly in the CNS. However, although Gln also appears to affect directly neurotransmission, it mainly participates in the brain as a precursor of the excitatory (Glu and Asp) and inhibitory (GABA) neurotransmitter amino acids **(Albrecht et al., 2010)**. Hence, the Gln synthesis is crucial to the Glu clearance in interstitial space and neuronal synthesis of Glu and GABA by participating in the Gln–Glu–GABA cycle between astrocytes and neurons. The deficiency of Gln synthesis due to the loss of Gln synthetase may result in a seizure or in the development of epilepsy **(Hu and Zhong, 2014)**.
Regarding glycine, it is the most primitive amino acid and has additional biochemical functions in carbon metabolism. In the hippocampal formation, glycine can exert opposing effects that depend on the activation of presynaptic or postsynaptic glycine receptors. It has been demonstrated that low concentrations of glycine (10 μM) exert proconvulsant effects, whereas higher glycine concentrations (100 μM) attenuate recurrent epileptiform discharges **(Boison, 2016)**.
Tau is also an inhibitory agent in the brain, causing hyperpolarization and inhibition of firing of neurons **(Saransaari and Oja, 2008)**. Changes in the levels of the inhibitory amino acid Tau may be related to changes in GABA levels, as GABAergic agents modify Tau release **(Bikjdaouene et al., 2003)**. A decrement in the concentration of Tau in the brain could increase the overall excitability of neuronal populations and thus contribute to the initiation of seizures **(Oja and Saransaari, 2013)**.
Althought GABA and Glu can play an important role in the neuronal excitability and probably in epileptogenesis and ictogenesis, in agreement to what has just been described, it is also true that many other neuroactive amino acids may be involved in these processes.

I.1.5.2. Catecholamines

As aforementioned in a more general way, other neurotransmitter systems, such as catecholamines, are also known to be involved in the epileptogenesis and ictogenesis. Therefore, it has been increasingly suggested that a neural network view needs to consider other connections beyond the hippocampus, thalamus and associated circuits, as part of a functional complex involving a multi-neurotransmitter system, in which the AEDs can act on multiple and different targets **(Werner and Coveñas, 2017)**.

Chemically, catecholamines are monoamines that contain an amino group linked by a two-carbon chain to a catechol ($C_6H_4(OH)_2$) group **(Gerald, 2010)**. The essential amino acid phenylalanine, derived from diet and endogenous protein catabolism, is converted to tyrosine (Tyr) via the enzyme phenylalanine hydroxylase **(Rodan et al., 2015)**. Through tyrosine hydroxylase, the Tyr is subsequently hydroxylated to L-3,4-dihydroxyphenylalanine (L-DOPA). Both tyrosine hydroxylase and phenylalanine hydroxylase are dependent on the cofactor tetrahydrobiopterin (BH4). Then, L-DOPA is converted to DA by the enzyme L-aromatic amino acid decarboxylase and DA is hydroxylated to NE by the enzyme dopamine ß-hydroxylase. NE is the final product in the central and peripheral adrenergic neuronal systems, whereas in the adrenal medulla it is further converted to epinephrine (E). Hence, DA and NE are primary amines, whereas E is a secondary amine. In the brain, DA is considered to be the most abundant catecholaminergic neurotransmitter and it is present in several brain regions **(Bicker et al., 2013; Peaston and Weinkove, 2004; Rodan et al., 2015)**.

The principal metabolic pathways for the catecholamines entering the circulation involve two key enzymes, monoamine oxidase (MAO) and catechol-*O*-methyltransferase (COMT), leading to a series of structurally related metabolites. After being taken up by the neuron, NE is inactivated by storage or by oxidative deamination through MAO to 3,4-dihydroxyphenylglycol (DOPEG) before reduction and *O*-methylation through COMT to 3-methoxy-4-hydroxyphenylglycol (MHPG or MOPEG) **(Bicker et al., 2013)**. Extraneuronal degradation of NE and E by the enzyme COMT leads to the formation of normetanephrine and metanephrine, respectively. Subsequent deamination and oxidation by MAO finally leads to the formation of 4-hydroxy-3-methoxymandelic acid or vanillylmandelic acid (VMA), the principal end-product of the metabolism of both NE and E **(Eisenhfer et al., 1996)**. The metabolism of DA closely resembles that of NE and E, with extraneuronal metabolism by COMT and MAO leading to the formation of 3-methoxytyramine, 3,4-dihydroxyphenylacetic acid (DOPAC) and finally homovanillic acid (HVA), which is the predominant end-product of DA metabolism **(Bicker et al., 2013; Peaston and Weinkove, 2004)**.

The adrenergic receptors are a class of GPCRs that are targets of catecholamines, particularly NE and E **(Cotecchia et al., 2012)**, but DA also stimulates α- and ß-adrenergic receptors as well as DA receptors **(Purves et al., 2003)**. Although other adrenergic receptors have been already identified, they are widely classified in four subtypes: α_1-, α_2-, β_1- and β_2-adrenergic receptors. Apparently, both proconvulsant and anticonvulsant actions have been identified for adrenergic

receptors, depending on the subtypes of adrenergic receptors involved and their presynaptic or postsynaptic localizations. More specifically, three α_1-adrenergic receptors subtypes known as α_{1A}, α_{1B}, and α_{1D} have been identified in CNS, being the α_{1A}-receptors the most abundant (~55%), while α_{1B}-receptors (35%) and α_{1D}-receptors (10%) have a more limited distribution. The thalamus and cortex are particularly enriched in α_1-receptors, and these receptors are also expressed in neurons and GABAergic interneurons. Changes in α_1-receptors density have been demonstrated in seizure-sensitive rodents and patients with epilepsy. The α_1-receptors are located as postsynaptic and generally have stimulatory actions in nature, but these receptors mediate inhibitory effect of NE on seizures, specifically seizures arising in the limbic system. The α_1-receptors inhibitory actions have often been attributed to their effects on the function of GABAergic interneurons: they increase interneuron firing and facilitate GABAergic interneuron transmission. In contrast to the α_{1A}-receptors, overactivity of α_{1B}-receptors may induce proconvulsant effects, since transgenic mice overexpressing the α_{1B}-receptors presented spontaneous epileptic seizures and widespread neurodegeneration, while lack of the α_{1B}-receptors protected against seizures induced by chemical convulsants (KA and PIL). The α_2-adrenergic receptors comprise three receptor subtypes α_{2A}-, α_{2B}- and α_{2C}-adrenergic receptors. Regional studies suggest that some forms of seizures are selectively associated with modifications of the α_2-adrenergic receptors binding sites in the brain. The α_{2A}-adrenergic receptors are widely distributed in brain regions regulating seizures and their activation suppresses epileptiform activity in the hippocampus, amygdala and entorhinal cortex. In brain, the α_{2A}- and α_{2C}-adrenergic receptors act as both presynaptic and postsynaptic receptors, but uncertainty remains about which receptors (pre or postsynaptic) are involved in anticonvulsant effect of α_2-adrenergic receptors agonists **(Ghasemi and Mehranfard, 2018)**. The antiepileptic actions of NE are very likely mediated by both α_1- and α_2-adrenoreceptors; indeed, α_2-adrenoreceptor agonists have shown to suppress seizures in kittens, whereas α_2-adrenoreceptor antagonists have promoted them **(Shouse et al., 2006)**. Therefore, NE plays a crucial role in suppression of seizure activity, as its depletion increases seizure susceptibility and promotes epileptogenesis **(Weinshenker and Szot, 2002)**. Actually, studies have demonstrated that NE at lower doses has a proconvulsant action and at higher doses an antiepileptic action **(Jurgens et al., 2005)**. In addition, a previous study revealed that agents acting to normalize NE levels have antiepileptic efficacy **(Freitas et al., 2010)**, which confirms the aspects discussed. However, also activated noradrenergic neurotransmission can be an etiological factor in some epilepsies **(Strac et al., 2016)**. For instance, NE depletion increased susceptibility to seizure induction **(Corcoran and Mason, 1980; Szot et al., 1999)** and NE loss increased neuronal damage in various limbic regions of rats after seizure induction **(Giorgi et al., 2003)**.

Regarding β_1 and β_2-adrenergic receptors, they are found on neurons and inhibitory interneurons in the cerebral cortex as well as in subcortical structures; however, among adrenergic receptors, the β_1-receptors have the lowest affinity for NE. An intense distribution of β-adrenergic receptors has been identified in the amygdala, and they likely mediate anticonvulsant actions in this area, since rats previously experiencing down-regulation of their

ß-receptors as result of long term antidepressant treatment more rapidly were kindled. Accordingly, decreased ß-adrenergic receptors observed in amygdala kindling model might contribute to the facilitation of epileptogenesis **(Ghasemi and Mehranfard, 2018)**. Other subtype of ß-adrenergic receptors is the $ß_3$-adrenergic receptor, which has been primarily associated with metabolic regulation, and there is some evidence that unlike the $ß_1$- and $ß_2$-adrenergic receptors, $ß_3$-adrenergic receptors may inhibit myocardial contractility **(Wachter and Gilbert, 2012).**

DA exerts an ambiguous and complex pathway in the pathogenesis of epilepsy. There are subtypes of DA receptors that seem to contribute to proconvulsant responses while others concur to anticonvulsant responses **(Bozzi et al., 2000; Gangarossa et al., 2014)**. Furthermore, activation of different DA receptor families (D_1 and D_2) may have diverse effects on neuronal excitability, wherein D_1 receptor mediates proconvulsant effects **(Barone et al., 1991)** and D_2 receptor mediates anticonvulsant effects **(Barone et al., 1991; Bozzi et al., 2000; Clinckers et al., 2004; Strac et al., 2016; Tripathi and Bozzi, 2015)**. Studies have also demonstrated that dopaminergic pathway is related to the pathophysiology of autosomal dominant nocturnal frontal lobe epilepsy and juvenile myoclonic epilepsy; however, in these conditions, a substantial reduction in D_1 receptor binding and a decrease in binding potential to the DA transporter occurs, respectively. Thus, a reduction in inhibitory dopaminergic activity leads to hyperexcitability and epilepsy **(Yin et al., 2013)**. Moreover, some studies reported that patients with juvenile myoclonic epilepsy showed a reduction in D_2/D_3 receptor binding restricted to the bilateral posterior putamen, suggesting an alteration of the dopaminergic system within this region **(Yin et al., 2013)**. The DA precursor L-DOPA improved the clinical outcome of a male patient suffering from intractable epileptic encephalopathy, again sustaining an overall anticonvulsant DA-mediated action **(Strac et al., 2016)**.

Hence, epilepsy is no longer considered only as a disturbance in the functioning of neuronal cells and their contact points (i.e. the synapses) but, instead, it is the result of a plethora of different deregulated mechanisms. Thus, a better understanding of the pathogenesis of epilepsy will likely offer the basis for new advances in epilepsy therapy, aiming to avoid the epileptogenesis or change the development of epilepsy in addition to symptomatic treatment.

I.1.6. Epilepsy treatment

The ultimate goal of epilepsy treatment should focus on the maintenance of a usual lifestyle, ideally freedom from seizures with minimal adverse effects, and reestablishment of the functional capacity, psychosocial and vocational activities of patients **(Shaju and Abraham, 2013)**.

Presently, AED therapy is the mainstream treatment for the patients with epilepsy **(Brown, 2016)**. Clinically, AEDs lead to an acceptable control of seizures and have a favorable risk-benefit relationship for 60-70% of diagnosed patients **(Schmidt, 2009)**. AEDs differ in various central pharmacological features, from the efficacy against diverse seizure types, adverse

effects profiles, potential for drug-drug interactions and comfort of use. Some non-pharmacological options are also available to aid in the treatment of epilepsy, which include surgery, vagus nerve stimulation (VNS) and ketogenic diet; these non-pharmacological therapeutic approaches are mainly considered upon AED failure **(Gschwind and Seeck, 2016; Koppel and Swerdlow, 2017; McGovern et al., 2016; Saxena and Nadkarni, 2011; Shorvon and Schmidt, 2016)**.

I.1.6.1. An overview of the historical development of antiepileptic drugs

The epilepsy and epileptic seizures have been treated for thousands of years with a variety of botanicals and herbs **(Raza and Choudhary, 2000; Schachter, 2009)**. However, the history of more conventional pharmacological treatments of epilepsy is already long. Bromides were introduced as a treatment for seizures in the 1850s by Sir Charles Locock, and they are considered as the first attempt for AED therapy **(Klitgaard H., 2005; Shorvon, 2009a)**. Because bromide salts were the only drug available for the treatment of epilepsy at that time, these compounds were used regularly in the next 50 years, despite their limited efficacy and undesirable side effects **(Wahab, 2010)**.

In the early 1900s it was discovered the phenobarbital (PB). Its anticonvulsant properties were accidently discovered in 1912 by Alfred Hauptmann, which was initially used as a hypnotic drug **(Brodie, 2010; Perucca, 2001; Shorvon and Schmidt, 2016; Wahab, 2010)**. Indeed, this drug is still currently the most broadly prescribed AED in the developing countries and remains as a popular choice in many industrialized countries, in part because of its low cost **(Brodie, 2010)**. Phenytoin (PHT), one of the drugs of first choice for generalized tonic-clonic and partial seizures was synthesized in 1908, and it was recognized as the first non-sedating AED after the pioneering studies of Merritt and Putnam using an electroshock-induced seizure model in cats. PHT and PB preserved their position as the best therapeutic options for patients with epilepsy for several years. In fact, both drugs have a broad-spectrum of anticonvulsant activity, but they are not active against absence seizures. Trimethadione (TMD) was the first drug specific for absence seizures and it was developed in 1940s following the nonclinical evaluation with the pentylenetetrazole (PTZ)-induced seizure model by Richards and Everett and the clinical evaluation by Lennox **(Wahab, 2010)**. Primidone (PRM) was introduced as an AED in the 1950s. PRM is metabolized into the active compounds PB and phenylethylmalonamide, but as it is associated with a higher incidence of side effects, other drugs have been preferred for clinical use **(Wahab, 2010)**. A few years later, specifically in 1960, ethosuximide (ESM) was introduced into clinical practice and it has been the AED of choice for children with absence seizures **(Bialer, 2012; Leppik, 2001)**.

During the 1960s the pharmaceutical companies significantly stopped the research and development of new products against epileptic seizures, in particular due to the small volume of sales of AEDs and also due to the more restricted requirements determined by the U.S. Food and Drug Administration (FDA) following the so-called "thalidomide tragedy" in Europe **(Dalkara**

and Karakurt, 2012). Consequently, only in the late 1960's a novel drug, the diazepam (DZP) [a 1,4-benzodiazepine (BZD)], was approved for use in treatment of SE **(Browne and Penry, 1973; Dalkara and Karakurt, 2012)**. Then, in 1974, carbamazepine (CBZ) was introduced in the market, and four years later valproic acid (VPA) appeared **(Dalkara and Karakurt, 2012)**. In the following years, several new AEDs were developed (known as second-generation AEDs), many of them by structural modification of the AEDs already used at that time (i.e. first-generation AEDs) (Table I.2) **(Dalkara and Karakurt, 2012)**.

Thus, in the period 1990-2010, several new drugs were introduced in the clinical practice aiming at improving the effectiveness, safety and/or tolerability of the classic AEDs. Over these years, zonisamide (ZNS), vigabatrin (VGB), oxcarbazepine (OXC), lamotrigine (LTG), felbamate (FBM), gabapentin (GBP), topiramate (TPM), tiagabine (TGB), levetiracetam (LEV) and pregabalin (PGB) were introduced in the market (Table I.2), while other molecules failed their clinical development due to toxic effects and/or low efficacy **(Jacob and Nair, 2016; Shorvon, 2009b)**. Unfortunately, this set of new AEDs did not provide noteworthy benefits in terms of efficacy in comparison with the older ones; nevertheless, they brought advantages in terms of tolerability, drug-drug interactions profile and pharmacokinetic properties **(Brodie, 2010; Gschwind and Seeck, 2016; Löscher and Schmidt, 2011)**.

Although the classification of AEDs in first-, second- and third-generation drugs is still a matter of debate, it is mostly accepted that the third-generation of AEDs started with the approval of lacosamide (LCM) in 2008, and also includes the drugs eslicarbazepine acetate (ESL), perampanel (PER), brivaracetam (BRV), rufinamide (RFM), retigabine (RTG) and stiripentol (STP) (Table I.2) **(Stefanović et al., 2017)**.

Table I.2. The main clinically approved antiepileptic drugs **(Stefanović et al., 2017)**.

First-generation	Second-generation	Third-generation
Phenobarbital	Zonisamide	Stiripentol
Phenytoin	Vigabatrin	Rufinamide
Primidone	Lamotrigine	Lacosamide
Ethosuximide	Oxcarbazepine	Eslicarbazepine acetate
Benzodiazepines	Felbamate	Retigabine
Carbamazepine	Gabapentin	Perampanel
Valproic acid	Topiramate	Brivaracetam
	Tiagabine	
	Levetiracetam	
	Pregabalin	

Newer generations of AEDs frequently show a superior pharmacological profile in experimental epilepsy models and are also better tolerated by epileptic patients and almost devoid of significant pharmacokinetic-based drug interactions; however, they still target voltage-gated

channels or GABA-mediated inhibition, without any important advance in terms of therapeutic targets **(Dalkara and Karakurt, 2012)**.

I.1.6.2. Basic mechanisms of action of antiepileptic drugs

As previously discussed, the pathophysiology of epilepsy is multifaceted, involving several neurotransmitter systems and many receptors, ion channels, intracellular signaling cascades, genes, and epigenetic modifications **(Moshé et al., 2014; Scharfman, 2007)**. Several of these possible mechanisms are targeted by current AEDs and are being targeted by new drug candidates under development. The majority of current AEDs target primarily either the classic voltage-gated ion channels (sodium, potassium and calcium) or the GABA system, whereas many AEDs have mixed mechanisms and others selectively inhibit more specific targets [e.g., neurotransmitter (Glu, GABA, DA, 5-HT) release by LEV, or AMPA receptors by PER] **(Brodie, 2010; Comai et al., 2012; Kikuyama et al., 2017; Rogawski and Löscher, 2004).**

Most AEDs currently available are thought to target several key mechanisms at the excitatory synapse (Figure I.2A). These mechanisms involve the voltage-gated sodium channels, synaptic vesicle glycoprotein 2A (SV2A), α2δ subunit of the voltage-gated calcium channels, AMPA receptors, and NMDA receptors. Many of the AEDs (PHT, CBZ, VPA, FBM, LTG, RFM, LCM, TPM, ZNS, OXC) can modulate voltage-gated sodium channels **(Lasoń et al., 2011)**. These mechanisms of action are expected to decrease depolarization-induced calcium influx and vesicular release of neurotransmitters. In addition, LCM is thought to enhance slow-inactivation of voltage-gated sodium channels. This effect is different from that of other AEDs listed, which are thought to enhance fast inactivation. The modulation of synaptic vesicle proteins is other mechanism of action recognized to some AEDs, which particularly modulate the synaptic vesicle protein SV2A that selectively enhances low-frequency neurotransmission and maintains the readily releasable pool of neurotransmitters **(Rowley et al., 2012)**. GBP and PGB bind to the α2δ subunit of voltage-gated calcium channels, which is thought to be associated with a decrease in neurotransmitter release. Excitatory neurotransmission at the postsynaptic membrane can also be limited by TPM, acting on AMPA and KA receptors, and by FBM, acting on NMDA receptors **(Bialer and White, 2010; Lasoń et al., 2011)**.

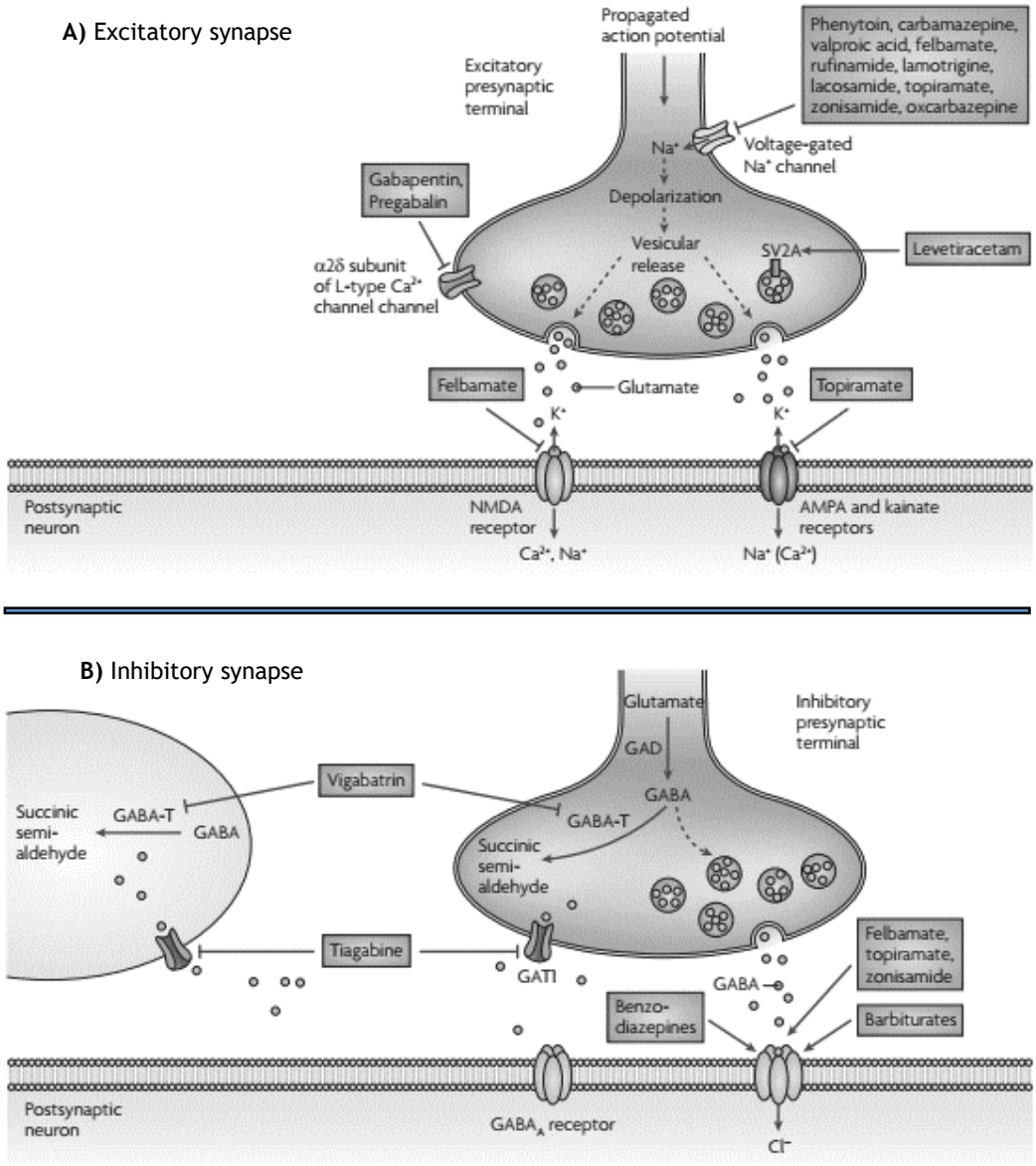

Figure I.2. Proposed mechanisms of action of currently available antiepileptic drugs at A) excitatory synapse and B) inhibitory synapse. AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; GABA-T, GABA transaminase; GAT, GABA transporter; NMDA, *N*-methyl-D-aspartate. Adapted from **(Bialer and White, 2010; Rho and Sankar, 1999).**

Several targets of AEDs at inhibitory synapses have also been proposed (Figure I.2B). These include the GABA transporter GAT1 (also known as SLC6A1), which is inhibited by TGB, leading to a decrease in GABA uptake into presynaptic terminals and surrounding glia; and GABA transaminase (GABA-T), which is irreversibly inhibited by VGB, decreasing the metabolism of GABA in presynaptic terminals and glial cells. The barbiturates, BZD, TPM and FBM have been found to enhance inhibitory neurotransmission by allosterically modulating $GABA_A$ receptor-mediated chloride currents. However, the action of each of these drugs is different and is dependent on the subunits conformation of the $GABA_A$ receptor complex **(Bialer and White, 2010; Greenfield, 2013; Lasoń et al., 2011)**.

Furthermore, it is also justified to mention in this context that other mechanisms of action involve the serotoninergic and dopaminergic systems; as example, it can be referred to the stimulation of brain DA turnover by VPA, and the increase of extracellular DA levels by CBZ, OXC, TPM and ZNS. In addition, VPA, CBZ, OXC, TPM and ZNS also increase the extracellular levels of 5-HT **(Perucca and Mula, 2013)**.

Most of AEDs in clinical use have been identified through compound screening programs in seizure models without considering their specific therapeutic mechanisms on ictogenesis and/or epileptogenesis **(Löscher et al., 2013)**. Thus, the mechanisms of action have been investigated after the discovery of anticonvulsant effects. Nevertheless, associations between efficacy against specific types of seizures or epileptic syndromes and some mechanisms of action have been established, which may help to preliminary predict the principal mechanism of action involved. For instance, focal seizures and primary generalized tonic-clonic seizures respond well to sodium channel blockers; T-type calcium channel blockers may be active against absence seizures; and GABAergic agents as well as AEDs with multiple mechanisms of action are more likely to present a broad-spectrum of activity against both focal and generalized seizure disorders **(Deckers et al., 2003; Perucca, 2011)**. On the other hand, certain AEDs that block selectively sodium channels or improve the synaptic availability of GABA are normally unsuccessful and they may even aggravate myoclonic and absence seizures, respectively **(Perucca, 2011)**.

In this context, numerous treatment guidelines have been published **(French et al., 2004; Glauser et al., 2006; National Institute for Health and Care Excellence, 2013)**, recommending a range of AEDs as potential first-line treatments for the variety of seizure types and epilepsy syndromes (Table I.3).

Table I.3. Antiepileptic drugs (AEDs) used in different seizure types and epilepsy syndromes **(Duncan et al., 2006; National Institute for Health and Care Excellence, 2013; Schmidt and Schachter, 2014; Shaju and Abraham, 2013)**.

	First-line AEDs	**Alternative AEDs**
Seizure type		
Focal	CBZ, LTG, LEV, OXC, VPA, TPM	GBP*, PGB, TGB, ZNS, CLB, CLZ, ESL, LCM, PB, PHT, VGB
Generalized		
Tonic-clonic	CBZ, LTG, OXC, VPA, TPM	CLB*, LEV*, ZNS, CLZ, PB, PHT
Absence	ESM, LTG, VPA	CLB, CLZ, TPM, ZNS
Myoclonic	LEV, VPA, TPM	CLB, CLZ, LTG, ZNS
Tonic	LTG, VPA	CLB, CLZ, TPM, ZNS, FBM, LEV, PB, PHT
Atonic	LTG, VPA	CLB, CLZ, TPM, ZNS, FBM, LEV, PB
Epileptic syndrome		
Childhood absence epilepsy	ESM, LTG, VPA	CLB, CLZ, LEV, TPM, ZNS
Dravet syndrome	VPA, TPM	CLB*, STP*
Benign epilepsy with centrotemporal spikes	CBZ, LTG, LEV, OXC, VPA	CLB*, GBP*, OXC*, TPM*, ESL, LCM, PB, PHT, PGB, TGB, VGB, ZNS
Panayiotopoulos syndrome	CBZ, LTG, LEV, OXC, VPA	CLB*, GBP*, OXC*, TPM*, ESL, LCM, PB, PHT, PGB, TGB, VGB, ZNS
Lennox-Gastaut syndrome	VPA	LTG*, FBM, RFM, TPM
Juvenile absence epilepsy	ESM, LTG, VPA	CLB, CLZ, LEV, TPM, ZNS
Juvenile myoclonic epilepsy	LTG, LEV, VPA, TPM	CLB, CLZ, ZNS
Epilepsy with generalized tonic-clonic seizures alone	CBZ, LTG, OXC, VPA	CLB*, LEV*, TPM*, CLZ, PB, PHT

* As adjunctive therapy. CBZ, carbamazepine; CLB, clobazam; CLZ, clonazepam; ESL, eslicarbazepine acetate; ESM, ethosuximide; FBM, felbamate; GBP, gabapentin; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; OXC, oxcarbazepine; PB, phenobarbital; PGB, pregabalin; PHT, phenytoin; RFM, rufinamide; STP, stiripentol; TGB, tiagabine; TPM, topiramate; VGB, vigabatrin; VPA, valproic acid; ZNS, zonisamide.

It is relevant to note that the antiepileptic efficacy of these drugs in initial add-on trials did not appear to vary considerably, which shows that comparable anticonvulsant activity can be achieved by different molecules acting in dissimilar therapeutic targets **(Löscher et al., 2013)**. Overall, the AEDs act by a variety of molecular mechanisms of action and probably many of them remain unknown. Also, the fact that many new AEDs have mechanisms of action analogous to those of older agents may be an explanation of why some patients who do not answer to older AEDs also do not respond to newer drugs **(Perucca, 2001)**.

I.1.6.3. Negative consequences of antiepileptic drug therapy

Undoubtedly, the AEDs currently available have a limited efficacy, and their negative properties restrict their use and cause difficulties in the management of patients with epilepsy. The long-term use of AEDs is limited due to their adverse effects, withdrawal symptoms, deleterious interactions with other drugs, and economic burden, especially in developing countries. Despite the huge funding and extensive premarketing testing for the adverse effects of new AEDs, they may still show severe adverse effects after being introduced in the market **(Wahab, 2010)**. Indeed, 15 to 20% of adult patients with epilepsy taking AEDs experience psychiatric and behavioral side effects; these include depressive mood, psychosis, increased irritability, and aggressive behavior. Psychiatric and behavioral side effects occur more frequently in patients taking LEV and ZNS, which leads to higher rates of intolerability **(Chen et al., 2017a)**. Also, a special warning with PER was issued concerning serious psychiatric and behavioral adverse reactions including aggression, hostility, irritability, anger and homicidal ideation; however, long-term safety studies have demonstrated good drug tolerability **(Gschwind and Seeck, 2016).**

Overall, multiple adverse effects have been manifested as result of AED therapy. Undoubtedly, some adverse effects depend on the dosage regimen and they may vary with the patient, such as lethargy, dizziness, and behavioral and cognitive impairment **(Laxer et al., 2014)**. These symptoms are more frequent with some AEDs, for example TPM is associated with impaired verbal fluency and confusion, and LEV with behavioral changes in children **(Gschwind and Seeck, 2016; Halma et al., 2014; Sommer et al., 2013)**. On the other hand, VGB, which can be an effective drug for a variety of epilepsies, may cause a peripheral retinopathy leading to (an often asymptomatic) visual field constriction **(Kinirons et al., 2006**; **Landmark and Patsalos, 2010; Laxer et al., 2014)**; in addition, VPA teratogenicity is currently well-recognized **(Vajda and Eadie, 2014)**, which limits the prescription to women of childbearing age. Even more, evidence refers that children born to mothers receiving VPA are more likely to have learning difficulties and autism spectrum disorders related to the exposure in utero to other AEDs such as CBZ, LTG, and LEV **(Bromley et al., 2013; Christensen et al., 2013)**.

The occurrence of adverse drug-drug interactions involving AEDs is another type of negative consequence ascribed to AED therapy, but it is more common with first-generation AEDs **(Landmark and Patsalos, 2010; Laxer et al., 2014)**.

Thus, clinical decisions taken regarding the prescription of AEDs should have into consideration the specific characteristics of patients, as well as the expected potential adverse effects and the likelihood of drug interactions. Sometimes the AED selection is also influenced by the presence of comorbid conditions, where a specific AED may also be beneficial; for example, in epilepsy patients with frequent migraine attacks, TPM and VPA can be useful **(Laxer et al., 2014)**.

I.1.6.4. Drug resistance

When the diagnosis of epilepsy is made, a single AED (monotherapy) is typically carefully chosen. The AED selection may be established based on some factors and the most significant is the correct identification of the seizure type or epilepsy syndrome. Also, the choice of the AED among the first-line agents always needs to be carefully individualized, mainly on the basis of the patient profile regarding age, gender, coexisting illnesses and concomitant medication. Tolerability, safety, ease of use, pharmacokinetic properties and cost are also fundamental elements that should be taken into account **(Schmidt, 2009)**.

If the first medication fails to control the seizures or induces unacceptable side effects, a second medication may be tested. Subsequently, the physician may choose to try other medications or initiate a combination of therapies (polytherapy). If polytherapy fails, the possibility of some combination of medications being able to stop the seizures is very low (1-4%) **(Karceski, 2007)**. When patients do not respond to medications and continue to have seizures, these conditions are referred to as refractory, drug resistant, or intractable epilepsy **(Kwan et al., 2010; Tang et al., 2017)**. It is currently recognized that up to 36% of epileptic patients have seizures that do not respond to AED medication(s) **(Karceski, 2007)**.

Recently, an ILAE Task Force commission defined drug resistance or pharmacoresistance as a *"failure of an adequate trial of two tolerated, appropriately chosen and used AED schedules (whether as monotherapies or in combination) to achieve sustained seizure freedom"* **(Kwan et al., 2010)**. However, it is important to have in mind that uncontrolled epilepsy is not always a drug-resistant condition; therefore, the occurrence of pseudoresistance due to inappropriate diagnosis, incorrect AED selection, or inappropriate dosage regimen must be ruled out before the patient's seizures are classified as drug-resistant **(Laxer et al., 2014)**.

Individuals with refractory epilepsy are more vulnerable to epilepsy progression than patients with a good seizure control. In addition, patients with refractory epilepsy present an increased risk of premature death, physical injury, cognitive impairments, psychosocial dysfunctions and a reduced quality of life **(Alexopoulos, 2013)**.

In general, three main mechanisms have been postulated to explain the refractoriness of epilepsy, which are disease-related, drug-related and genetic-related mechanisms **(Schmidt and Löscher, 2005)**. The theories based on the target hypothesis and the multidrug-transporter hypothesis have gained an increasing scientific support **(Basic, 2016; Schmidt and Löscher, 2005; Tang et al., 2017)**. The target hypothesis postulates that acquired alterations in the structure and/or functionality of target ion channels and neurotransmitter receptors result in loss of AED affinity and efficacy. On the other hand, the multidrug-transporter theory proposes that an overexpression of drug efflux transport proteins at the BBB impairs the drug uptake into the brain leading to insufficient AED levels at their target sites **(Löscher and Schmidt, 2011; Remy and Beck, 2006)**. A proof-of-concept trial with an inhibitor of the efflux transporter P-glycoprotein (also known as MDR1 or ABCB1) reversed the resistance to AED treatment in a rat model of pharmacoresistant TLE. However, as several AEDs are not apparently P-glycoprotein substrates **(Löscher et al., 2011; Zhang et al., 2012)**, other mechanisms seem

to contribute to the overall problem of AED resistance. These two key hypothesis are the most cited theories trying to explain refractory epilepsy, but no theory alone fully explains the neurobiological basis of pharmacoresistance **(Tang et al., 2017)**.

