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Ciências

Nonclinical assessment of the potential for herb-drug interactions between herbal extracts present in weight loss supplements and lamotrigine

Sandra Cristina do Espírito Santo Ventura

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Orientador: Prof. Doutor Gilberto Alves
Coorientador: Prof. Doutor Amílcar Falcão

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Não sou nada.
Nunca serei nada.
Não posso querer ser nada.
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O mundo é para quem nasce para o conquistar
E não para quem sonha que pode conquistá-lo, ainda que tenha razão.
Tenho sonhado mais que o que Napoleão fez.
Tenho apertado ao peito hipotético mais humanidades do que Cristo,
Tenho feito filosofias em segredo que nenhum Kant escreveu.
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Ainda que não more nela;
Serei sempre o que não nasceu para isso (...)

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Resumo

Resumo

As plantas têm sido, e continuarão ainda a ser, uma das fontes mais importantes de princípios ativos. Na realidade, as plantas constituem ainda a "espinha dorsal" das farmacopeias modernas e continuam a ser uma fonte de novos candidatos a fármacos. A utilização de plantas medicinais ou de preparações medicinais à base de plantas está também a aumentar em muitos países desenvolvidos como uma forma alternativa e complementar para o tratamento de doenças. Por conseguinte, o uso concomitante de plantas e medicamentos convencionais é uma prática comum em doentes com hipertensão, diabetes, epilepsia, depressão e doenças oncológicas, assim como em pessoas com obesidade e excesso de peso. Recentemente, a obesidade e a epilepsia têm sido consideradas comorbilidades com uma elevada prevalência, particularmente em doentes com epilepsia refratária e polimedicados. O tratamento de doentes com epilepsia deve, portanto, ter em consideração que a presença de comorbilidades pode comprometer a eficácia e a segurança dos fármacos antiepiléticos, os quais constituem a principal estratégia terapêutica na epilepsia. A lamotrigina (LTG) é um fármaco antiepilético bem tolerado e amplamente utilizado na epilepsia, mas que apresenta uma margem terapêutica estreita e uma variabilidade interindividual considerável na sua farmacocinética. Por isso, o foco de investigação considerado nesta tese foi a avaliação não-clínica do potencial de interação entre extratos de plantas presentes em suplementos à base de plantas para emagrecimento e a LTG, usando o rato como modelo animal. Após a otimização e a validação de métodos bioanalíticos seletivos, precisos e exatos para a quantificação da LTG em amostras humanas (plasma e saliva) e em amostras de rato (plasma e cérebro), as condições para prosseguir com os estudos não-clínicos estavam reunidas. Portanto, de seguida, um conjunto de estudos não-clínicos foi realizado em ratos Wistar machos adultos com o objetivo principal de avaliar os efeitos de extratos padronizados de *Paullinia cupana* (guaraná), de *Garcinia cambogia* (tamarindo de Malabar), de *Citrus aurantium (*laranja-amarga) e de *Fucus vesiculosus* (bodelha) na cinética da LTG. Para tal, pelo menos dois estudos farmacocinéticos independentes foram realizados para avaliar os efeitos de cada extrato na farmacocinética da LTG; o primeiro estudo teve como objetivo avaliar os efeitos após a coadministração do extrato e da LTG, e o segundo estudo foi realizado para avaliar os efeitos de um período de pré-tratamento de 14 dias com cada extrato na farmacocinética da LTG administrada subsequentemente ao 15º dia. Globalmente, os resultados dos estudos farmacocinéticos envolvendo os quatro extratos de plantas revelaram que o extrato de *P. cupana* é aquele que tem maior potencial para interagir com a LTG, enquanto que os extratos de *G. cambogia*, *C. aurantium* e *F. vesiculosus* tiveram poucos ou nenhuns efeitos na farmacocinética da LTG. A coadministração do extrato de *P. cupana* e LTG causou, em particular, um decréscimo significativo da concentração plasmática máxima (C_{max}) e da extensão de exposição sistémica à LTG nas primeiras 24 h (AUC_{0-24}). Com base nos resultados obtidos nestes estudos não-clínicos,

uma interação farmacocinética importante entre o extrato de *P. cupana* e a LTG foi aqui descrita pela primeira vez, a qual potencialmente pode ter impacto clínico em doentes tratados com a LTG. Além disso, a administração repetida dos extratos testados durante um período de 14 dias não teve efeitos relevantes sobre o ganho de peso corporal dos ratos, o que levanta dúvidas sobre a eficácia deles na redução do peso corporal. Assim, em conclusão, a avaliação não-clínica de interações planta-fármaco é de extrema importância para antecipar os efeitos potenciais de preparações à base de plantas na farmacocinética de fármacos de índice terapêutico estreito como a LTG, constituindo esses dados o ponto de partida para confirmação posterior e investigação da relevância dessas interações a nível clínico.

Palavras-chave

Extratos de plantas, fármacos antiepiléticos, farmacocinética, interações planta-fármaco, lamotrigina, rato.

Abstract

Abstract

Plants have been and still continue to be one of the most important sources of active ingredients. Actually, plants are still the backbone of modern pharmacopoeias and remain as a source of new drug candidates. The use of medicinal plants or plant-based medicinal products is also increasing in many developed countries as an alternative and complementary form for the treatment of diseases. Thus, the concomitant use of plants and conventional medications is emerging as a common practice in patients with hypertension, diabetes, epilepsy, depression, and oncological diseases, as well as in people with obesity and being overweight. Recently, obesity and epilepsy have been related as comorbid conditions with a high prevalence, particularly in patients with refractory epilepsy and under polytherapy. Treatment of patients with epilepsy should, therefore, take into account that the presence of comorbid conditions may compromise the efficacy and safety of antiepileptic drugs, which constitute the main therapeutic approach in epilepsy. Lamotrigine (LTG) is a well-tolerated antiepileptic drug widely used in epilepsy; however, it has a narrow therapeutic range and a considerable interindividual variability in its pharmacokinetics. Therefore, the focus of research addressed in this thesis was the nonclinical assessment of the potential for herb-drug interactions between herbal extracts present in weight loss supplements and LTG, using the rat as whole animal model. After optimization and validation of selective, precise and accurate bioanalytical methods for the quantification of LTG in human samples (plasma and saliva) and in rat samples (plasma and brain), the conditions for proceeding with nonclinical studies were met. Therefore, then a number of nonclinical studies were performed in adult male Wistar rats with the main objective of evaluating the effects of standardized extracts of *Paullinia cupana* (guarana), *Garcinia cambogia* (malabar tamarind), *Citrus aurantium* (bitter orange) and *Fucus vesiculosus* (bladderwrack) on the kinetics of LTG. To this end, at least two independent pharmacokinetic studies were carried out to evaluate the effects of each herbal extract on the pharmacokinetics of LTG; the first study aimed to evaluate the effects after the co-administration of the extract and LTG, and the second one aimed to evaluate the effects of a 14-day pre-treatment period with the extract on the pharmacokinetics of LTG subsequently administered on the 15th day. Globally, the results of the pharmacokinetic studies involving the four herbal extracts pointed out that *P. cupana* extract is the one that has higher potential to interact with LTG, while *G. cambogia, C. aurantium* and *F. vesiculosus* extracts had minor or no effects on LTG pharmacokinetics. The co-administration of *P. cupana* extract and LTG caused, in particular, a significant decrease in the peak plasma drug concentration (C_{max}) and in the extent of systemic exposure to LTG over the first 24 h (AUC_{0-24}). Based on the findings achieved in these nonclinical studies, an important pharmacokinetic interaction between *P. cupana* extract and LTG was herein described for the first time, which potentially may have clinical impact in patients treated with LTG. Moreover, the repeated administration of the tested herbal extracts during

a 14-day period did not have relevant effects on the body weight gain of rats, which raises doubts about their effectiveness in reducing body weight. So, in conclusion, the nonclinical assessment of herb-drug interactions is of utmost importance to anticipate the potential effects of herbal preparations in the pharmacokinetics of narrow therapeutic index drugs like LTG, constituting these data the starting point for further confirmation and investigation of the relevance of these interactions at a clinical level.