Other disease-related factors such as etiology of seizures, progression of the disease, structural brain variations and/or network alterations such as seizure-induced synaptic reorganization may also contribute to the altered clinical patterns of drug resistance (Figure I.3) **(Löscher et al., 2013; Schmidt and Löscher, 2005; Tang et al., 2017)**. Failure of anticonvulsant efficacy (or pharmacological tolerance) in the course of treatment, is a possible mechanism related with insufficient seizure control **(Schmidt and Löscher, 2005; Wahab, 2010)**. Genetic modifications such as polymorphisms in genes encoding for drug targets, multidrug transporters or proteins involved in pharmacokinetics and pharmacodynamics may also explain the differences found in response to AEDs **(Löscher et al., 2013; Schmidt and Löscher, 2005; Tang et al., 2017)**.

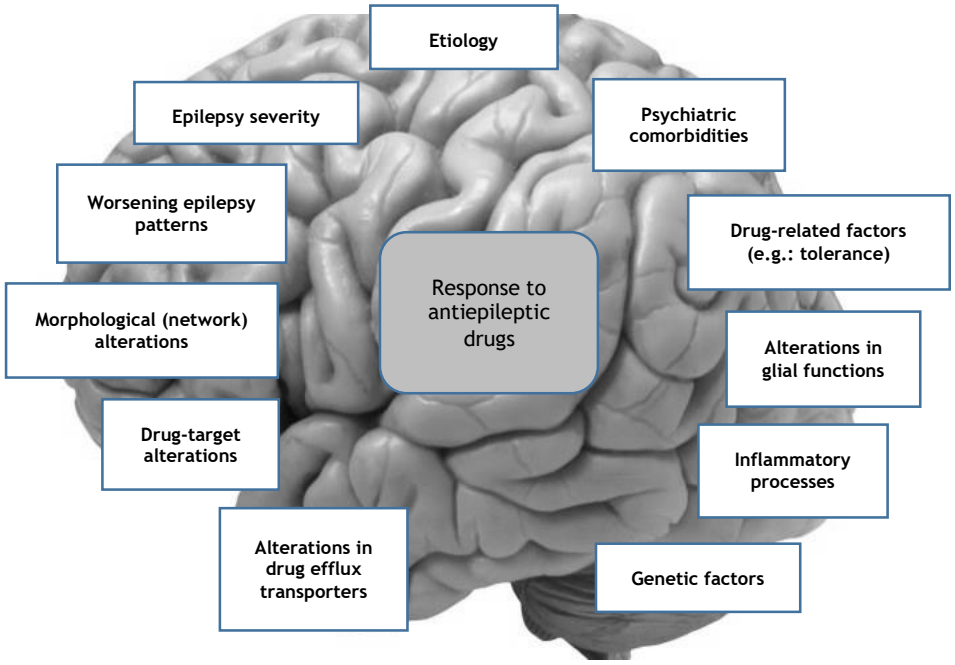

Figure I.3. Possible determinants of antiepileptic drug resistance in human and experimental epilepsies **(Löscher et al., 2013)**.

The multifaceted nature of pharmacoresistance in epilepsy dictates a multidisciplinary approach to future research and therapeutic interventions. The identification of causes that lead to therapeutic failure is vital for the development of new therapeutic approaches. Thus, despite the partial understanding of the cellular and molecular mechanisms underlying pharmacoresistance, it is believed that the next generation of therapies must necessarily include therapeutic targets that specifically contribute to epileptogenesis and drug resistance in relevant epilepsy models **(Löscher and Schmidt, 2011; Weaver, 2013)**. Hence, it is expected that a major breakthrough in the pharmacological treatment of refractory epilepsy

will only be achieved when the development of new drugs is directed towards the underlying causes of the disease **(Löscher et al., 2013)**.

I.1.6.5. Antiepileptogenic therapies: Where are we now?

Nowadays, it is widely accepted by the scientific community that the AEDs currently available in the clinical practice are not antiepileptogenic compounds but rather anticonvulsant or antiseizure agents, which actually act clinically as symptomatic treatments, preventing or alleviating seizure activity **(Łukawski et al., 2018)**. However, some nonclinical evidence has emerged supporting that, in addition to being able to suppress seizure activity, many AEDs also show neuroprotective properties, protecting discrete brain structures against seizure-related brain damage. Apart from AEDs with neuroprotective activity (e.g., BZDs, PB, LTG, TPM and VPA), there are also other AEDs that display much less potential in terms of neuroprotection such as CBZ and PHT **(Botting and Kuhn, 2012; Trojnar et al., 2002)**.

Considering that neurodegeneration is one of the pivotal mechanisms responsible for the process of epileptogenesis **(Pitkänen and Lukasiuk, 2011)**, an important question to consider is whether AEDs that share neuroprotective activity can efficiently inhibit the cascade of events that determines the conversion of a healthy into an epileptic brain tissue **(Łukawski et al., 2018)**. Indeed, the neuroprotection provided by AEDs following SE-induced brain damage is likely to counteract epileptogenesis. However, this is not obvious, as some neuroprotective AEDs failed to prevent remote, post-SE, seizure activity.

Some of the AEDs for which antiepileptogenic potential has been described are DZP and ESL **(Doeser et al., 2014; Pitkänen et al., 2005)**. Nevertheless, the correlation between neuroprotection and inhibition of epileptogenesis is not evident; therefor, the prophylactic use of neuroprotective agents in patients with active epileptogenesis is limited because there is no clinical biomarker for epileptogenesis **(Łukawski et al., 2018)**. Moreover, the results of clinical studies concerning the antiepileptogenic effects of older AEDs (PHT, PB, DZP, CBZ, VPA) have been unsatisfactory **(Mani et al., 2011; Pitkänen and Lukasiuk, 2011; Temkin, 2001, 2009)**. As possible reasons for the unsatisfactory results obtained with the studied AEDs may be the fact that these drugs do not interfere in any important pathway involved in the "epileptogenic" process and/or because they may have been tested at inappropriate doses, for an inappropriate duration, or at an inappropriate time after the brain injury has occurred **(Łukawski et al., 2018)**.

Furthermore, several strategies targeting various central mechanisms of action with therapeutic agents not related to AEDs, namely rapamycin (an immunosuppressive and antiproliferative drug that inhibits the mTOR pathway) **(Shima et al., 2015; Sosanya et al., 2015; Van Vliet et al., 2012)** or plant-derived agents such as resveratrol **(Saha and Chakrabarti, 2014; Wu et al., 2009)** have been explored to improve the pharmacological therapy of epilepsy.

In addition, novel targets for the development of new AEDs or antiepileptogenic drugs may involve, for example, inflammatory pathways, since inflammation is implicated in both epileptogenesis and ictogenesis, making anti-inflammatory drug targets promising for innovative epilepsy therapies **(Löscher et al., 2013)**.

Also, BBB barrier dysfunctions may participate in epileptogenesis, such that treatments targeting mechanisms at the level of BBB may offer a novel strategy for preventing or modifying the development of epilepsy **(Friedman et al., 2009)**. Furthermore, mechanisms of drug resistance involve alterations in the structure and/or functionality of AED targets. $GABA_A$ receptors or voltage-dependent sodium channels, also involved in epileptogenesis increase the expression and functionality of drug efflux transporters such as P-glycoprotein at the BBB, which lead to insufficient AED concentrations in the brain and, thus, they can also be possible therapeutic targets **(Löscher et al., 2013)**. Moreover, cation chloride co-transporters, such as the bumetanide-sensitive sodium-(potassium)-chloride co-transporter 1, that can undergo dramatic changes in expression within epileptic brain tissue, causing a shift from hyperpolarizing to depolarizing GABA currents in adult neurons, may also crucially contribute to the chronic hyperexcitability of epileptic neurons **(Löscher et al., 2013)**.

The monoaminergic system has a central role in psychiatric diseases, including mood disorders, anxiety and psychoses, but it is also involved in the regulation of the seizure threshold. Thus, noradrenergic, dopaminergic or serotonergic neurotransmission systems offer hopeful targets for new epilepsy therapies that not only block seizures but also improve epilepsy comorbidities **(Löscher et al., 2013)**.

Given the small advances already made in the discovery and development of antiepileptogenic therapies, it is expected that in the coming years the search for effective antiepileptogenic drugs will continue and be intensified at both nonclinical and clinical levels **(Łukawski et al., 2016)**. It is also likely that in a near future drugs in clinical use other than AEDs will be evaluated as potential antiepileptogenic therapies. For instance, losartan, which is an angiotensin II receptor type 1 antagonist, is an example of an already registered non-AED with antiepileptogenic potential and a proposal for its evaluation into clinical trials has been already made **(Radzik et al., 2015)**.

I.1.6.6. Non-pharmacologic treatment strategies

Nowadays, the pharmacological therapy with available AEDs is established as the first therapeutic option for the control and prevention of epileptic seizures; however, other non-pharmacological therapies such as surgery, vagus nerve stimulation and ketogenic diet have appear as alternative or complementary approaches to consider in conditions of drug-resistant epilepsy **(Sharma et al., 2015)**.

In fact, surgery can be the most successful non-pharmacological therapeutic option for some patients with refractory epilepsy **(Basic, 2016; Tang et al., 2017)**. The aim of surgery is to eliminate the epileptogenic focus of the brain, thus ending the epileptic seizures **(Karceski,**

2007). Risk–benefit consideration for surgery includes not only the risks, but also the expectations. For instance, the risks related with a temporal resection are superior to the risks associated with a neurostimulation procedure, but in the first case there is a greater chance of achieving seizure freedom **(Laxer et al., 2014)**.

A novel hope for patients with intractable epilepsy appeared in 1997 when the FDA approved the use of the first device for the treatment of refractory partial epilepsy, the vagus nerve stimulator **(Hosain et al., 2000; Labar et al., 1998)**. VNS has been applied to generalized forms of epilepsy as well, including some epilepsy syndromes **(Binnie, 2000; Ng and Devinsky, 2004)**. VNS symbolizes the "low-risk, low-reward" paradigm **(Laxer et al., 2014)**. Moreover, deep-brain stimulation that uses intracranial electrodes to stimulate specific brain structures in order to restrict seizure activity, and responsive neurostimulation that employs subdural and/or intraparenchymal electrodes in a closed-loop approach have been also used to seizure control. Responsive neurostimulation was permitted in November 2013 by the FDA and has the capacity to treat two epileptogenic foci providing useful diagnostic information. Remarkably there are no considerable differences in terms of efficacy between the three neurostimulation approaches mentioned above, but the complications are greater for the two intracranial techniques. At present, VNS is certainly the preferable option due to its favorable risk–benefit relationship **(Benbadis et al., 2014)**.

The ketogenic diet is another recognized non-pharmacological therapy for more problematic epilepsies, mainly in children **(Neal et al., 2008)**. In other neurological disorders this alternative or complementary approach has also been studied **(Paoli et al., 2014)**. Indeed, dietary manipulations to treat epilepsy date back to the time of Hippocrates. Effectively, in a period prior to the discovery of AEDs, the ketogenic diet rapidly became prevalent among medical practices. Posteriorly, the ketogenic diet fell out of favor because the oral medication was easier than the firm and rigorous dietary routine **(Rogawski et al., 2016)**. The ketogenic diet is a high-fat diet in which carbohydrates are almost completely eliminated, so that the body has minimal dietary sources of glucose. Fatty acids are thus an essential source of cellular energy production by peripheral tissues and the brain. The hallmark of ketogenic diet is the high circulating levels of ketone bodies (β-hydroxybutyrate, acetoacetate, and acetone), which are largely produced by the liver **(Freeman and Kossoff, 2010)**. In the absence of glucose, ketone bodies are used as the preferred source of energy for extrahepatic tissues, including the brain. The ketone bodies are oxidized, releasing acetyl-coenzyme A, which enters the tricarboxylic acid cycle **(Gasior et al., 2006)**. At present, the basic mechanisms underlying the therapeutic benefits of ketogenic diet remain only moderately understood. Nevertheless, the efficacy of ketogenic therapies to improve functional outcomes in epilepsy has increased interest in translating their potential to treat other neurologic disorders, including Alzheimer's disease and stroke rehabilitation **(Koppel and Swerdlow, 2017)**.

Furthermore, other alternative and/or complementary non-pharmacological therapies of epilepsy include yoga, acupuncture, chiropractic, massage therapy, electroencephalography biofeedback, aromatherapy, homeopathy, herbal medicines (traditional Chinese medicine),

among others **(Saxena and Nadkarni, 2011; Sharma et al., 2015)**. Apparently, the epilepsy patients who present more frequent seizures under more stressful conditions may benefit from learning stress management and relaxation techniques, such as progressive muscle relaxation and deep diaphragmatic breathing **(Saxena and Nadkarni, 2011)**.

I.1.7. Experimental models of epilepsy and seizures

Up today, several *in vitro* and *in vivo* epilepsy and seizure models have been developed. Although useful, *in vitro* models cannot completely replicate the complexity of a living being and their potential is more limited than that of whole-animal models **(Campos et al., 2018)**. Nevertheless, *in vitro* models, including brain slices, cell culture and molecular assays, allow more detailed investigations of cellular and molecular mechanisms of epileptogenesis while still preserving the critical network phenotypic features of epilepsy, particularly the development of spontaneous seizures. Thus, *in vitro* models may be advantageous because they offer the opportunity to study unique mechanistic insights into epilepsy. In fact, intact hippocampal preparations or acute brain slices maintain much of the needed circuitry to generate electrographic seizures **(Wong, 2011)**.

Regarding *in vivo* models of epilepsy, it is undoubtedly that animals most closely mimic the clinical features of human epilepsy since they can exhibit actual behavioral and electroencephalographic seizures. Therefore, it is not surprising that a large variety of animal models of seizures and epilepsy that have been developed, which involve the administration of chemoconvulsants (e.g. strychnine, PTZ, picrotoxin, isoniazid, lithium PIL, yohimbine, bicuculline, 4-aminopyridine, KA and penicillin), the electrical stimulation (e.g., maximal electroshock and kindling), genetic changes (e.g., knock-out mice), and other injurious stimuli (e.g., trauma, hypoxia, stroke); this large number of whole-animal models is important to match the equally numerous types and causes of human epilepsy **(Kasthuri et al., 2013)**. Also, with regard to the used species, animal models range from drosophila and zebrafish to nonhuman primates **(Easter et al., 2009; Sarkisian, 2001)**. However, perhaps due to practical reasons, rodents have been the most frequently used species in the discovery and development of new AEDs **(Löscher, 2011)**.

Hence, innumerable animal models of epilepsy and epileptic seizures have been described and they can be allocated to different categories, e.g. models with spontaneously occurring seizures *versus* chemically or electrically induced seizures, models with recurrent seizures *versus* models with single seizures (i.e., chronic *versus* acute models), models with partial seizures *versus* models with generalized seizures, among others **(Löscher, 2011)**. In this context, an important distinction that is also often made is the separation between acute seizure models and chronic epilepsy models. In acute seizure models, an electrical or chemical stimulus is used for induction of seizures in naïve heathy rodents or other animal species; therefore, these models are called as induced seizure models. Otherwise, to develop chronic models of epilepsy an electrical or chemical repetitive stimulation is usually required to induce epilepsy in healthy

animals. Alternatively, animal models of disease that intrinsically exhibit spontaneous recurrent unprovoked seizures can also be used, mimicking more closely the phenomenology observed in human epilepsy **(Campos et al., 2018)**. Hence, each whole-animal model of epileptic seizures or epilepsy has its own characteristics, and the suitable animal model to be selected for a particular experiment depends on the objectives of the study itself **(Löscher, 2011; Stables et al., 2003)**. In the following sections some of the most commonly reported animal models and especially those of greater interest within the scope of the present thesis are discussed in more detail.

I.1.7.1. Acute seizure models

As aforementioned, in acute seizure models, the seizures are usually induced in rodents (mice and rats) through a chemical or electrical stimulation **(Simonato et al., 2014)**. Historically, the majority of AEDs were identified through screening assays for anticonvulsant activity in animal models of acute seizures, of which the maximal electroshock (MES) test and the subcutaneous pentylenetetrazole (scPTZ) test were the most commonly used **(Liu et al., 2010)**.

In the MES model, bilateral transcorneal or transauricular electrodes are used for the application of a high frequency but short duration electrical stimulus (50 mA in mice and 150 mA in rat), inducing initially a severe tonic phase which then evolves into clonic seizures **(Castel-Branco et al., 2009)**. The MES test enables the identification of anticonvulsant agents with activity against generalized tonic-clonic seizures and focal seizures (Table I.4) **(French et al., 2013)**.

Recently, the 6-Hz model, another model involving electrical stimulation, has re-emerged as an acute seizure model with a unique pharmacologic profile, which allowed the identification of a number of clinically important AEDs (e.g. LEV and CLZ) inactive in the MES test (Table I.4) **(French et al., 2013; Löscher, 2011).** In the 6-Hz model, electrical stimulation by low-frequency (6-Hz) and long duration (3 s) is delivered through corneal electrodes, inducing seizures that are reminiscent of "psychomotor seizures" occurring in human limbic epilepsy **(Löscher, 2016)**. The 6-Hz seizure test has also been re-evaluated as a potential screening model for drug-resistant epilepsy, and it is perhaps the best suited as an acute seizure model that can be used to evaluate promising investigational AEDs **(French et al., 2013)**.

Table I.4. Correlation of the efficacy of antiepileptic drugs in whole-animal models and human epilepsy **(French et al., 2013)**.

Animal model	Seizure phenotype	Human correlation	AEDs active in the model
Maximal electroshock	Tonic–extension seizure	Generalized tonic-clonic seizures, focal seizures	PHT, CBZ, OXC, VPA, PB, FBM, GBP, LTG, LCM, TPM, ZNS, RTG
Subcutaneous pentylenetetrazole	Minimal clonic seizure	Generalized myoclonic seizures	ESM, VPA, BZD, RTG, FBM, GBP, PB[a], TGB[a], VGB[a]
6-Hz (32/44 mA)	Limbic seizures secondarily generalized	Pharmacoresistant limbic seizures	CLZ, FBM, LCM, LEV, RTG, VPA
Kindled rodent	Limbic seizures secondarily generalized	Limbic seizures	CBZ, OXC, PHT, VPA, PB, BZD, FBM, GBP, PGB, LCM, LTG, TPM, TGB, ZNS, LEV, VGB, RTG
GAERS, Lethargic mouse, and Wistar rat	Spike–wave discharges[b]	Absence seizures	ESM, VPA, BZD, LTG, TPM, LEV

BZD, benzodiazepines; CBZ, carbamazepine; CLZ, clonazepam; ESM, ethosuximide; FBM, felbamate; GAERS, generalized absence epilepsy rat from Strasbourg; GBP, gabapentin; LCM, lacosamide; LEV, levetiracetam; LTG, lamotrigine; OXC, oxcarbazepine; PB, phenobarbital; PGB, pregabalin; PHT, phenytoin; RTG, retigabine; TGB, tiagabine; TPM, topiramate; VGB, vigabatrin; VPA, valproic acid; ZNS, zonisamide.
[a]PB, TGB, and VGB block clonic seizures induced by subcutaneous pentylenetetrazole but are inactive against generalized absence seizures and may exacerbate spike-wave seizures.
[b]Models of spike-wave seizures not routinely employed in initial evaluation of investigational drugs.

The scPTZ test, also known as subcutaneous metrazol test, was also extensively used to discover drugs with efficacy against non-convulsive absence seizures or myoclonic seizures **(Löscher, 2002; Łukawski et al., 2016)**. The scPTZ model is based on the subcutaneous administration of PTZ, a convulsant compound, in a dose sufficient to produce a minimal clonic seizure of the vibrissae and/or forelimbs that persist for at least 5 seconds **(Campos et al., 2018)**. The absence of seizures induced by scPTZ (85 mg/kg in mice and 56.4 mg/kg in rats) advocates that the test substance raises the seizure threshold **(Liu et al., 2010)**.

As a chemoconvulsant agent, PTZ seems to act as a selective antagonist of the $GABA_A$ receptor/chloride ionophore complex and it affects GABAergic and glutamatergic systems in many brain regions, including hippocampus **(Patsoukis et al., 2004; Psarropoulou et al., 1994; Rubio et al., 2010)**. Other chemoconvulsant agents that have been used to induce acute seizures consist of a variety of $GABA_A$ receptor-related substances (e.g., bicuculline, picrotoxin), glutamic acid decarboxylase inhibitors (e.g., isonicotinehydrazide, 3-mercaptopropionic acid, allylglycine), excitatory amino acid-related substances (e.g., KA, *N*-methyl-D,L-aspartate, homocysteine, quisqualic acid), and acetylcholine-related substances (e.g., PIL) **(Liu et al., 2010)**. Although these chemoconvulsant compounds can be used to induce acute seizures, the observed phenotype is highly dependent of the dose level administered and the number of administrations; in fact, repeated administrations of

subconvulsant doses of some convulsant agents (e.g., PTZ, KA, tetanus toxin and penicillin) can result in the development of chronic epilepsy models.

One of the main limitations of these acute seizure models is the fact that they do not represent the development of epilepsy. Thus, the drug candidates identified using these classic models exert a seizure-suppressing effect but they do not affect or prevent the mechanisms underlying epilepsy or associated comorbidities **(Simonato et al., 2014)**.

I.1.7.2. Chronic epilepsy models

The models of chronic epilepsy are important because the goal is generally to treat epilepsy rather than symptomatic seizures in normal brain. Therefore, some chronic models of epilepsy do not exhibit seizures at an early stage but are instead characterized by a latency period of one or two weeks during which the neuronal circuits undergo epileptogenesis that then leads to spontaneous seizures. In contrast, several other chronic epilepsy models are based on an initial very prolonged period of seizures known as *''status epilepticus (SE)''*, which can be induced by KA, PIL or prolonged electrical stimulation; in all these cases epileptogenesis also occurs during a latent period of a week or two before spontaneous seizures start, but in these models there is a substantial neuronal loss caused by SE at the outset. Actually, the concept of latency period in acquired, chronic models of epilepsy is important, since it models the clinical concept that acquired epilepsies commonly occur months or years after a precipitating injury **(Jefferys, 2010)**. Hence, the chronic models are also very useful to understand the process of epileptogenesis and the progressive nature by which epilepsy is developed; indeed, they can be used to investigate all of the causal factors, including the initial insult, latency phase and spontaneous seizures **(Liu et al., 2010)**. Currently, a major priority continues to be finding new therapeutic ways to stop the process of epileptogenesis. In fact, as previously mentioned most AEDs have minimal impact on epileptogenesis, so other classes of drugs are being tested.

I.1.7.2.1. Kindling models

The kindling models of epilepsy usually involve a progressive stimulation of brain excitability by application of repeated excitatory stimuli (electrical or chemical) preferentially in structures of the limbic system, such as amygdala or hippocampus, thus increasing seizure susceptibility **(Erkeç and Arihan, 2015)**. The repeated stimulation eventually culminates in emergence of spontaneous seizures and in the establishment of a permanent epileptic state. The kindling model is one of the most common models of complex partial seizures with secondary generalization, and it has been used expansively to model TLE **(Liu et al., 2010; Löscher, 2016)**. Electrical or chemical kindling are epileptogenic models used for understanding the epileptogenic process and for evaluating molecules that could preventing this phenomenon **(Erkeç and Arihan, 2015)**.

Different stages of seizures are perceived during kindling process. Racine's grading of convulsive stages of kindling was established on electrical stimulation of amygdala, which comprises five stages. The stages 1 and 2 are primarily associated with facial and oral activities which include ipsilateral eye closure and blinking followed by head bobbing and drooling. Forelimb clonus eventually appears in the stage 3. Afterward, in stage 4, the seizures generalize with stronger clonus and rearing. Then dramatic rearing and falling behavior is observed in stage 5. Researchers continue to stimulate the animal in most kindling studies till the development of stage-5 seizures. When more carefully examined, it should be observed that the amygdala kindled stage-5 seizures are not merely clonic but rather tonic-clonic seizures, and they involve all four limbs. An animal (usually a rodent) is considered as fully kindled if there is development of seizures with phenotypic characteristics of stage 5 **(Liu et al., 2010)**. Accordingly, kindling is an expensive and time-consuming process, implicating long periods of handling and stimulation events. Furthermore, in electrical kindling exists the risk of losing or damaging the chronic implants **(Kandratavicius et al., 2014)**. Also, concerning chronic models of epilepsy, the limbic kindling features are different from other models, such as KA or PIL, which begins by severe SE related to prominent temporal and extratemporal damage **(Kandratavicius et al., 2014)**.

I.1.7.2.2. Models based on *status epilepticus*

Clinically, SE is a medical emergency characterized by a continuous seizure activity or multiple seizures without regaining consciousness for more than 30 minutes, that if untreated, can lead to brain damage and even death **(Reddy and Kuruba, 2013)**. Common experimental models of SE in rodents involve the administration of the chemoconvulsants KA or PIL. These models of SE are used to investigate the transition from a single episode of SE to chronic epilepsy, the mechanisms of neuronal injury and susceptibility, the synaptic reorganization (sprouting), the hippocampal sclerosis, the seizure-induced changes in gene expression and neurogenesis, and new drugs as well **(Reddy and Kuruba, 2013)**.

I.1.7.2.2.1. Kainic acid models

Kainic acid or KA, which in Japanese means ''monster from the sea or ghost of the sea'', is a structural analog of Glu **(Liu et al., 2010)**. It was originally isolated from the seaweed *Digenea simplex*, which was used in Japanese folk medicine as an ascaridial preparation. The KA model of epilepsy has been used since it was described **(Olney et al., 1974)**, and it is a powerful neuronal excitatory compound with high affinity for KA receptors of the neurotransmitter Glu **(Reddy and Kuruba, 2013)**.

The administration of KA can be performed in rats by intracerebroventricular (0.4–0.8 μg) or systemic routes (8–12 mg/kg by subcutaneous or intraperitoneal route); in mice, the convulsant

dose of KA usually used is of 20–40 mg/kg by intraperitoneal route, but this dose is somewhat variable depending on the strain employed **(Reddy and Kuruba, 2013)**.

In terms of phenotypic features, within five minutes after systemic injection of KA, rats exhibit a catatonic posture with staring; nine minutes after injection, the first epileptic signs are identified in the EEG (spikes or bursts), followed by wet-dog shakes around twenty minutes post-dose; later, animals show some motor signs (sniffing, masticatory movements, head nodding, rearing, and loss of postural control); about one hour after KA injection, the onset of continuous epileptiform spiking activity is observed, which is considered to be a marker for the onset of SE. Then, separate recurrent seizures will become more protuberant, and culminate in SE that may last for several hours **(Liu et al., 2010)**.

Regarding mice, in some inbred strains it has been found that they do not display the characteristic pattern of hippocampal neurodegeneration observed in rats following seizures induced by the systemic administration of KA. However, the differences in hippocampal neurodegeneration among the strains do not appear to be correlated with the strain differences in the pharmacokinetics (uptake or metabolism) of KA, or strain differences in the distribution of Glu receptors **(McLin and Steward, 2006)**.

KA has the benefit of producing usually hippocampus-restricted injuries, in contrast to PIL, which can also produce lesions in neocortical areas **(Kandratavicius et al., 2014; Rubio et al., 2010; Sharma et al., 2007)**. However, extra-hippocampal areas are also pointedly compromised in human TLE **(Bonilha et al., 2010)**, making PIL another useful chemoconvulsant.

I.1.7.2.2.2. Pilocarpine models

PIL is a cholinergic muscarinic-type agonist that provokes seizures in rodents after systemic or intracerebral administration **(Curia et al., 2008; Kandratavicius et al., 2014; Rubio et al., 2010; Turski et al., 1989)**. PIL can be administered in high doses (normally 320–400 mg/kg), or, if lithium (3 mEq/kg, corresponds to 127 mg/kg) is given 24 hours before, it can be administered in a smaller dose (30 mg/kg) **(Curia et al., 2008)**. Lithium pre-treatment, followed by low doses of PIL, produces SE with a lower mortality than the observed with higher doses **(Glien et al., 2001)**. In addition, other less common variations of the PIL model have been established by combining this convulsant with other drugs picrotoxin, cycloheximide, MK-801 and *N*-omega-nitro-L-arginine methyl ester **(Curia et al., 2008)**.

PIL produces several behavioral alterations, including staring spells, facial automatisms, salivation, piloerection, and behavioral automatisms, such as stereotypic scratching, grooming, sniffing, and chewing that resemble stage 1- or 2-kindled seizures. These behavioral features are followed by limbic motor seizures that develop approximately 30 minutes after PIL injection. Stage 4 or 5 seizures start 10 minutes later, and seizure activity progresses to limbic SE that would last for several hours. In the development of PIL model, SE is allowed to continue for 90 minutes, period after which seizure activity is stopped with DZP to reduce the high mortality rate characteristic of this model. The most sensitive regions for epilepsy-related

damage after PIL-induced convulsions are amygdala, thalamus, olfactory cortex, hippocampus, neocortex, and *substantia nigra* **(Liu et al., 2010)**.

PIL model is widely used because it results in severe SE, and eventually develops an epileptic phenotype with features very similar to those of human TLE, resulting in its widespread use for studying both of these conditions **(Benke and Brooks-kayal, 2006)**. Nevertheless, mortality rates in the PIL model are higher, since approximately 30–40% of treated animals will not survive to SE **(Curia et al., 2008)**.

I.1.7.3. Genetic models of epilepsy

Various genetic whole-animal models of epilepsy have been characterized over the last decades **(Rubio et al., 2010)**. Genetic animal models of epilepsy comprise genetically predisposed animal species in which seizures occur either spontaneously or in response to sensory stimulation. The major advantage of these models as whole-animal models of human epilepsy is that they better mimic the clinical condition than any other experimental model of epilepsy. Models with spontaneous recurrent seizures include epileptic dogs, tottering mice, and rats with spike-wave absence seizures. Models with reflex seizures comprise photosensitive baboons (*Papio papio*) and fowl, audiogenic seizure-susceptible mice and rats, and gerbils with seizures in response to different sensory stimuli **(Rubio et al., 2010)**. More specifically, these genetic models include, for example, DBA/2 mice with audiogenic seizures and rats mutants with spontaneously occurring spike-wave absences in the EEG, such as the genetic absence epilepsy rat from Strasbourg (GAERS) **(Loscher and Schmidt, 1988; White et al., 2006)**.

Actually, the particular pharmacologic profiles of the GAERS, the inbred Wistar Albino Glaxo Rat from Rijswijk (WAG/Rij), and the Lethargic mouse support their usefulness as important and validated models of human absence seizures (Table I.4) **(French et al., 2013)**.

I.2. Bioanalysis

I.2.1. An overview

Overall, bioanalysis comprises all aspects related to the identification and quantification of analytes (drugs, metabolites, proteins, peptides, and biomarkers) in biological samples (e.g. blood, plasma, serum, saliva, urine, feces, skin, hair, organ tissue) **(Moein et al., 2016)**.

Nowadays, many important areas involving human health are dealing with the analysis of chemical or biochemical substances present in living organisms or biological fluids. At the same time, the detection and quantification of biomarkers is gaining an increasing attention in medical research, including for diagnostic purposes. A typical biomarker measures a given characteristic that is considered as a representative of biological or pathological status and it should be an unambiguous indicator of exposure or disease. Moreover, biomarkers cover a range of compounds from gases to biological macromolecules, and they are often found in the nanomolar range in complex biological samples **(Hamidi et al., 2018).** According to **Hill (2009)**,*"the cutting edge of analysis is based around the ability to identify, quantify and locate analytes at the cellular level"*. In fact, the identification of biomarkers and progress in related bioanalytical methodologies for the diagnosis of several diseases in early stages is currently a hot topic in the preclinical and clinical research. For instance, aldehydes are lipid peroxidation byproducts that have been introduced as biomarkers of tissue damage caused by oxidative stress **(Hamidi et al., 2018).**

Bioanalysis is an important discipline in research areas such as forensic analysis and doping control and is also an essential part in drug discovery and development. Actually, bioanalysis is well established in pharmaceutical industry to support the discovery and development of new drugs, having an important role to perform toxicokinetic, pharmacokinetic and pharmacodynamic studies **(Moein et al., 2016)**. However, biological samples are difficult to process due to their inherent complexity. This complexity usually hinders the direct analysis, as most of the biological fluids are not amenable with analytical instruments and, therefore, appropriate sample preparation procedures are required to a reliable identification and quantification of the analytes of interest, often present in sample at sub ng/mL levels **(Hamidi et al., 2018; Moein et al., 2016).**

Over the last few years, considerable progresses in the development of more powerful analytical and detection systems have occurred, which have continuously changed bioanalytical demands, emerging more sensitive, reliable and automated bioanalytical assays as a result of the combination of miniaturized sample preparation techniques with advanced analytical systems **(Alves et al., 2013)**. Thus, we are certainly not making a mistake when referring to the fact that, over time, bioanalysis has reflected the technical progresses and developments and has contributed to advances in multiple scientific areas. Consequently, today's reality is quite different from that of the past, even if not very distant.

Historically, the first studies to measure the existence of drugs in biological fluids were to determine potential overdosing as part of the emergence of the new science of forensic medicine/toxicology. The necessity to measure drug levels in biological fluids was further motivated by the development of pharmacokinetics as a scientific branch of pharmacology in the 1930s. The advances of chromatographic techniques such as the paper chromatography initiated in the 1940s and allowed separation of a drug from its metabolites. Ten years later, thin layer chromatography was used to determine drugs in biological fluids, but the sensitivity of these technologies was not suitable for the determination of new drugs, such as the tricyclics drugs that had therapeutic levels in the range of ng/mL **(Hill, 2009)**.

In the early 1950s, gas chromatography (GC) became widely used in the pharmaceutical industry. This technique improved the selectivity and sensitivity with the development of more sensitive detectors, such as the flame ionization detector. Later, a variety of additional detectors became commercially available, such as detectors of mass spectrometry (MS) **(Hites, 2016)**.

The rise of high-pressure liquid chromatography (HPLC) initiated in the late 1960s and early 1970s; unlike GC, it did not involve laborious sample preparation procedures and analyte derivatization, or difficulties caused by thermal lability or existence of water in the sample. Thus, the popularity of HPLC increased as the diversity of detectors increased, allowing this methodology to become more sensitive in line with the increasing potency of new therapeutic agents **(Hill, 2009)**. Hence, the bioanalytical methodologies, mainly the chromatographic ones, have crossed many scientific disciplines where drug analysis in biological fluids is important **(Pandey et al., 2010)**.

At this point, it is important to highlight that the implementation of a bioanalytical method for use in routine firstly requires its appropriate development, optimization and validation. However, each analytical method has its own features that depending on the target analyte(s) and appropriateness of the method may be influenced by the purposes of the studies **(Shah et al., 2000)**. Accordingly, before starting the development of a bioanalytical method there are many points to consider. These points are analyte chemical structure, pKa value, solubility properties; stability and adsorption properties to plastic or glass devices; nature of the matrix; sensitivity required and instrumentation. Bioanalytical method development includes two main parts, sample preparation and analyte(s) detection. Sample preparation has an important role in bioanalysis to get clean extracts with high extraction efficiency. Additionally, the choice of the detection system is dependent on the analyte concentration range **(Pandey et al., 2010)**.