Keywords

Antiepileptic drugs, herbal extracts, herb-drug interactions, lamotrigine, pharmacokinetics, rat.

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List of Abbreviations

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A	
AED	Antiepileptic drug
ADME	Absorption, distribution, metabolism, and excretion
AUC	Area under the concentration-time curve
AUC_{0-24}	AUC from time zero to 24 h
AUC_{0-t}	AUC from time zero to the last measurable concentration
$AUC_{0-\infty}$	AUC from time zero to infinite
B	
Bias	Deviation from nominal concentration
C	
C_{last}	Last quantifiable concentration
C_{max}	Peak plasma concentration
CNS	Central nervous system
CV	Coefficient of variation
CYP	Cytochrome P450
D	
DAD	Diode array detector
DGAV	Portuguese National Authority for Animal Health, Phytosanitation and Food Safety *(Direção Geral de Alimentação e Veterinária)*
E	
EMA	European Medicines Agency
F	
FDA	Food and Drug Administration
G	
GABA	γ-Aminobutyric acid
GACP	Good Agricultural and Collection Practice
H	
HCA	Hydroxycitric acid
HDI	Herb-drug interaction

HPLC	High-performance liquid chromatography
HMPC	Committee on Herbal Medicinal Products
5-HT	5-Hydroxytryptamine

I

ICH	International Conference on Harmonisation
ILAE	International League Against Epilepsy
i.p.	Intraperitoneal
IS	Internal standard

K

k_{el}	Apparent terminal elimination rate constant
K_m	Half-maximal rate of metabolism

L

LD_{50}	Median lethal dose
LLE	Liquid-liquid extraction
LOQ	Limit of quantification
LOD	Limit of detection
LTG	Lamotrigine

M

MEPS	Microextraction by packed sorbent
MRT	Mean residence time
MS	Mass spectrometry

O

OAT	Organic anion transporters
OATP	Organic anion transporting polypeptides
OCT	Organic cations transporters

P

PEPT	Peptide transporters
P-gp	P-glycoprotein
p.o.	*Per os*
PP	Protein precipitation

Q

QC	Quality control

R

r^2	Determination coefficient
Rpm	Rotations per minute

S

SEM	Standard error of the mean
SPE	Solid-phase extraction
SPME	Solid-phase microextraction

T

TDM	Therapeutic drug monitoring
t_{max}	Time to reach C_{max}
TSH	Thyroid-stimulating hormone
$t_{1/2el}$	Apparent terminal elimination half-life

U

UGT	Uridine diphosphate-glucuronosyltransferase
UV	Ultraviolet

V

V_d	Apparent volume of distribution
V_{max}	Maximal rate of metabolism

W

WHO	World Health Organization

List of Publications

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Ventura, S., Rodrigues, M., Pousinho, S., Falcão, A., Alves, G. (2016). An easy-to-use liquid chromatography assay for the analysis of lamotrigine in rat plasma and brain samples using microextraction by packed sorbent: Application to a pharmacokinetic study. Journal of Chromatography B 1035: 67–75.

Ventura, S., Rodrigues, M., Pousinho, S., Falcão, A., Alves, G. (2017). Determination of lamotrigine in human plasma and saliva using microextraction by packed sorbent and high performance liquid chromatography–diode array detection: An innovative bioanalytical tool for therapeutic drug monitoring. Microchemical Journal 130: 221–228.

Ventura, S., Rodrigues, M., Falcão, A., Alves, G. (2018). Effects of *Paullinia cupana* extract on lamotrigine pharmacokinetics in rats: A herb-drug interaction on the gastrointestinal tract with potential clinical impact. Food and Chemical Toxicology 115: 170-177.

Ventura, S., Rodrigues, M., Falcão, A., Alves, G. Administration of *Garcinia cambogia* and lamotrigine: Safety evidence from non-clinical pharmacokinetic studies in Wistar rats *(submitted for publication)*.

Ventura, S., Rodrigues, M., Falcão, A., Alves, G. (2018). Evaluation of the effects of *Citrus aurantium* (bitter orange) extract on lamotrigine pharmacokinetics: Insights from *in vivo* studies in rats. Food and Chemical Toxicology 121: 166-172.

Ventura, S., Rodrigues, M., Falcão, A., Alves, G. (2018). Safety evidence on the administration of *Fucus vesiculosus* L. (bladderwrack) extract and lamotrigine: Data from pharmacokinetic studies in the rat. Drug and Chemical Toxicology, https://doi.org/10.1080/01480545.2018.1518454.

Chapter I.

General introduction: plants, obesity and epilepsy

I.1. Plants: the medicines from nature

Plants have been one of the most important and primary sources of medicines throughout the history of mankind. They have never been old or dispensable, and their importance in modern civilizations is as remarkable as it was in ancient times when men depended on them to survive, either as a food source or in cultural and religious traditions. In this new era of herbal renaissance, plants are still the backbone of modern pharmacopoeias, and constitute an emerging source of new drug candidates, as prototypes or lead compounds (Mishra and Tiwari 2011; Pferschy-Wenzig and Bauer 2015; Sponchiado et al. 2016). The first pure natural product drug prototypes were identified through the investigation of vascular plants. These plant-derived medicines include morphine (from *Papaver somniferum*), digitoxin (from *Digitalis purpurea*), salicylic acid (from *Salix alba*), quinine (from *Cinchona* sp), atropine (from *Atropa belladonna*) and paclitaxel (from *Taxus brevifolia*). Terrestrial animals were also a source of therapeutics like captopril (from the viper *Bothrops jararaca*) and epibatidine (from the frog *Epipedobates anthonyi*) (Bernardini et al. 2017; Mushtaq et al. 2018). Indeed, over the last decades, about 40% of the new chemical entities approved by the Food and Drug Administration (FDA) were natural products or natural-based products (including semi-synthetic derivatives, synthetic compounds based on natural pharmacophores and natural products mimetics), and mostly from plant origin (Chen et al. 2015; Katz and Baltz 2016). Despite the intensive investigation of the terrestrial flora, it is estimated that only 6% of the approximately 300,000 species of higher plants have been systematically and pharmacologically investigated, and only 15% phytochemically investigated (Cragg and Newman 2013; Mushtaq et al. 2018).

The diversity and richness of secondary metabolites in plants have been exploited along the years, following the development of powerful analytical tools based upon genomics, proteomics, metabolomics and bioinformatics (Harvey et al. 2015; Ngo et al. 2013; Sharma and Shrivastava 2016). The biosynthesis of secondary metabolites occurs mainly through the shikimate, acetate–malonate and acetate–mevalonate pathways, usually in response to environmental stress (caused by temperature, water, salinity, radiation, chemical or mechanical signals), allowing plants to survive, protect themselves (from pathogens and predators) and be adapted to hostile conditions (Bernardini et al. 2017; Patra et al. 2013). Despite the enormous variety of chemical substances produced, these metabolites are often produced in low quantities and its production strongly depends on the growing and developmental stage of the plant (Ramakrishna and Ravishankar 2011). These specialized substances exhibit a variety of biological activities according to their chemical structures and are mainly organized in two groups: nitrogen-containing molecules (alkaloids) and nitrogen-deficient molecules (terpenoids and phenolics) (Patra et al. 2013).

Alkaloids, found in over than 20% of plant species, are low-molecular weight containing nitrogen substances that have strong pharmacological activities such as anti-tumoral (vincristine and vinblastine, from *Catharanthus roseus*), antimalarial (quinine, from *Cinchona*

sp.), cholinergic (pilocarpine, from *Pilocarpus* sp.), anticholinergic (atropine, from *Atropa belladonna*), adrenergic (ephedrine, from *Ephedra*), anaesthetic (cocaine, from *Erythroxylum coca*) and analgesic (like morphine from *Papaver somniferum*), among others (Shitan 2016). Additionally, many alkaloids mimic, block or modulate neurotransmitter activity or interfere with basic neurological functions. Biosynthetically, true and non-heterocyclic alkaloids are derived from amino acids, such as phenylalanine, ornithine, arginine, tyrosine, and tryptophan (from shikimate and acetyl-coenzyme A pathways). However, others are originated from alternative precursors (e.g. purine-derived caffeine) (Staniek et al. 2013).