Subsequently to the development and optimization of analytical conditions, the method must be correctly validated. Bioanalytical method validation is the methodical process of defining the analytical requirements and confirming that the method under consideration has performance capabilities consistent with the demands of its intended application. Thus, each methodological step should be explored to determine the extent to which environment, matrix or multiple variables can affect the estimation of the analyte from the time of sample collection

up to the analysis. Indeed, the success of validation depends on the performance achieved in the development and optimization of the bioanalytical method **(Chandran and Singh, 2007)**.
It is essential to guarantee that the analytical methods generate accurate, precise and consistent data to enable a correct interpretation of the studies they support. In this context, one of the first formal regulatory guidances on how to carry out bioanalysis was published in 1977 by the U.S. FDA as part of the "Bioequivalence and Bioavailability" regulations **(Hill, 2009)**. Since that time, the need to define widely accepted bioanalytical criteria to be used in the measurement of drugs has driven the regulatory process. These bioanalytical criteria were discussed at the meeting "Analytical Methods Validation, Bioavailability, Bioequivalence and Pharmacokinetic Studies" in 1990 **(Shah et al., 1992)**. Later other conferences were held concerning this issue, resulting in the publication of the relevant report "Guidance for Industry on Bioanalytical Method Validation", in 2001 **(FDA, 2001)**. Originally issued in 2001, the U.S. FDA guidance was revised to reflect the advances in bioanalytical science and technology and, in September 2013, an updated draft of the document was made available for public review and comments before its implementation **(FDA, 2013)**. The final version resulting from this review took years to be finalized, and its final version was only recently published **(FDA, 2018)**. Other international guidelines have been published by various regulatory agencies, such as the European Medicines Agency (EMA); for instance, the current version of the EMA guideline for validation of bioanalytical methods applied in the quantitative analysis of samples from animal and human studies was released in July of 2011 **(EMA, 2011)**.

I.2.2. Chromatographic analysis of neurotransmitters in brain

The brain is the most refined organ in nature. This complex organ is responsible for the control of the body activities, and for perception, behavior, cognition, memory, and consciousness. Thus, there are more than 1000 disorders related with dysfunctions of the CNS, such as neurological and psychiatric disorders **(Liu et al., 2010)**. The human CNS consists of 10^{10}–10^{11} neurons and most likely a 10-fold higher number of glial cells **(Temburni and Jacob, 2001)**. In general, it can be assumed that each neuronal cell type in the brain has a distinct physiological role and their individual characterization is indispensable for the understanding of brain function **(Liu et al., 2010)**.
In the human brain, neurons communicate with each other at junctions called synapses, by releasing chemical messengers called neurotransmitters **(Snyder, 2009)**. Prior to their release into the synaptic cleft, neurotransmitters are stored in secretory vesicles (called synaptic vesicles) near the plasma membrane of the axon terminal. Indeed, the release of neurotransmitters occurs most often in response to the arrival of an action potential at the synapse. At synapse, an action potential triggers the presynaptic neuron to release neurotransmitters. This induces postsynaptic electrical responses by binding to members of a diverse group of proteins called receptors **(Gemperline et al., 2014; Purves et al., 2003)**. At this level there are two major classes of receptors: the ionotropic and the metabotropic

receptors. Ionotropic receptors are responsible for fast synaptic neurotransmission and decode chemical signals into electrical responses. They respond very rapidly to the transient release of neurotransmitters, thereby affecting the excitability of cells. In contrast, the postsynaptic responses triggered by the activation of metabotropic receptors occur much more slowly, over seconds or minutes, because these receptors regulate the opening and closing of ion channels indirectly **(Smart et al., 2012)**.

Virtually, all neurotransmitters undergo a similar cycle of use, which involves synthesis and packaging into synaptic vesicles; release from the presynaptic cell; binding to postsynaptic receptors; and, finally, rapid removal and/or degradation. Three primary criteria have been used over the years to establish that a molecule acts as a neurotransmitter: 1) the substance must be present within the presynaptic neuron, 2) be released in response to presynaptic depolarization and the release must be calcium-dependent, and 3) act on specific receptors on the postsynaptic cell **(Purves et al., 2003)**. Moreover, they are rapidly inactivated by highly specific transporter systems or enzymes in order to prevent continuous activation of the postsynaptic nerve cells **(Gemperline et al., 2014)**.

More than 100 substances have been identified as neurotransmitters since 1914, when the first neurotransmitter was discovered – the Ach **(Gemperline et al., 2014)**. The biogenic amines (DA, NE, E, 5-HT and histamine) were well established as neurotransmitters in 1970. Later on, amino acids were identified as neurotransmitters, with GABA being accepted after a lengthy process and glycine strongly suspected as inhibitory neurotransmitters; however, additional evidence had to be produced for Glu **(Engidawork et al., 2015)**.

Overall, neurotransmitters can be differentiated into two broad categories simply based on size (Figure I.4).

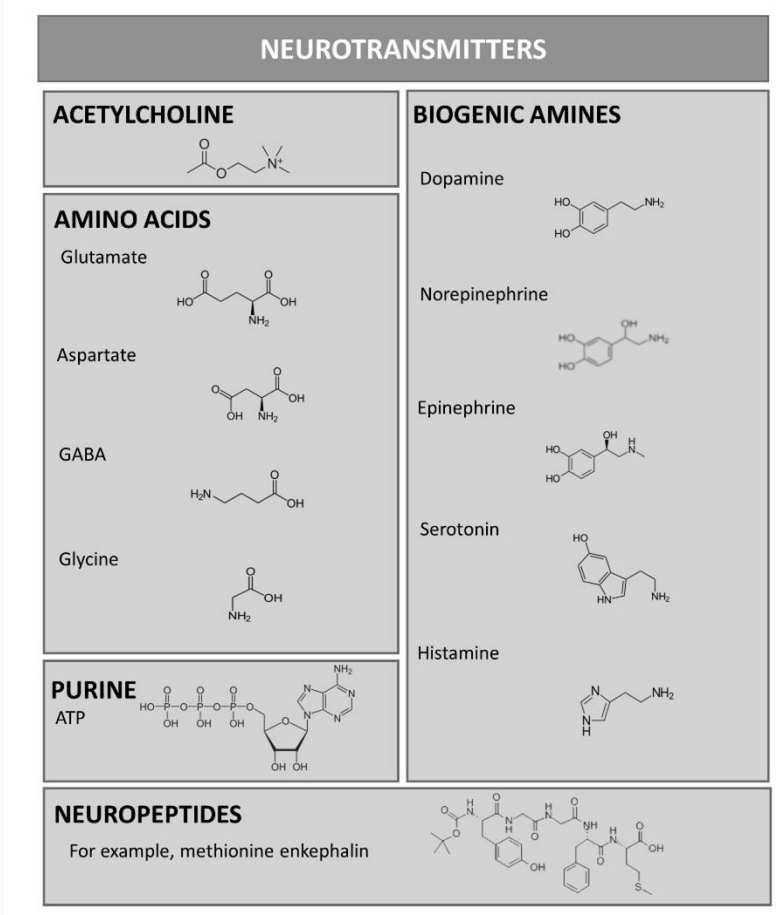

Figure I.4. Examples of small-molecule and peptide neurotransmitters. Small-molecule neurotransmitters can be subdivided into acetylcholine, amino acids, purines, and biogenic amines. Adapted from **Purves et al. (2003)**.

As neurotransmitters of small molecular size can be mentioned the individual amino acids (e.g. Glu, Asp, GABA and glycine) as well as the Ach and biogenic amines, whereas neuropeptides are relatively lager molecules composed of 3 to 36 amino acids **(Purves et al., 2003)**. Most of small-molecule neurotransmitters modulate activities requiring fast responses **(Engidawork et al., 2015)**. On the other hand, peptides and some small-molecule neurotransmitters tend to modulate slower and ongoing actions, and these compounds have slower turnover rates **(Gemperline et al., 2014; Purves et al., 2003)**. Due to differences in modes of action, neuropeptides are sometimes considered to be neuromodulators. Thus, neuropeptides with neurotransmitter functions are often defined as putative neurotransmitters or co-transmitters **(Gemperline et al., 2014)**. In Table I.5 is presented a summary of particular aspects related to functional activity, synthesis, release, and removal mechanism of important neurotransmitters.

Table I.5. Main functional features of important neurotransmitters **(Purves et al., 2003)**.

Neurotransmitter	Postsynaptic effect	Precursor	Rate-limiting step in synthesis	Removal mechanism
Acetylcholine	Excitatory	Choline + Acetil-CoA	Choline acetyltransferase	Acetylcholinesterase
Catecholamines	Excitatory	Tyrosine	Tyrosine hydroxylase	Transporters, MAO, COMT
Serotonin	Excitatory	Tryptophan	Tryptophan hydroxylase	Transporters, MAO
Histamine	Excitatory	Histidine	Histidine decarboxylase	Transporters
Glutamate	Excitatory	Glutamine	Glutaminase	Transporters
GABA	Inhibitory	Glutamate	Glutamic acid decarboxylase	Transporters
Glycine	Inhibitory	Serine	Phosphoserine	Transporters
Adenosine triphosphate	Excitatory	Adenosine diphosphate	Mitochondrial oxidative phosphorylation, glycolysis	Hydrolysis to adenosine monophosphate and adenosine
Neuropeptides	Excitatory and inhibitory	Amino acids	Synthesis and transport	Proteases

Acetil-CoA, acetyl coenzyme A; COMT, Catechol-*O*-methyltransferase; MAO, Monoamine oxidase.

It is essential the availability of consistent approaches and techniques to better understand the brain activity in physiological conditions, as well as in dysfunctional conditions and even in "disease" states **(Bosse et al., 2013)**. Indeed, a deeper neurochemical knowledge of neurological systems provides the opportunity to design new tactics and drug treatments and to identify the basic mechanisms of action of drugs.

In recent decades, neurochemical measurements have led to many advances in understanding the association between chemistry in the CNS and the functional state of an organism. In this scope, neurotransmitters have been actively researched regarding their roles in various diseases and applications. Making such measurements, particularly in the brain tissue, is fraught of difficulties related with the complex and delicate nature of this tissue, beyond the requirements needed to achieve high selectivity and suitable resolution **(Perry et al., 2009)**.

The structure determination and quantitation of neurotransmitters in brain tissue can be performed by GC, but the complexity of sample preparation, analysis time and cost has shifted the focus toward liquid chromatography (LC). The particular features of most neurotransmitters and their properties explain the extensive history of conventional HPLC methods for their analysis. Also, the design of HPLC assays is straightforward, which allows the development of improved methods **(Jong et al., 2011)**.

The bioanalytical methods used for detection and quantification vary with structure, location, endogenous concentration, manner of degradation of the analyte(s), among others. Except for most peptides, the other neurotransmitters are produced and stored in nerve terminals, being readily available for release. Thus, they can be present in quite high concentrations in brain extracts (10 pmol/g), but once released their degradation is generally rapid (within seconds) and their concentrations in perfusates and body fluids (e.g. plasma and urine) can fall to femtomole levels **(Bovingdon and Webster, 1994)**.

In this context, most recent analytical techniques published in literature (2010-2018) for the determination of specific neurochemicals in brain samples, including biogenic amines, amino acids and other mediators, were summarized and are presented in Appendix I. For more than 50 years, biogenic amines have dominated thinking about the role of neurotransmitters in the pathophysiology of several brain disorders. Thus, research has been largely focused on monoamines, particularly on catecholamines and, consequently, from a bioanalytical perspective they are the most commonly targeted analytes, as can be seen in Appendix I. In the following sections are highlighted and discussed in more detail relevant bioanalytical aspects related to the analytical methods summarized in Appendix I.

I.2.2.1. Sample preparation: Brain tissue

The brain has multiple functions and each different brain region controls a diversity of complex behaviors in human beings and animals. Thus, distribution of small-molecules (drugs, metabolites or biomarkers) in brain tissue has physiological and pharmacological significance due to their different influence on such brain functions. Quantitative studies of small molecules in brain tissue can be useful for multiple purposes such as, to study tissue distribution of drug candidates; to assess pharmacokinetic profiles of drugs; to measure biomarker levels; or to investigate the neurotoxicity of chemicals **(Li and Bartlett, 2014)**.

Nowadays, it is possible to evaluate the concentrations of neurotransmitters by direct sampling under *in vivo* conditions from different parts of the brain using specialized techniques; microdialysis is the most common technique. Neurotransmission can also be studied in brain tissue, which enables the study of different regions of the brain, but it has to be performed *post mortem* in most cases. In addition, cerebrospinal fluid (CSF) can also be analyzed, but CSF concentrations reflect average concentrations accumulated from all brain regions, and analysis of a specific brain area is not possible **(Suominen, 2015)**.

The brain microdialysis technique is the chosen method to monitor several neurotransmitters and their alterations during the brain activity, which can be associated with a specific behavior or brain-altered function. Notwithstanding the fact that microdialysis procedure can be considered as an excellent technique to monitor neurotransmitters in brain, it has some serious drawbacks, such as the small sample volumes that can be collected. Furthermore, the limited length of the microdialysis membrane leads to low concentrations of recovered substances **(Yoshitake et al., 2006)**. Therefore, this requires that the microdialysis has to be coupled to

refined and highly sensitive analytical systems. Another obvious disadvantage is the insertion of the probe that induces a local injury **(Plock and Kloft, 2005; Zhang and Beyer, 2006)**.

As a singular organ with exceptional functions and anatomy, the brain has a different matrix composition that makes it more complex in terms of sample preparation **(Li and Bartlett, 2014)**. Analyzing neurotransmitters from the brain samples involves extraction and purification procedures in order to safeguard that the analyte(s) are present and free from significant impurities in sample extracts. Selecting the best conditions for a sample clean-up procedure will depend on the detection system and it is imperative to achieve a fast, reproducible and cost-effective method **(Perry et al., 2009)**.

The main challenge for brain tissue analysis is its high lipid composition, which constitutes about half of the dry weight of brain. The brain tissue contains phospholipids, sterols, sphingolipids, gangliosides, cerebrosides, sulfatides and phosphoinositides. Due to the nonpolar phosphate ester group and the long alkyl chain, lipid molecules are very hydrophobic and thus they may be extracted together with the analytes when sample preparation methods based on hydrophobicity are used **(Li and Bartlett, 2014)**.

As is usually the case with other solid biological tissues, the first step in processing solid brain tissue samples involves homogenization procedures in order to transform the sample into a liquid form. Homogenization is a process that uses mechanical power to disrupt the tissue and disperse it into a certain solution (usually a buffer) to form a stable suspension. Tissue homogenization can be achieved by diverse types of techniques, including grinding, rotating blade, bead beating and ultrasonication. Rotating blade is the most typically used homogenization technique for brain tissue samples, particularly due to its low cost and easy operation. Nevertheless, attention is required to ensure the cleaning of the blades, because the occurrence of cross-contamination can be a problematic issue. Regularly, these procedures of sample handling are carried out on ice or at low temperatures (approximately 4 °C). Another aspect affecting the outcome of homogenization is the medium in which the brain tissue is dispersed; it can be pure water, organic acids, aqueous buffers, organic solvents or mixtures of more than one of these agents. Usually, to one unit weight of brain tissue, at least one to two volumes of the homogenization medium is added **(Xue et al., 2012)**.

Organic acids, formic acid or inorganic acids, such as perchloric acid, are also commonly used aqueous homogenization media **(De Benedetto et al., 2014; Cui et al., 2017; Fitoussi et al., 2013; Fonseca et al., 2017, 2018; Hong et al., 2013; Parrot et al., 2011; Perucho et al., 2015; Stragierowicz et al., 2017; Tsunoda and Funatsu, 2012; Zhu et al., 2011)**. These homogenization media facilitate the breakdown of cell membranes, adjusting the pH of the homogenate for the ionization/deionization or stability of the analyte as well. In the case of strong acids, they can also irreversibly denature and precipitate the proteins of the brain samples **(Li and Bartlett, 2014)**.

In some circumstances, ascorbic acid, sodium bisulphite, ethylenediaminetetraacetic acid (EDTA) or cysteine have been added to increase the stability of the analytes **(De Benedetto et**

al., 2014; Fonseca et al., 2017, 2018; Maldonado and Maeyama, 2013; Parrot et al., 2011; Redondo-Castro et al., 2014). For example, cysteine was used in a study published by De Benedetto et al. **(2014)** for the determination of DOPAC, NE, DA and 5-HT in brain tissue samples by HPLC coupled to fluorescence detection (FLD). The addition of cysteine to the brain tissue led to the stabilization of DA and 5-HT in their hydrochloride form. Therefore, based on the work of this author and reasoning, standard solutions and samples were also prepared in the same way in our research works **(Fonseca et al., 2017, 2018)**.

Methanol and acetonitrile have been the most commonly used organic solvents for homogenization of brain tissue **(Allen et al., 2017; Bathena et al., 2012; Wei et al., 2014; Xu et al., 2011; Zheng et al., 2012)**, perhaps due to their moderate hydrophobicity and precipitating strength. Instead of using organic solvents that have strong precipitating abilities, a mixture of organic solvents (acetonitrile or methanol) and water or aqueous solutions has also been used as homogenization medium **(Falasca et al., 2012; de Freitas Silva et al., 2009; He et al., 2013; Huang et al., 2014; Şanlı et al., 2015; Xu et al., 2014)**.

Impurities such as lipids, proteins and salts are responsible for a number of difficulties in the chromatographic analysis, including but not limited to matrix effects, peak shape distortion, column clogging and instrument contamination **(Li and Bartlett, 2014)**. Therefore, in addition to brain tissue homogenization, further sample preparation procedures are often required before the injection into chromatographic system in order to achieve an acceptable sensitivity and selectivity in the analysis as well as to preserve the performance of the instruments. To this end, the most commonly used sample preparation approaches are protein precipitation (PPT), liquid-liquid extraction (LLE), or solid-phase extraction (SPE) **(Li and Bartlett, 2014)**.

Insoluble proteins can be easily removed by filtration or centrifugation, thus the main target interferences in sample preparation are soluble proteins which need to be precipitated. PPT is a simple and fast sample preparation approach. It usually involves three steps: (1) addition of precipitating agents to the sample, (2) mixing, and (3) removal of the precipitated proteins. When miscible organic solvents are added to the brain tissue homogenate, the solvation layer around the protein will decrease as the organic solvent molecules displace water molecules from the protein surface. With a smaller solvation layer, proteins can get closer and form interactions with each other via attractive electrostatic or dipole interactions, leading to the aggregation **(Li and Bartlett, 2014)**.

For the analyses of neurotransmitters in brain tissue, commonly used organic solvents for PPT are acetonitrile and methanol **(Bathena et al., 2012; Peng et al., 2011; Xu et al., 2014)**. Acetonitrile has a strong precipitating ability and methanol is a milder organic precipitating agent, yielding flocculent protein sediments. The temperature is another factor that should be considered in PPT procedures, as ice-cold organic solvents can be used to keep the stability of analytes. After the addition of a precipitating agent to sample, the resulting mixture needs to be completely mixed by vortexing. Depending on the volume of samples, usually at least 1 to 10 min of vortexing is required. During vortexing mixing the molecules of precipitating agents

are uniformly dispersed across the fluid eddies and interact with proteins, and thus analytes bound to proteins will be released into the solution because protein conformations are changed. The next step is a physical process to separate precipitated proteins from the supernatant, in which the small-molecule analytes remain dissolved. Typically, a centrifugation force of 3000 to 12000 g is adequate to settle the protein precipitates and form a solid pellet **(Li and Bartlett, 2014)**.

Since sometimes centrifugation procedures are not capable to eliminate insoluble impurities, particularly those with lower densities, filtration has also been commonly used to aid in the purification of the supernatant **(Dam et al., 2014; Inoue et al., 2016; Monge-Acuña and Fornaguera-Trías, 2009; Park et al., 2013; Vermeiren et al., 2014; Wu et al., 2014)**, by loading the supernatant onto a filter tube with a submicrometer-sized (0.2 or 0.45 μm in most applications) membrane. Dilution or evaporation can also be used to reduce the impact of acids or organic solvents **(Li and Bartlett, 2014)**. As it can be seen in Appendix I, acid deproteinization of samples in combination with a filtration step or dilution are the most frequent choices for the pretreatment of brain tissue homogenate samples for chromatographic assays of neurotransmitters.

In general, LLE or SPE are not carried out for the analysis of neurotransmitters. This happens due the physicochemical characteristics of neurotransmitters and also because PPT approaches become faster and less expensive procedures. Furthermore, these operations are simple to perform, leaving less chance for error resulting from inter-batch or inter-operator differences. Moreover, PPT procedures are still associated with a high recovery efficiency. However, a weakness of simple PPT is the fact that the extracts obtained still contain many interfering components **(Li and Bartlett, 2014)**.

I.2.2.2. Chromatographic assays

The specific physicochemical characteristics of most neurotransmitters help to clarify the long history of HPLC methods developed for the determination of these compounds. Few authors have used GC coupled to MS detection for analysis of neurotransmitters, particularly due the time-consuming sample preparation procedures required **(Farthing et al., 2017; Hong et al., 2013; Pinto et al., 2014; Wang et al., 2012)**.

Despite the abundant variety of available chromatographic columns, reversed-phase octadecylsilane (ODS or C_{18}) columns are the standard choice (see Appendix I); indeed, C_{18} columns enable the fast elution of polar compounds and relatively short run times. However, there is a risk of too fast elution of the analytes and/or poor peak resolution, owing to the poor retention of polar compounds in the hydrophobic stationary phase. To overcome this constraint has emerged the hydrophilic interaction liquid chromatography (HILIC) columns **(Bicker et al., 2013)**. In this case analytes will interact with the mobile phase and hydrophilic environment of stationary phase by hydrogen and electrostatic bonding. The HILIC columns have been

frequently used in methods involving MS detection **(Bathena et al., 2012; Falasca et al., 2012; Forgacsova et al., 2018; Inoue et al., 2016; Peng et al., 2011)**.

The composition of the mobile phase is another important aspect to establish during the development and optimization of a method. The nature and proportion of the organic modifier, the composition of the aqueous solution and the final pH value are key variables to consider. For instance, the mobile phase pH influences the degree of ionization of the analytes which is determined by their pKa values **(Bicker et al., 2013)**. Consequently, the degree of ionization of the compounds determines different chromatographic behaviors. For example, compounds such as HVA and DOPAC, as acidic compounds, are very sensitive to pH **(Zapata et al., 2010)**. The pH of mobile phases used for neurotransmitters quantification has been found in range from 2.6 to 6.5 with most of methods presenting values lower than 4.5 (Appendix I).

For example, Wu et al. **(2014)** developed a chromatographic method with ultraviolet (UV)/ visible detection for the determination of five amino acid neurotransmitters (Asp, Glu, glycine, Tau and GABA) in rat hippocampi with pre-column derivatization. The mobile phase that enabled good peak shapes, satisfactory resolution and relatively short analysis time was composed of 16% acetonitrile and 84% phosphate buffer (0.02 mol/L, pH 6); this pH of the mobile phase was carefully selected since it affects the separation of the derivatization products. On the other hand, the addition of ion-pairing agents to mobile phases is frequently used to improve the chromatographic separation of ionizable compounds and the retention time of basic neurotransmitters in reversed-phase columns; on the contrary, these agents have a minor effect on the retention of acidic neurotransmitters. However, ion-pairing agents can increase the column equilibration time and worsen peak shape **(González et al., 2011)**. For instance, González et al. **(2011)** added heptafluorobutyric acid (HFBA), a volatile ion-pairing reagent, to the mobile phase for the analysis of Glu, GABA, choline, ACh, DA, 5-HIAA, 5-HT, DOPAC and HVA with UHPLC-MS/MS, because some of these compounds (i.e. Glu, ACh, GABA and choline) were poorly retained in the column.

Concerning to the elution mode of mobile phase, isocratic elution is simpler than gradient elution. Nevertheless, as it can be observed from Appendix I, gradient elution has been applied in a great proportion (approximately 50%) of chromatographic methods used to quantify several neurotransmitters in brain samples.

Chemically, some neurotransmitters are monoamines that comprehend one amino group that is linked to an aromatic ring by a two-carbon chain (–CH2–CH2–). This ring structure makes the compounds naturally fluorescent and easily oxidizable **(Cai et al., 2010)**. Amino acids are amphiprotic molecules containing both amine and carboxyl functional groups. Most amino acids are little aliphatic molecules, and they are neither fluorescent nor have strong absorbance in the UV/visible region **(Kang et al., 2006)**.

Several detection systems have been used for the analysis of neurotransmitters; for example, catecholamines and their metabolites are electroactive compounds and therefore can be quantified by electrochemical detection (ECD) **(Bicker et al., 2013)**. In turn, amino acids in

their native form do not possess electrochemical activity **(Lü et al., 2010)**. ECD is considered a highly selective and sensitive detection system **(Bicker et al., 2013)**. Coulometry and amperometry are the two forms of ECD and, in both the current produced by the reaction is proportional to the concentration of the analyte in solution. However, while in coulometry the majority the electroactive species suffer oxidation or reduction, in amperometry only a fraction of electroactive compounds reacts **(Sabbioni et al., 2004)**.

To successfully apply ECD, it is necessary to define the potential required to produce the electrochemical reaction. The selection of electrochemical settings is normally done based on hydrodynamic voltammograms, which relate the generated current with applied potentials **(Schwarz, 2004)**. Furthermore, optimal pH conditions should be determined based on the reversible redox behavior of the target compounds. While a lower pH value is better for a sensitive determination based on reduction efficiency, oxidation efficiency is greater for higher pH values **(Unceta et al., 2001)**. ECD is also vulnerable to pumping rate fluctuations, thus increasing the signal-to-noise ratio **(Peaston and Weinkove, 2004)**. Moreover, deposition of oxidation products in the electrode surface leads to an attenuation of the signal and, consequently, to a drastic reduction of the overall sensitivity **(Manica et al., 2003)**. Another aspect that has influence on the sensitivity of electrochemical detectors is the composition of the mobile phase, which must be electrically conductive **(Peaston and Weinkove, 2004)** and make it better to operate in isocratic elution mode **(Bicker et al., 2013)**.

Given the characteristics of acidic catecholamine metabolites, as well as 5-HT and its metabolite, they are sometimes determined by HPLC-ECD **(Dam et al., 2014; Redondo-Castro et al., 2014; Vermeiren et al., 2014)**. In turn, the amino acid analysis by HPLC-ECD is less frequent because derivatization processes are required **(Cui et al., 2017; Fonseca et al., 2018; Monge-Acuña and Fornaguera-Trías, 2009; Stragierowicz et al., 2017)**.

FLD is widely used to neurotransmitters, however is frequently linked to derivatization step, because, for example, in native form amino acids do not have chromophores groups or in case of the catecholamines emission is low **(De Benedetto et al., 2014; Lü et al., 2010)**. According to De Benedetto et al. **(2014)**, native fluorescence of catecholamines provides reproducible results, which enabled the development and validation of a fast and simple isocratic HPLC-FLD method for the determination of DA, NE, 5-HT, and DOPAC in mouse brain, more specifically in the striatum region. The major advantage of this procedure was the easy sample preparation, which reduces assay time and chances for technical errors. Taking into account these aspects, a similar protocol was used by us in the development of a bioanalytical method for the determination of catecholamines **(Fonseca et al., 2017)**.

When using derivatization processes to improve sensitivity and selectivity, the reaction conditions can be optimized relatively to several factors, such as derivatizing agent concentration, pH, temperature, time of the reaction, among others. Some of these derivatization reactions can be lengthy and a degree of automation would be desirable for the accuracy of reaction time and to reduce laboratory workload **(Adegoke, 2012)**.

The derivatizing reagents that have been most commonly employed in FLD or even ECD for the determination of neurotransmitters (mainly amino acids) in brain samples are the 4-fluoro-7-nitrobenzofurazan (NBD-F) **(Wu et al., 2014)**, *o*-phtalaldehyde (OPA) **(Cui et al., 2017; Fonseca et al., 2018; de Freitas Silva et al., 2009; Maldonado and Maeyama, 2013; Monge-Acuña and Fornaguera-Trías, 2009; Perucho et al., 2015; Stragierowicz et al., 2017)** and 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde **(Şanlı et al., 2015)**. These derivatization reactions can occur in pre-column mode or post-column mode. In pre-column mode (i.e. before the analytical separation), the reaction is generally performed manually in vials before HPLC injection but can also be automated. In post-column mode, the reaction is performed automatically by adding the derivatizing reagent after chromatographic separation, and before detection, by means of a second HPLC pump **(Elbashir et al., 2011)**.
OPA reacts with primary amino groups in the presence of a thiol-containing compound, typically 2-mercaptoethanol, to produce isoindole derivatives that are both fluorescent and electroactive **(Hows et al., 2004)**. The reaction of amines with OPA and 2-mercaptoethanol takes place at basic pH (8.5–10.7) and at room temperature. For instance, Perucho et al. **(2015)** reported a bioanalytical methodology to obtain higher sensitivity and better resolution through the study of fluorometric excitation (λex) and emission (λem) spectrum wavelengths of OPA–amino acids.

Overall, methods employing FLD systems are able to provide enough sensitivity and selectivity. In fact, comparatively, FLD is typically more consistent and simpler to operate than ECD. Regarding MS detection, one of the major advantages is its capacity to quantify and identify compounds, associating retention times with structural information. The mass transition between the precursor ion and the product ions in tandem MS identifies the analytes of interest and contributes to the MS/MS specificity **(Bicker et al., 2013)**.
Multiple reaction monitoring (MRM) chromatograms of the transitions are extracted after positive or negative electrospray ionization (ESI). Usually, positive ESI is used for the ionization of basic compounds while negative ESI is chosen for acidic analytes **(Törnkvist et al., 2004)**. Indeed, Fenli et al. **(2013)** quantified levels of 5-HT, NE, DA and their metabolites 5-HIAA, 3-methoxy-4-hydroxyphenylglycol sulfate, DOPAC and HVA in rat brain tissue samples. LC-MS/MS analyses were carried out with a quadruple mass spectrometer equipped with ESI. MS acquisition of 5-HT, NE and DA was performed in positive ESI MRM mode, while negative ESI MRM mode was used to monitor their metabolites **(Fenli et al., 2013)**.
The sensitivity achieved by a LC-MS or LC-MS/MS system can be influenced by the efficiency of compound ionization which can be affected by the characteristics of the analyte, chromatographic variables and matrix effect **(Kushnir et al., 2002)**. For instance, the sensitivity of ESI is not ideal for highly polar and small molecules and are more susceptible to ion suppression **(Annesley, 2003; Cech and Enke, 2000)**. For samples that exhibit a poor electrospray response, atmospheric pressure chemical ionization can be considered. Furthermore, the influence of the mobile phase on the ionization of neurotransmitters should

be considered in method development, and the suppression or enhancement of ionization caused by matrix effect **(Gu et al., 2008)**.

He et al. **(2013)** developed a method where analytes and internal standard (IS) were separated on an Inertsil ODS-EP column (150 × 4.6 mm, 5 µm particles) and analyzed in less than 9.0 min, using gradient elution with a mobile phase consisting of methanol and 0.01% acetic acid in water at a flow rate of 1.2 mL/min. The optimal conditions for the triple-quadrupole analyzer were determined and MS parameters were optimized. Thus, the most intensive product ion of the precursor ion was used for quantification, and the second was for the qualification of the compounds. The MRM transitions named mass-to-charge ratio (*m/z)* are 146.200→87.200, 205.20→188.00, 170.10→152.20, 154.10→137.20, 148.20→84.10, 192.20→146.00, 104.20→87.10 and 177.10→160.20 for ACh, Trp, NE, DA, Glu, 5-HIAA, GABA and 5-HT, respectively.

The mass spectral characteristics of the majority of neurotransmitters are poor. This can be improved by introduction of a suitable group during derivatization. For example, Zheng et al. **(2012)** to achieve a better chromatographic behavior and mass-detecting sensitivity, resorted to a chemical derivatization with benzoyl chloride. Benzoyl chloride was chosen as the derivatizing reagent because it can react with primary and secondary amines and phenols under mild conditions with a high yield. The developed LC-MS/MS method enables a rapid quantification of 11 metabolites spanning DA, 5-HT, and kynurenine metabolic pathways within 10.5 minutes **(Zheng et al., 2012)**.

During the development and validation of a LC-MS or LC-MS/MS technique is relevant the use of an IS to correct ion suppression effects **(Bicker et al., 2013)**. Furthermore, quantification in all chromatographic methods the use of an adequate IS should be considered**.** Indeed, depending on the detection system, numerous compounds have been used as IS in different analytical techniques developed to quantify neurotransmitters (Appendix I). The IS most often selected has been 3,4-dihydroxybenzylamine (DHBA). In MS detection, the IS can co-elute with the analyte of interest and be differentiated by *m/z*. These compounds offer a better accuracy and precision since their ionization is identical to the unlabeled analyte **(Vogeser, 2003)**. In spite of requiring complex and expensive instrumentation, MS and MS/MS detection are seen as irrefutable and versatile detection methodologies, because unequivocally identify analytes and is associated with a high sensitivity **(Bicker et al., 2013)**.

For example, Huang et al. **(2014)** developed a MS method that showed good linearity over the concentration range of 1 ng/mL to 1 µg/mL for 5-HT, E, NE, 5-HIAA and DA, and 0.5 µg/mL to 50 µg/mL for GABA and Glu. In addition, Wei et al. **(2014)** validated an ultra-fast liquid chromatography (UFLC)-MS/MS method using rat brain homogenate samples that showed a good linearity over a wide concentration range, with a limit of quantification (LOQ) of 4 to 16 ng/mL for GABA, NE, DA, DOPAC, HVA, 5-HT and 5-HIAA. Also, Peng et al. **(2011)** developed a LC-MS/MS method to measure endogenous ACh in specific brain regions. The limit of detection (LOD) was 0.2 ng/mL and linearity was maintained over the concentration range of 10–1000 ng/mL.

I.2.2.3. Challenges in the quantification of endogenous compounds

Since the beginning of the application of chromatographic techniques that they played a key role in the quantitative determination of drugs and their metabolites in biological samples. Classically, these target compounds are xenobiotics and, as such, they do not usually occur in the biological matrix of interest. This makes the development and application of such bioanalytical methods moderately straightforward. However, the quantitative determination of endogenous compounds in biological samples is more complex, both analytically and from a validation perspective. With respect to method validation the main challenge is perhaps the difficulty to obtain analyte-free samples of the authentic biological matrix or samples with accurately known analyte concentrations and, consequently, the preparation of reference samples has to be addressed in a different way **(Van de Merbel, 2008)**.