Terpenoids are lipid-soluble substances, synthetized mostly from the mevalonate pathway, which include an enormous variety of compounds classified according to the number of isoprene units. They exhibit a broad range of pharmacological activities, including antimicrobial (essential oils, from *Thymus* sp. and *Mentha piperita*), sedative and anxiolytic (valepotriates, from *Valeriana officinalis),* antioxidant *(*essential oil, from *Melissa officinalis*), anti-tumoral (diterpenes, from *Taxus* sp.), nootropic (ginkgolides and bilobalide, from *Ginkgo biloba*), ionotropic (cardenolides, from *Digitalis purpurea*), analgesic (cannabinoids, from *Cannabis sativa*), neuroprotective (ginsenosides, from *Panax ginseng*), among others (Kennedy and Wightman 2011; Staniek et al. 2013; Staniek et al. 2014).

Plant phenols are one of the most common and widespread group of substances, formed by the shikimate/phenylpropanoid pathways. They have at least one aromatic ring, with one or more hydroxyl substituents, and include several subgroups: phenylpropanoids, coumarins, lignans, flavonoids, and tannins. From these, flavonoids represent the largest and most diverse group, encompassing some 6000 compounds, which include anthocyanins, proanthocyanidins, chalcones, flavones, flavonols, flavanones, and isoflavones. Similarly, they have different biological effects such as antibacterial and scavenger of free radicals (polyphenols, from *Camellia sinensis*), antidepressant effects (flavonoids and hipericins, from *Hypericum perforatum*), anti-inflammatory effects (salicylic acid, from *Salix* sp.) and cardioprotective and oestrogen-like activity (isoflavones from *Glycine max*), among others (Bernardini et al. 2017; Bjørklund et al. 2017; Kennedy and Wightman 2011; Latteef 2016).

Besides the important bioactivities of secondary metabolites, plant primary metabolites are also important in food and pharmaceutical industries, as well as in traditional medicine. For example, fatty acids (in olive oil, as cardioprotective) and carbohydrates (honey, as antibacterial and wound dressing) play an important role in modern medicine (Molan and Rhodes 2015; Schwingshackl and Hoffmann 2014; Waterman and Lockwood 2007).

Pharmaceutical investigations have learned from plant chemistry and have targeted their knowledge to synthetize and extract both secondary and primary metabolites as drugs to treat diseases. Despite the sophisticated advances in chemically synthetic approaches to drug design and structure-activity relationship analysis, pharmaceutical investigations are again focused in the potential of plants as source of novel compounds with unique mechanisms of action. Most of the new drug discovery research programs are based on traditional medicine-based strategies to increase success and ensure the safety of new drugs (Yuan et al. 2016). There is no doubt

that traditional medicines truly protected human society since antiquity and the "learning from nature, learning from our ancestors" are still supporting society in medication, research and development (Leonti and Verpoorte 2017).

I.1.1. Regulatory perspective on the use of herbal medicines

From the descriptions and therapeutic knowledge of the *De Materia Medica* (from *Dioscorides*, 1st century CE) it is easy to understand the importance of herbal medicines (Atanasov et al. 2015; Chinou et al. 2014; Staub et al. 2016). Several plants have been used for thousands of years and are still used across all over the world. Plants and herbal medicines are important for human health care as an integral part of traditional medical systems. It is estimated that 70% of the world's population does not have access to conventional medicines and, therefore, rely on traditional treatments as their primary source of healthcare (Pferschy-Wenzig and Bauer 2015).

Herbal medicines differ in many features from conventional medicines, and several aspects bring a variety of challenges in what concerns to their pharmacovigilance, namely the plant substitution and adulteration, the lack of uniformity in nomenclature, quality control and standardization and the lack of monitoring the adverse reactions (de Boer et al. 2015; Osathanunkul et al. 2016).

The World Health Organization (WHO) defined herbal medicines as plant-derived products or preparations (like extracts and tinctures) constituted by one or more ingredients from one or more plants, used with therapeutic or other human health benefits (Pferschy-Wenzig and Bauer 2015). Due to the complex phytochemical mixtures that are present in some of the plant-derived products or preparations, the European Medicines Agency (EMA) differentiated the herbal medicinal products in three categories: (a) standardized products, with a given content of chemically defined substances or substances with known therapeutic activity; (b) quantified products, with a defined range of active markers, that are constituents or groups of constituents that generally contribute to the therapeutic activity; and (c) other herbal products, in which neither constituents with therapeutic activity nor active markers are known. In this case, the extract as a whole is regarded as the active principle (Pferschy-Wenzig and Bauer 2015).

Recognizing that there is a worldwide variation in what concerns the regulatory policies on the use of herbal medicines, one of the most acceptable approaches to quality assurance is the primary standardization of the active constituents and/or analytical markers. Phytochemical profiling and metabolite fingerprinting are already increasing tools to ensure this goal. Nevertheless, the quality assurance in terms of chemical composition alone does not bring consistent therapeutic efficacy and/or safety of the herbal medicines (Bansal et al. 2016). Indeed, international regulatory guidelines have been redefined to ensure the manufacturing and controlling of herbal medicines in terms of quality, safety and efficacy. But, like other drugs, herbal medicines must also ensure stability, consistent therapeutic efficacy and safety throughout the defined herbal medicinal products shelf life (Bansal et al. 2016). Guidelines

from EMA, FDA and WHO already require stability data for the finished herbal product (EMA 2011b; ICH 2003; WHO 2006). It is important to emphasize that efficacy and safety of these finished products are directly dependent on the quality and chemistry of medicinal herb raw materials (Govindaraghavan and Sucher 2015). To ensure both safety and efficacy of herbal medicines, implementation of good agricultural and collection practices (GACP), good plant authentication and identification practices, and good manufacturing practices before and during the manufacturing process, as well as good laboratory practices are mandatory. The authenticity of herbal medicines starting materials free of impurities (heavy metals contamination, pesticide residues, and aflatoxins/mycotoxins) is another major step to ensure the quality of finished herbal medicinal products (Govindaraghavan and Sucher 2015). In Europe, there are specific guidelines of GACP (EMEA/HMPC/246816/2005) and on efficacy, safety and quality of herbal medicinal products (Peschel 2014). Additionally, the International Conference on Harmonisation (ICH) provides ground rules for validation of analytical methods, which in turn ensure the method suitability and ruggedness as a quality measure across multiple laboratories (Govindaraghavan and Sucher 2015; ICH 2005).

Complementary sources of information about herbal medicines are available in specific herbal medicines databases (such as THINKherb and TCMID), and in different collections of monographs on selected medicinal herbs, like in the American Herbal Pharmacopoeia, the British Herbal Pharmacopoeia, the German Commission E, the United States Pharmacopoeia, and the European Pharmacopoeia (Chinou et al. 2014; Pan et al. 2013; Shaw et al. 2012). The WHO had published 117 monographs in four volumes (WHO 1999–2009) and the European Pharmacopoeia include already more than 250 general methods on analysis, being 166 of herbal substances, and more than one hundred of herbal preparations (i.e. extracts, tinctures, essential oils, starches, fatty oils and waxes) (Chinou et al. 2014; Peschel 2014; Vlietinck et al. 2009). European Union herbal monographs, formerly known as Community herbal monographs, are important to support herbal medicinal products registration and authorization. These herbal monographs comprise the scientific opinion of the Committee on Herbal Medicinal Products (HMPC), an EMA committee responsible for compiling and assessing scientific data on herbal substances, that supports the harmonization of the European market on herbal medicinal products. Each monograph includes information about qualitative and quantitative composition, pharmaceutical form(s), therapeutic indication(s), posology and method of administration, contraindications, special warnings and precautions for use, information about interactions with other medicinal products, uses in special populations, effects on ability to drive and use machines, undesirable effects and toxicity information, as well as pharmacodynamic and pharmacokinetic properties and existing preclinical safety data (Chinou et al. 2014).