For the study of sensitivity and selectivity, blank samples of the biological matrix should be acquired from at least six sources and any interfering components with analytes and IS should be less than 20% of the response at the LOQ of the analytes and less than 5% of the response of the IS. Accuracy and precision should be measured at LOQ, low quality control (QC) (within three times the LOQ), middle QC (in the midrange), and high QC (approaching the high end) samples using a minimum of five determinations per concentration, and they should be within 15%, except for the LOQ that can be up to 20%. A calibration curve should consist of a blank matrix, a zero standard, and at least six non-zero standards covering the expected dynamic range. Other criteria regarding stability, recovery, matrix effect, and incurred sample reanalysis are also discussed in the guidelines **(EMA, 2011; FDA, 2013, 2018)**. However, guidelines are mainly planned to the validation of exogenous compounds (xenobiotics) and following their validation criteria for the quantification of endogenous compounds is a challenge, in particular due to the lack of blank matrix needed to prepare all the calibration standards and QC samples that are required to evaluate all those validation parameters **(Thakare et al., 2016)**.

This has triggered the FDA to dedicate a section for the quantification of endogenous compounds in its most recent report (2018), which was not addressed in the previous 2001 version **(FDA, 2001)**. The FDA guideline stated that *"the biological matrix used to prepare calibration standards should be the same as the study samples and should be free of the endogenous analytes. The suitability of analyte-free biological matrices needs to be assured, by demonstrating no measurable endogenous analytes and no matrix effect or interference when compared to the biological matrices of the study samples"* **(FDA, 2018)**. The guidelines suggested that the QCs should be prepared in the same biological matrix as study samples and that the concentrations of these QCs should account for the background endogenous concentrations in the biological matrix, i.e. using the method of background subtraction. The guidelines also referred to the use of alternate (surrogate) matrices such as buffers and dialyzed serum but recommend this approach only in the case of an analyte-free biological matrix is not readily available or cannot be prepared. If this approach is used, a suitable justification and evidence of similar matrix effect to the original matrix are required **(Thakare et al., 2016)**.

I.2.3. The role of bioanalysis in the context of epilepsy

Globally it is argued that an imbalance between excitatory and inhibitory neurotransmission in the brain is involved in the generation of seizures in epilepsy patients, as well as in animal models of seizures and/or chronic epilepsy **(Bradford, 1995; Peña and Tapia, 2000)**. The different whole-animal models of seizures and epilepsy are widely used as they reproduce behavioral and electroencephalographic changes that are similar to those seen in human epilepsy. Therefore, these models are useful to study the pathophysiological mechanisms that determine the behavioral, histopathological and neurochemical changes related to epileptic activity **(Löscher, 2011)**.

As aforementioned, the mechanisms involved in the epileptogenesis and ictogenesis are not fully unraveled, but the participation of neuromediators (neurotransmitters and neuromodulators) in the initiation and propagation of these processes is increasingly accepted, suggesting that changes in excitatory and inhibitory neurotransmission can contribute to modify neuronal excitability. Nevertheless, the exact mechanisms of how these neuromediators influence the epileptogenesis and ictogenesis are not yet fully elucidated. However, given the involvement of neuromediators in epilepsy, such as catecholamines and amino acids, the determination of these endogenous substances is fundamental to deepen knowledge in this scope. Therefore, the quantitative determination of these neuromediators is essential in many studies, and thus bioanalysis plays a vital role in this scientific area.

From another point of view, the knowledge of the pharmacokinetic properties of both AEDs and convulsant agents often used to experimentally induce seizures and/or chronic epilepsy has also a great relevance. Therefore, the development of bioanalytical methods to quantify AEDs and the convulsant agents themselves is important to relate the concentration levels of AEDs and convulsants with seizure activity at nonclinical level, aiming to improve the therapeutic strategies.

Actually, the determination of plasma or serum levels of certain AEDs is an already well-established practice in clinical routine to support therapeutic drug monitoring and individualization of dosing regimens. In fact, many of the AEDs are good candidates for therapeutic drug monitoring based on drugs concentration levels because they exhibit high inter-individual variability in pharmacokinetics, are essentially used as prophylactics drugs for long-term epileptic seizure control, and there is no validated biomarker that can be predictive of drug efficacy and useful in the decision-making process **(Johannessen et al., 2003)**. Accordingly, plasma or serum drug concentrations correlate better than drug daily doses with clinical response **(Anderson, 2008)**.

Hence, keeping in mind all that has been mentioned before, it is indisputable the interest in the development of simple and reliable bioanalytical tools for multiple purposes in the scope of epilepsy.

I.3. Global aims of this thesis

For each type of epilepsy, it is likely that specific and different changes occur in the neurochemistry nature, histopathological and neurophysiological circuits of the epileptogenic area. Such peculiarities may be associated with different clinical and neurophysiological characteristics, patterns of spread of epileptic seizures, and different responses to pharmacological treatments.

Over the years, the pharmaceutical industry has developed several drugs capable of partially or completely reduce the occurrence of seizures. Despite the significant advances in the development of new drugs for the treatment of epilepsy, many patients continue to have epilepsy disorders that are refractory to available pharmacological therapies.

Thus, a more in-depth study of the biochemical phenomena underlying pathomechanisms involved in epilepsy, as well as the neurobiochemical changes induced by AEDs, can be very important for further therapeutic advances. Indeed, a deeper understanding of the biochemical processes underlying epileptic disorders may contribute to the identification of novel promising pathways and potential new therapeutic targets, and this can be a way to discover new drugs that may change the natural course of epileptogenesis and/or that are more effective in the management of refractory epilepsy.

Thus, the global aims of this project were the development of a series of bioanalytical tools for the quantitative determination of important endogenous neuromediators, potentially involved in epileptogenesis and ictogenesis, as well as AEDs, and chemoconvulsants of interest in the development of whole-animal models of seizures and epilepsy. The availability of such different and complementary bioanalytical tools will certainly be useful to support many nonclinical research studies that may be carried out in the field of neurosciences.

Therefore, the specific aims outlined for the implementation of this work were as follows:

- Development and validation of a simple and reliable HPLC-FLD method to simultaneously quantify catecholamines (DA, NE and E) and related compounds (L-DOPA, HVA and 3-*O*-MD) in rat brain tissue.
- Development and validation of a novel and straightforward HPLC-FLD method to simultaneously determine important neuroactive amino acids (Glu, Asp, Tau, Gln and GABA) in rat brain tissue.
- Development and validation of an innovative HPLC method coupled to diode array detection (DAD) for the simultaneous determination of some AEDs (LEV, ZNS and LTG) and chemoconvulsant agents (PTZ and PIL) in rat plasma and brain tissue.

CHAPTER II - Determination of catecholamines and endogenous related compounds in rat brain tissue exploring their native fluorescence and liquid chromatography

The content of this chapter is included in the following publication:

Fonseca, BM., Rodrigues, M., Cristóvão, AC., Gonçalves, D., Fortuna, A., Bernardino, L., Falcão, A., and Alves, G. (2017). Determination of catecholamines and endogenous related compounds in rat brain tissue exploring their native fluorescence and liquid chromatography. J Chromatogr B Analyt Technol Biomed Life Sci *1049-1050*, 51-59. doi: 10.1016/j.jchromb.2017.02.028

II.1. Introduction

The most plentiful endogenous catecholamines (DA, NE and E; Figure II.1) act as neurotransmitters or hormones that play an important role in different physiological functions of the endocrine and nervous systems, as well as in several diseases **(Bicker et al., 2013)**. Therefore, catecholamines, together with their metabolites and precursors, may constitute useful biomarkers for the diagnosis, therapy, prognosis, and investigation of the development and progression of a wide array of pathological disorders, such as neuroendocrine tumours (e.g. pheochromocytoma and paraganglioma) **(Fishbein, 2016)**, Parkinson's disease **(Goldstein, 2013; LeWitt, 2012)**, Alzheimer's disease **(Liu et al., 2011)**, heart failure **(Tousoulis et al., 2012)**, hypertension **(Saxena et al., 2014)**, stroke **(Husseini and Laskowitz, 2014)**, obesity **(Zouhal et al., 2013)**, depression **(Huang et al., 2014)** and epilepsy **(Freitas et al., 2006)**.
Chemically, catecholamines are a class of bioamines possessing an amino function on a side chain linked to the catechol group (Figure II.1).

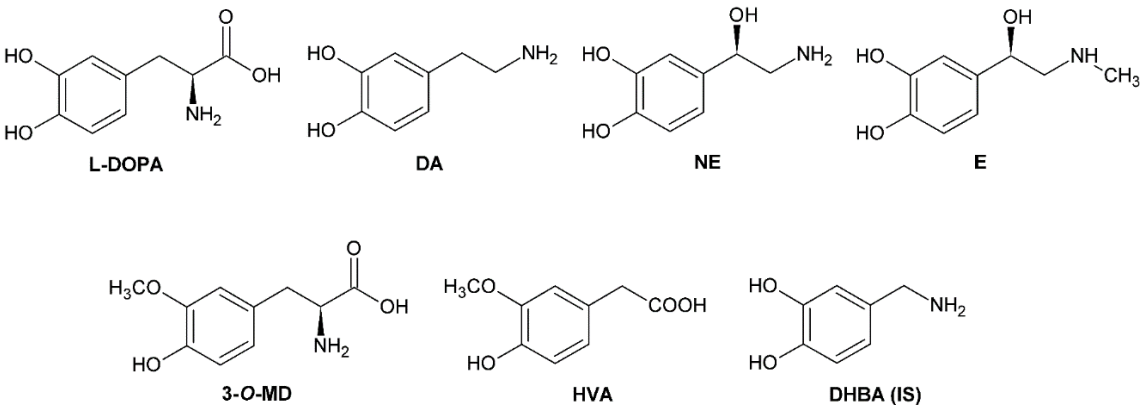

Figure II.1. Chemical structures of levodopa (L-DOPA), dopamine (DA), norepinephrine (NE), epinephrine (E), 3-*O*-methyldopa (3-*O*-MD), homovanillic acid (HVA) and the 3,4-dihydroxybenzylamine (DHBA) which was used as internal standard (IS).

A schematic representation of the main biosynthetic and metabolic pathways of catecholamines was recently published by Bicker and co-workers **(Bicker et al., 2013)**. As the pathophysiological levels of catecholamines and their metabolites are dependent of the catalytic activity of biosynthetic and metabolic enzymes, the simultaneous determination of these endogenous compounds can be of particular interest to differentiate physiological and pathological processes involving the neural functions in both clinical and nonclinical arenas.

Although gas chromatography, capillary electrophoresis and immunoassays have been employed for the analysis of catecholamines and their metabolites **(Christensen et al., 2011; Fiehn, 2016; Hao et al., 2013; Naccarato et al., 2014; Peaston and Weinkove, 2004; Pussard et al., 2014; Zhang and Gong, 2016)**, the liquid chromatography (LC) coupled with various detection systems, such as ultraviolet (UV) detection **(Muzzi et al., 2008)**, fluorescence detection (FLD) **(Kanamori et al., 2015, 2016; Yamaguchi et al., 1998)**, electrochemical detection (ECD) **(Chen et al., 2016b; Dam et al., 2014; Zhang et al., 2016)** and mass spectrometry (MS) or tandem mass spectrometry (MS/MS) **(Cho et al., 2012; Li et al., 2014; Wei et al., 2014)**, has been the most used analytical methodology for the determination of this kind of compounds. In this context, our research group extensively reviewed **(Bicker et al., 2013)** the LC methods available in literature for the quantification of catecholamines and their metabolites in several human and animal fluids (e.g. plasma, urine, saliva, cerebrospinal fluid, blood dialysate, brain dialysate, myocardial dialysate) and tissues (e.g. brain, adrenal gland, liver, kidney). Despite the great diversity of methods that have been developed throughout the years, the measurement of catecholamines and their metabolites in biological samples remains a current analytical challenge since almost all methods have their own advantages and limitations. Thus, in each circumstance, it is important to implement the most suitable bioanalytical method that fit-for-purpose.

Nevertheless, to better understand disease processes, establish animal models of neurological diseases, and evaluate the action of specific drugs, it is often essential the quantitative determination of biogenic amines and their metabolites, which are of great interest in

neurosciences. Hence, the availability of an easy-to-use analytical method that enables the high-throughput measurement of specific catecholamines and some of their metabolites in brain tissue of experimental animal models is of paramount importance. Several methods have been developed to detect catecholamines in brain tissue, such as gas chromatography **(Hong et al., 2013)** or LC combined with ECD **(Dam et al., 2014)**, MS **(Huang et al., 2014; Wei et al., 2014)** and FLD **(De Benedetto et al., 2014)**. However, all these methods have their own constraints. For instance, MS and MS/MS detection systems can offer benefits in terms of analytical selectivity as detection is based on molecular mass and chemical structure, but for the analysis of biological samples often occur interfering peaks in the low-m/z region resulting in poor sensitivity and specificity and higher detection limits in comparison with ECD **(Bicker et al., 2013)**; additionally, using LC-MS or LC-MS/MS methods is hard to detect some catecholamines and their metabolites in a single method because of their differences of polarity and acid–base properties **(Lv et al., 2015)**. LC-ECD is used most frequently due its high sensitivity and selectivity, but it has trend to fail in reproducibility mostly because of hysteretic degradation of the electrode **(Bicker et al., 2013; Lakshmana and Raju, 1997; Yoshitake et al., 2007)**. Hence, overall, LC-FLD is regarded as a sensitive and selective technique, and it is an analytical system more consistent and easier to operate **(Bicker et al., 2013; Yoshitake et al., 2006)**. The LC-FLD assays that have been reported for determination of catecholamines and their metabolites are based on the derivatization of the target analytes with a fluorescent reagent (e.g. 1,2-diphenylethylenediamine, *o*-phthalaldehyde, benzylamine) **(Fujino et al., 2003; Liu et al., 2011; Muzzi et al., 2008; Zhao and Suo, 2008)**, or have explored the native fluorescence of the compounds **(Baranowska and Płonka, 2008; De Benedetto et al., 2014; Muzzi et al., 2008; Sakaguchi et al., 2011)**. The pre or postcolumn derivatization offers the required sensitivity, but it implicates complex and labour intensive sample preparation procedures, and analytical complications such as instability of the generated products, long chromatographic runs, and the use of internal standard is mandatory **(De Benedetto et al., 2014; Fang et al., 2012)**. Thus, an often feasible alternative approach is to take advantage of the native fluorescence of catecholamines and some of their metabolites, affording reproducible results and using straightforward sample preparation procedures **(De Benedetto et al., 2014; Forster and Macdonald, 1999; Yoshitake et al., 2007)**.

Taking into account all the aforementioned aspects, the purpose of the current work was to develop and validate a reliable, fast and easy-to-use LC-FLD method, based on the native fluorescence of the target analytes, for the simultaneous quantification of DA, NE, E, levodopa (L-DOPA), homovanillic acid (HVA) and 3-*O*-methyldopa (3-*O*-MD) (Figure II.1) in rat brain tissue. Besides the main catecholamines (DA, NE, E) and the HVA metabolite, this bioanalytical assay also enables the quantitative determination of L-DOPA and 3-*O*-MD, the latter being accumulated at high levels after exogenous administration of L-DOPA **(Lee et al., 2008)**. Moreover, to the best of our knowledge, this is the first bioanalytical work reporting the quantification of this specific set of compounds based on measurement of their natural

fluorescence, which may support the research of neurological disorders associated with alterations of brain catecholamine profile.

II.2. Material and methods

II.2.1. Chemicals and reagents

The analytical standards of L-DOPA, DA hydrochloride, DL-NE- hydrochloride, (-)-E-(+)-bitartrate salt, 3-*O*-MD monohydrate and 3,4-dihydroxybenzylamine (DHBA) used as internal standard (IS) (Figure II.1), as well as L-cysteine, were obtained from Sigma-Aldrich (St Louis, MO, USA). HVA was acquired from Acros Organics (Geel, Belgium). HPLC-grade methanol and octane sulfonic acid (OSA) was purchased from Fisher Scientific (Leicestershire, United Kingdom). Sodium dihydrogen phosphate (NaH_2PO_4), perchloric acid and 85% *ortho*-phosphoric acid were purchased from Panreac Química SA (Barcelona, Spain). Ultra-pure water (HPLC-grade, >18 MΩ.cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA).

II.2.2. Stock solutions, calibration standards and quality control samples

Stock solutions (1 mg/mL) of L-DOPA, DA, NE, E, 3-*O*-MD and HVA were individually prepared by dissolving the appropriate amount of each compound in water. These solutions were then adequately diluted in 0.2 M perchloric acid containing 3 mM L-cysteine to obtain the corresponding appropriate working solutions. Afterwards, seven combined spiking solutions with final concentrations 25, 50, 150, 250, 500, 200 and 3750 ng/mL for L-DOPA; 35, 70, 150, 250, 1500, 3750 and 5000 ng/mL for DA; 125, 250, 1000, 1500, 1750, 2500 and 3750 ng/mL for NE; 12.5, 25, 50, 250, 500, 2000 and 3750 ng/mL for E; 20, 40, 150, 250, 500, 2000 and 3750 ng/mL for 3-*O*-MD, and 10, 20, 50, 250, 500, 2000 and 3750 ng/mL for HVA were prepared. Each one of these combined solutions was daily used in order to prepare seven calibration standards in the concentration ranges of 5-750 ng/mL for L-DOPA; 7-1000 ng/mL for DA; 25-750 ng/mL for NE; 2.5-750 ng/mL for E; 4-750 ng/mL for 3-*O*-MD and 2-750 ng/mL for HVA. The stock solution of the IS was also prepared in water (1.0 mg/mL) and the working solution (1.0 µg/mL) was obtained after diluting an appropriate volume of the stock solution with 0.2 M perchloric acid containing 3 mM L-cysteine. All solutions were stored at 4 °C and protected from light.

Quality control (QC) samples at three concentration levels were also independently prepared in the supernatant of rat brain tissue homogenate to obtain final concentrations of 25, 375 and 675 ng/mL for L-DOPA; 250, 500 and 900 ng/mL for DA; 225, 375 and 675 ng/mL for NE; 20, 375 and 675 ng/mL for 3-*O*-MD, and 45, 375 and 675 ng/mL for E and HVA.

II.2.3. Rat brain tissue

Adult male Wistar rats aged approximately 10 weeks were obtained from local animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). The rats were maintained under controlled environmental conditions (temperature 20±2 ºC; relative humidity 55±5%; 12-h light/dark cycle) with free access to a standard rodent diet and tap water *ad libitum*. All animal procedures were conducted in compliance with protocols approved by the national ethical requirements for animal research and the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Union Directive number 2010/63/EU) **(EU, 2010)**. To obtain the brain tissue, rats not subjected to any experimental procedures were anesthetized with pentobarbital (60 mg/kg) and then decapitated. After exsanguination, the brain was quickly removed and frozen using liquid nitrogen. Thereafter, the brain tissue was homogenized in ice-cold 0.2 M perchloric acid containing 3 mM L-cysteine (4 mL per g of tissue) and sonicated for 5 min. The brain homogenates were centrifuged at 17000 rpm (30000 g) for 4 min at 4 ºC and the corresponding supernatants were collected and stored at −20 ºC until used.

II.2.4. Apparatus and chromatographic conditions

Chromatographic analysis was carried out in an HPLC system (Shimadzu Corporation, Japan) equipped with a DGU-20A5R automatic degasser, a LC-20AD quaternary solvent pump, a SIL-20ACHT refrigerated automatic injector, a CTO-10AS VP columns oven and a RF-20AXS fluorescence detector. LabSolutions software was used to control the HPLC components and to process data.

The chromatographic separation was achieved on a Grace Smart RP C_{18} chromatography column (150 x 4.6 mm, 5 µm). The column was maintained at 25 ºC and the mobile phase was pumped at the flow rate of 1.0 mL/min. The mobile phase consisting of 75 mM NaH_2PO_4 with 1.5 mM OSA in water (pH 3.5) (solvent A), 75 mM NaH_2PO_4 with 1.5 mM OSA in water pH 5.0 (solvent B) and methanol (solvent C) eluting under the pH gradient conditions shown in Table II.1. The pH of solvent A and B was adjusted with 85% *ortho*-phosphoric acid. The mobile phase was filtered through a 0.45 µm filter. The fluorescence was monitored at excitation and emission wavelengths of 279 nm and 320 nm, respectively.

Table II.1. Mobile phase pH gradient elution program. Solvents A, B and C correspond to 75 mM sodium dihydrogen phosphate with 1.5 mM octane sulfonic acid (OSA) in water pH 3.5, 75 mM sodium dihydrogen phosphate with 1.5 mM OSA in water pH 5, and methanol, respectively.

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0.00	97	0	3
2.00	97	0	3
2.10	0	97	3
3.40	0	97	3
3.50	97	0	3
12.00	97	0	3

II.2.5. Sample preparation

Each aliquot of 100 µL of supernatant of brain homogenate, spiked with 20 µL of IS working solution (1.0 µg/mL), was added of 20 µL of 2.0 M perchloric acid; the final mixture was vortex-mixed for 1 min and then centrifuged at 14000 rpm (18620 g) for 4 min at 4 °C to precipitate proteins in order to minimize sample interferences. The resulting clear supernatant was collected and an aliquot of 20 µL was injected into the HPLC system.

II.2.6. Method validation

The validation assays were carried out according to the bioanalytical method validation guidelines of the Food and Drug Administration (FDA) **(FDA, 2001)** and the European Medicines Agency (EMA) **(EMA, 2011)**, with some unavoidable adjustments due to the endogenous nature of the target analytes. The method validation included the assessment of the following parameters: linearity, limits of detection and quantification, precision, accuracy, recovery, stability of samples and selectivity.

As the target analytes are endogenous compounds, the linearity and sensitivity of the assay was validated by using appropriate standard solutions spiked with the IS. The linearity of the analytical method for each analyte was determined from the calibration curves constructed with standard solutions at seven concentrations and assayed on three different days (n = 3). The calibration curves were constructed by plotting analyte-IS peak height ratio as function of the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis **(Almeida et al., 2002)**. The limit of quantification (LOQ) was evaluated by analyzing five replicates (n = 5), and it was defined as the lowest concentration of the calibration curve that can be measured with appropriate inter and intra-day precision and accuracy **(EMA, 2011; FDA, 2001)**. For the LOQ, the precision criterion was defined as a coefficient of variation (CV) value lower than or equal to 20%, and for the accuracy was considered to be acceptable a *bias* value within ±20%. Limit of detection (LOD) is the lowest

concentration that can be reliably distinguished from background noise, and it was defined as the concentration that yields a signal-to-noise ratio of 3:1 **(FDA, 2001)**.

The inter-day precision and accuracy of the assay were assessed using QC samples analyzed on three consecutive days (n = 3), while the intra-day precision and accuracy were evaluated by analysing five sets of the QC samples in a single day (n = 5). Precision values (% CV) must be lower than or equal to 15% for replicate measurements and accuracy values (% *bias*) should be within ±15%.

The absolute recovery of the analytes from the supernatant of rat brain homogenate samples was determined at three concentration levels (QC_1, QC_2 and QC_3) on the same day (n = 5). For this purpose, brain homogenate supernatant samples were spiked with the appropriate standard solutions, and the recovery (R) was calculated as follows $R = 100 \times [(C_{spiked} - C_{sample\ (endogenous)})/C_{standard}]$, where C_{spiked} is the concentration in the spiked brain homogenate supernatant sample, $C_{sample\ (endogenous)}$ is the concentration in the brain homogenate supernatant sample without spiking, and $C_{standard}$ is the added concentration.

Stability of the analytes in biological matrix during the sample handling and storage conditions is an essential parameter for method validation. The stability assays of the analytes in the supernatant of rat brain homogenate samples were conducted in replicate (n = 4) at two concentration levels (low and high QC values). Then, the data of the QC samples analyzed before (reference samples) and after being exposed to the experimental circumstances for stability evaluation (stability samples) were compared. The stability/reference samples ratio of 85-115% was accepted as stability criterion **(Chen et al., 2005; EMA, 2011)**. Specifically, the stability was assessed at room temperature for 4 h, 4 °C for 24 h, −20 °C for 5 days in order to mimic sample handling and storage time in the refrigerator before analysis; post-preparative stability of the analytes in processed samples was also evaluated at room temperature during 12 h, simulating the period that samples may be in the auto-sampler before analysis; the influence of three freeze–thaw cycles was also studied; aliquots of the supernatant of rat brain homogenate samples were stored at −20 °C for 24 h, defrosted at room temperature, and when entirely thawed, samples were refrozen for 24 h under the same conditions until concluding the three cycles.

The selectivity of the method was evaluated by analyzing a set of endogenous compounds, such as DOPAC, histamine, 5-HT, L-tryptophan, L-tyrosine, in order to ascertain the potential interference with the target analytes and IS.

II.2.7. Method application

To demonstrate the applicability of the proposed method, Wistar rats (n = 3) were anesthetized, sacrificed by decapitation, and the brain was promptly excised and frozen using liquid nitrogen. Then, the brain was carefully dissected into nine brain regions (cerebellum, amygdala, cortex, hippocampus, striatum, mesencephalon, medulla oblongata, *substantia nigra* and ventral tegmental area) on ice. Subsequently, the different brain regions were individually

homogenized as previously described in *section II.2.3.*, and the homogenate supernatants were taken and stored at −20 ºC until the LC analysis of the target analytes (L-DOPA, DA, NE, E, HVA and 3-*O*-MD).

II.3. Results and discussion

II.3.1. Method development

A key point to consider in the development of any bioanalytical assay is to ensure the stability of the analytes in the intended biological matrices not only during the sample storage period but also throughout all steps of the analytical processing. Thus, the selection of a suitable sample pre-treatment procedure is a critical aspect to successfully achieve the validation of an analytical method. Having in mind the thermal and photochemical instability of catecholamines and endogenous related compounds (precursors and metabolites), the sample handling is usually done in the dark at low temperature and low pH values; moreover, in this case, to improve sample integrity, it is common the addition of antioxidants such as L-cysteine and ascorbic acid or by using a combination of sodium bisulphite and/or metal chelating agent disodium edetate **(Patel et al., 2005)**. So and based on Benedetto et al. **(2014)** both standard solutions and samples were prepared with a solution of 0.2 M perchloric acid containing 3 mM L-cysteine as stabilizer. On the other hand, in what concerns the sample preparation, a simple protein precipitation step with different concentrations of perchloric acid was tested; at the end, considering the assays performed, 2.0 M perchloric acid was selected as the protein precipitation agent, providing not only a straightforward sample preparation procedure but also a high and reproducible recovery for the analytes.

The chromatographic variables were optimized to achieve an adequate separation between the analytes and interference from the background of samples of the supernatant of rat brain homogenates, taking also into account an optimal compromise in terms of resolution and symmetric peak shapes, as well as a short run time. For this purpose, some important chromatographic conditions were investigated. The influence of mobile phase pH on the separation of catechol compounds within the range of 2.5–5.5 was studied. The effects of the mobile phase pH are dependent on the pKa values and related to the degree of ionization of the compounds. The more-acidic carboxylic groups which are protonated at low pH will dissociate with pH increasing. The retention of compounds such as L-DOPA, 3-*O*-MD and HVA was dramatically reduced when pH was increased and the retention time of latest-eluted compound decreases. The peak intensities also change with pH, due to the increasing degree of ionization. Retention time, sensitivity and chromatographic resolution of L-DOPA, DA, NE and 3-*O*-MD increased at pH 3.5, but the intensity of HVA peak decreased extensively and a delay in the elution occurred. Thus, to improve the peak sharp, reduce the retention time and

enhance HVA sensitivity, a pH gradient elution with a less acidic pH (pH 5) was executed. As it is well recognized, the addition of an ion-pairing reagent (e.g. OSA) to the mobile phase is commonly used to increase the retention of amines at acid pH values, preventing their elution with the solvent front. In this case, the concentration of OSA was increased up to 1.5 mM, showing this to be necessary to improve NE retention factor from the solvent front. Organic modifiers (e.g. methanol, acetonitrile) have impact in retention of all sample components and affect particularly the enhancement of DA fluorescence **(Wang et al., 2002)**. Catecholamines and their metabolites are highly polar molecules and, therefore, they are weakly retained in reversed-phase chromatographic columns. Thus, in this study, different mobile phases with low percentages of methanol and/or acetonitrile used as organic modifiers were tested. The optimal conditions were achieved with a methanol content of 3%.

The fluorimetric parameters were also optimized. The pairs of excitation/emission wavelengths (220/320 nm and 279/320 nm, respectively) were tested because these wavelength pairs have been widely used and some catecholamines have a maximum fluorescence emission response near at 320 nm **(De Benedetto et al., 2014; Siva et al., 2012)**. Indeed, in our experimental conditions, the intensity of fluorescence of the studied analytes was confirmed to be largely higher when monitored at 279/320 nm excitation/emission wavelengths.

Considering the set of experiments carried out to optimize the chromatographic separation and detection conditions, the analytical and instrumental parameters established for this method were described in detail in *section II. 2.4.* Under such conditions all target analytes and the IS were appropriately resolved and eluted in a short run time (less than 12 min) (Figure II.2). Throughout the analysis the column temperature was controlled and maintained at 25 ºC, thus overcoming temperature fluctuations of the laboratory and getting greater reproducibility.

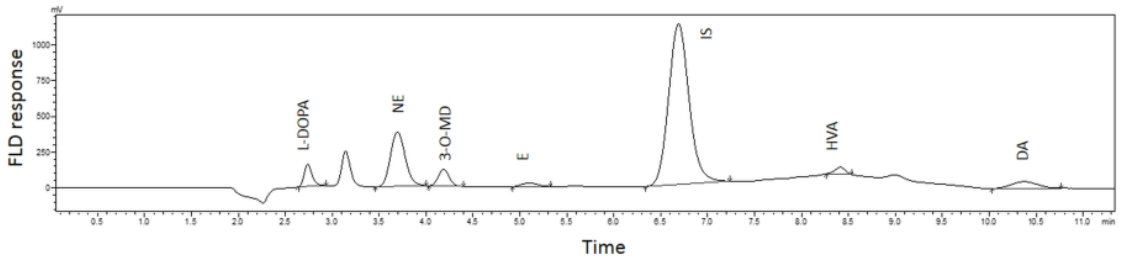

Figure II.2. Typical chromatogram obtained by the developed LC-FLD assay during the analysis of standard solutions containing the analytes (L-DOPA, NE, 3-*O*-MD, E, HVA and DA) at the concentrations of the limit of quantification (LOQ) and spiked with the internal standard (IS). L-DOPA, levodopa; NE, norepinephrine; 3-*O*-MD, 3-*O*-methyldopa; E, epinephrine; IS, internal standard; HVA, homovanillic acid; DA, dopamine.

II.3.2. Method validation

The quantitative determination of endogenous compounds in biological samples of limited size (e.g. rat or mouse specimens) is undoubtedly a more complex process from an analytical and method validation point of view **(Wenying et al., 2013)**. In these circumstances a crucial part of the method development is the choice of an appropriate approach to prepare calibration

and QC samples. In fact, for the quantitative measurement of endogenous compounds, as catecholamines and related compounds (precursors and metabolites), the authentic biological matrix typically contains an unknown concentration of the analytes, making it often inadequate for the preparation of calibration and QC samples **(Van de Merbel, 2008)**. Some approaches for the analysis of endogenous compounds involve, for instance, the use of a surrogate matrix, which is a matrix that is as analogous as possible to the authentic biological matrix but not containing the target analytes **(Flaherty et al., 2005)**. Nevertheless, surrogate matrix may lack certain enzyme systems, such as proteases or esterase, which mediate degradation of certain types of analytes in authentic matrix **(Wenying et al., 2013)** and, in this case, a truly representative surrogate cannot be obtained. Also charcoal stripping may be feasible for certain purposes, but this strategy has some disadvantages as an incomplete removal of the endogenous analytes of interest and it can be a time-consuming process **(Van de Merbel, 2008)**. Another simple approach to avoid this tricky point is the application of the method of standard addition, by adding know concentrations of the analyte to individual aliquots of the sample. A calibration curve is made and the endogenous concentration in the sample is calculated from the intercept of this calibration curve **(Van de Merbel, 2008)**. However, proceeding in this way, a large amount of biological matrix would be required, and therefore, it would be necessary to sacrifice many animals.

Given all these difficulties to obtain analyte-free samples of the authentic biological matrix, the preparation of reference samples has to be held differently and, as a consequence, validation also becomes less straightforward. Thus, this validation process was carried out based on the bioanalytical method validation guidelines of the FDA **(FDA, 2001)** and EMA **(EMA, 2011)**, introducing, however, some modifications previously mentioned in *section II.2.6 Method validation* due to the inherent challenges of validating a quantitative analytical method for endogenous substances.

Thus, since the rat brain homogenate matrix has significant endogenous levels of catecholamines and their precursors/metabolites, seven-point calibration curves were prepared by the analysis of spiked aliquots of 0.2 M perchloric acid containing 3 mM L-cysteine. Calibration curves were constructed based on the analyte-IS peak height ratios versus the corresponding nominal concentrations. The calibration model was selected based on the analysis of the data by weighted linear regression analysis, and the best linear fit and least-squares residuals were achieved with a $1/x^2$ weighting factor. Hence, as shown in Table II.2, all calibration curves exhibited good linearity with determination coefficients (r^2) equal or higher than 0.9941.

The LOQs were established at 5 ng/mL for L-DOPA, 25 ng/mL for NE, 4 ng/mL for 3-*O*-MD, 2.5 ng/mL for E, 2 ng/mL for HVA, and 7 ng/mL for DA. The LOQs of the analytes were established experimentally and they were defined as the lowest concentration of the calibration ranges (Table II.2) determined with acceptable precision (CV ≤ 8.76%) and accuracy (*bias* varied from −13.49% to 13.86%). The LODs were established at 0.5 ng/mL for L-DOPA, 5 ng/mL for NE, 1 ng/mL for 3-*O*-MD, E and HVA, and 4 ng/mL for DA.

Table II.2. Mean calibration parameters (*n* = 3) of levodopa (L-DOPA), norepinephrine (NE), 3-*O*-methyldopa (3-*O*-MD), epinephrine (E), homovanillic acid (HVA) and dopamine (DA).

Analyte	Calibration parameters		
	Concentration range (ng/mL)	Equation[a]	r^2
L-DOPA	5-750	y = 0.0164x + 0.0079	0.9975
NE	25-750	y = 0.0069x - 0.0023	0.9992
3-*O*-MD	4-750	y = 0.0226x + 0.0150	0.9960
E	2.5-750	y = 0.0042x - 0.0003	0.9983
HVA	2-750	y = 0.0137x + 0.0523	0.9960
DA	7-1000	y = 0.0026x - 0.0097	0.9941

[a] y represents analyte-IS peak height ratio; x represents analyte concentration (ng/mL).