Herbal medicinal products can be regarded to as well-established and traditional use products in European Union according to Directive 2004/24/EC amending Directive 2001/83/EC (Chinou et al. 2014; Peschel 2014). A traditional medicinal herbal product must follow standards of safety and quality and there must be an evidence that the product (or an equivalent product)

has been in use as a traditional medicinal product for at least 30 years (15 years of which must be in the European Union). HMPC responsibilities, in this matter, include the establishing of herbal monographs, covering the therapeutic uses and safety conditions of well-established and traditional use, and also to list the herbal substances, preparations and combinations thereof for use in traditional herbal medicinal products [Commission implementing decision (EU) 2016/1658, amending Decision 2008/911/EC]. Until December 2012, a total of 1015 registrations as traditional use and 514 authorisations as well-established use have been registered for about 200 different herbal drugs (Peschel 2014). So, it is highly probable that Europe leads the market of herbal products due to the development of effectiveness, quality, and safety standards, based on the robust control of the manufacture and delivery of plant medicines through all stages of production (Alonso-Castro et al. 2015).

In Portugal, herbal products consumption and commercialization are legally regulated according to their categories as dietary supplements (Decreto-Lei n.º 118/2015), plant or plant-based preparations, traditional herbal medicines or plant-based medicines (Decreto-Lei n.º 176/2006).

I.2. Herb-drug interactions

The use of herbal medicines is increasing in many countries. However, it is difficult to evaluate the extent of its use since they can be marketed as dietary supplements, functional foods or cosmetics (Pferschy-Wenzig and Bauer 2015). Concomitant intake of herbal medicines and prescribed medication is also a very common practice, especially in patients with hypertension, diabetes, cancer, epilepsy and depression (Awortwe et al. 2018). The incidence of herb-drug interactions (HDIs) is not really known and the lack of these data may be due to under-reporting or unrecognized interactions. Patients can apparently tolerate adverse effects remarkably well, and many interactions can be accommodated and the effects may not consciously be recognized as the result of an interaction (Williamson et al. 2009).

The under-reporting use of herbal medicines by consumers can also contribute to the under-reporting adverse effects since they believe that herbal medicines are safe due to its natural origin, even if they are consumed at the same time than prescribed drugs. Particularly, elderly and polymedicated patients are more susceptible to the use of these herbal medicines and, therefore, they are also more likely to suffer from potential interactions between herbs and drugs (Alissa 2014; Li et al. 2016). However, the risk of HDIs is already recognized as a public health problem that can lead to life-threatening adverse drug events, prolonged hospitalization and even death (Awortwe et al. 2018).

Due to the increasing use of herbal medicines and supplements by the general population, the research about HDIs has been accelerated (Choi et al. 2016). A review of the literature by Posadzki *et al*. (2013) identified fifty systematic reviews reporting adverse effects of herbal medicines, although associated with moderately severe or minor adverse effects. In another

review were identified adverse drug reactions due to HDIs in fifteen of the reviewed case reports and observational studies. From these, eight patients had central nervous system (CNS) diseases, including two with epilepsy (Awortwe et al. 2018).

As it was previously mentioned, herbal products can contain more than one plant or plant-product, with a complex mixture of bioactive substances or phytochemicals that may act synergistically or additively to exhibit a proper biological and pharmacological activity (Alissa 2014; Brantley et al. 2014; Carmona and Pereira 2013; Oga et al. 2016). So, it is quite difficult to evaluate the individual contribution of each herbal substance for the interaction with other drugs.

Additionally, herbal substances are often administered in combination with conventional drugs, raising the potential of pharmacokinetic and/or pharmacodynamic interactions (He et al. 2010). These HDIs may have clinical relevance when the metabolizing enzymes and transporters responsible for the fate of many drugs are induced or inhibited, resulting in unexpected, and sometimes, fatal consequences (Oga et al. 2016). Indeed, HDIs can be the result of a combination of acute effects (inhibition) and repeated effects (induction) on the drug-metabolizing enzymes and/or transporters.

I.2.1 Pharmacokinetic herb-drug interactions

Contrary to the development of conventional medicines, herbal medicines are usually not studied from a pharmacokinetic point of view, but it seems that most of the pharmacokinetic principles are applied to them. Pharmacokinetic interactions involving herbal medicines can occur at the absorption, distribution, metabolism and excretion levels, the so-called ADME interactions (Alissa 2014), with direct effects on the extent of systemic exposure to drugs and/or metabolites, that is generally observed in the dose-response relationship. Pharmacokinetic-based HDIs can become clinically significant when considerable changes occur in the pharmacokinetic parameters of the co-administered drug that are directly related to the drug's efficacy and toxicity, such as the area under the concentration-time curve (AUC), peak plasma concentration (C_{max}) or time to reach C_{max} (t_{max}) (Tarirai et al. 2010). AUC is indeed the pharmacokinetic parameter that primarily reflects the extent of drug bioavailability (Hsueh et al. 2017). So, the risk of pharmacokinetic interactions entails two major challenges: pharmacotoxicity and treatment failure (Fasinu et al. 2012).

Most herbal medicines are administered orally in a chronic regimen (He et al. 2010). The bioavailability of herbal substances is dependent on many pre-systemic processes that include solubility in the gastrointestinal fluid and membrane permeability, degradation in the gastrointestinal tract, transporter-mediated intestinal efflux, pre-systemic gut wall and hepatic metabolism. Flavonoids, for example, are poorly absorbed due to their large molecular weight and poor solubility in the lipid-rich outer membranes of the enterocytes. Additionally, some flavonoids can be effluxed by P-glycoprotein (P-gp) and others can be extensively susceptible to first-pass metabolism in the gut or in the liver (He et al. 2010).

Herbal medicines can also alter the absorption of concomitantly administered drugs mostly by causing changes in the gastrointestinal pH and in other biochemical factors that can alter dissolution properties and the absorption of pH-dependent drugs. Other mechanisms like complexation and chelation may lead to the formation of insoluble complexes and competition at the sites of absorption, changing the extent of absorption of drugs. The concomitant use of drugs with anthranoid-containing plants like cassia (*Cassia senna*), cascara (*Rhamnus purshiana*), rhubarb (*Rheum officinale*), and soluble fibres can decrease drug absorption by decreasing gastrointestinal transit time (Fasinu et al. 2012).

The extent of protein binding is another factor that affects the ability of one drug to be distributed and have therapeutic or toxic effects. If an herbal substance has affinity to bind serum proteins, it may compete with other drugs for binding to proteins. Displacement of therapeutic agents from binding sites on serum proteins will increase their rates of elimination, and sudden displacement of drugs from serum proteins can increase the free drug concentration to toxic levels (Sprouse and Van-Breemen 2016).

The metabolism of herbal substances shares the same drug-metabolizing proteins, including cytochrome P450 (CYP) enzymes and uridine diphosphate-glucuronosyltransferases (UGTs). It is also true that phytochemicals and drugs share the same transport proteins such as P-gp, from the ATP-binding cassette (ABC) family of transporters, influencing the drug biodisposition. Indeed, some herbal substances are known to influence both transporters and enzymes function. Due to the high expression of P-gp on the epithelial cells lining the intestine, these transporters may change the absorption and bioavailability of herbal substances, being so determinant on its pharmacokinetics, efficacy and toxicity. Additionally, P-gp can be found in the canalicular membranes of hepatocytes, kidney proximal tubules, and brain endothelial cells (Kumar et al. 2010). P-gp has been shown to exhibit up to five substrate binding sites involved in the transport of numerous hydrophobic, amphipathic, cationic and neutral molecules. Thus, P-gp is responsible for extruding a variety of drugs from cells, potentiating multidrug resistance (Han 2011). In the kidneys and liver, P-gp is related to the excretion of toxic substances and in the intestine P-gp-mediated efflux can reduce the bioavailability of drugs that are administered orally. At the blood-brain barrier (BBB), P-gp prevents the entrance of drugs into the CNS. Kaempferol and quercetin are some of the herbal substances that can modulate P-gp activity (Kumar et al. 2010). Other herbal compounds have been reported to be substrates of other drug transporters such as multidrug-resistance associated proteins (MRPs) or breast cancer resistance protein (BCRP). Although less documented, solute-carrier (SLC) protein transporters responsible for the uptake of organic anions [organic anion transporting polypeptides (OATPs) and organic anion transporters (OATs)], organic cations [organic cation transporters (OCTs)], peptides [peptides transporters (PEPTs)], and multidrug and toxic compound extrusion (MATE) transporters implicated in the efflux of metabolic waste products and xenobiotics have also been related to HDIs. For instance, the flavonoids apigenin, quercetin and kaempferol block the transporter functions of OATP1A2 and OATP2B1, which are localized in the apical membrane of the intestinal lumen (Husain et al. 2016). Ellagic acid, caffeic acid and rhubarb

anthraquinones are strong inhibitors of OATs, being the rhubarb anthraquinones strong inhibitors of the human OAT1 and OAT3, which are almost exclusively expressed in kidneys (Lu et al. 2017).