Considering the major catecholamines (DA, NE and E), the LOQs achieved with the present method can be directly compared with those obtained without derivatization by the LC-FLD assay reported by Benedetto et al. **(2014)**; overall, the LOQs for DA, NE and E afforded by our method were approximately two–three times lower, and so, more sensitive. Moreover, comparing the current method with the LC-FLD assay developed by Tsunoda et al. **(2010)**, which employed derivatization procedures, it was found that the LOQ of DA provided by our method is approximately 6-fold lower, despite the native fluorescence has been used herein to monitor the analytes. In other studies **(Hong et al., 2013; Park et al., 2013)** LOQs ranging 1 to 5 ng/mL were achieved for the main compounds studied in mouse brain, but they employed complex sample preparation procedures that included derivatization reactions. Therefore, even without derivatization process, this LC-FLD method compared with previous methods is more convenient and enables the faster and easier determination of the target analytes with satisfactory limits.

Precision and accuracy assays were carried out using rat brain homogenates samples at three different concentrations (QC_1, QC_2 and QC_3) and the results obtained for all analytes are summarized in Table II.3. At this point, it should be noted that the analyte concentrations set for QC_1 samples were selected based on the mean concentration found for each analyte in several aliquots of the authentic brain matrix used in the validation procedures.

Table II.3. Precision (% CV) and accuracy (% *bias*) for the determination of levodopa (L-DOPA), norepinephrine (NE), 3-*O*-methyldopa (3-*O*-MD), epinephrine (E), homovanillic acid (HVA) and dopamine (DA) in brain tissue homogenate supernatant samples at the low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges.

Analyte	Nominal concentration (ng/mL)	Inter-day		Intra-day	
		Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)
L-DOPA	25	6.98	-0.03	4.56	0.97
	375	5.52	9.28	2.55	0.15
	675	3.47	6.55	4.64	-1.06
NE	225	6.40	2.31	4.30	-2.24
	375	5.12	0.17	2.37	3.74
	675	4.06	1.99	2.99	7.42
3-*O*-MD	20	0.93	10.60	5.95	14.48
	375	5.81	-7.01	2.70	-12.41
	675	1.47	-10.00	2.55	-14.42
E	45	6.73	5.30	6.29	1.45
	375	1.89	-4.62	2.01	-5.57
	675	2.22	-5.80	2.57	0.29
HVA	45	0.67	4.43	5.45	14.65
	375	7.36	-3.46	3.00	-8.61
	675	1.82	-8.39	2.23	-12.48
DA	250	8.67	0.97	3.08	-7.45
	500	1.88	-5.33	2.14	-5.56
	900	2.97	-4.05	2.62	-0.86

bias, deviation from nominal concentration value; CV, coefficient of variation.

As shown in Table II.3, the overall intra- and inter-day precision (CV) was lower than or equal to 8.67%, while the accuracy (*bias*) ranged from −14.42 to 14.65%. Precision and accuracy values on both intra- and inter-day were all within the acceptable range, indicating that the current method was reliable and reproducible, demonstrating that the 0.2 M perchloric acid containing 3 mM L-cysteine based calibration curve can be used to accurately quantify these target analytes in the authentic biological matrix.

Similarly to precision and accuracy, the experiments to evaluate recovery were also performed using rat brain homogenate supernatant samples containing the analytes of interest at three concentration levels (QC_1, QC_2 and QC_3). Table II.4 summarizes the obtained data for the mean absolute recovery of all the analytes, which ranged from 96.16% to 114.62%, being the CV values equal or lower than 7.71%. Similarly to the yields achieved for the analytes, the absolute recovery of the IS was 99.70%. Hence, the *bias* to the theoretical concentration values and the recoveries demonstrate that the proposed method allows simultaneous determination of L-DOPA, NE, 3-*O*-MD, E, HVA and DA, demonstrating the absence of significant matrix effects.

Table II.4. Mean absolute recovery of levodopa (L-DOPA), norepinephrine (NE), 3-*O*-methyldopa (3-*O*-MD), epinephrine (E), homovanillic acid (HVA) and dopamine (DA) from brain tissue homogenate supernatant samples (*n* = 5).

Analyte	Nominal concentration (ng/mL)	Absolute recovery (%)[a]	Precision (% CV)
L-DOPA	25	109.43 ± 2.44	2.23
	375	110.27 ± 2.66	2.41
	675	108.92 ± 5.13	4.71
NE	225	98.24 ± 2.83	2.89
	375	103.63 ± 2.30	2.22
	675	106.53 ± 3.23	3.03
3-*O*-MD	20	115.32 ± 2.43	2.11
	375	103.63 ± 2.48	2.40
	675	103.00 ± 2.66	2.58
E	45	98.49 ± 2.37	2.41
	375	97.48 ± 2.71	2.78
	675	100.73 ± 2.56	2.54
HVA	45	114.62 ± 2.77	2.39
	375	110.18 ± 2.70	2.45
	675	108.91 ± 2.45	2.25
DA	250	96.97 ± 3.16	3.26
	500	96.16 ± 2.15	2.23
	900	98.32 ± 2.58	2.62

[a] Mean ± standard deviation, *n* = 5.

The stability of L-DOPA, NE, 3-*O*-MD, E, HVA and DA in rat brain tissue homogenate supernatant samples was investigated under the conditions previously stated in the *section II.2.6.*, and the results obtained are reported in Table II.5. From the stability data obtained it is evident that no significant loss of the target analytes was observed in unprocessed brain homogenate supernatants at 4 ºC for 24 h, −20 ºC for 5 days, and after three freeze–thaw cycles at −20 ºC, or in processed samples at room temperature during 12 h. Nevertheless, a slight deviation of the stability acceptance criterion was found for the HVA and 3-*O*-MD when the stability was evaluated at room temperature for 4 h. However, these results do not comprise the application of the methodology described herein, as long as rat brain homogenate supernatant samples are always prepared on ice.

Finally, regarding the selectivity, the analysis of test compounds confirmed the absence of interferences in the retention times of target analytes and the IS with the established chromatographic and detection conditions.

Table II.5. Stability (values in percentage) of levodopa (L-DOPA), norepinephrine (NE), 3-*O*-methyldopa (3-*O*-MD), epinephrine (E), homovanillic acid (HVA) and dopamine (DA) in brain tissue homogenate supernatant samples under different conditions of sample handling and storage (n = 4).

Analyte	Nominal concentration (ng/mL)	Stability (%)				
		Unprocessed sample				Processed sample
		Room temperature (4 h)	4 °C (24 h)	Freeze-thaw cycles (3 cycles; −20 °C)	−20 °C (5 days)	Room temperature (12 h)
L-DOPA	25	87.15	93.13	103.42	103.11	93.64
	675	85.70	91.12	102.59	103.88	97.48
NE	225	107.84	109.84	108.50	114.40	97.88
	675	95.74	99.06	100.55	108.42	96.25
3-*O*-MD	20	84.18	95.85	94.39	94.40	94.09
	675	102.94	91.73	101.73	99.67	98.64
E	45	114.62	111.25	94.39	112.72	101.07
	675	92.67	95.56	105.83	106.69	95.38
HVA	45	81.80	90.62	90.05	88.65	102.96
	675	80.17	85.95	103.59	98.96	95.89
DA	250	97.51	97.87	105.50	113.60	99.22
	900	90.82	93.60	103.77	109.44	93.43

II.3.3. Method application

The proposed LC-FLD method was developed aiming at its application in the broad field of neurosciences to support many pre(non)clinical studies that would benefit from the quantitative determination of L-DOPA, NE, 3-*O*-MD, E, HVA and/or DA in whole or specific rat brain regions. Thus, a pilot study was performed with a small number of rats (n = 3), and it was carried out, particularly, with the purpose of demonstrating the applicability of the method for analysis of target compounds in specific rat brain regions. To this end, measurement of L-DOPA, NE, 3-*O*-MD, E, HVA and/or DA was tested in nine different rat brain regions (cerebellum, amygdala, cortex, hippocampus, striatum, mesencephalon, medulla oblongata, *substantia nigra* and ventral tegmental area), and the results are shown in Table II.6. Typical chromatograms of the rat brain samples are also shown in Figure II.3.

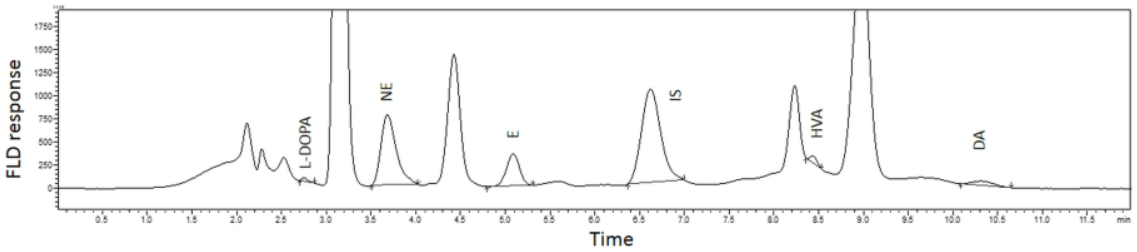

Figure II.3. Representative LC-FLD chromatogram of the analysis of a rat brain sample. Concentrations of catecholamines and endogenous related compounds were measured in ventral tegmental: 0.027 ng/mg for L-DOPA; 1.153 ng/mg for NE; 3-*O*-MD was not detected; 0.790 ng/mg for E; 0.183 ng/mg for HVA; and 0.144 ng/mg for DA. L-DOPA, levodopa; NE, norepinephrine; 3-*O*-MD, 3-*O*-methyldopa; E, epinephrine; IS, internal standard; HVA, homovanillic acid; DA, dopamine.

As it can be seen in Table II.6, NE was determined in all brain regions except the striatum, in which NE levels were found to be below the limit of quantification (BLQ); additionally, it is worthy of mention that relative homogeneity displayed in the concentration of NE in all those brain regions where it was possible to measure it quantitatively, with levels ranging from 0.253 ng/mg in medulla oblongata to 0.636 ng/mg in ventral tegmental area. In contrast, DA tissue content was extremely heterogeneous ranging from high levels in the striatum (3.896 ng/mg) to undetectable levels in cerebellum, hippocampus, mesencephalon and medulla oblongata. Indeed, the DA content measured in these samples is consistent with the dopaminergic innervation density **(Fitoussi et al., 2013; Vasconcelos et al., 2004)**. L-DOPA levels were found in striatum, *substantia nigra* and ventral tegmental area, while the 3-*O*-MD was not detected in any brain region. Regarding HVA, the maximum concentration (0.597 ng/mg) was found in the striatum, such as dopamine. The E concentrations were lower in the cerebellum and amygdala and the maximum concentration (0.656 ng/mg) was reached in the ventral tegmental area.

Table II.6. Tissue contents of levodopa (L-DOPA), norepinephrine (NE), 3-*O*-methyldopa (3-*O*-MD), epinephrine (E), homovanillic acid (HVA) and dopamine (DA) in various rat brain regions.

Brain region	Concentration (mean ± SD; ng/mg)					
	L-DOPA	NE	3-*O*-MD	E	HVA	DA
Cerebellum	ND	0.280 ± 0.037	ND	0.056 ± 0.004	ND	ND
Amygdala[#]	ND	0.394 ± 0.092	ND	0.062 ± 0.004	0.040 ± 0.009	0.077 ± 0.004
Hippocampus	ND	0.271 ± 0.112	ND	ND	ND	ND
Cortex	ND	0.378 ± 0.009	ND	0.067 ± 0.010	ND	0.065 ± 0.021
Striatum	0.061 ± 0.010	BLQ	ND	0.267 ± 0.010	0.597 ± 0.144	3.896 ± 0.351
Mesencephalon	ND	0.382 ± 0.033	ND	0.118 ± 0.028	ND	ND
Medulla oblongata	ND	0.253 ± 0.019	ND	0.073 ± 0.006	ND	ND
Substantia nigra	0.026 ± 0.002	0.426 ± 0.044	ND	0.260 ± 0.086	0.059 ± 0.018	0.146 ± 0.074
Ventral tegmental	0.027 ± 0.020	0.636 ± 0.408	ND	0.656 ± 0.404	0.106 ± 0.080	0.097 ± 0.047

Results expressed as mean ± standard deviation of three independent samples (n = 3), unless otherwise noted; [#]n = 2. BLQ, below the limit of quantification; ND, not detected.

At this point, it should be highlighted that the absence of measurable concentrations of 3-*O*-MD in all the studied brain areas does not invalidate the convenience of the current method to support the determination of this analyte in other kind of studies, particularly those involving research in Parkinson's disease. For instance, the exogenous administration of L-DOPA remains the primary pharmacological intervention in Parkinson's disease, and it is well known that the metabolite 3-*O*-MD is accumulated at high levels under treatment with L-DOPA **(Lee et al., 2008)**. Thus, we can anticipate that in such experimental conditions, our LC-FLD method will also be able to provide quantitative levels of 3-*O*-MD. As a result, our data suggest that this novel bioanalytical assay may represent a valuable tool to support many research works, especially focused on the nigrostriatal system associate disorders, like Parkinson's disease.

CHAPTER III - An easy-to-use liquid chromatography method with fluorescence detection for the simultaneous determination of five neuroactive amino acids in different regions of rat brain

The content of this chapter is included in the following publication:

Fonseca, BM., Cristóvão, AC., and Alves, G. (2018). An easy-to-use liquid chromatography method with fluorescence detection for the simultaneous determination of five neuroactive amino acids in different regions of rat brain. J Pharmacol Toxicol Methods. *91,* 72-79. doi: 10.1016/j.vascn.2018.02.002

III.1. Introduction

An efficient communication between neuronal circuits across the brain is essential for the normal functioning of the central nervous system (CNS) and several neurotransmitter/neuromodulator systems are involved in this process **(Halassa and Haydon, 2010; Mangia et al., 2012; Naef et al., 2015; Valenzuela et al., 2011)**. Indeed, different well-known neurotransmitters and/or neuromodulators, such as amino acids [e.g., Glu, Asp, Gln, Tau, GABA, glycine], biogenic amines (e.g., 5-HT, DA, NE, E, histamine), neurosteroids (e.g., allopregnanolone, allotetrahydrodeoxycorticosterone), neuropeptides (e.g., ghrelin) and other (e.g., Ach, adenosine, nitric oxide) have attracted a great attention in the last few years **(Albrecht et al., 2010; Barker et al., 2016; Benson et al., 2015; Džoljić et al., 2015; Granger et al., 2016; Halassa and Haydon, 2010; Mangia et al., 2012; Naef et al., 2015; Rahmani et al., 2016; Saransaari and Oja, 2010; Shah et al., 2002; Valenzuela et al., 2011; Wu and Prentice, 2010; Zlomuzica et al., 2016)**. Among these endogenous compounds, amino acids play pivotal roles in neuronal signaling pathways, therefore changes in neuronal amino acid levels may lead to a variety of brain disorders as epilepsy **(DiNuzzo et al., 2014)**, stroke

(Carmichael, 2012), schizophrenia **(Perez and Lodge, 2014)**, Alzheimer's disease **(Lin et al., 2014)**, Parkinson's disease **(Amalric, 2015)**, depression **(Benson et al., 2015)**, and attention-deficit/hyperactivity disorder **(Bollmann et al., 2015)**. Amino acid neurotransmitters are usually classified into excitatory amino acids including Glu and Asp, and inhibitory amino acids, as GABA, glycine and Tau **(Perry et al., 2009)**. As the major excitatory neurotransmitter in the mammalian CNS, Glu is present in more than half of all CNS synapses, which also underlines its involvement in important functions as learning, memory, sleep, movement and feeding **(Rawls et al., 2006)**. On the other hand, 10–40% of nerve terminals in the hippocampus and cerebral cortex may use GABA as a neurotransmitter to transmit "closure" signals **(Şanlı et al., 2015)**. Gln is also found abundantly in the CNS; however, although Gln also appears to directly affect neurotransmission, it participates in the brain mainly as a precursor of the excitatory (Glu and Asp) and inhibitory (GABA) neurotransmitter amino acids **(Albrecht et al., 2010)**. Hence, Gln may be involved with inhibitory and excitatory activity as it has been implicated in the replenishment of GABA and Glu neurotransmitter pools through the GABA/Glu-Gln cycle **(Walls et al., 2015)**.

Thus, taking into account the aforementioned data, it can be anticipated that the quantitative measurement of neuroactive amino acids has potential in the diagnosis and understanding of the etiology and progression of several CNS disorders **(de Freitas Silva et al., 2009; Marc et al., 2011)**. Nevertheless, the cerebral concentrations of amino acids are in the micromolar to submicromolar range and, therefore, to reliably quantify these endogenous compounds highly selective and sensitive bioanalytical methods are required **(de Freitas Silva et al., 2009)**.

Liquid chromatography (LC) coupled with ultraviolet (UV) detection **(Kang et al., 2006; Kubíčková et al., 2011; Wu et al., 2014)**, fluorescence detection (FLD) **(Clarke et al., 2007; de Freitas Silva et al., 2009; Perucho et al., 2015; Rawls et al., 2006; Şanlı et al., 2015)**, electrochemical detection (ECD) **(Acosta, 1998; Canevari et al., 1992; Clarke et al., 2007; Mckenzie et al., 2002; Monge-Acuña and Fornaguera-Trías, 2009; Murai et al., 1989; Rowley et al., 1995)** or tandem mass spectrometry (MS/MS) detection **(Bathena et al., 2012; González et al., 2011; He et al., 2013; Huang et al., 2014; Zhu et al., 2011)** has been the most used analytical instrumentation for the analysis of amino acids.

As most of the neuroactive amino acids (e.g., Glu, Asp, Gln, GABA, Tau) in its native form are structurally devoid of chromophores and do not possess fluorescent or electroatractive properties, their analysis by LC remains challenging. On the one hand, pre- or post-column derivatization of amino acids is required to provide enough sensitivity and selectivity to the determination of these compounds by LC-UV, LC-FLD or LC-ECD **(Dai et al., 2014; Shah et al., 2002)**. On the other hand, although the direct measurement of amino acids by LC-MS/MS is possible, and it has been increasingly employed in recent years, due to the highly hydrophilic nature of amino acids their appropriate chromatographic retention in reversed-phase columns is a difficult task **(González et al., 2011; Zhu et al., 2011)**; thus, for this reason, the use of LC-MS/MS methods employing high cost hydrophilic interaction liquid chromatography columns has been considered **(Bathena et al., 2012)**. Despite the great advantages afforded by LC-

MS/MS methods in terms of simplicity of sample preparation, sensitivity and selectivity, only a restricted number of neuroactive amino acids (Glu and GABA) has been often targeted by these assays **(González et al., 2011; He et al., 2013; Huang et al., 2014; Zhu et al., 2011)** and the chromatographic runtime was not as short as would be expected (8-15 minutes) **(Bathena et al., 2012; González et al., 2011; He et al., 2013; Huang et al., 2014; Zhu et al., 2011)**. So, overall, bearing in mind the pros and cons, it seems to be very reasonable, in many situations, the selection of a LC-FLD system for the bioanalysis of neuroactive amino acids **(Clarke et al., 2007; de Freitas Silva et al., 2009; Perucho et al., 2015; Rawls et al., 2006; Şanlı et al., 2015)**.

Despite the diversity of LC-FLD methods already developed for the measurement of amino acids in biological samples, all these methods have their own advantages and limitations. Therefore, in each circumstance, it is important to implement the most suitable bioanalytical method that fit-for-purpose. Nevertheless, aiming to ensure reliable quantitative analysis of amino acids, a critical concern that should be noted in relation to previously developed LC-FLD assays is the absence of an internal standard (IS) **(Clarke et al., 2007; de Freitas Silva et al., 2009; Perucho et al., 2015; Rawls et al., 2006)**; in fact, in quantitative bioanalysis the use of an IS is essential to improve the precision and accuracy of results, and it is particularly important in bioanalytical processes involving complex sample preparation procedures (e.g., analyte derivatization, extraction) and where volume errors (e.g. injection errors) are hard to predict and control. Also to emphasize the importance of the addition of IS to both calibration standards and samples, in order to minimize analytical errors, is the fact that almost all the recently developed LC-MS/MS methods to quantify neuroactive amino acids have considered its use **(Bathena et al., 2012; González et al., 2011; He et al., 2013; Huang et al., 2014; Zhu et al., 2011)**.

Howsoever, the availability of reliable and straightforward bioanalytical methods that enable the determination of important neuroactive amino acids in brain tissue of experimental animal models is of great interest in neurosciences both to differentiate physiological and pathological processes involving the neural functions and evaluate the action of specific drugs or drug candidates. Thus, considering all the above discussed aspects, the aim of this work was to develop a simple, accurate and economical LC-FLD method for the reliable and simultaneous quantification of Asp, Glu, Gln, Tau and GABA in rat brain tissue samples. Although other LC-FLD methods have been developed for the measurement of neuroactive amino acids, such methods only included a few number of amino acids and/or did not employ an IS. Accordingly, to the best of our knowledge, the present report provides the first LC-FLD assay reporting the simultaneous quantification of this specific set of neuroactive amino acids (Asp, Glu, Gln, Tau and GABA), employing an appropriate IS (methyl-L-arginine); the molecular structures of the compounds are shown in Figure III.1.

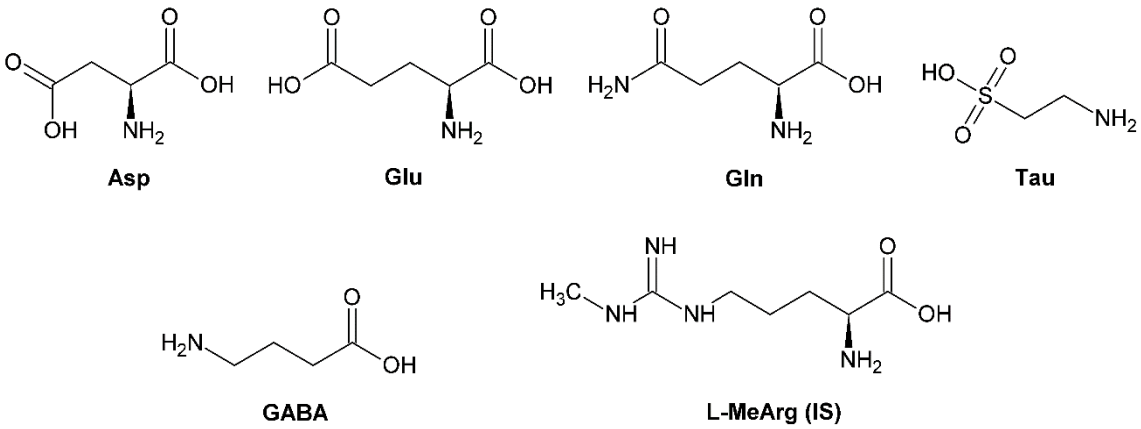

Figure III. 1. Chemical structures of aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), taurine (Tau), γ-aminobutyric acid (GABA), and the methyl-L-arginine (L-MeArg) which was used as internal standard (IS).

Thus, this bioanalytical tool may be valuable to support the nonclinical research of neurological disorders associated with alterations of brain amino acid profile and to better understand the amino acid dynamics in different brain areas. To assess the usefulness of the current method, it was applied to the measurement of Asp, Glu, Gln, Tau and GABA levels in five distinct brain regions (frontal cortex, amygdala, hippocampus, cerebellum and striatum) of rats.

III.2. Material and methods

III.2.1. Standards and chemicals

Standards of Asp, Glu, Gln, Tau, GABA, and methyl-L-arginine (L-MeArg) used as the IS (Figure III.1) were purchased from Sigma-Aldrich (Steinheim, Germany). HPLC-grade acetonitrile and acetic acid glacial 99-100% were purchased from Chem Lab (Zedelgem, Belgium) and sodium acetate was obtained from Sigma-Aldrich (Steinheim, Germany). Perchloric acid was acquired in Panreac Química SA (Barcelona, Spain), L-cysteine in Sigma-Aldrich (Steinheim, Germany), *o*-phthalaldehyde (OPA) in Acros Organics (Geel, Belgium), ß-mercaptoethanol (BME) and di-sodium tetraborate in Merck (Darmstadt, Germany), methanol in Chem Lab (Zedelgem, Belgium) and sodium hydroxide in Vencilab (Porto, Portugal). Ultra-pure water (HPLC grade, >18 MΩ.cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA).

III.2.2. Rat brain tissue

Adult male Wistar rats of approximately 10 weeks were obtained from local animal facilities (Faculty of Health Sciences, University of Beira Interior, Covilhã, Portugal). The rats were maintained under controlled environmental conditions (temperature 20±2 ºC; relative humidity 55±5%; 12-h light/dark cycle). The animals were allowed free access to a standard rodent diet and water was available ad libitum. All animal procedures were conducted in compliance with protocols approved by the national ethical requirements for animal research and the European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes (European Union Directive number 2010/63/EU) **(EU, 2010)**. In order to obtain the biological matrix of interest (rat brain tissue), which is required for the development of the bioanalytical method, rats were anesthetized with isoflurane and then immediately sacrificed by decapitation. After exsanguination, the whole brain of each animal was rapidly removed and frozen using liquid nitrogen. Thereafter, the brain tissue was homogenized in ice-cold 0.2 M perchloric acid containing 3 mM L-cysteine (4 mL per g of tissue) and sonicated for 5 minutes. The brain homogenate was centrifuged at 17000 rpm (30000 g) for 4 minutes at 4 ºC and the resulting supernatant was separated, aliquoted and stored at –20 ºC until used.

III.2.3. Stock solutions, calibration standards and quality control samples

Stock solutions (1.0 mg/mL) of Asp, Glu, Gln, Tau and GABA were individually prepared by dissolving the appropriate amount of each compound in ultra-pure water **(de Freitas Silva et al., 2009; Monge-Acuña and Fornaguera-Trías, 2009)**. These solutions were adequately diluted in ultra-pure water to obtain the corresponding appropriate intermediate solutions (100 µg/mL and 10 µg/mL). Then, six combined spiking solutions with final concentrations 400, 800, 2000, 4000, 12000 and 20000 ng/mL for Asp, Gln, Tau and GABA, and 800, 1600, 4000, 8000, 24000 and 40000 ng/mL for Glu were prepared. Aliquots (10 µL) of these combined solutions were used to spike the diluted supernatant of rat brain homogenate in order to obtain six calibration standards in the concentration ranges of 25-1250 ng/mL for Asp, Gln, Tau and GABA, and 50-2500 ng/mL for Glu. The stock solution of the IS was also prepared in ultra-pure water (1.0 mg/mL) and the corresponding spiking solution (7.5 µg/mL) was obtained after diluting an appropriate volume of the stock solution with ultra-pure water. The stock and intermediate solutions of amino acids and IS were stored at –20 °C, while the spiking solutions, once prepared, were aliquoted and also stored at –20 °C; when required, spiking solutions were daily thawed and kept at approximately 4 ºC during the time of use.

Quality control (QC) samples at three representative concentration levels, representing the low (QC_1) medium (QC_2) and high (QC_3) ranges of the calibration curves, were also independently prepared in the diluted supernatant of rat brain tissue homogenate to achieve final

concentrations of 75, 625 and 1125 ng/mL for Asp, Gln, Tau and GABA; and 150, 1250 and 2250 ng/mL for Glu.

III.2.4. Sample preparation and derivatization

The sample preparation and derivatization steps were previously optimized and the final conditions were as follows. The supernatant of rat brain tissue homogenate was diluted (1/50) in ultra-pure water. Then, 10 µL of diluted sample were spiked with 10 µL of IS (7.5 µg/mL) and vortex-mixed for 15 seconds. Afterwards, the resulting mixture was added with 20 µL of the derivatization reagent [25 mg OPA diluted in 1500 µL of methanol, 100 µL of borate buffer 0.1 M (pH 9) and 30 µL of BME], which was prepared weekly, kept at 4 ºC and protected from light. The derivatization mixture was vortexed for 1 minute at room temperature, added of 120 µL of ultra-pure water and, then, 20 uL of this final solution were injected into the HPLC system.

III.2.5. Instrumentation and chromatographic conditions

For chromatographic analysis an HPLC system (Shimadzu Corporation, Japan) was used. The system is equipped with a DGU-20A5R automatic degasser, a LC-20AD quaternary solvent pump, a SIL-20ACHT refrigerated automatic injector, a CTO-10AS VP columns oven and a RF-20AXS fluorescence detector. LabSolutions software was used to control the HPLC components and to process data.

Chromatographic separation was achieved on a reversed-phase LiChroCART® Purospher Star column (C_{18}, 55 mm × 4 mm; 3 µm particle size) protected by a LiChroCART® Purospher Star precolumn (C_{18}, 4 mm × 4 mm; 5 µm particle size) purchased from Merck KGaA (Darmstadt, Germany). The column temperature was maintained at 25 ºC and the mobile phase was pumped at a flow rate of 1.0 mL/min using gradient elution conditions. The mobile phase components consisted of aqueous acetate buffer 25 mM (pH 5.4) (A) and acetonitrile (B), and the gradient elution program was carried out as follows: 0-10 minutes 30% (B), 10-11 minutes 13% (B). The aqueous component of mobile phase was filtered through a 0.45 µm filter. The fluorescence was monitored at excitation and emission wavelengths of 340 nm and 448 nm, respectively.

III.2.6. Method validation

The validation assays were conducted based on the bioanalytical method validation guidelines of the Food and Drug Administration **(FDA, 2001)** and European Medicines Agency **(EMA, 2011)**. As the analytes were endogenous compounds, the linearity of the detector response for the target analytes was assessed with a set of six calibration standards prepared in the diluted matrix (1/50) spiked with known concentrations of amino acids (25, 50, 125, 250, 750 and 1250

ng/mL for Asp, Gln, Tau and GABA and 50, 100, 250, 500, 1500 and 2500 ng/mL for Glu) and added with a fixed amount (75 ng) of IS (10 µL of 7.5 µg/mL solution). The calibration curves were assayed on three different days (*n* = 3) and were constructed by plotting the corrected analyte-IS peak height ratio as function of the corresponding nominal concentrations. Due to the presence of endogenous analytes in diluted matrix, blank values of the target amino acids were subtracted from each calibration point **(Bathena et al., 2012; He et al., 2013)**. As a result, the mean value of analyte-to-IS peak height ratios of blank samples (*n* = 3) was defined as Y_{BS} and analyte-to-IS peak height ratio of calibration standards was defined as Y_{CS} (CS = 1, 2, 3, 4, 5 and 6), and then, the calibration curve for each analyte was constructed using the difference between Y_{CS} and Y_{BS} versus the nominal standard concentration. The data were subjected to a weighted linear regression analysis **(Almeida et al., 2002)**. The limit of quantification (LOQ) was defined as the lowest concentration of the calibration curve that can be measured with acceptable precision [a coefficient of variation (CV) value lower than or equal to 20%] and accuracy (a *bias* value within ±20% of the nominal concentration) **(EMA, 2011; FDA, 2001)**. The LOQ was evaluated by analyzing a set of samples with known concentrations of analytes in five replicates (*n* = 5). The inter-day precision and accuracy of the assay were determined using QC samples at three concentration levels (QC_1, QC_2 and QC_3), analyzed on three consecutive validation days (*n* = 3), using freshly prepared calibration curves. The intra-day precision and accuracy were assessed by analyzing five replicates (*n* = 5) of QC samples on the same day. Precision was established as a CV value that must be lower than or equal to 15% for replicate measurements, whereas accuracy was established as a *bias* value that should be within ±15%.

The stability of the analytes was investigated in the diluted supernatant of rat brain tissue homogenate samples at two different concentration levels (low and high QC values) and the analyses were performed in replicates (*n* = 4). The data of the QC samples analyzed before (reference samples) and after being exposed to the experimental conditions for stability evaluation (stability samples) were compared. The stability/reference samples ratio of 85-115% is accepted as stability criterion **(Chen et al., 2005; EMA, 2011)**. To mimic sample handling and storage before analysis, stability was evaluated in different conditions: at room temperature for 5 h, 4 °C for 24 h, −20 °C for 5 and 30 days; the influence of three freeze–thaw cycles was also investigated at −20 °C.

III.2.7. Method application

To demonstrate the application of this new LC-FLD method, it was employed for the simultaneous measurement of the levels of the target neuroactive amino acids (Asp, Glu, Gln, Tau and GABA) in authentic samples of different rat brain tissue regions. For this purpose, a group of three adult male Wistar rats (*n* = 3) was anesthetized with isoflurane, sacrificed by decapitation and, after exsanguination, the brain was promptly excised and frozen using liquid nitrogen. Later, the brain was carefully dissected into five brain regions (frontal cortex,

amygdala, hippocampus, cerebellum and striatum) on ice. Subsequently, the different brain regions were individually homogenized as previously described in *section III.2.2*. The resulting supernatants were collected, appropriately diluted (1/50) with ultra-pure water and stored at −20 ºC until the LC analysis.

III.3. Results and discussion

III.3.1. Method development

A LC-FLD method was developed and successfully validated for the simultaneous determination of five neuroactive amino acids (i.e. Asp, Glu, Gln, Tau and GABA) in samples of rat brain tissue, using L-MeArg as IS (Figure III.1). To promote the success of the validation process, a set of preliminary studies was carried out in order to optimize different bioanalytical procedures related to sample pretreatment, derivatization reaction and instrumental analysis. The final sample preparation/derivatization and chromatographic conditions correspond to those previously mentioned in *section III.2.4* "*Sample preparation and derivatization*" and *section III.2.5* "*Instrumentation and chromatographic conditions*", respectively.

III.3.2. Sample pretreatment

The selection of a suitable sample pretreatment procedure is an essential part of any bioanalytical assay as it has a significant impact in the subsequent bioanalytical steps and in data quality **(Alves et al., 2013)**. The sample pretreatment should firstly ensure the stability of the target analytes in the samples, avoiding losses during sample handling and storage **(Shah et al., 2002)** and, if possible, the sample processing conditions should be practical, environmentally friendly and cost-efficient.

Similarly to other classic neurotransmitters (e.g. DA, NE and DA), most neuroactive amino acids and their precursors and/or metabolites are polar compounds **(He et al., 2013)** and several approaches have been reported for the pretreatment of brain tissue samples aiming at measuring amino acid levels. The first step typically consists of brain tissue homogenization and a large variety of homogenization media have been employed such as deionized water **(Monge-Acuña and Fornaguera-Trías, 2009)**, 0.9% saline solution **(Wu et al., 2014)**, methanol **(Bathena et al., 2012)**, methanol–water mixture **(de Freitas Silva et al., 2009)**, acidified methanol **(He et al., 2013; Huang et al., 2014; Şanlı et al., 2015)**, formic acid **(González et al., 2011; Zhu et al., 2011)** and perchloric acid **(Canevari et al., 1992; Kang et al., 2006; Perucho et al., 2015)** or perchloric acid containing L-cysteine and EDTA **(Shah et al., 1999)**. Furthermore, it has been reported that some amino acids undergo rapid degradation after homogenizing brain tissues in water, likely due to residual activity of endogenous enzymes

(Bathena et al., 2012); thus, the homogenization of brain tissue in an organic solvent (methanol) or aqueous acidic solution is preferred also allowing the simultaneous deproteinization of the sample.