Biotransformation reactions mediated by metabolic enzymes are classified into two categories, namely, phase I biotransformation reactions (such as oxidation, reduction, hydrolysis and hydration) and phase II biotransformation reactions (such as sulfation, methylation, acetylation, glutathione conjugation, fatty acid conjugation and glucuronidation) (Tarirai et al. 2010). The biotransformation of herbal substances in phase I is performed mostly in liver by the isoforms of CYP enzymes (CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A). The phase I enzymatic reactions make an herbal substance more susceptible to phase II reactions, which are conjugation reactions, originating molecules more easily excreted, either by biliary or renal route (Choi et al. 2011). UGTs enzymes are differentially expressed in tissues, with liver and intestine being the main sites, and these enzymes are responsible for drug glucuronidation reactions. As many phytochemicals are primarily glucuronidated by UGT1A enzymes, there is a potential for HDIs through competition with drug substrates for this metabolic pathway (Mohamed and Frye 2011). For example, UGT1A enzymes mediate the conjugation of many flavonoids, anthraquinones, coumarins, catechins, and curcuminoids (He et al. 2010).

Herbal substances can either inhibit or induce the enzymes responsible for the metabolism of therapeutic drugs or their transporters (**Figure I.1**). By inhibiting the action of specific drug-metabolizing enzymes, herbal substances can prolong the half-life of drugs that depend on the same enzymes for their degradation, deactivation, or conjugation prior to excretion. Longer half-lives result in prolonged action or even toxicity, especially if drug levels rise unexpectedly after multiple doses (Sprouse and Van-Breemen 2016). The ursolic acid, a natural triterpene, is an inhibitor of CYP3A, and *Echinacea purpurea* is known to inhibit CYP1A2 activity in humans (Oga et al. 2016; Sprouse and Van-Breemen 2016). *Citrus aurantium* is also known to inhibit the P-gp and the CYP3A4 isoenzyme, a major drug-metabolizing enzyme (Tarirai et al. 2010). Valerian has demonstrated potential for HDIs through inhibition of UGTs (Fasinu et al. 2012). On the other hand, enzyme induction can shorten drug half-lives, which may result in subtherapeutic levels of drugs in the body (Sprouse and Van-Breemen 2016).

Clinically, induction phenomena increase clearance or decrease bioavailability of the victim drug, leading to a decrease in systemic drug exposure. Induction usually requires time and should be monitored upon chronic exposure to the herbal substance in order to identify time-dependent changes in systemic drug exposure (Brantley et al. 2014). *Hypericum perforatum* (St. John's wort), quercetin and rutin (flavonoids), are known to induce both P-gp and CYP3A4. These changes of normal P-gp efflux and CYP activity may have impact on the pharmacokinetic disposition of P-gp and CYP3A4 substrate drugs, leading to lower efficacy of the victim drug (Oga et al. 2016; Vieira and Huang 2012; Zhang et al. 2017).

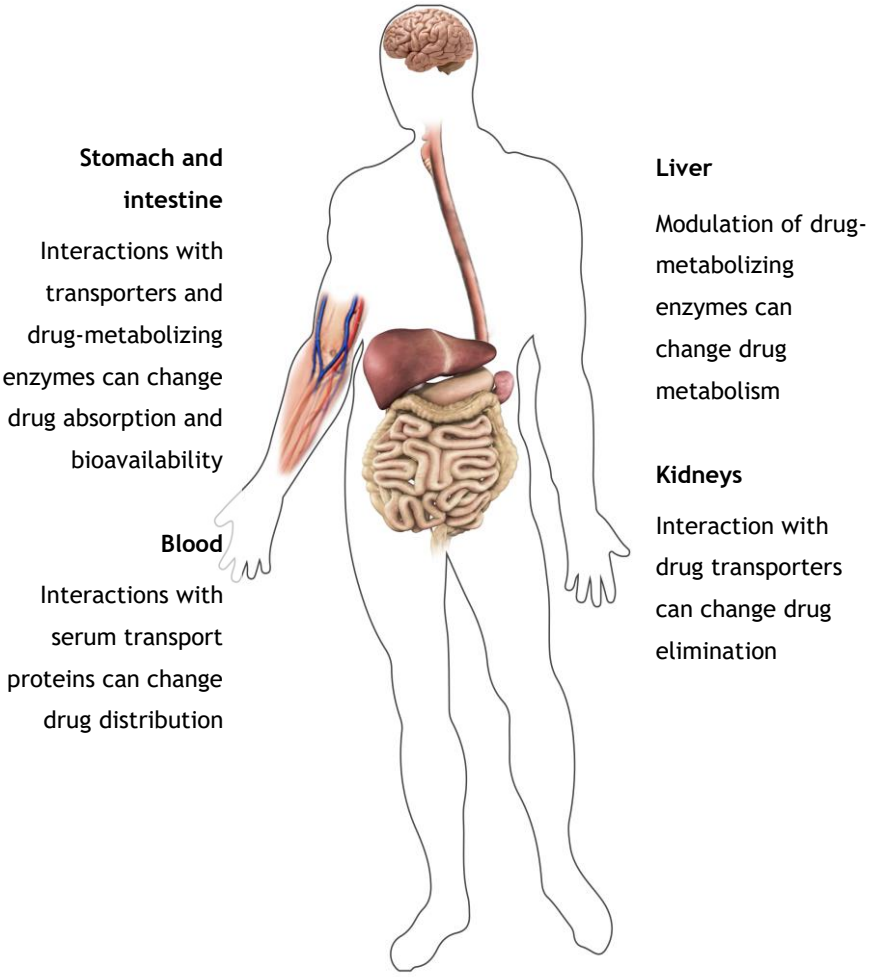


Figure I.1. Main sites for potential pharmacokinetic-based herb-drug interactions (Sprouse and Van-Breemen 2016).

HDIs involving enzyme induction can be delayed in its onset and more slowly resolved, but this type of interactions is less common than those based on inhibition mechanisms. This last phenomenon can occur within two to three days, resulting in the rapid development of toxicity. Inhibition can be reversible, being additionally competitive [perpetrator binds to the active site of the enzyme, preventing the victim drug from binding, so changing the half-maximal rate of metabolism (K_m)]; non-competitive [(perpetrator does not bind at the same active site of the enzyme, decreasing the maximal rate of metabolism (V_{max})]; and uncompetitive (perpetrator binds to the enzyme–victim drug complex, modulating both K_m and V_{max}) (Brantley et al. 2014).

Excretion of herbal substances can be performed by the same excretion routes of free drugs or metabolites, usually via urine and faeces, and rarely by skin or lungs, being kidneys the main route of excretion of herbal substances (Jha 2010). Excretion is mediated by passive glomerular filtration of small molecules not bound to plasma proteins and by active tubular secretion in the proximal tubule, which is a key site of reabsorption of salts, small molecules and proteins

(Lepist and Ray 2016). Renal clearance is normally considered the net result of glomerular filtration, tubular secretion, and reabsorption. Considering that many substances are mainly cleared by kidneys, HDIs involving changes in transporters activity can affect their systemic and tissue concentrations. One of the reasons for limited reports on renal elimination is that many drugs are subject to parallel elimination pathways including passive glomerular filtration and hepatic elimination. Indeed, many enzymes and transporters are expressed in kidneys, such as some UGT isoforms and OAT1 and OAT3. These OATs have been already identified as targets for HDIs. *Geranium tuberosum* extract, used in folk medicine, has been identified as a potent OAT1 inhibitor and *Glycyrrhiza glabra* and *Juniperus oblonga* extracts, among others, have been related to OAT3 inhibition (Lu et al. 2017).

I.2.2. Pharmacodynamic herb-drug interactions

Pharmacodynamic interactions are those interactions that causes changes in the pharmacological response (e.g., changes in the physiological effect and mechanism of action of the drug on the body). The mechanisms of pharmacodynamic interactions may result in augmentation or reduction of the pharmacological activity of a co-administered drug. Pharmacodynamic-based HDIs can, therefore, involve changes in the pharmacological effects of the drug through additive, synergistic or antagonistic actions (Alissa 2014; Fasinu et al. 2012; Tarirai et al. 2010). One example of a particular additive effect is the one that occurs between the *Hypericum perforatum* (St. John's wort) and conventional medicines. St. John's wort inhibits the reuptake of 5-hydroxytryptamine (5-HT, serotonin) and this effect may result in a clinically important pharmacodynamic interaction known as serotonin syndrome. The reasons for this effect are not fully understood, but the serotonin syndrome is thought to occur as a result of the overstimulation of 5-HT1A and 5-HT2A receptors and possibly in other serotonin receptors of the CNS. This syndrome can develop shortly after one serotonergic drug is added to another, or even if one is replaced by another without allowing a long enough washout period in between (Williamson et al. 2009).

In contrast to additive interactions, antagonistic interactions occur when a drug has an activity that is opposed to that of another drug. For instance, coumarin anticoagulants can prolong the blood clotting time by competitively inhibiting the effects of dietary vitamin K. If the intake of vitamin K is increased, the effects of the coumarin anticoagulants are reduced and the prothrombin time can return to normal values, thereby cancelling out the therapeutic benefits of anticoagulant treatment. There is some evidence that high doses of some individual flavonoids, such as hesperidin and baicalin, may have additive anxiolytic effects with benzodiazepines, suggesting a possible pharmacodynamic interaction. Additive loss of potassium and water by anthraquinone-containing substances and potassium-depleting diuretics has already been identified (Williamson et al. 2009).

I.2.3. Herb-drug interactions evaluation

Prediction and evaluation of HDIs may ideally prevent and minimize the severity of the adverse effects potentially caused by these interactions. Information about HDIs is usually based on the scientific literature and translated into general recommendations regarding the use of herbal products containing a specific substance. However, the interaction potential of one specific herbal substance is difficult to extrapolate to other products with origin from the same raw source material. So, for traditional and well-established herbal preparations, the potential for interaction should be clarified if reports point to clinically relevant HDIs in humans (EMA 2012).

Data from *in vitro* experiments, preclinical and clinical studies, and *in silico* simulations can provide a mechanistic framework to address the potential for HDIs. The number of scientific reports related to HDIs has increased in the past decade, although most of them involved the use of *in vitro* systems such as hepatic microsomes or cytosolic fractions (Roe et al. 2016).

Actually, *in vitro* systems are useful tools to estimate the contribution of drug-metabolizing enzymes and transporters in the disposition of a drug. These metabolic systems include microsomes and recombinant enzymes and they are commonly used to assess the potency and mode of enzyme inhibition. On the other hand, induction assays must be performed on intact cells, since induction assessment is dependent upon measurement of mRNA or protein expression for both metabolic enzymes and transporters. Human hepatocytes are adequate for analysing induction response since in the immortalized cells (e.g., HepG2) the expression of transcription factors or nuclear receptors can be altered (Brantley et al. 2014).

Appropriate animal models have several advantages in the investigation of HDIs when compared to *in vitro* systems, as they can provide more reliable estimates of the exposure to drug and/or metabolites after administration of the drug itself. Additionally, animal models are critical in the drug development process, even for herbal substances. Several key characteristics of drug disposition can only be determined in *in vivo* conditions, particularly the contribution of metabolic and excretory routes. Moreover, the contribution of an enzymatic pathway to overall elimination can only be estimated using *in vivo* data (Brantley et al. 2014; Roe et al. 2016). However, animal models have a major disadvantage when compared to human models. Animals may have different metabolic and transport pathways and differences in tissue expression or substrate specificity can also exist (Brantley et al. 2014).

Despite the limitations associated with the costs involved, phytochemical variability in commercial supplements, phytochemical bioavailability and biomarkers detection, among others, *in vivo* human studies are undoubtedly the most reliable and realistically studies for assessing HDIs (Gurley 2012). In this last decade, modelling and simulation-based approaches have also become useful tools. Sophisticated approaches, such as physiologically-based pharmacokinetic modelling and simulation, are preferable when compared to the single kinetic approach (Brantley et al. 2014).

I.3. Plants, overweight and obesity

Obesity is defined as a phenotypic manifestation of abnormal or excessive fat accumulation. Primary obesity is normally caused by an increased intake of high-fat diets. Iatrogenic or secondary obesity can be related to drug treatments (like antidepressants, antiepileptics, steroids, insulin), or to certain diseases (like Cushing syndrome, hypothyroidism, hypothalamic defects) (González-Castejón and Rodriguez-Casado 2011). At a cellular level, obesity is characterized by an increase in the number (hyperplasia) and size (hypertrophy) of adipocytes (Jayarathne et al. 2017). Additionally obesity is directly related to a body mass index (BMI) higher than 30 kg/m^2 (de Freitas Junior and de Almeida 2017; González-Castejón and Rodriguez-Casado 2011). The pathophysiology of obesity and overweight is complex and involves the interaction of various factors including genetic, metabolic, environmental, and behaviour determinants (Bahmani et al. 2016). Obesity is one of the major risk factors for type 2 diabetes, cardiovascular disease, hypertension, dyslipidaemias, musculoskeletal diseases and cancer (Cercato et al. 2015; de Freitas Junior and de Almeida 2017; Esteghamati et al. 2015; Jayarathne et al. 2017; Mopuri and Islam 2017). Recent data indicate that about 13% of the world's adult population (11% of men and 15% of women) were obese in 2016, and 39% of adults aged 18 years and over (39% of men and 40% of women) were overweight (WHO 2017).

The use of herbal extracts for weight loss is rapidly growing (Astell et al. 2013) as an alternative and complementary therapy to treat obesity (Ríos-Hoyo and Gutiérrez-Salmeán 2016). In a review about the use of medicinal plants for obesity treatment, seventy-six plant species have been used to treat obesity through pharmacological approaches as well as in pre-clinical and clinical trials. These species were from among fifty-two botanical families, the most prominent being *Asteraceae* with approximately 13.16% of species, and *Fabaceae* with 7.89%. Moreover, phenolic compounds, especially flavones, flavanols, flavanones, catechins, anthocyanins, isoflavones and chalcones, were presented as the main secondary metabolites responsible for anti-obesity action. Among others, *Citrus aurantium*, *Fucus vesiculosus, Garcinia cambogia and Paullinia cupana (var. sorbilis)* were described, in this review, as anti-obesity substances (de Freitas Junior and de Almeida 2017). Several other authors have also reviewed the anti-obesity activity of plants and phytochemicals with potential use in obesity treatment, considering data obtained from *in vitro* assays, and non-clinical and clinical studies (Hasani-Ranjbar et al. 2013; Martel et al. 2017; Mopuri and Islam 2017; Patra et al. 2015).

Herbal substances having anti-obesity activity can be distinguished by their mechanisms of action and effects, acting in particular as appetite suppressants, decreasing lipid absorption and energy intake, increasing energy expenditure, decreasing pre-adipocyte differentiation and proliferation, decreasing lipogenesis or increasing lipolysis (de Freitas Junior and de Almeida 2017; Mopuri and Islam 2017).