Considering all the aforementioned information, and taking also into account that recently we have developed a new quantitative assay for determination of catecholamines and endogenous related compounds in rat brain tissue **(Fonseca et al., 2017)**, in which the brain tissue samples were homogenized in ice-cold 0.2 M perchloric acid containing 3 mM L-cysteine, the same sample processing conditions were applied in the current work, enabling, in the future, the quantitative analysis of catecholamines and neuroactive amino acids from a single sample of rat brain tissue. This specific aspect seems to be of value for routine nonclinical research in neurosciences, and this is clearly supported by the recent trends in the development of LC-MS/MS methods for the simultaneous analysis of neurotransmitters belonging to different classes (amino acids, catecholamines, indolamines) **(González et al., 2011; He et al., 2013; Huang et al., 2014; Zhu et al., 2011)**.

In this case, due to the high brain levels of most neuroactive amino acids, the homogenate supernatant of brain tissue samples cannot be directly treated with the derivatization agent and, therefore, towards obtaining an appropriate chromatographic response a dilution (1/50) of the homogenate supernatant with ultra-pure water was required before the derivatization step. The basal levels of endogenous compounds, including neuroactive amino acids, are subject to fluctuations **(Barth et al., 2015; Godfrey et al., 2017; Young, 2017)**, which are dependent of multiple factors (e.g. age, sex, physiological conditions, circadian rhythm, etc). Therefore, there is the need to check the levels of the target analytes in the biological matrix of interest prior to the preparation of calibration and QC samples.

III.3.3. Derivatization reaction conditions

Structurally, in their native form, the target neuroactive amino acids (Asp, Glu, Gln, Tau and GABA) do not have chromophore or fluorophore groups, making necessary a derivatization step prior to ultraviolet, fluorescence or even electrochemical detection in order to improve the sensitivity and selectivity in biological samples. Indeed, a variety of derivatization reagents has been used for analysis of amino acids in brain or spinal cord tissues: naphthalene-2,3-dicarboxaldehyde **(Clarke et al., 2007)**, 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde **(Şanlı et al., 2015)**, dansyl chloride **(Kang et al., 2006)**, 4-fluoro-7-nitrobenzofurazan **(Wu et al., 2014)** and OPA **(Canevari et al., 1992; de Freitas Silva et al., 2009; Mckenzie et al., 2002; Monge-Acuña and Fornaguera-Trías, 2009; Perucho et al., 2015; Tcherkas et al., 2001)**.

Knowing the large number of LC techniques for analysis of amino acids that involve the pre-column derivatization with OPA, this derivatization reagent was considered as the first choice for development of the current method. Even so other alternatives available have also been tried, the dansyl chloride and the 4-chloro-7-nitrobenzofurazan, the best results were obtained

using OPA. In fact, the derivatization reaction with OPA generated less chromatographic interferences. Additionally, OPA reacts rapidly at room temperature with primary amines and amino acids (except cystein or cystine) in the presence of a thiol reagent (e.g. BME) to form highly fluorescent derivatives **(Dai et al., 2014; Mckenzie et al., 2002)**. However, substantial differences were found in the composition of the OPA-containing derivatization reagents used from group to group **(Canevari et al., 1992; de Freitas Silva et al., 2009; Mckenzie et al., 2002; Monge-Acuña and Fornaguera-Trías, 2009; Perucho et al., 2015; Tcherkas et al., 2001)**. Thus, in this work has been taken as starting point the OPA-containing derivatization reagents of some authors as Allison et al. **(1984)**, Canevari et al. **(1992)**, Lindroth and Mopper **(1979)** and Tcherkas et al. **(2001)**. After several preliminary tests, to obtain the best performance between selectivity and derivatization potential, the selected composition for the derivatization reagent is as follows: 25 mg OPA diluted in 1500 µL of methanol, 100 µL of borate buffer 0.1 M (pH 9) and 30 µL of BME.

III.3.4. Liquid chromatography

The chromatographic conditions were developed based on those used by Perucho et al. **(2015)**, which evaluated several neuroactive amino acids in rat brain samples employing a gradient elution program. Based on these initial conditions, several factors were then optimized to simplify and achieve an appropriate separation of the target amino acids and potential endogenous interferences present in samples, obtain symmetric peaks and a running time as short as possible.

The pre-column derivatization offers the advantage of sufficiently increasing the hydrophobicity of the analytes to be retained on a reversed stationary phase **(Płotka-Wasylka et al., 2015)**. For this purpose, the composition of the mobile phase (acetate buffer concentration, pH, organic modifiers) was investigated using a C_{18} stationary phase column; in addition, different conditions of column temperature and flow rate were also tested. Firstly, regarding the acetate buffer concentration, 25 mM and 50 mM were used, but no improvements were found for most of the amino acid derivatives; thus, taking into account the benefits of lower salt concentrations for the chromatographic column, 25 mM acetate buffer was selected. The influence of mobile phase pH within the range of 4.8-6.4 was also studied. The results showed that the chromatographic behavior of OPA derivatives were very sensitive to the mobile phase pH, as the pH increased the fluorescence intensity increased but the retention time decreased. Thus, a pH value of 5.4 was considered preferable for the overall separation with satisfactory sensitivity. The choice of the organic modifier (e.g. methanol, acetonitrile) was also taken into account, since differences in the chemical properties of these organic solvents influence the separation of the target compounds. For the chromatographic separation of the neuroactive amino acids of interest and to avoid interferences from other concomitant amino acids present in the sample, a good peak resolution was achieved with a gradient elution program using acetonitrile as organic modifier. Finally, aiming at achieving a good compromise

between peak resolution and sample run time, a mobile phase composed of acetate buffer 25 mM (pH 5.4) and acetonitrile (13-30%) pumped at a flow rate of 1.0 mL/min under gradient elution conditions was established. To overcoming temperature fluctuations of the laboratory and getting greater reproducibility, separation was achieved with the column temperature controlled and kept at 25 ºC.

Under the above chromatographic conditions the total analysis time was within 11 minutes, and a typical chromatogram obtained after analysis of a spiked sample is shown in Figure III.2. An appropriate resolution was observed between all compounds of interest with retention times of approximately 1.3, 2.0, 3.8, 6.2, 8.2 and 9.1 minutes for Asp, Glu, Gln, L-MeArg (IS), Tau and GABA, respectively.

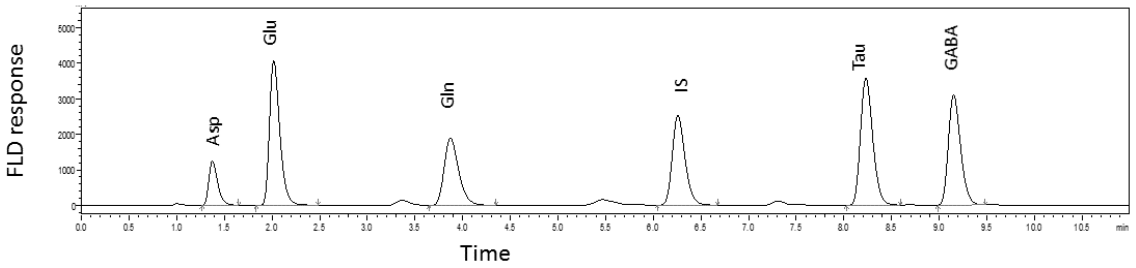

Figure III. 2. Typical chromatogram obtained by the developed LC-FLD assay after the analysis of a spiked sample containing the analytes (Asp, Glu, Gln, Tau and GABA) and the internal standard (IS). Asp, aspartic acid; Glu, glutamic acid; Gln, glutamine; Tau, taurine; GABA, γ-aminobutyric acid.

III.3.5. Method validation

For the quantification of endogenous compounds, such neuroactive amino acids, the authentic biological matrix typically contains an unknown concentration of the analytes. Thus, to resolve this delicate issue the application of the method of standard addition, by adding know concentrations of the analyte to individual aliquots of the sample, can be a useful strategy. However, this approach was not followed by some authors, such as Perucho et al. **(2015)** or de Freitas Silva et al. **(2009)**, that evaluated the linearity of the method by construction of calibration curves using only standard solutions. Nevertheless, in this context it is important to highlight that the derivatization procedure can occur differently in biological samples and in standard solutions. This issue was studied by Dorresteijn and collaborators and they concluded that to obtain a reliable method, care must be taken with the treatment of the samples and the standard solutions because the linearity of the calibration curve depends on the solvent being used; they showed that the use of linear calibration curves does not always coincide with the real response of the OPA amino acid derivatives **(Dorresteijn et al., 1996)**. Taking this aspect into account, in the current method the calibration standards and QCs were prepared by spiking the biological matrix of interest with the appropriate standard solutions of the analytes, and the basal response ascribed to the endogenous levels of amino acids in the unspiked samples was subtracted.

Calibration curves were constructed based on the analyte-IS peak height ratios versus the corresponding nominal concentrations and were established using a weighted linear regression analysis of the data, being the best linear fit and least-squares residuals achieved with a $1/x^2$ weighting factor. Hence, as shown in Table III.1, this method showed good linearity with determination coefficients (r^2) equal to or higher than 0.9920. The LOQs of the analytes were established experimentally and they were defined as the lowest concentration of the calibration ranges determined with acceptable precision (CV ≤ 11.81%) and accuracy (*bias* varied from -2.60% to 5.80%), which were defined as 25 ng/mL for Asp, Gln, GABA and Tau and 50 ng/mL for Glu.

Table III.1. Mean calibration parameters (n = 3) of aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), taurine (Tau) and γ-aminobutyric acid (GABA).

Analyte	Calibration parameters		
	Concentration range (ng/mL)	Equation[a]	r^2
Asp	25-1250	y = 0.000087x + 0.0008	0.992
Glu	50-2500	y = 0.000163x + 0.0006	0.992
Gln	25-1250	y = 0.000241x + 0.0002	0.992
Tau	25-1250	y = 0.000352x + 0.0052	0.993
GABA	25-1250	y = 0.000378x - 0.0005	0.996

[a] y represents analyte-IS peak area ratio; x represents analyte concentration (ng/mL).

Meanwhile, the LOQs of the present method can be directly compared with other LC-FLD methods that used OPA as derivatization reagent. For example, de Freitas Silva et al. **(2009)** reported LOQs for GABA and Glu of 100 and 1000 ng/mL respectively, demonstrating the better sensitivity of our method for these compounds. When compared the current method with the LC-FLD method developed by Sanlı et al. **(2015)**, in which 3-(4-carboxybenzoyl)-2-quinolinecarboxaldehyde was employed in the derivatization step, it was found that the LOQs established for Tau, GABA and Glu were better than those obtained by us; however, the method developed by Sanlı et al. **(2015)** requires a slow derivatization step. LC-MS/MS methods can usually afford higher selectivity and sensitivity than LC-FLD methods, but the instrumentation is more complex and expensive. Nevertheless, it also is worthy of note that Huang et al. **(2014)** using a LC-MS/MS method presented LOQs in the order of 500 ng/mL for GABA and Glu in mouse hippocampus, which are much higher when compared with our method. Therefore, the current method compared with previous methods is more convenient, and allows the fast determination of the levels of neuroactive amino acids in samples of brain tissue.

Intra- and inter-day precision and accuracy were evaluated using diluted rat brain tissue homogenate supernatant samples by replicate analyses of QC samples at three concentration levels within the same day and on three consecutive days, and the results obtained are summarized in Table III.2. As a result, the overall intra- and inter-day precision (assessed by CV) was lower than or equal to 13.64%, while the accuracy (assessed by *bias*) ranged from -

14.60–12.10%. Hence, precision and accuracy values on both intra- and inter-day were all within the acceptable range, indicating that the current method was reliable and reproducible, demonstrating that it can be used to quantify these target analytes in the real biological matrix. For the stability studies, diluted brain tissue homogenate supernatants were processed and analyzed as described above, simulating the handling and sample storage conditions that are likely to be encountered during the analytical process. The stability of compounds was guaranteed at room temperature for 5 h, at 4 ºC for 24 h, at −20 ºC for 5 and 30 days, and also after three freeze–thaw cycles at −20 ºC.

Table III.2. Precision (% CV) and accuracy (% *bias*) for the determination of aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), taurine (Tau) and γ-aminobutyric acid (GABA) in diluted brain tissue homogenate supernatant samples at the low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges.

Analyte	Nominal concentration (ng/mL)	Inter-day		Intra-day	
		Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)
Asp	75	2.56	-14.60	12.99	11.54
	625	12.78	7.95	11.48	12.10
	1125	2.21	-2.77	11.08	10.51
Glu	150	13.10	1.57	9.09	1.76
	1250	5.58	4.24	8.29	5.85
	2250	0.37	-3.28	8.13	8.11
Gln	75	10.78	1.50	9.80	-0.89
	625	2.43	-2.40	4.70	-3.57
	1125	3.70	-5.39	2.57	-2.43
Tau	75	7.27	4.87	13.64	-9.16
	625	2.66	-0.29	6.22	-3.38
	1125	4.35	-4.40	3.52	-0.30
GABA	75	6.71	1.44	7.25	-5.27
	625	3.09	-0.82	3.81	-6.02
	1125	4.66	-6.57	5.18	-5.74

bias, deviation from nominal concentration value; CV, coefficient of variation.

III.3.6. Method application

The proposed LC-FLD method was established aiming its application in the field of neurosciences to support nonclinical studies that could benefit with the quantitative determination of neuroactive amino acids in whole or specific regions of rat brain. Thus, a preliminary study was performed with three rats aiming at demonstrating the applicability of the method in the analysis of Asp, Glu, Gly, Tau and GABA in specific brain regions (frontal cortex, amygdala, hippocampus, cerebellum and striatum) and the results are presented in

Table III.3. In Figure III.3 is shown a representative chromatogram of the analysis of a sample of frontal cortex of Wistar rat.

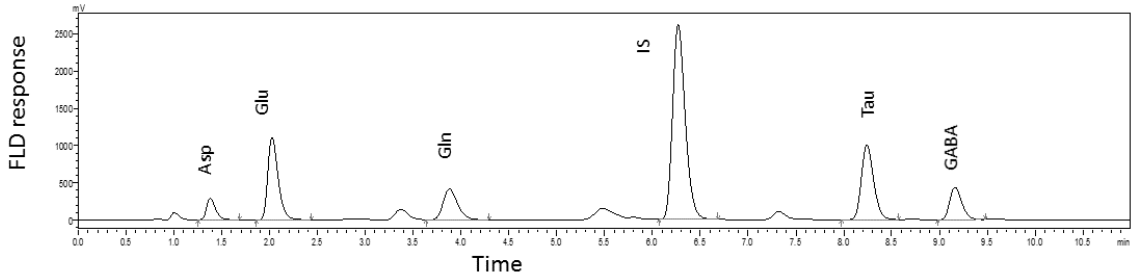

Figure III.3. Representative chromatogram of the analysis of a rat brain sample. Concentrations of neuroactive endogenous compounds measured in frontal cortex: 2104.68 ng/mg for Asp, 2356.75 ng/mg for Glu, 673.69 ng/mg for Gln, 818.16 ng/mg for Tau, and 347.58 ng/mg for GABA. Asp, aspartic acid; Glu, glutamic acid; Gln, glutamine; Tau, taurine; GABA, γ-aminobutyric acid; IS, internal standard.

Table III.3. Tissue contents of aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), taurine (Tau) and γ-aminobutyric acid (GABA) in various specific regions of the brain of Wistar rat.

Brain region	Concentration (mean ± SD; ng/mg)				
	Asp	Glu	Gln	Tau	GABA
Frontal cortex	1842.91 ± 228.91	2327.42 ± 111.33	732.88 ± 58.15	929.34 ± 113.78	386.49 ± 43.31
Amygdala	1179.57 ± 137.06	1811.31 ± 389.56	842.01 ± 26.60	775.72 ± 120.58	489.12 ± 73.04
Hippocampus	1260.06 ± 25.57	2093.17 ± 58.53	650.71 ± 53.08	909.03 ± 31.27	368.68 ± 55.99
Cerebellum	1118.30 ± 341.20	2143.13 ± 499.19	750.96 ± 88.77	717.37 ± 103.70	278.33 ± 20.74
Striatum	1054.62 ± 181.96	1493.34 ± 134.97	697.66 ± 24.84	1095.30 ± 54.12	448.71 ± 45.20

Results expressed as mean ± standard deviation of three independent samples (*n* = 3).

Glu is the most abundant excitatory amino acid neurotransmitter and it is widely distributed in the mammalian brain. Glutamatergic neurons make up about 80% of the total population of neurons in the brain cortex **(Somogyl et al., 1998)**. As can be seen in Table III.3, the average concentration of Glu in the frontal cortex was 2327.42 ng/mg of tissue, and this value was found to be superior to other brain regions, and therefore it is in accordance with the literature. Asp also displayed its highest concentration (1842.91 ng/mg) in the frontal cortex. Gln has on average concentrations of 734.84 ng/mg in brain and Tau concentrations were lower in the cerebellum and the maximum concentration (1095.30 ng/mg) was reached in the striatum. GABA is presented in all parts of the brain, most highly concentrated in the amygdala 489.12 ng/mg. The results herein obtained in different brain regions are similar to those found in previous reports **(Acosta, 1998; Clarke et al., 2007; Zhu et al., 2011)**

CHAPTER IV - First HPLC method for the simultaneous quantification of levetiracetam, zonisamide, lamotrigine, pentylenetetrazole and pilocarpine in rat plasma and brain samples

The content of this chapter is included in the following publication:

Fonseca, BM., Rodrigues, M, and Alves, G. (2018). First HPLC method for the simultaneous quantification of levetiracetam, zonisamide, lamotrigine, pentylenetetrazole and pilocarpine in rat plasma and brain samples. Anal Methods. *10*, 515-525. doi: 10.1039/C7AY02602A

IV.1. Introduction

Epilepsy is a serious chronic and often progressive neurological disorder affecting more than 70 million people worldwide **(Singh and Trevick, 2016)**. Although the occurrence of repeated seizures is the clinical hallmark of epilepsy, the epileptogenesis begins before the first seizure and may continue or even be potentiated with the onset of seizures. Nowadays, despite the wide armamentarium of antiepileptic drugs (AEDs) in clinical use, commonly referred to as antiseizure drugs, they fail the seizure control in 20–30% of patients and no AED seems to clinically prevent the development of epilepsy **(Löscher et al., 2013)**. In fact, it is not surprising that almost all the presently used AEDs seem to purely act as anticonvulsant agents once they were discovered as a result of their ability to protect against seizures in animal models **(Kaminski et al., 2014; Löscher et al., 2013)**. However, there is some preclinical evidence that certain AEDs have some antiepileptogenic properties, including the older agents VPA and ethosuximide and the newer agents LEV, LTG and ZNS **(Chen et al., 2017b; Hashimoto et al., 2003; Kaminski et al., 2014; Stratton et al., 2003)**. Hence, aside from their antiseizure activity, it is of interest to assess whether other newer AEDs could potentially have antiepileptogenic effects and to further investigate the antiepileptogenic properties of LEV, LTG and ZNS. Among the newer generation of AEDs, LEV, LTG and ZNS have been the most frequently used for the treatment of many types of seizures and epilepsy syndromes; indeed,

these drugs seem to act by a number of different mechanisms of action which may determine their broad-spectrum of efficacy **(Goldenberg, 2010; Kaminski et al., 2008; Lynch et al., 2004; Lyseng-williamson, 2011; Mula, 2013; Patsalos, 2013; Romigi et al., 2015; Sasa, 2006; Stefan and Feuerstein, 2007)**.

Undoubtedly, rodent animal models of seizures and epilepsy have played an essential role in the discovery of AEDs and in the understanding of basic mechanisms underlying ictogenesis and epileptogenesis **(Löscher, 2011)**. In fact, amongst the diversity of chemical convulsant agents that have been used experimentally to induce seizures or epilepsy models that mimic different clinical seizures types and acute *versus* chronic epilepsy phenomena are the pentylenetetrazole (PTZ) and pilocarpine (PIL) **(Hedlund and Bartfai, 1981; Löscher et al., 2013; Marchi et al., 2014; Maslanski et al., 1994; Turski et al., 1989)**. Surprisingly, in the last few years many research works have employed PTZ- and/or PIL-induced epilepsy models to study the pathophysiological events underlying epileptogenesis, as well as the antiepileptogenic and neuroprotective effects of potential therapeutic agents **(Abdel-Wahab et al., 2017; Chen et al., 2017b; Doeser et al., 2015; Gross et al., 2017; Kandeda et al., 2017; Kaur et al., 2017; Lee et al., 2017; Mazhar et al., 2017; Pitsch et al., 2017; Zgrajka et al., 2010)**. Thus, in order to better understand the effects of these convulsant agents (PTZ and PIL) in the progression of epileptogenesis and to evaluate the impact of specific AEDs on such events, it is of paramount importance to support the qualitative findings with the simultaneous quantitative determination of convulsants and AEDs. Hence, the availability of an easy-to-use bioanalytical method that enables the simultaneous measurement of PTZ, PIL, LTG, ZNS and LEV (Figure IV.1) in plasma and brain tissue of rodents may be of great interest to elucidate the epileptogenic-antiepileptogenic phenomena.

Actually, until now, some high-performance liquid chromatography (HPLC) methods have been used for the separated quantification of PTZ **(Ramzan, 1988; Soto-Otero et al., 1987)**, PIL **(Epps et al., 2012; Römermann et al., 2015)**, LTG **(Castel-Branco et al., 2001; Liu et al., 2014; Ventura et al., 2016; Walker et al., 2000; Walton et al., 1996; Yamashita et al., 1997; Yang et al., 2013)**, ZNS **(Toyota et al., 2001)** and LEV **(Doheny et al., 1999; Luo et al., 2013; Tong and Patsalos, 2001)** in rat matrices, but none permits the concurrent determination of these compounds. Therefore, the purpose of this work was to develop and validate an HPLC method coupled to diode array detection (DAD) to simultaneously quantify three AEDs (LEV, LTG and ZNS) and two convulsant agents (PTZ and PIL) in rat plasma and brain tissue samples.

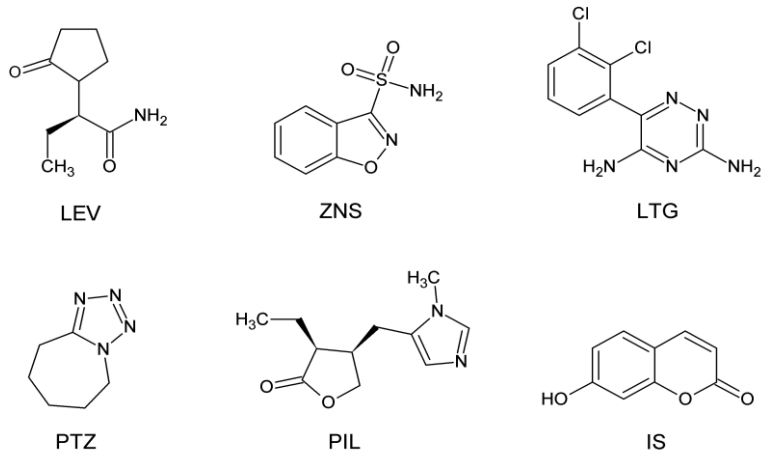

Figure IV.1. Chemical structures of levetiracetam (LEV), zonisamide (ZNS), lamotrigine (LTG), pentylenetetrazole (PTZ), pilocarpine (PIL), and 7-hydroxycoumarin which was used as internal standard (IS).

IV.2. Material and methods

IV.2.1. Standards and reagents

LEV, ZNS and PIL hydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan), PTZ was acquired from Sigma–Aldrich (St Louis, MO, USA) and 7-hydroxycoumarin, used as internal standard (IS), was purchased from Supelco Analytical (Bellefonte, DA, USA). LTG was kindly provided by Bluepharma (Coimbra, Portugal). Acetonitrile of HPLC gradient grade and dichloromethane of analytical grade were purchased from Fisher Scientific (Leicestershire, United Kingdom). Ultrapure water (HPLC grade, >18 MΩ.cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). Triethylamine was acquired from Merck KGaA (Darmstadt, Germany) and the 85% *ortho*-phosphoric acid was purchased from Panreac Química SA (Barcelona, Spain). Pentobarbital (Eutasil® 200 mg/ml, Ceva Saúde Animal) used as the anaesthetic drug was also commercially acquired.

IV.2.2. Blank rat matrices: Plasma and brain tissue

Adult Wistar rats of approximately 10 weeks were obtained from local animal facilities as a source of the blank matrices (plasma and brain tissue) required for the bioanalytical method validation. All procedures were conducted in accordance with the European Directive (2010/63/EU) regarding the protection of laboratory animals used for scientific purposes. To obtain the blank rat matrices, naïve rats were firstly anaesthetized with pentobarbital [60 mg/kg; intraperitoneal (i.p.) injection] and then exsanguinated by decapitation. Blood samples were collected into EDTA tubes and the brain was quickly excised and kept on ice until further processing. The blood samples were centrifuged at 4000 rpm for 10 min at 4 ºC and then the

plasma was immediately collected and separated from the blood cells. The brain tissue was weighed and homogenised in ultrapure water (4 mL per g of tissue) using an Ultra-Turrax® tissue homogenizer and centrifuged at 17000 rpm for 4 min at 4 ºC. The plasma and brain homogenate supernatants were collected and stored at −20 ºC until used.

IV.2.3. Stock solutions, calibration standards and quality control samples

Stock solutions of LEV, PTZ, PIL, ZNS and LTG (10 mg/mL) were individually prepared by dissolving the appropriate amount of each compound in methanol. These solutions were then adequately diluted with water-methanol (50:50, v/v) to obtain the corresponding appropriate intermediate solutions (1 mg/mL and 100 µg/mL). Afterwards, these solutions of drugs were properly mixed to afford seven combined spiking solutions with final concentrations 2, 4, 12.5, 50, 200, 350 and 500 µg/mL for LEV; 10, 25, 75, 150, 200, 350 and 500 µg/mL or PTZ; 3.75, 7.5, 25, 50, 200, 350 and 500 µg/mL for PIL; 0.75, 2.5, 12.5, 50, 200, 350 and 500 µg/mL for ZNS and 1.5, 3, 12.5, 25, 45, 150 and 250 µg/mL for LTG. Each one of these combined solutions was daily used for spiking aliquots of blank rat plasma and brain in order to prepare seven calibration standards in the concentration ranges of 0.4-100 µg/mL for LEV, 2-100 µg/mL for PTZ, 0.75-100 µg/mL for PIL, 0.15-100 µg/mL for ZNS, and 0.3-50 µg/mL for LTG. The stock solution of the IS was prepared in methanol (1 mg/mL) and the working solution (25 µg/mL) was obtained by diluting an appropriate volume of the stock solution with water-methanol (50:50, v/v). Stock and working solutions were stored at –20 °C and protected from light, and spiking solutions were stored at 4 °C and protected from light.

Quality control (QC) samples at three representative concentration levels, representing the low (QC_1), medium (QC_2) and high (QC_3) ranges of the calibration curves, were also independently prepared in rat plasma and brain samples. With that purpose, aliquots of blank rat samples were spiked to attain final concentrations of 1.2, 50 and 90 µg/mL for LEV; 6, 50 and 90 µg/mL for PTZ; 2.25, 50 and 90 µg/mL for PIL; 0.45, 50 and 90 µg/mL for ZNS; and 0.9, 25 and 45 µg/mL for LTG. An additional QC sample was also prepared at the concentration of the limit of quantification (QC_{LOQ}): 0.4 µg/mL for LEV, 2 µg/mL for PTZ, 0.75 µg/mL for PIL, 0.15 µg/mL for ZNS and 0.3 µg/mL for LTG.

IV.2.4. Chromatographic system and conditions

The analysis was performed using an HPLC system (Shimadzu LC-2010A HT Liquid Chromatography) coupled with a DAD detector (Shimadzu SPD-M20A). All instrumental parts were automatically controlled by the LC solution software (Shimadzu, Kyoto, Japan). The separation of the analytes and IS was carried out at 30 °C on a reversed-phase LiChroCART® Purospher Star column (C_{18}, 55 mm × 4 mm; 3 µm particle size) with a 5 µm C_{18} Purospher guard

column purchased from Merck KGaA (Darmstadt, Germany). An isocratic elution was applied at a flow rate of 1 mL/min with a mobile phase composed of acetonitrile (7.5%) and a mixture (92.5%) of water–triethylamine (99.5:0.05%, v/v) at pH 6.4 adjusted with 85% *ortho*-phosphoric acid. The mobile phase was filtered through a 0.2 µm filter and degassed ultrasonically for 15 min before use. The injection volume was 20 µL and wavelengths of 205 nm for LEV, PTZ, LTG and IS, 215 nm for PIL and 240 nm for ZNS were selected for the detection of compounds.

IV.2.5. Sample extraction procedure

Each aliquot (100 µL) of rat plasma or brain homogenate supernatant was added with 20 µL of IS working solution and then with 1 mL of dichloromethane. Afterwards, the mixture was vortex-mixed for 1 min and centrifuged at 13500 rpm (3 min). The organic layer was transferred to a glass tube and evaporated to dryness under a gentle nitrogen stream at 55 °C. The resulting dry extract was reconstituted with 100 µL of mobile phase and an aliquot (20 µL) of this final sample was injected into the chromatographic system.

IV.2.6. Method validation

The method was validated according to the international accepted recommendations for bioanalytical method validation **(EMA, 2011; FDA, 2001)**.

The selectivity of the method was evaluated by analyzing blank plasma and brain samples from six different rats to assess the interference of matrix endogenous substances at the same retention times of the analytes (LEV, PTZ, PIL, ZNS and LTG) and IS. In addition, interference from several anaesthetics (pentobarbital, xylazine and ketamine) commonly used in nonclinical *in vivo* studies were also evaluated by injecting standard drug solutions at a concentration of 50 µg/mL under the optimised chromatographic conditions.

To evaluate the linearity of the method, calibration curves were generated using seven calibration standards prepared in rat plasma and brain homogenate samples and assayed on three different days (n = 3). These curves were constructed by plotting analyte-IS peak area ratio as a function of the corresponding nominal concentrations over the range of 0.4-100 µg/mL for LEV, 2-100 µg/mL for PTZ, 0.75-100 µg/mL for PIL, 0.15-100 µg/mL for ZNS, and 0.3-50 µg/mL for LTG. The data were subjected to a weighted linear regression analysis **(Almeida et al., 2002)**. The limit of quantification (LOQ) was evaluated for each analyte by analyzing plasma and brain samples prepared in five replicates (n = 5) at the lowest concentration level of the calibration curve. As acceptance criteria, the precision which is given by the value of coefficient of variation (CV) cannot exceed 20%, while accuracy expressed by the deviation from nominal concentration value (*bias*) should be within ±20%.

The interday precision and accuracy of the assay were investigated using rat plasma and brain QC samples analyzed on three consecutive days (n = 3) at four concentration levels (QC_{LOQ}, QC_1,

QC_2 and QC_3) representative of the calibration range. Similarly, the intraday precision and accuracy were also assessed analyzing five sets of the QC samples in a single day (n = 5). Intra and interday precision (expressed as the percentage of CV) must be lower than or equal to 15% (or 20% in the LOQ) and intra and interday accuracy (expressed as the percentage of *bias*) must be within ±15% (or ±20% in the LOQ).

The absolute recovery of the analytes and IS from rat plasma and brain samples was calculated for the three QC samples (QC_1, QC_2 and QC_3), comparing the analytes peak areas from extracted QC samples with those obtained after direct injection of non-extracted solutions at the same nominal concentrations (n = 5).

Rat plasma and brain stability of the five analytes was investigated for QC_1 and QC_3 (n = 3), comparing the data of the QC samples analyzed before (reference samples) and after being exposed to the experimental conditions for stability assessment (stability samples). The stability/reference samples ratio of 85-115% is accepted as the stability criterion. The stability of the analytes in the biological matrices was evaluated at room temperature for 4 h, 4 °C for 24 h and –20 °C for 15 days in order to simulate sample handling and storage conditions before analysis; the influence of three freeze–thaw cycles on the stability of the compounds was also studied at –20 °C; for that, aliquots of spiked plasma and brain samples were stored at –20 °C for 24 h, thawed unassisted at room temperature, and when completely thawed, samples were refrozen for 24 h under the same conditions until completing the three cycles. Additionally, the post-preparative stability of the analytes in processed samples was assessed at room temperature during 12 h.

IV.2.7. Method application

To assess the applicability of the proposed method a preliminary pharmacokinetic study was conducted specifically designed to this end, which involved the concomitant administration of the AEDs (LEV, ZNS and LTG) followed by the administration of a convulsant agent (PTZ or PIL). More precisely, four Wistar rats received a single dose of a mixture of LEV (40 mg/kg), ZNS (10 mg/kg) and LTG (2.5 mg/kg) by i.p. injection (10 mL/kg of body weight). After the administration of the AEDs, specifically 30 min later, two rats were treated with PTZ (25 mg/kg, i.p.) and the other two rats with PIL (10 mg/kg, i.p.). To determine the concentration-time profiles for the AEDs (LEV, ZNS and LTG) and convulsant agents (PTZ and PIL), multiple serial blood samples (~0.3 mL) were collected into EDTA tubes, at several pre-defined post-dose time-points, through a cannula introduced into the lateral tail vein of each rat and by decapitation at the end of the experiment. Having as reference the time of the AEDs administration, blood samples were harvested before (pre-dose sample considered as 0 min) and at 10, 20, 30, 40, 50, 60, 75, 90, 120, 180, 240, 360, 480 and 720 min after administration; the blood samples taken from 40 min, inclusive, were used for the simultaneous analysis of AEDs and convulsant (PTZ or PIL). In each of the two groups of rats, the brain was collected at the end of the study

for one of the rats whereas for the other rat the brain was harvested earlier (at 120 min post-AEDs administration which corresponds to 90 min after the administration of PTZ or PIL).

IV.3. Results and discussion

IV.3.1. Method development and optimisation

Several preliminary tests were carried out to optimise the analytical method for the quantitative analysis of the selected AEDs and convulsant agents in rat plasma and brain samples.

Firstly, the chromatographic conditions were optimised aiming at providing a symmetric peak shape and good resolution of all the analytes and the IS, within the shortest run time. Thus, different conditions were tested to find a suitable mobile phase and the most appropriate wavelength values in order to achieve a reasonable relationship between selectivity and sensitivity. Initially different percentages of a mixture of acetonitrile and water were tested at a flow rate of 1 mL/min. The use of very low percentages of acetonitrile increased the run time, whereas higher percentages did not allow a good separation of the peaks of different analytes. Due to the very polar nature of LEV, it was found that a reasonable retention of this analyte was only achieved with a low percentage of organic modifier in the mobile phase (less than 10%). The influence of the pH of the mobile phase on resolution and retention times was also evaluated. Considerable differences were observed in the pH range of 3.5-7.0 for PIL that demonstrate to have a pH-dependent chromatographic behavior. Thus, the aqueous portion of the mobile phase adjusted to pH 6.4 was selected due to the more favourable retention times for all the analytes enabling its chromatographic separation. Furthermore, triethylamine (an amine additive) was added to the aqueous mobile phase allowing a decrease of the asymmetry and peak tailing phenomenon **(McCalley, 2010)**. The optimised mobile phase consisted of acetonitrile (7.5%) and a mixture (92.5%) of water–triethylamine (99.5:0.05, v/v), pH 6.4, adjusted with 85% *ortho*-phosphoric acid, which was pumped isocratically at a flow rate of 1 mL/min. The effect of column temperature was also tested, being the temperature of 30 °C selected. A lower temperature allowed a better resolution between the peaks, but it increased significantly the run time, making the technique less practical.