Suppression of appetite is a complex process regulated by gut, brain and adipose tissues. When stomach is empty, the hormone ghrelin is released by the gastrointestinal tract and induces hunger by acting on hypothalamic brain cells in the CNS. In opposition, the presence of

food in the gastrointestinal tract activates the vagus nerve afferent pathway, leading to inhibition of the hunger centre in the brain. Similarly, food intake induces the release of cholecystokinin by epithelial cells of the small intestine, which inhibits the activity of hunger-stimulating neuropeptide Y in the hypothalamus (Martel et al. 2017). Noradrenaline, dopamine, 5-HT and endocannabinoids also regulate appetite and satiety. The hormone leptin, released from adipocytes upon stimulation with insulin, inhibits the activity of neuropeptide Y and the hunger-stimulating fatty acid neurotransmitter anandamide and also activates the hunger-suppressing peptide α-melanocyte-stimulating hormone (Martel et al. 2017). For instance, celastrol, a pentacyclic triterpenoid compound found in the roots of the thunder god vine, has appetite-suppressing activity (**Figure I.2**). *Garcinia cambogia, Hoodia gordonii* and *Camellia sinensis* have also shown appetite-repression activity (Martel et al. 2017; Yun 2010).

Adipocytes, the main cellular component of adipose tissues, have important endocrine functions as they release hormones and cytokines (adipokines) that regulate homeostatic processes including satiety, energy levels and immune function (Martel et al. 2017). Hypertrophied adipocytes secrete more pro-inflammatory adipokines, such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6), than adipocytes of normal size. These pro-inflammatory adipokines interfere with insulin signalling, glucose and lipid metabolism in muscles, liver and adipose tissues and also induce chronic inflammation (González-Castejón and Rodriguez-Casado 2011; Wan-Loy and Siew-Moi 2016).

Inhibition of lipid accumulation in adipocytes and inhibition of pancreatic lipase activity are considered major anti-obesity strategies (Alonso-Castro et al. 2015; Yun 2010). Brown algae containing fucoidans and fucoxanthins have demonstrated inhibitory effects on pre-adipocyte differentiation (Yun 2010). Other phytochemicals, including genistein (an isoflavone found mainly in soy), glycyrrhizin (found in liquorice), capsaicin (found in chilli peppers) and quercetin have similar antiproliferative and pro-apoptotic effects on adipocytes (**Figure I.2**) (Martel et al. 2017). Pancreatic lipase is a key enzyme in dietary triacylglycerol absorption, hydrolysing triacylglycerols to monoacylglycerols and fatty acids, being responsible for the hydrolysis of 50–70% of total dietary fats (Birari and Bhutani 2007). Pancreatic lipase inhibitory phytochemicals include carbohydrates (like chitin and chitosan), saponins (tea saponins), polyphenols, flavonoids, and caffeine. Marine algae extracts also have demonstrated lipase inhibitory activity (Birari and Bhutani 2007; Yun 2010).

Lipid accumulation and energy storage might also be reduced by the induction of thermogenesis in adipocytes and muscles. Thermogenesis is normally activated by cold, which stimulates transient receptor potential channels on sensory neurons, transmitting signals to the brain and activating the sympathetic nervous system. Several herbal substances including capsaicin and catechins also activate transient receptor potential channels on neurons, thus promoting thermogenesis. Ephedrine and caffeine (present in *Camellia sinensis, Paullinia cupana* and *Ilex paraguariensis*) have also been linked to increased energy expenditure and lipolysis induction (Martel et al. 2017; Yun 2010).

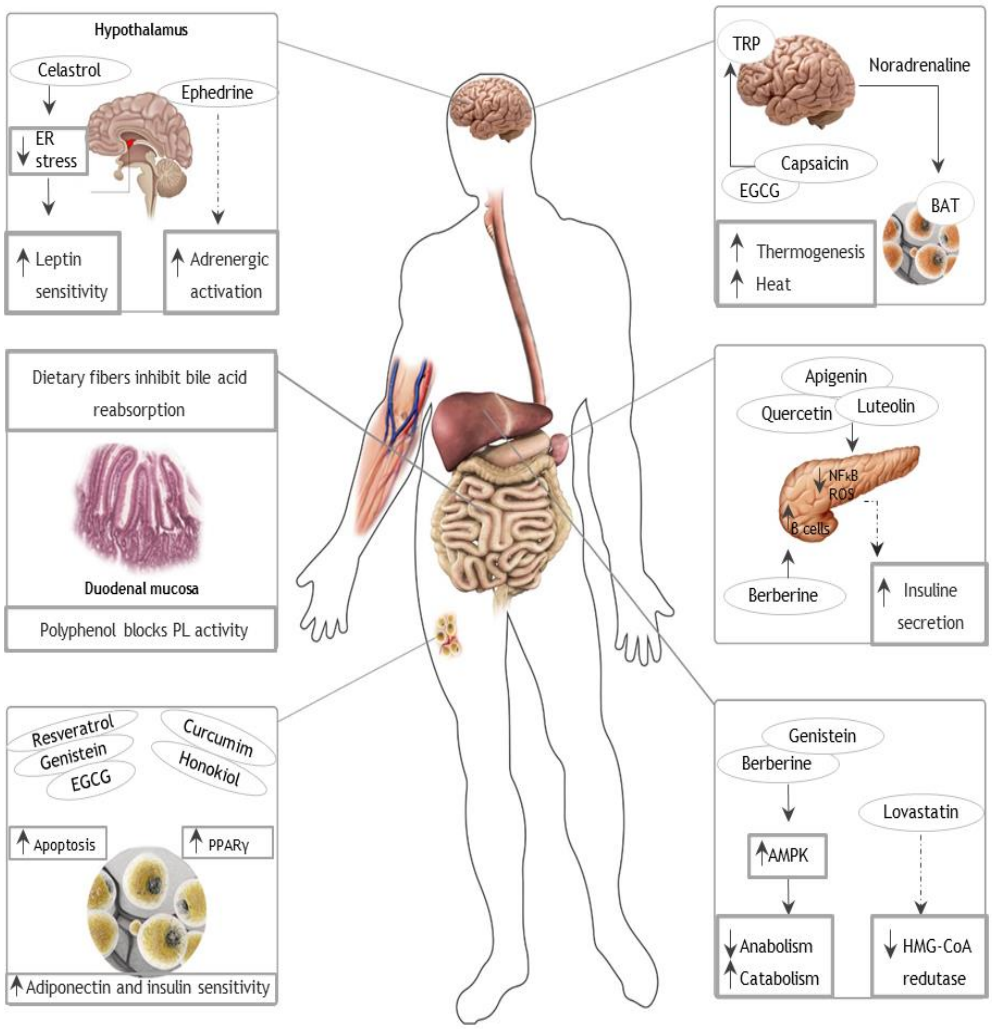


Figure I.2. Anti-obesity effects of some plant substances (AMPK, AMP-activated protein kinase; BAT, brown adipose tissue; EGCG, epigallocatechin gallate; ER, endoplasmatic reticulum; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; NFκB, nuclear factor κ-light-chain-enhancer of activated B cells; PL, pancreatic lipase; PPARγ, peroxisome proliferator-activated receptor γ; ROS, reactive oxygen species; TRP, transient receptor potential channels on sensory nerves) (Martel et al. 2017).

In obesity several mechanisms can lead to thyroid dysfunction such as the influence of leptin, thyroid hormone resistance, and mitochondrial dysfunction (Witkowska-Sędek et al. 2017). Thyroid-stimulating hormone (TSH) has receptors in pre-adipocytes and induces differentiation of pre-adipocytes into adipocytes and expansion of the adipose tissue. Additionally, leptin and TSH are closely related to the regulation of thyroid and fat metabolism. On the one hand, leptin stimulates the transcription of pro-thyrotropin-releasing hormone, which leads to the increase of thyrotropin-releasing hormone and TSH concentrations.