Moreover, for the accurate and precise quantification of the analytes, it is important the use of a suitable IS. The compound 7-hydroxycoumarin was selected as IS due to its favourable performance in the optimised chromatographic conditions in comparison with other tested compounds, namely levamisole, chloramphenicol, phenobarbital, theophylline, among others.

For the detection of the compounds, some wavelengths in the range of 200 nm to 300 nm were evaluated but the best compromise in terms of sensitivity and selectivity was achieved at 205 nm for LEV, PTZ, IS and LTG, at 215 nm for PIL and at 240 nm for ZNS.

Under the optimised chromatographic conditions, the order of elution of the compounds was LEV, PTZ, PIL, ZNS, IS and LTG, and the peaks showed a symmetric shape and were well separated in a running time shorter than 19 min.

In regard to the sample preparation, it has been recognised that it is a crucial and indispensable step that characteristically takes the majority of the total analysis time and affects the quality of all posterior bioanalytical steps **(Nováková and Vlcková, 2009)**. Thus, during the optimisation of the sample preparation, procedures as simple and economical as possible were taken into account. Protein precipitation with organic solvents (acetonitrile and methanol) and acids (perchloric or trichloroacetic acid) were tested but they did not provide enough selectivity. Then, in order to achieve an acceptable recovery of the analytes and an appropriate sample clean-up a single step of liquid-liquid extraction with different solvents (e.g. dichloromethane and ethyl acetate) was tested. Dichloromethane enabled a better selectivity than ethyl acetate and showed in general better recoveries for the majority of the compounds. Thus, dichloromethane was selected as the extraction solvent in both matrices (plasma and brain tissue homogenate).

IV.3.2. Method validation

IV.3.2.1. Selectivity

The chromatograms of blank and spiked rat plasma and brain homogenate samples are shown in Figures IV.2 and IV.3, respectively. The analysis of blank plasma and brain samples from six rats confirmed the absence of endogenous interferences in the retention times of selected analytes (LEV, PTZ, PIL, ZNS and LTG) and the IS using the established analytical conditions. Considering the chromatographic behaviour of the anaesthetic drugs tested, it was found that xylazine elutes approximately to the retention time of LTG. Thus, in future studies involving the determination of LTG is desirable to avoid the use of this anaesthetic. In contrast, none of the other two anaesthetics tested (pentobarbital and ketamine) interferes with any of the compounds of interest.

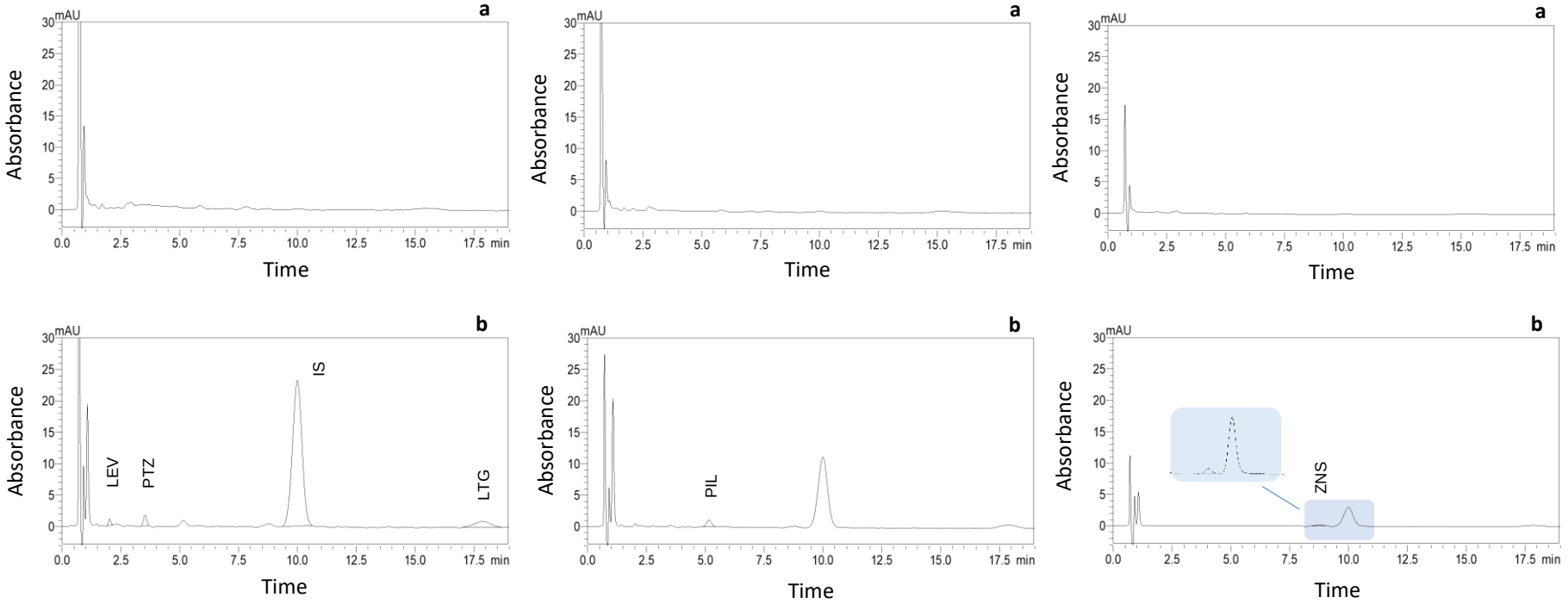

Figure IV.2. Typical chromatograms of extracted rat plasma samples obtained by the HPLC-DAD assay developed: rat blank plasma at 205 nm (a1), 215 nm (a2) and 240 nm (a3); and rat plasma spiked with internal standard (IS) and the target analytes at concentrations of the limit of quantification at 205 nm (b1), 215 nm (b2) and 240 nm (b3). LEV, levetiracetam; PTZ, pentylenetetrazole; PIL, pilocarpine; IS, internal standard; ZNS, zonisamide; LTG, lamotrigine.

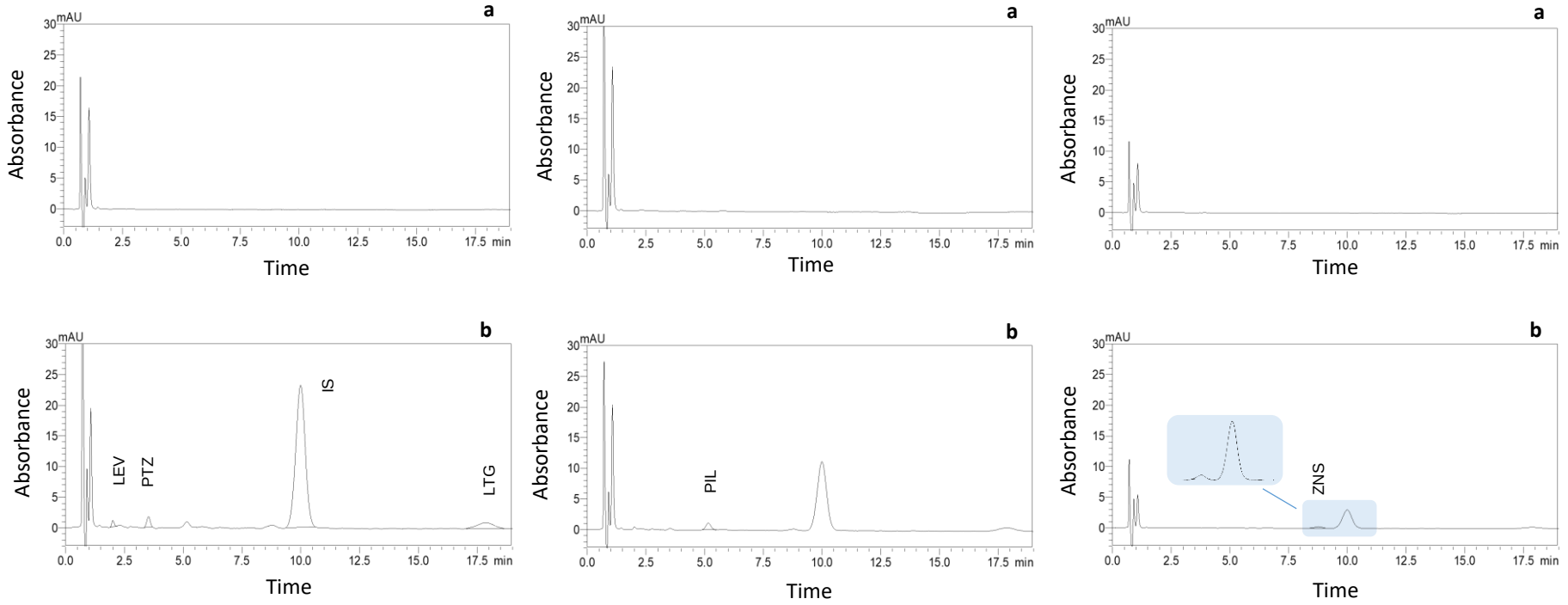

Figure IV.3. Typical chromatograms of extracted rat brain homogenate samples obtained by the HPLC-DAD assay developed: rat blank brain homogenate at 205 nm (a1), 215 nm (a2) and 240 nm (a3); and rat brain homogenate spiked with internal standard (IS) and the target analytes at concentrations of the limit of quantification at 205 nm (b1), 215 nm (b2) and 240 nm (b3). LEV, levetiracetam; PTZ, pentylenetetrazole; PIL, pilocarpine; IS, internal standard; ZNS, zonisamide; LTG, lamotrigine.

IV.3.2.2. Calibration curves and LOQs

The calibration curves obtained in rat plasma and brain homogenate for all the analytes were shown to be linear ($r^2 \geq 0.991$) in the concentration ranges previously defined and showed a consistent correlation between analyte-IS peak area ratios and corresponding nominal concentrations. A weighted linear regression analysis was used due to the wide calibration ranges, enabling to compensate for the presence of heteroscedasticity. Taking into account the weighted linear regression analysis performed, the best-fit weighting factor for the analytes was shown to be $1/x^2$. Table IV.1 summarises the regression equations of the calibration curves and the corresponding determination coefficients (r^2) achieved for each compound. The LOQs were experimentally defined as 0.4 µg/mL for LEV, 2 µg/mL for PTZ, 0.75 µg/mL for PIL, 0.15 µg/mL for ZNS and 0.3 µg/mL for LTG in rat plasma and brain matrices.

Table IV.1. Mean calibration parameters (n = 3) of levetiracetam (LEV), pentylenetetrazole (PTZ), pilocarpine (PIL), zonisamide (ZNS) and lamotrigine (LTG) in rat plasma and brain matrices.

Analyte	Calibration parameters		
	Concentration range (µg/mL)	Equation[a]	r^2
Plasma			
LEV	0.4-100	y = 0.0278x + 0.0015	0.996
PTZ	2-100	y = 0.0105x - 0.0008	0.991
PIL	0.75-100	y = 0.0331x + 0.0072	0.994
ZNS	0.15-100	y = 0.0513x - 0.0031	0.995
LTG	0.3-50	y = 0.1839x - 0.0010	0.994
Brain			
LEV	0.4-100	y = 0.0265x - 0.0018	0.994
PTZ	2-100	y = 0.0093x - 0.0028	0.991
PIL	0.75-100	y = 0.0325x - 0.0006	0.993
ZNS	0.15-100	y = 0.0524x - 0.0003	0.994
LTG	0.3-50	y = 0.1882x + 0.0061	0.994

[a] y represents analyte-IS peak area ratio; x represents analyte concentration (µg/mL).

In addition, as shown in Table IV.2, the sensitivity achieved with the current method seems to be very good considering in particular the previously reported LOQs of HPLC-UV/DAD methods. In our method it was necessary to meet a compromise between sensitivity, selectivity and feasibility in order to be possible the separation and quantification of the five analytes using a simple and cost-effective process.

Table IV.2. Comparison of determinant bioanalytical aspects between the current method and previous methods used for the bioanalysis of levetiracetam (LEV), pentylenetetrazole (PTZ), pilocarpine (PIL), zonisamide (ZNS) and lamotrigine (LTG) from rat plasma/brain and related biological matrices.

Matrix	Analytical system	Analyte	Sample volume	Extraction procedure	LOQ	Reference
Plasma	**HPLC-DAD**	**LEV**	**100 µL**	**LLE**	**0.40 µg/mL**	**Current method**
Brain						
Serum	HPLC-UV	LEV	15 µL	LLE	-	Doheny 1999
Cerebrospinal fluid						
Serum	HPLC-UV	LEV	25 µL	LLE	-	Tong and Patsalos 2001
Microdialysate			10 µL			
Dried blood spot	HPLC-MS/MS	LEV	-	PP	0.067 µg/mL	Luo 2013
Plasma	**HPLC-DAD**	**PTZ**	**100 µL**	**LLE**	**2.0 µg/mL**	**Current method**
Brain						
Plasma	HPLC-UV	PTZ	200 µL	LLE	5.0 µg/mL	Soto Otero et al. 1987
Brain			1000 µL			
Serum	HPLC-UV	PTZ	50 µL	PP	50 µg/mL	Ramzan 1988
Cerebrospinal fluid					50 µg/mL	
Brain					50 µg/g	
Plasma	**HPLC-DAD**	**PIL**	**100 µL**	**LLE**	**0.75 µg/mL**	**Current method**
Brain						
Brain	HPLC-UV	PIL	500 µL	LLE	-	Epps et al. 2012
Plasma	HPLC-UV	PIL	50 µL	PP	0.2 µg/mL	Römermann et al. 2015
Brain			200 µL		1.1 µg/g	
Plasma	**HPLC-DAD**	**ZNS**	**100** µL	**LLE**	**0.15 µg/mL**	**Current method**
Brain						
Serum	HPLC-UV	ZNS	20 µL	SPE	-	Toyota 2001
Plasma	**HPLC-DAD**	**LTG**	**100** µL	**LLE**	**0.30 µg/mL**	**Current method**
Brain						

Brain	HPLC-UV	LTG	100 µL	LLE	-	Walton et al. 1996
Plasma	HPLC-UV	LTG	20 µL	SPE	-	Yamashita et al. 1997
Serum	HPLC-UV	LTG	50 µL	PP	-	Walker et al 2000
Brain	HPLC-UV	LTG	1000 µL	PP+LLE	0.1 µg/mL	Castel-Branco et al. 2001
Plasma	HPLC-MS	LTG	100 µL	PP	0.01 µg/mL	Yang et al. 2013
Plasma	HPLC-UV	LTG	100 µL	LLE	0.5 µg/mL	Liu et 2014
Brain				PP	0.25 µg/g	
Plasma	HPLC-DAD	LTG	100 µL	PP+MEPS	0.1 µg/mL	Ventura et al. 2016
Brain						

DAD, Diode array detection; HPLC, High performance liquid chromatography; LLE, Liquid-liquid extraction; LOQ, Limit of quantification; MEPS, Microextraction by Packed sorbent; MS, mass spectrometry; MS/MS, Tandem mass spectrometry; PP, Protein precipitation; SPE, Solid-phase extraction; UV, Ultraviolet.

IV.3.2.3. Precision and accuracy

In Table IV.3 are shown the data for intra and interday precision and accuracy obtained from QC plasma and brain samples at the four different concentration levels (QC_{LOQ}, QC_1, QC_2 and QC_3). In rat plasma, the intra and interday CV values did not exceed 14.88%, and the intra and interday *bias* values varied between -13.89 and 12.16%. Similarly, in rat brain, the intra and interday CV values did not exceed 14.73%, between -9.21 and 15.29% (this last value was found at the concentration of the LOQ – QC_{LOQ}). These data evidently demonstrate that the HPLC–DAD method herein described is reliable and accurate.

Table IV.3. Precision (% CV) and accuracy (% *bias*) for the determination of levetiracetam (LEV), pentylenetetrazole (PTZ), pilocarpine (PIL), zonisamide (ZNS) and lamotrigine (LTG) in rat plasma and brain samples at the concentrations of the limit of quantification (*) and at the low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges.

Analyte	Nominal concentration (μg/mL)	Interday (*n* = 3)		Intraday (*n* = 5)	
		Precision (% CV)	Accuracy (% *bias*)	Precision (% CV)	Accuracy (% *bias*)
Plasma					
LEV	0.4*	14.58	8.14	1.03	-10.93
	1.2	9.52	1.59	6.42	-3.40
	50	4.28	-7.52	8.63	0.11
	90	6.78	-1.53	3.42	1.73
PTZ	2*	11.60	-3.84	13.45	-0.15
	6	14.88	-13.23	13.74	-3.25
	50	3.99	-13.89	10.63	5.68
	90	11.29	4.29	2.18	12.16
PIL	0.75*	13.99	1.23	5.31	-12.51
	2.25	9.73	9.55	3.56	5.18
	50	6.31	-4.44	6.39	-0.23
	90	7.35	1.73	5.40	5.00
ZNS	0.15*	8.41	9.32	3.47	9.95
	0.45	4.85	-5.41	4.31	-9.89
	50	7.67	-0.80	4.59	3.00
	90	7.62	-0.69	4.86	2.52
LTG	0.3*	2.04	9.62	1.03	-10.93
	0.9	2.29	11.00	3.00	8.62
	25	6.15	3.31	4.88	3.54
	45	4.06	3.98	3.42	5.44
Brain					
LEV	0.4*	11.08	12.31	13.03	15.29
	1.2	8.96	-1.46	10.44	12.54
	50	7.74	-3.19	6.04	-2.15
	90	2.05	-2.50	8.47	1.93

PTZ	2*	14.66	-3.77	13.17	7.59
	6	8.03	-4.51	10.93	5.24
	50	12.12	-0.02	8.78	12.26
	90	10.19	0.65	12.79	10.44
PIL	0.75*	12.53	3.52	7.03	7.49
	2.25	2.24	8.55	4.15	8.08
	50	4.72	0.51	6.45	2.60
	90	5.83	6.51	3.17	9.39
ZNS	0.15*	6.72	2.51	10.31	7.62
	0.45	7.23	-6.63	10.03	-3.14
	50	4.89	1.33	6.52	-1.05
	90	5.42	0.73	3.48	1.27
LTG	0.3*	14.73	-9.21	10.69	4.66
	0.9	7.28	5.92	5.01	10.54
	25	5.53	5.93	6.70	3.57
	45	5.19	10.49	1.81	12.63

bias, deviation from nominal concentration value; CV, coefficient of variation.

IV.3.2.4. Recovery

The overall absolute recovery of the analytes from rat plasma and brain samples tested at three different concentration levels (QC_1, QC_2 and QC_3) was evaluated and the results are presented in Table IV.4. The absolute mean recovery values ranged from 52.97 to 93.91% in rat plasma with CV values equal to or lower than 14.87% and ranged from 48.57 to 89.40% in brain homogenate with maximal CV values of 14.89%. LEV showed the lower recovery values probably due to the higher hydrophilicity of the drug, but the extent of recovery of this analyte is consistent, precise, and reproducible. Similarly to the yields achieved for the analytes, the average of absolute recovery of the IS in rat plasma and brain homogenate samples was 75.31% with a CV value of 7.00%.

Table IV.4. Absolute recovery of levetiracetam (LEV), pentylenetetrazole (PTZ), pilocarpine (PIL), zonisamide (ZNS) and lamotrigine (LTG) from rat plasma and brain matrices (*n* = 5).

Analyte	Nominal concentration (μg/mL)	Absolute recovery (%)[a]	CV (%)
Plasma			
LEV	1.2	53.95 ± 2.27	4.20
	50	57.21 ± 5.58	9.76
	90	55.59 ± 2.09	3.76
PTZ	6	52.97 ± 7.88	14.87
	50	69.35 ± 10.29	14.84
	90	72.28 ± 2.72	3.76
PIL	2.25	86.95 ± 8.75	10.06
	50	93.91 ± 5.80	6.18
	90	91.48 ± 7.35	8.04
ZNS	0.45	77.31 ± 9.90	12.81
	50	90.14 ± 4.31	4.78
	90	87.24 ± 3.99	4.57
LTG	0.9	67.10 ± 9.06	13.50
	25	85.08 ± 5.40	6.35
	45	81.11 ± 5.62	6.92
Brain			
LEV	1.2	48.57 ± 1.65	3.41
	50	52.02 ± 3.17	6.09
	90	52.19 ± 5.05	9.68
PTZ	6	60.20 ± 13.83	13.83
	50	64.35 ± 5.83	5.83
	90	71.36 ± 13.85	13.85
PIL	2.25	85.61 ± 5.52	6.45
	50	89.40 ± 4.52	5.05
	90	86.43 ± 3.40	3.93
ZNS	0.45	84.06 ± 9.10	10.82
	50	84.67 ± 4.25	5.02
	90	78.24 ± 11.25	14.38
LTG	0.9	77.63 ± 3.61	4.65
	25	80.99 ± 6.25	7.72
	45	76.79 ± 11.44	14.89

[a] Mean ± standard deviation, *n* = 5.

IV.3.2.5. Stability

The stability of the compounds in rat plasma and brain samples achieved under the studied conditions, which simulated the handling and storage of samples likely to be found in the course of the analytical process are shown in Table IV.5. According to these results, the analytes of interest (LEV, PTZ, PIL, ZNS and LTG) were found to be stable in unprocessed and processed samples of rat plasma and brain matrices under the various conditions tested.

Table IV.5. Stability (values in percentage) of levetiracetam (LEV), pentylenetetrazole (PTZ), pilocarpine (PIL), zonisamide (ZNS) and lamotrigine (LTG) in rat plasma and brain samples under different handling and storage conditions (n = 3).

Analyte	Nominal concentration (μg/mL)	Unprocessed sample				Processed sample
		Room temperature (4 h)	4 °C (24 h)	Freeze-thaw cycles (3 cycles; −20 °C)	−20 °C (15 days)	Room temperature (12 h)
Plasma						
LEV	1.2	99.34	93.55	92.71	94.99	114.65
	90	101.84	98.62	96.19	98.86	102.08
PTZ	6	109.97	95.44	102.24	97.65	107.88
	90	99.84	94.85	91.58	99.34	102.20
PIL	2.25	104.39	88.00	86.60	96.59	107.20
	90	99.75	93.43	93.11	96.67	100.92
ZNS	0.45	112.71	90.86	93.04	98.60	112.17
	90	100.93	94.02	93.38	98.48	102.87
LTG	0.9	103.68	90.56	87.88	97.26	99.13
	45	99.49	94.53	94.31	100.81	100.32
Brain						
LEV	1.2	90.14	90.68	108.62	94.82	98.45
	90	104.31	105.30	88.59	100.51	97.28
PTZ	6	114.28	109.78	114.49	109.00	94.48
	90	103.82	99.13	93.94	106.56	96.97
PIL	2.25	100.08	100.83	90.54	98.34	95.68
	90	101.94	97.73	85.51	92.49	96.13
ZNS	0.45	110.75	113.85	99.47	104.22	107.73
	90	101.49	98.46	85.97	94.24	97.05
LTG	0.9	102.18	104.09	92.44	99.58	94.80
	45	102.83	100.55	87.33	97.05	99.19

IV.3.3. Method application

The validated method was applied to the analysis of the target compounds in plasma and brain homogenate samples obtained from Wistar rats that received a single i.p. dose of a combination of AEDs (LEV, ZNS and LTG) and that 30 min later were treated with one convulsant agent (PTZ or PIL). Representative chromatograms of the analysis of real samples of rat plasma and brain are shown in Figure IV.4. During the different analyses no interferences were observed in any of the samples analysed, which reinforces the selectivity of the method.

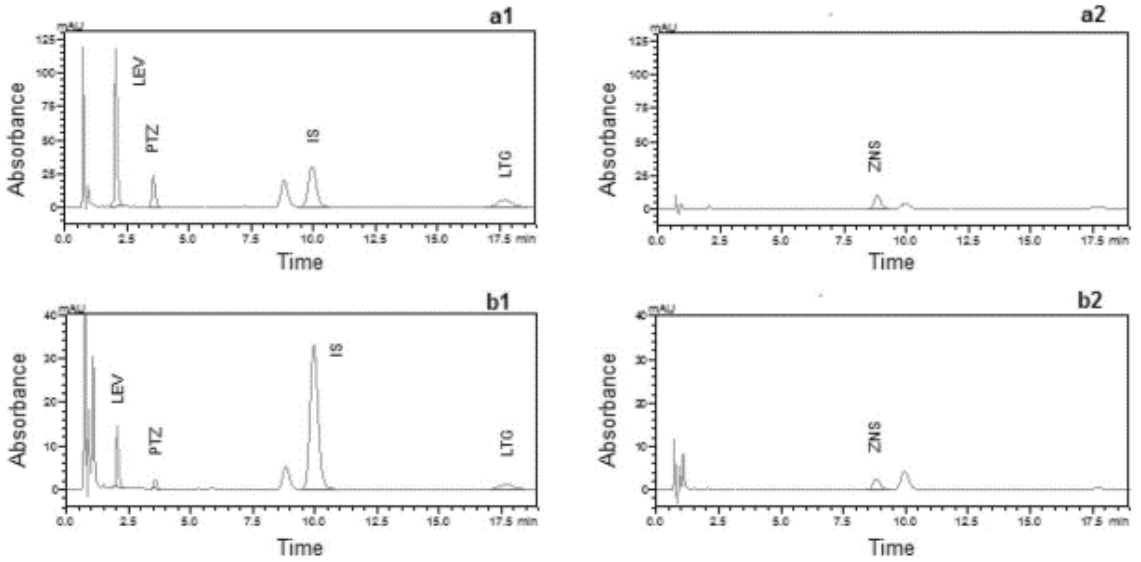

Figure IV.4. Representative chromatograms resulting from the analysis of real plasma and brain samples after the administration to rats of antiepileptic drugs [levetiracetam (LEV), zonisamide (ZNS), lamotrigine (LTG)] and the convulsant agent pentylenetetrazole (PTZ): in plasma at 205 nm (a1) and 240 nm (a2); and in brain homogenate at 205 nm (b1) and 240 nm (b2). LEV, levetiracetam; PTZ, pentylenetetrazole; IS, internal standard; ZNS, zonisamide; LTG, lamotrigine.

The plasma concentration–time profiles obtained for the target compounds are depicted in Figure IV.5, and whenever possible, are also represented the concentrations measured in the brain samples collected at 120 and 720 min after AEDs administration and at 90 and 680 min after treatment with the convulsant agents. The PIL was the only compound that was not quantified in rat brain tissue at both time-points (90 and 680 min), being the PIL levels in brain below the LOQ (BLQ) of the method in both circumstances. However, it is worthy to mention that the BLQ levels of PIL found in the brain tissue do not compromise the application of the method, since the dose of PIL usually used is higher (360–400 mg/kg) **(Curia et al., 2008)** than that considered by us in this study (only a dose of 10 mg/kg was used in order to reduce the animal suffering in this preliminary study).

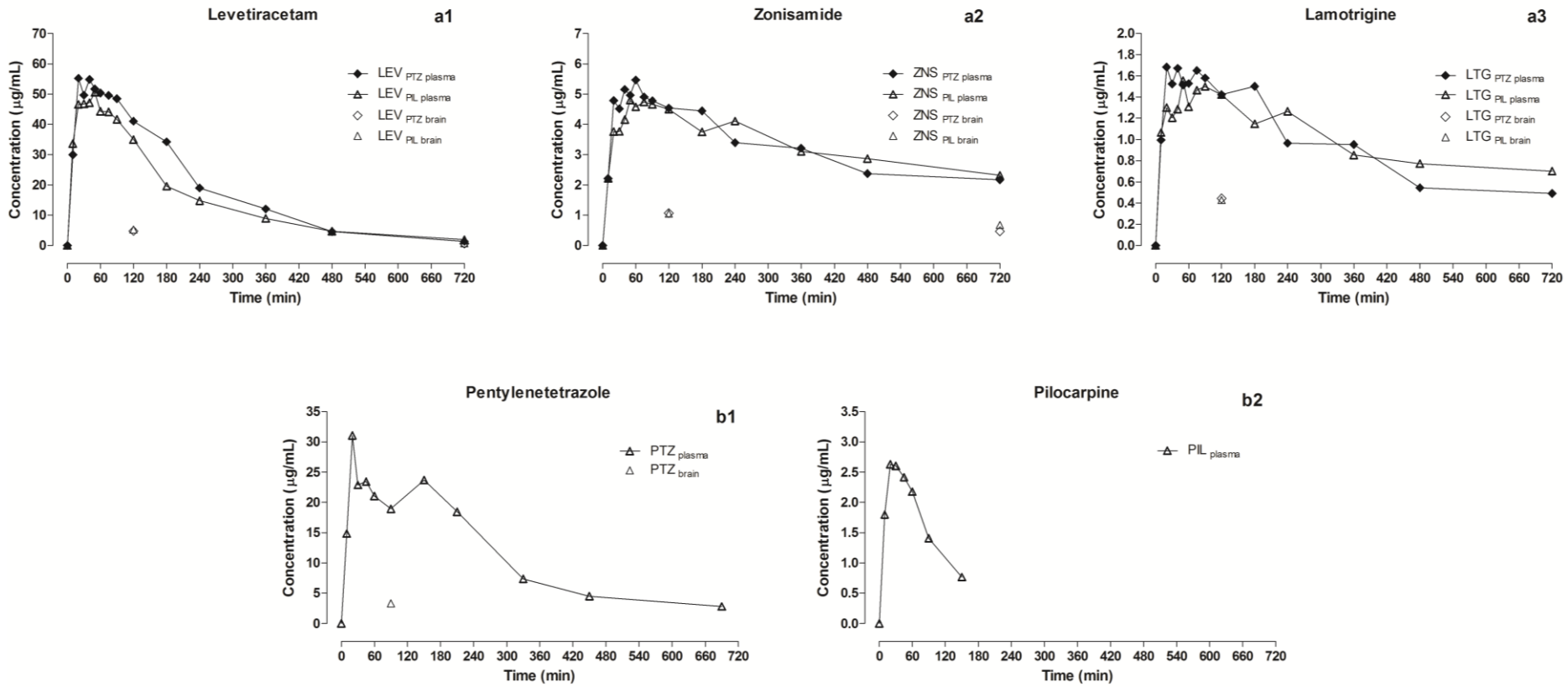

Figure IV.5. Representative plasma concentration-time profiles of the target antiepileptic drugs (AEDs) [levetiracetam (LEV; a1), zonisamide (ZNS; a2), lamotrigine (LTG; a3)], and convulsant agents [pentylenetetrazole (PTZ; b1) and pilocarpine (PIL; b2)]. Whenever possible, the concentration of the AEDs (LEV, ZNS and LTG) and convulsant agents (PTZ or PIL) quantified in brain homogenate samples at two post-dose time points were also indicated.

Hence, the simple chromatographic and extraction conditions established will allow the easy implementation of this assay in any laboratory to conduct nonclinical pharmacokinetic-based studies that involve these AEDs and convulsant agents. In addition, this bioanalytical method may also be a useful tool to support future studies aimed at understanding the seizures activity and the molecular basis of epileptogenesis in rodent models. Indeed, the simultaneous determination of systemic and brain concentrations of AEDs and convulsant agents and the recording of epileptogenic/seizure activity in whole-animal models of disease is certainly essential to establish robust pharmacodynamic-pharmacokinetic relationships.

CHAPTER V – General discussion

Considering that each of the chapters presented in this thesis involving experimental work already have its own discussion about the specific content that each one contains, this section was prepared with the purpose of discussing in a more integrated and broader way the different topics dealt within the previous chapters. Thus, in this section, it will be provided a critical overview of the key issues covering the overall research performed in order to reach the main goals proposed in the work plan leading to this thesis.

The development of bioanalytical tools for the determination of important endogenous neuromediators involved in epileptogenesis and ictogenesis, as well as AEDs and chemoconvulsants of interest in the development of whole-animal models of seizures and epilepsy, will be certainly useful to support many nonclinical research studies that may be carried out in the field of neurosciences. Actually, it has been hypothesized that a better understanding of the biochemical processes underlying epileptic disorders could enable the identification of novel and promising therapeutic pathways and the discovery of new drug candidates that could change the natural course of epileptogenesis and/or be more effective in the management of refractory epilepsy.

This project started with the development and validation of two distinct but complementary methodologies suitable for the quantification of two groups of endogenous compounds in brain tissue and their performance was demonstrated by the determination of the target analytes in different rat brain regions. One method allowed the quantification of catecholamines and other endogenous related compounds, such as L-DOPA, DA, NE, E, 3-*O*-MD and HVA (Chapter II), while the other method enabled the determination of important neuroactive amino acids as Asp, Glu, Gln, Tau and GABA (Chapter III). These two methods will be certainly key bioanalytical tools to support further *in vivo* nonclinical studies directed to investigate the biochemical and molecular phenomena that lead to neuronal excitability disorders, such as epilepsy.

Undoubtedly, the quality of the data provided by analytical methods used for the quantification of compounds in biological samples plays a critical and decisive role in the evaluation and interpretation of results obtained from several studies. Therefore, a lot of practical aspects are important to be considered during the iterative process of method development and validation in order to obtain reliable data **(Summerfield, 2016)**. Indeed, in the process of development and validation of a bioanalytical method several decisions have to be made according to the purposes for which the method is intended; thus, early decisions have to be taken regarding the biological matrix to be used, the procedure for sample collection and processing until the analysis, the chromatographic separation of the target analyte(s), the detection system, and data processing **(Bressolle et al., 1996; Shah et al., 2000)**.

Among the analytical systems most widely employed in the quantitative assessment of endogenous compounds, LC remains as the first choice since it allows the separation and quantification of numerous compounds present in complex samples, and it also enables the determination of analytes present in samples in very small levels.

Regarding the detection system, some authors **(Zhu et al., 2011**; **He et al., 2013**; **Huang et al., 2014**; **Kim et al., 2014**; **Wei et al., 2014**; **Bergh et al., 2016)** have employed MS or MS/MS detectors to simultaneously determine the target neuromediators of this work (catecholamines and neuroactive amino acids) in brain samples; however, it must be borne in mind that this kind of expensive instrumentation may not be available for routine use in many research laboratories. Thus, the development of these two simple and reliable HPLC methods coupled to FLD was delineated as a reasonable strategy to overcome existing constraints related to the availability of more expensive analytical systems. Structurally, as catecholamines are monoamines that comprehend a catechol group linked to an amine group by a two-carbon chain, these structural properties make these compounds naturally fluorescent and easily oxidizable **(Cai et al., 2010)**. On the contrary, most amino acids are low molecular weight aliphatic molecules, devoid of fluorescence and strong absorbance in the UV/visible region **(Kang et al., 2006)**. Consequently, for the determination of amino acids a derivatization step with OPA (a widely used derivatizing agent) was considered, whereas for the determination of catecholamines in brain tissue their native fluorescence was shown to be enough for FLD.

Another key point to study in the development of any bioanalytical assay is to ensure the stability of the analytes of interest in the biological matrices during the sample storage period and throughout all steps of the analytical procedure. Similarly to catecholamines, most neuroactive amino acids and their precursors and/or metabolites are polar compounds **(He et al., 2013)** and numerous approaches have been described for the pretreatment of brain tissue samples before analysis. Having in mind, particularly the thermal and photochemical instability of catecholamines and related endogenous compounds, the sample handling was performed in the dark at low temperature, low pH values, and to improve sample integrity the addition of antioxidants such as L-cysteine was made. The procedure for rat brain tissue homogenization involved the addition of ice-cold 0.2 M perchloric acid containing 3 mM L-cysteine in a proportion of 4 mL per g of tissue, sonication for 5 min and centrifugation at 17000 rpm for 4 min at 4 ºC. At this point, it should be noted that this basic sample processing procedure is common for the determination of the selected neuromediators (catecholamines and neuroactive amino acids), thus enabling the quantitative analysis of catecholamines and neuroactive amino acids from a single sample of rat brain tissue and this specific aspect seems to be of value for routine nonclinical research in neurosciences. In fact, although the brain homogenate is taken as the common starting point for the analysis of catecholamines and neuroactive amino acids, due to the very high levels of amino acids found in brain, the resulting brain tissue homogenate supernatant was diluted (1/50) with ultra-pure water before the derivatization step in order to obtain an appropriate chromatographic response.

Another crucial part of the method development is the choice an appropriate approach to make calibration and QC samples, and some approaches for the analysis of endogenous compounds have been reported. In these two different HPLC-FLD methods developed different strategies were applied in the evaluation of the validation parameters. For the determination of catecholamines and related compounds in samples of brain tissue homogenate supernatant, the calibration curves were made in 0.2 M perchloric acid containing 3 mM L-cysteine. In contrast, for the determination of amino acids the study of all validation parameters was performed using the biological matrix itself (in this case a smaller sample volume is required for analysis).
Knowing that the brain is somehow compartmentalized, complex behaviors and functions are mediated by different functional areas **(Karceski, 2007)**. Hence, in many nonclinical studies it is convenient to dissect the brain in order to analyze individually different brain regions of interest. Therefore, the applicability of the HPLC-FLD assays developed for the quantification of the selected endogenous neuromediators was checked in different rat brain regions, such as cortex frontal, amygdala, hippocampus, cerebellum and striatum. Actually, the analysis of the neurochemical distribution pattern of endogenous compounds, as catecholamines and neuroactive amino acids, can illustrate specific correlations between brain regions and behavioral and neurological responses, which may be important when using experimental models.
Furthermore, these HPLC-FLD techniques demonstrated several common improvements over the assays previously published for the quantification of catecholamines and endogenous related compounds and neuroactive amino acids in brain samples. Our bioanalytical methods involve easy and fast sample preparation procedures, convenient analysis times (less than 12 min), satisfactory sensitivity and the use of an appropriate internal standard. Also, these bioanalytical assays may support research in epilepsy as well as in several other neurological diseases, making these methods valuable bioanalytical tools.

Within the scope of this doctoral thesis was also developed an HPLC-DAD method for determination of some AEDs (i.e. LEV, ZNS and LTG) and widely used chemoconvulsant agents (i.e. PTZ and PIL) in rat plasma and brain samples (Chapter IV). This HPLC-DAD method employs isocratic elution of a mobile phase essentially composed of water and yet still allowed to reach the chromatographic separation of all five compounds in 19 min. Moreover, the use LLE resulted in a fast and reproducible sample preparation procedure, affording satisfactory values of recovery. This bioanalytical method, which uses simple instrumentation and uncomplicated chromatographic conditions, allows a reliable and cost-effective analysis of possible samples derived from pharmacokinetics or pharmacokinetics/pharmacodynamics-based experiments. An important advantage of this method is the fact that only a small sample volume (100 µL) is needed for each analysis, being this aspect essential when a serial blood sampling is required during pharmacokinetic studies performed in small laboratory animals (usually rodents). Therefore, this bioanalytical assay allows the simultaneous determination of the target AEDs and chemoconvulsants, being potentially useful for a better understanding of the effects of

these compounds. Moreover, it may also be useful to provide a more extensive and comprehensive characterization at nonclinical level of the pharmacokinetics of these established AEDs (i.e. LEV, ZNS and LTG) and chemoconvulsants (i.e PTZ and PIL), as well as to support the development of new therapeutic strategies for the management of epilepsy.

CHAPTER VI – Conclusion & future perspectives

The experimental work presented in this thesis consisted in the development and full validation of a set of bioanalytical methods for the quantification of several endogenous compounds of interest (selected neuromediators) and exogenous compounds (selected AEDs and chemoconvulsant agents).

Hence, in summary, the most relevant achievements and conclusions obtained throughout the work underlying the present thesis will be succinctly provided below:

- A simple HPLC-FLD method to simultaneously quantify six different catecholamines or endogenous related compounds (DA, NE, E, HVA, 3-*O*-MD, L-DOPA) using their native fluorescence in rat brain tissue was developed and fully validated. The analytical procedure is easy and fast once it does not require derivatization or complex pre-treatment steps. Moreover, this assay demonstrated suitability to support many (non)clinical studies in the broad field of neurosciences that benefit from the quantitative analysis of these bioamines and their precursors or derivatives.
- A reliable and sensitive HPLC-FLD method using a precolumn derivatization step was developed and fully validated to simultaneously quantify five neuroactive amino acids (Glu, Asp, Tau, Gln and GABA) in rat brain tissue. Although this assay involves a derivatization step of the target analytes with OPA, the derivatization occurs rapidly. Moreover, the technique has the benefit of measuring simultaneously neuroactive amino acids that are interconverted *in vivo* and have distinctive neurochemical activities in diverse regions of brain. Thus, this methodology is valuable for evaluating the role of the target neuroactive amino acids in the pathophysiology and treatment of neurological disorders, including epilepsy.
- A new HPLC-DAD method using LLE was developed and fully validated to simultaneously quantify of three antiepileptic drugs (LEV, ZNS, LTG) and two chemoconvulsant agents (PTZ and PIL) in rat matrices (plasma and brain tissue homogenates). Its major advantages include the simple instrumentation and uncomplicated chromatographic conditions employed. Moreover, the small sample volume required is also an important advantage as it allows the collection of multiple blood samples from a single rat, reducing the number of animals used in pharmacokinetic studies.

In conclusion, the findings reported along this thesis showed the relevance of bioanalysis in different experimental contexts particularly related to epilepsy. In addition, it is certainly useful an integrated application of these bioanalytical techniques to afford novel and more

robust nonclinical data on the neurochemical mechanisms underlying epileptogenesis and ictogenesis phenomena. Thus, the work described in this thesis represents a small but eventually important contribution to help unravel part of the puzzle of the complex biochemical phenomena involved in epilepsy.

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APPENDICES

Appendix I

Appendix I - Chromatographic techniques for quantitative determination of neurotransmitters in brain samples.

ANALYTES	INTERNAL STANDARD	ANALYTICAL METHOD	STATIONARY PHASE	MOBILE PHASE	FLOW RATE (mL/min) AND ELUTION	RUN TIME (MINUTES)	SAMPLE	TISSUE PREPARATION	DERIVATIZATION	RECOVERY (%)	LOD	CALIBRATION RANGE	REFERENCE
NE, DA DOPAC, 5-HT, 5-HIAA	NR	HPLC-ECD	Hypersil C_{18} (150 x 4.6 mm, 5 µm)	Sodium dihydrogen phosphate (60 mM), disodium EDTA (0.1 mM), OSA (2 mM), methanol (7%), pH 3.9	1.2 mL/min Isocratic	30	Brain	Homogenized in 0.05 mL of 0.1 N perchloric acid, and centrifuged.	No	NR	0.1-0.8 ng/mL	NR	(Fitoussi et al., 2013)
NE, DA, DOPAC, HVA, 3-MT, 5-HT, 5-HIAA	DHBA	UHPLC-ECD	Kinetex C_{18} (100 × 4.6 mm, 2.6 µm)	Potassium phosphate (70 mM), EDTA (0.1 mM), OSA (1.1 mM), TEA (3.1 mM), methanol (14%), pH 3.1	1.6 mL/min Isocratic	8	Brain	Homogenized in 0.1 M perchloric acid containing 1.34 mM EDTA and 0.05% sodium bisulfite and centrifuged.	No	NR	0.1-1.8 ng/mL	0.2-19 ng/mL	(Parrot et al., 2011)
L-DOPA, DA, NE, 5-HT	NR	HPLC-ECD	Waters xBridge C_{18} (100 × 4.6 mm, 3.5 µm)	Potassium dihydrogen phosphate (0.1 mM), EDTA (0.1 mM) and OSA (4.5%), pH 2.6	0.8 mL/min Isocratic	NR	Brain	Homogenized in buffer - 0.1% sodium metabisulphite, 0.01% EDTA-sodium, 0.1% cysteine and 3.5% perchloric acid and centrifuged.	NR	NR	NR	NR	Ledondo-Castro et al., 2014)

5-HT, DA, NE, E, DOPAC, HVA, 5-HIAA, MHHG	DHBA, 5-HMT	HPLC-ECD	ALF-125 (250 × 1 mm, 3 µm)	Potassium chloride (8 mM), phosphoric acid (50 mM), citric acid (50 mM), disodium EDTA (0.1 mM), OSA (1.8 mM)	0.04 mL/min Isocratic	40	Human brain (Cerebellar cortex)	Homogenized in 4 mL phosphoric-citric buffer and centrifuged. The supernatant was filtered.	No	87-100%	0.0001-0.0004 ng/mL	0.04-58.4 ng/mL	(Dam et al., 2014)
GABA, Glu, Gln	NR	HPLC-ECD	Waters Spherisorb (250 × 4.6 mm, 5 µm)	Methanol (23%), sodium dihydrogen phosphate (100 mM), EDTA (0.5 mM), pH 5.5	0.3 mL/min Isocratic	35	Brain (Hippocampus)	Homogenized in water and centrifuged. Supernatants were filtered and diluted in 990 µL of deionized water.	OPA/sodium sulfite	NR	3200 ng/mL	0.00005-0.150 ng/mL	(Monge-Acuña and Fornaguera-Trías, 2009)
DA, NE, 5-HT, DOPAC, HVA, 5-HIAA	DHBA	HPLC-ECD	HR-80 (RP-C_{18}) (80 × 4.6 mm, 5 µm)	Lithium dihydrogen phosphate (100 mM) OSA (1.5 mM), methanol (10%)	NR Isocratic	18	Brain (Striatum)	Homogenized in perchloric acid 0.1 M and centrifuged.	No	96–105%	NR	20-400 ng/mL	(Yang and Beal, 2011)
ADO, ATP, ADP, AMP	No	HPLC-ECD	Thermo Fischer (250 × 3.2 mm, 5 µm)	Sodium perchlorate (19.0 mM), acetonitrile (4.0%), pH 2.7	0.5 mL/min Isocratic	7	Brain (Striatum, cortex, hippocampus, olfactory bulb, *substantia nigra*, cerebellum)	Homogenized in sodium perchlorate (19.0 mM), acetonitrile (4.0%), pH 2.75 (75%) with 0.2 N perchloric acid (25%) and centrifuged. The supernatant was filtered.	No	90%	NR	NR	(Pani et al., 2014)
DA, DOPAC, HVA, NE, 5-HIAA	DHBA	HPLC-ECD	Thermo Fischer (150 × 3.2 mm, 3 µm)	Sodium acetate (100 mM), citric acid (20 mM), sodium octyl sulfate (0.38mM), EDTA (0.15 mM), acetonitrile, pH 3.3	0.6 mL/min Isocratic	9	Brain (frontal cortex, striatum, nucleus accumbens, hippocampus, *substantia nigra* pars compacta, and ventral tegmental area)	Homogenized in methanol and centrifuged. Homogenate and water was vortexed, centrifuged and evaporated.	No	≥ 90%	0.13 ng/mL	0.30-30 ng/mL	(Allen et al., 2017)

HA, Ns-MHA	N-MHA	HPLC-ECD, HPLC-FLD	TSK-gel ODS 80Ts (150 × 4.6 mm, 1.7 µm)	Methanol (30%), potassium phosphate buffer (50 mM), disodium EDTA (0.5 mM), pH 6.5	0.5 mL/min Isocratic	25	Brain (Hypothalamus, cortex)	Homogenized with 3% perchloric acid containing 0.5 mM EDTA.	OPA	97-95%	0.13 pmol	0.13-5 pmol	(Maldonado and Maeyama, 2013)
L-DOPA, DA, NE, E, 3-*O*-MD, HVA	DHBA	HPLC-FLD	C_{18} (150 × 4.6 mm, 5 µm)	A) Sodium phosphate monobasic (75 mM) with OSA (1.5 mM) in water (pH 3.5) B) Sodium phosphate monobasic (75 mM) with (1.5 Mm) OSA in water (pH 5.0) C) methanol	1 mL/min Gradient	12	Brain (cerebellum, amygdala, cortex, hippocampus, striatum, mesencephalon, medulla oblongata, *substantia nigra* and ventral tegmental area)	Homogenized in ice-cold 0.2 M perchloric acid containing 3 mM L-cysteine (4 mL per g of tissue), sonicated and centrifuged.	No	96- 114%,	2-25 ng/mL	2-1000 ng/mL	(Fonseca et al., 2017)
Asp, Glu, Gln, Tau, GABA	Methyl-L-arginine	HPLC-FLD	C_{18} (55 × 4 mm, 3 µm)	Acetate buffer (25 mM pH 5.4) and acetonitrile	1 mL/min Gradient	11	Brain (frontal cortex, amygdala, hippocampus, cerebellum and striatum)	Homogenized in ice-cold 0.2 M perchloric acid containing 3 mM L-cysteine (4 mL per g of tissue), sonicated and centrifuged.	OPA/MPE	No	25-50 ng/mL	25-2500 ng/mL	(Fonseca et al., 2018)
Glu, GABA	No	HPLC-FLD	C_{18} (100 × 2.1 mm, 3.5 µm)	Sodium acetate buffer (100 mm, pH 6.0) and methanol	0.3 mL/min Isocratic	3.5	Brain (cerebellum, brain stem and basal nucleus)	Homogenized in 4 equivalents of water, and again after the addition of 4 M perchloric acid, centrifuged.	OPA	101%	100 ng/mL	100-10000 ng/mL	(Forgacsova et al., 2018)
NE, E, DA	N-methyl DA	HPLC-FLD	CAPCELL PAK SCX UG80 (150 × 2 mm, 5 µm)	Potassium acetate (65 mM)/potassium phosphate (75 mM) (95:5), pH 3.5	0.2 mL/min Isocratic	30	Brain	Homogenized in 0.1 M perchloric acid and centrifuged.	Ethylenediamine imidazole in acetonitrile–ethanol–water (80:10:10, v/v/v)	95-102%	0.04-0.6 pmol	0.2-200 pmol	(Tsunoda and Funatsu, 2012)
Glu, Gly, Tau, GABA	2-aminoadipic acid	HPLC-FLD	Synergi Hydro-RP (150 × 2 mm, 4 µm)	A) tetrahydrofuran with ammonium acetate buffer (50 mM) (95:5, v/v) B) methanol	0.35 mL/min Gradient	20	Brain	Homogenized in 500 µL of 0.1 M perchloric acid–methanol, vortexed and then centrifuged.	CBQCA/DMSO	97-104.5%	0.03-0.06 µM	0.50-50 µM	(Şanlı et al., 2015)

DOPAC, NE, DA, 5-HT	No	HPLC-FLD	Microsorb-MV100-5 C_{18} (250 × 4.6 mm, 5 µm)	Acetic acid (12 mM), disodium EDTA (0.26 mM) pH 3.5, methanol (14%)	1 mL/min Isocratic	16	Brain (Striatum)	Homogenized in 0.2 mL of 0.2 M perchloric acid containing 3 mM cysteine and centrifuged.	No	97-102%	NR	0.06-250 ng/mL	(De Benedetto et al., 2014)
Asp, Glu, Ser, Gln, HD, Gly, Thr, Arg, Tau, Ala, Tyr, GABA, Trp, Met, Val, Phe, Isoleu, Leu	No	HPLC-FLD	Ultrasphere ODS (150 × 4.6 mm, 5 µm)	A) sodium acetate (50 mM)/methanol (95:5), B) methanol (70%)	0.5 mL/min Gradient	30	Brain (Cortex, cerebellum)	Homogenized in ice-cold 0.4 N perchloric acid and then centrifuged. The supernatants were diluted 1/750 in double-distilled water.	OPA/MPA	No	0.001-0.02 pmol/µL	0.017-8.6 pmol/µL	(Perucho et al., 2015)
GABA, Glu	No	HPLC-FLD	C_{18} (150 × 4.6 mm, 3 µm)	Sodium acetate (50 mM), tetrahydrofuran and methanol (50:1:49, v/v) pH 4	1 mL/min Isocratic	9	Brain (Hippocampus, thalamus, prefrontal cortex)	Homogenized in 15 volumes of methanol/water (85:15, v/v) and centrifuged.	OPA/MPA	100-102%	NR	100-10000 ng/mL	de Freitas Silva et al., 2009)
Asp, Glu, Ser, Gln, Gly, Tau, GABA	Homoserine	HPLC-FLD	BDS (250 × 4.6 mm, 5 µm)	Sodium acetate buffer (50 mM, pH to 6.5) and methanol	1 mL/min Gradient	23	Brain (Hippocampus, cortex)	Homogenized in ice-cold 0.4 N perchloric acid, centrifuged and filtered.	OPA/ ß-thiofluor	85.5-99.7%	50-100 ng/mL	50-10000 ng/mL	(Cui et al., 2017)
5-HT, DA, DOPAC, 3-MT, HVA	DHBA	HPLC-MS	ZORBAX Eclipse C_{18} (150 × 4.6 mm, 5 µm)	A) 0.1% formic acid in water, pH 3 B) 0.1% formic acid in acetonitrile (40:60, v/v)	0.2 mL/min Isocratic	30	Brain	Homogenized in ice-cold lysis buffer. The homogenate was centrifuged and filtered through a 0.2 µm filter.	Ethanol-pyridine ethyl (4:1), chloroformate and diethyl ether	90%	1-5 ng/mL	1-700 ng/mL	(Park et al., 2013)

Gln, MI, GABA, Glu, NAA, Asp, Tau, Cho, CR, PC	Glu-d5	LC-(ESI)-MS/MS	HILIC silica (150 × 2.1 mm, 5 µm)	A) 0.1% formic acid, B) Acetonitrile	0.3 mL/min Gradient	15	Brain	Homogenized in methanol, diluted with distilled water, extracted by protein precipitation using 2 mL methanol and centrifuged.	No	70%	NR	2.5-20 to 500-4000 ng/mL	(Bathena et al., 2012)
Glu, GABA, Cho, ACh, DA, 5-HIAA, 5-HT, DOPAC, HVA	No	LC-(ESI)-MS/MS	BEH C_{18} (50 × 2.1 mm, 1.7 µm)	A) methanol, B) 0.05% formic acid with 1mM of HFBA	0.2 mL/min Gradient	8	Brain (Prefrontal cortex, striatum, nucleus accumbens, amygdala)	Nucleus accumbens and amygdala tissues were homogenized in a 20-fold volume of a solution of formic acid (0.1 M), whereas prefrontal cortex and striatum tissues were homogenized in a 10-fold volume of the same solution. The homogenates were centrifuged.	No	68-112%	0.05-7.3 ng/mL	1 (2 or 5)-200 ng/mL	(González et al., 2011)
5-HT, 5-HIAA, Try, DA, NE, GABA, Glu, ACh	Isoproterenol hydrochloride	HPLC-(ESI)-MS/MS	Inertsil ODS-EP (150 × 4.6 mm, 5 µm)	A) 0.01% acetic acid, B) methanol	1.2 mL/min Gradient	9	Brain	Homogenized in a 20-fold volume of 2% formic acid in methanol and centrifuged.	No	80%	0.02-0.2 ng/mL	1000-50000 µg/mL	(He et al., 2013)
GABA, Glu, E, NE, DA, 5-HT, 5-HIAA	No	LC-(ESI)-MS/MS	BEH C_{18} (100 × 2.1 mm, 1.7 µm)	A) 0.1% formic acid, B) acetonitrile	0.1 mL/min Gradient	9	Brain (Hippocampus)	Homogenized in ice-cold methanol (0.1% formic acid) and 10 µL IS. The homogenate was centrifuged and the supernatant was evaporated to dryness reconstituted in mobile phase.	No	87-107%	1-5 ng/mL	50-50000 ng/mL	(Huang et al., 2014)
BH4, DA, 5-HT, NE, E, Glu, GABA	DA-d4, 5-HT-d4, NE-d6, E-d3, Glu-d5,	LC-(ESI)-MS/MS	BH4, DA Sepax Polar-Imidazole (100 × 2.1 mm, 3 µm)	BH4, DA Ammonium formate (10 mM) in an acetonitrile/water (75:25, v/v) mixture, pH 3	BH4, DA 0.3 mL/min Isocratic	5.5	Brain	Homogenized with acetonitrile and centrifuged.	No	NR	10-200 ng/mg	NR	(Kim et al., 2014)

	GABA-d6		5-HT, NE, E, Glu, GABA Luna C_{18} (150 × 3 mm, 3 µm)	5-HT, NE, E, Glu, GABA Acetonitrile/water (20:80, v/v) mixture	5-HT, NE, E, Glu, GABA 0.35 mL/min Isocratic								
Glu, GABA, NE, DA, 5-HT, 5-HIAA, DOPAC, HVA	DHBA	UFLC-(ESI)-MS/MS	Synergi Fusion-RP 80A ODS (150 × 2 mm, 4 µm)	A) 0.05% formic acid, B) acetonitrile	0.2 mL/min Gradient	11	Brain	Homogenized in methanol and centrifuged. To the supernatant was added 10 µL of the IS, 10µL water and vortexed. Next, these samples were centrifuged. The supernatants were evaporated to dryness and reconstituted in 100 µL water.	No	85%	4-16 ng/mL	4-4000 ng/mL	(Wei et al., 2014)
NE, 5-HT, L-ENK, M-ENK	Isoproterenol, DHBA	LC-(ESI)-MS/MS	Alltima C_{18} (250 × 4.6 mm, 5 µm)	A) acetonitrile, B) 0.05% formic acid	1 mL/min Gradient	15	Brain (Hypothalamus)	Homogenized in cold methanol, vortexed and then centrifuged. The supernatant was evaporated. The residue was reconstituted with 200 µL water. Then 300 µL of chloroform-isopropanol was added and vortexed. The mixture was centrifuged to get the water layer B. The eluent A and the water layer B were mixed and evaporated under nitrogen and then residue was reconstituted in 200 µL water and the solution was centrifuged.	No	10-79%	2-1000 ng/mL	4 ng/mL	(Xu et al., 2011)
Glu, Asp	Glu-d5	HPLC-(ESI)-MS/MS	Restek C_{18} (150 × 2.1 mm, 5 µm)	Water–acetonitrile (40:60, v/v), 0.1% formic acid	0.3 mL/min Isocratic	5	Brain (Hippocampus)	Homogenized with methanol in an ice-water mixture. The homogenate was diluted 450 times with water, finally 700 µL acetonitrile was added for protein precipitation. Then the samples were vortexed and centrifuged.	No	89-113%	10 ng/mL	10-1000 ng/mL	(Xu et al., 2014)

DA, DOPAC, HVA, NE, Trp, 5-HT, 5-HIAA, KYN, 3-HK, 3-HAA, KA	Caffeic acid	LC-(ESI)-MS/MS	Kromasil C_{18} (150 × 2.1 mm)	A) 0.1% formic acid, ammonium acetate (2 mM), B) acetonitrile	0.2 mL/min Gradient	13	Brain (Cortex)	Homogenized and sonicated in an ice bath. Ice-cold acetonitrile was then added to the homogenate, and the mixture was vortexed followed by centrifugation. The supernatant was then evaporated to dryness under vacuum.	Benzoyl chloride	81-115%	0.1-5 ng/mL	10-39200 nM	(Zheng et al., 2012)
5-HT, DA, E, NE	4-chlorophenyl-alanine	UPLC-(ESI)-MS/MS	ACQUITY UPLC BEH (210 × 5 mm, 1.7 μm)	A) 0.1% formic acid in water–acetonitrile (95:5, v/v), ammonium acetate (1 mM), B) 0.1% formic acid acetonitrile–water (95:5, v/v), ammonium acetate (1 mM)	0.5 mL/min Gradient	6	Brain (Cortex, brainstem, hypothalamus)	Homogenized with 0.2% formic acid in cold methanol–acetonitrile and centrifuged.	No	91–120%	0.3-3 nM	1-3000 nM	(Zhou et al., 2015)
DA, E, GABA, Glu, 5-HT, NE	DA-d4, E-d6, GABA-d6, Glu-d5, 5-HT-d4, NE-d6	LC-(ESI)-MS/MS	ACE C_{18} (100 × 2.1 mm, 3 μm)	A) 0.1% formic acid in water, B) 0.1% formic acid in acetonitrile	0.2 mL/min Gradient	6	Brain	Homogenized in ice-cold 0.5 M formic acid.	No	81-106%	0.03-50 ng/mg	0.0075-1 pg/mg	(Zhu et al., 2011)
Glu, Gln, pGlu, GABA, Thea	Glu-d5, Gln-d5, pGlu-d5, Thea-d5	LC-(ESI)-MS/MS	TSK-gel Amide-80 (150 × 2 mm, 5 μm)	A) 0.1%formic acid in water, B) 0.1%formic acid in acetonitrile	0.3 mL/min Gradient	15	Brain (Hippocampus, central cortex)	The brain tissue was added to 10 mL of 0.1% formic acid in water–methanol, then the extraction solution with two zirconia beads (3.0 mm) in the tubes was homogenized and centrifuged. The supernatant was prepared by dissolving 0.1% formic acid in water–acetonitrile. The sample solution was filtered.	No	83-99%	1.3-5.2 ng/mL	10.3-15 ng/mL	(Inoue et al., 2016)
Cho, ACh, Cart, ACart	ACh-d4, Cho-d9, Cart-d3,	LC-(ESI)-MS/MS	HILIC (250 × 2 mm, 5 μm)	A) 0.2% formic acid, B) acetonitrile	0.4 μL/min Gradient	45	Brain (Prefrontal cortex, striatum, hippocampus)	Homogenized in ice-cold acetonitrile containing 0.3% formic acid. The supernatants were filtered and diluted four times with water containing 0.3% formic acid.	No	NR	NR	NR	(Falasca et al., 2012)

	ACart-d9, OctCart-d3, PalmCart-d3												
Ach	Cho-d9	LC-(ESI)-MS/MS	HILIC silica (150 × 2.1 mm, 3 µm)	Ammonium formate (20 mM) in water–acetonitrile (30:70, v/v), pH 3	0.4 mL/min Isocratic	6	Brain	Homogenized with 1 mM physostigmine on ice and using dual homogenizer. Acetonitrile was added to the homogenate, vortexed and deproteinized by centrifugation.	No	99-103%	0.2 ng/mL	10-1000 ng/mL	(Peng et al., 2011)
DA, HVA, 3-MT, 5-HT, 5-HIAA, NE, Ach, Glu, GABA	DA-d3, 5-HT-$^{13}C_2{}^{15}N$, GABA-d6, DA-$^{13}C_6$, 5-HT- d4, NE-d6, ACh-d4, DL-Glu-d4, 5-HIAA-d5	UHPLC-MS/MS	Acquity HSS T3 C_{18} (100 × 2.1 mm, 1.8 µm)	A) formic acid (25 mM) B) methanol	0.5 mL/min Gradient	5.2	Brain	Homogenized in water. Ice-cold formic acid (250 mM) was added to homogenate vortexed, and deproteinized by centrifugation.	No	88-101%	1.0-4000 nM	1-4000 nM	(Bergh et al., 2016)
Asp, Aspa Glu, Gln, GABA, Nacetyl-L-Asp, pyroglutamic acid, ACh, Cho	Cho-d9, GABA-d6-4-, Asp-d3, N-Acetyl-L-Asp-d5, Glu-d3, Gln-d3, ACh-d9, pyroglutamic acid-d5, Aspa-d5	UHPLC-MS/MS	HILIC silica (10 × 2.1 mm, 1.7 µm)	A) ammonium formate (50 mM) B) acetonitrile	0.7 mL/min Gradient	9	Human brain	Homogenized in acetonitrile and 0.1% formic acid, centrifuged diluted with 80% acetonitrile (1:1, v/v) and centrifuged.	No	83-113%	12-1930 ng/mL	25-250000 ng/mL	(Forgacsova et al., 2018)
DA, 5-HT, 5-HIAA, DOPAC, HVA	Salicin	HPLC-PDA	Hypersil Gold C_{18} (250 x 2.1 mm, 5 µm)	Perchloric acid (5 mM), acetonitrile (5%)	0.25 mL/min Isocratic	60	Brain (Cortex, hippocampus, striatum, *substantia nigra*, hypothalamus, amygdalae, cerebellum)	Homogenized in ice-cold 0.2 M perchloric acid and centrifuged. The supernatants were filtered.	No	94-102%	0.4-8.4 pg	0.1-1000 ng/mL	(Gu et al., 2015)

Asp, Glu, Gly, Tau, GABA	No	HPLC-UV	Diamonsil C_{18} (250 × 4.6 mm, 5 µm)	Acetonitrile/phosphate buffer (20 mM) (16:84, v/v), pH 6	1 mL/min Isocratic	20	Brain (Hippocampus)	Homogenized in 4 mL of chilled saline solution (0.9%), centrifuged and filtered.	NBD-F	95-105%	0.02-0.2 µmol/L	0.500-500 µmol/L	(Wu et al., 2014)
HVA, DA, L-DOPA, E, NMN, 5-HT, 5-HIAA, NE, VMA, MHPG, DOPAC	HVA-d5, DA-d3, E-d6	GC-(EI)-MS/MS	HP-1 capillary (30 m × 0.25 mm, 0.25 µm)	Helium	1 mL/min Gradient	12	Brain	Homogenized in 1 mL of 0.1% formic acid, sonicated in an ice-bath and centrifuged.	HMDS and MBHFBA (TMS)	88-92%	0.7-3.7 ng/mL	1-200 ng/mL	(Hong et al., 2013)
Ala, Aspa, Asp, Cys, Gln, Glu, Isoleu, Leu, Lys, Met, Phe, Pro, Sar, Ser, Val, Thr	NR	GC-(EI)-MS	Rtx-5MS fused-silica capillary (30 m × 0.25 mm, 0.25 µm)	Helium	0.1 mL/min Gradient	20	Brain (Prefrontal cortex, striatum, hippocampus, cerebellum)	Homogenized in lysis buffer solution and centrifuged. 100 µL of supernatants was precipitate with 900 µL of methanol.	BSTFA (TMCS 1%)	89-129%	10-7070 nmol/L	20-100000 nmol/L	(Pinto et al., 2014)
Glu, GABA	Glu-d5, GABA-d4	GC-MS/MS	DB-5 ms (15 m × 0.25 mm, 0.25 µm)	Helium	Gradient	6	Brain	Homogenized, centrifuged and evaporated.	MethElute™	NR	100 - 250 ng/mL	500-10000 ng/mL	(Farthing et al., 2017)

3-HAA: 3-hydro-xyanthranilic acid; 3-HK: 3-hydroxykynurenine; 3-MT: 3-methoxytyramine; 5-HIAA: 5-hydroxyindole-3-acetic acid; 5-HMT: 5-hydroxy-N-methyl tryptamine oxalate; 5-HT: 5-hydroxytryptamine (serotonine); ACart: Acetylcarnitine; ACh: Acetylcholine; ADO: Adenosine; ADP: Adenosine diphosphate; Ala: Alanine; AMP: Adenosine monophosphate; Arg: Arginine; Asp: Aspartic acid; Aspa: Asparagine; ATP: Adenosine triphosphate; Cart: Carnitine; CBQCA: 3-(4-carboxybenzoyl)-2-quinoline carbox-aldehyde; Cho: Choline; CR: Creatine; DA: Dopamine; DHBA: Dihydroxybenzylamine; DMSO: Dimethyl sulfoxide; DOPAC: 3,4-dihydroxyphenylacetic acid; ECD: Electrochemical detector; EDTA: Ethylenediaminetetraacetic acid; ESI: electrospray ionization; E: Epinephrine; FLD: Fluorescence detector; GABA: γ-aminobutyric acid; Gln: Glutamine; Glu: Glutamate; Gly: Glycine; HA: Histamine, HFBA: Heptafluorobutyric acid; HILIC: Hydrophilic interaction liquid chromatography; HD: Histidine; HMDS: Hexamethyldisilazane; HPLC: High-performance liquid chromatography; HVA: Homovanillic acid; Isoleu: Isoluecine; KA: Kynurenic acid; KYN: Kynurenine; LC: Liquid chromatography; L-DOPA; 3,4-dihydroxy-l-phenyl-alanine; L-ENK: Leucine-enkephalin; Leu: Leucine; LOD: Limit of detection; LOQ: Limit of quantification; Lys: Lysine; MBHFBA: N-methyl-bis-heptafluorobutyramide; M-ENK: Methionine- encephalin; Met: Methionine; MHPG: 3-methoxy-4-hydroxyphenylglycol; MI: Myo-inositol; MPA: 3-mercaptopropionic acid; MS/MS: Mass spectrometry in tandem; MS: Mass spectrometry, NAA: N-acetyl aspartic acid; NBD-F: 4-fluoro-7-nitrobenzofurazan; NE: Norepinephrine; NMN: Normetanephrine hydrochloride; NR: not reported; Ns-MHA: Ns-methylhistamine; OctCart: Octanoylcarnitine; OPA: O-Phtalaldehyde; OSA: 1-octanesulfonic acid; PalmCart: Palmitoylcarnitine; PC: Phospho- choline; pGlu: Pyroglutamic acid; Phe: Phenylalanine; Pro: Proline; S: Sulfate; Sar: Sarcosine; Ser: Serine; Tau: Taurine; TEA: Triethylamine; Thea: Theanine; Thr: Threonine; TMCS: Trimethylchlorosilane; TMS: Trimethylsilane; Trp: Tryptophan; Tyr: Tyrosine; UHPLC: Ultra-high-performance liquid chromatography; Val: Valine; VMA: 4-hydroxy-3- methoxymandelic acid.