On the other hand, TSH may directly stimulate the differentiation of pre-adipocytes into adipocytes and the production of leptin by adipocytes. The secretion of inflammatory cytokines by adipose tissue is thought to be another mechanism responsible for increasing the TSH concentrations in obese patients. Additionally, TSH plays a role in the regulation of adipokines

synthetized in mature adipocytes and in the induction of pre-adipocyte proliferation and differentiation. So, thyroid hormones and adipocytes are intrinsically related and interdependent. Particularly, the triiodothyronine hormone, also known as T3, controls metabolic and energy homeostasis and can influence body weight, thermogenesis, and lipid metabolism (Witkowska-Sędek et al. 2017).

Considering the anti-obesity effects traditionally claimed for several plants, in the work underlying this doctoral thesis were mainly addressed extracts of four herbal species: *Paullinia cupana (P. cupana), Garcinia cambogia (G. cambogia), Citrus aurantium (C. aurantium) and Fucus vesiculosus (F. vesiculosus)*.

I.3.1. *Paullinia cupana*

P. cupana from *Sapindaceae* family, also known as Guarana, is being consumed all over the world in herbal supplements and stimulating drinks (Portella et al. 2013). It is also an ingredient in some soft drinks, non-alcoholic beverages and cosmetics (Hamerski et al. 2013). Guarana, uarana or varana is related to "vine" in various indigenous dialects, and it was so named due to the liana growth habit of this perennial plant. This native climbing Amazonian plant has been described since 1669 as having stimulant and medicinal properties and has been used for centuries by indigenous communities of this region.

Guarana consumption had been reported in Europe and in the United States in the 18th and 19th centuries, and in Brazil guarana became more popular in the beginning of the 20th century. In the last two decades guarana has increased its popularity as a key ingredient in various 'sports' and energy drinks particularly among adolescents and young adults (Portella et al. 2013). About 70% of the brazilian production of guarana seeds is used for beverage production, 20% is used in the pharmaceutical and cosmetic fields and about 10% is sold as guarana powder (Schimpl et al. 2013).

Seeds of *P. cupana* are one of the most important parts of the plant due to its high caffeine content (2-8%) **(Figure I.3).** The two other methylxanthines theophylline and theobromine **(Figure I.3)** are also found in small amounts (< 0.3%) in seeds, as well as in guarana bark, flowers and leaves (Ashihara et al. 2008; Schimpl et al. 2013).

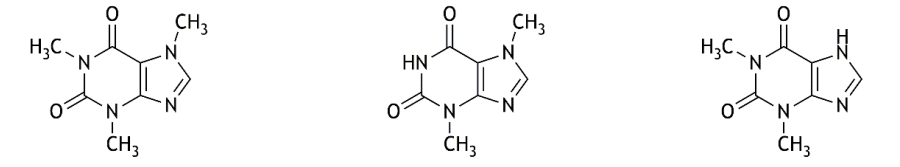


Figure I.3. Chemical structures of methylxanthines present in *Paullinia cupana* (Schimpl et al. 2014).

Others constituents found in *P. cupana* seeds are starch, polysaccharides, tannins, catechins, epicatechins, proanthocyanidins, lipids, saponins, proteins, choline and pigments (Lima et al. 2017; Schimpl et al. 2013). In addition to reserve polysaccharides, the seeds contain structural polysaccharides from the cell wall (Dalonso and Petkowicz 2012). Tannins are believed to be related to the brown colour of guarana-based energetic tonics that results from the interaction of tannins and caffeine (Schimpl et al. 2013).

Among the species that produce caffeine, *P. cupana* has the higher natural content when compared to coffee (*Coffea arabica),* tea *(Camellia sinensis)* and mate *(Ilex paraguariensis)* (Ashihara and Crozier 2001; Ashihara et al. 2008). In fact, depending on how the extract is prepared, *P. cupana* extracts can contain more than four times the amount of caffeine found in coffee beans (Moustakas et al. 2015). Mostly due to the presence of caffeine the traditional use of *P. cupana* has been related to fatigue and weakness (EMA 2013).

In addition to caffeine stimulating activity on the CNS, other effects have been attributed to guarana, such as improvement of alertness, reaction time, speed of information processing, memory, mood and performance in physical exercises (Schimpl et al. 2013). Also due to the presence of caffeine, some studies have demonstrated that products containing *P. cupana* affect lipid metabolism, enhance weight loss, and increase basal energy expenditure, acting as thermogenic or metabolic stimulant products (Glade 2010; Hamerski et al. 2013; Portella et al. 2013). In fact, caffeine increases the excitability of adenosine-sensitive sympathetic nervous system, stimulating fat lipolysis, increasing energy expenditure and satiety, and decreasing hunger (**Figure I.4**) (Glade 2010; Harpaz et al. 2017).

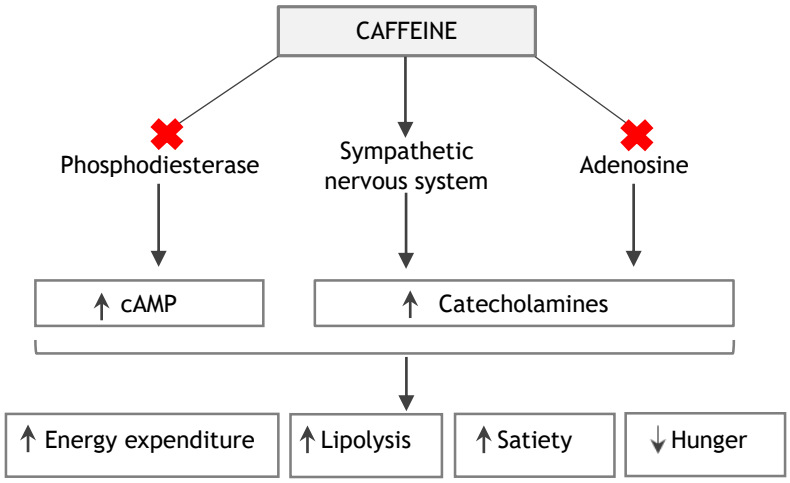


Figure I.4. Caffeine effects in thermogenesis and energy intake (cAMP, cyclic adenosine monophosphate) (Harpaz et al. 2017).

The increased lipolysis, heat production in skeletal muscle and satiety signals in the liver are dependent on the production and presence of cyclic adenosine monophosphate (cAMP). Caffeine can inhibit phosphodiesterase which result in the increase of cAMP response. Caffeine

can also antagonize the effect of adenosine in the presynaptic nerve terminals, and thus enhancing the release of catecholamines. These effects of caffeine potentiate postsynaptic neurotransmission in the sympathetic nervous system and so the effects in thermogenesis and energy intake (Glade 2010; Harpaz et al. 2017).

Several other pharmacological effects are related with *P. cupana* consumption including antiplatelet aggregation, cardioprotective, antioxidant, antidepressant, antimicrobial and chemopreventive (Hamerski et al. 2013). A weak diuretic effect is probably related to the presence of theobromine. Theophylline also has stimulant effect similar to caffeine, but to a lesser extent, and is characterised as a bronchodilator (Schimpl et al. 2013). The extracts of *P. cupana* showed antidepressant, anxiolytic and anti-amnesic effects. The antidepressant activity after long term treatment was comparable to that of the tricyclic antidepressant imipramine and it had a beneficial effect on cognition without altering locomotor activity (Hamerski et al. 2013). Catechins and pectic polysaccharides exhibit an important antioxidant activity (Dalonso and Petkowicz 2012; Schimpl et al. 2013) and catechins also modulate the expression of several genes associated with adipogenesis (Lima et al. 2017). Additionally, experimental *in vitro* and *in vivo* studies suggested that catechins were able to inhibit the intestinal absorption of dietary lipids. These molecules have potential to inhibit the glycerol-3-phosphate dehydrogenase that catalyses the ß-nicotinamide adenine dinucleotide (NADH)-dependent reduction of dihydroxyacetone phosphate to yield glycerol-3-phosphate, which is one of the major precursors of triacylglycerols (Suleiman et al. 2016).

In what concerns the safety use of *P. cupana*, there is no data regarding its use in children and adolescents under eighteen years old and so consumption should be avoided in these age groups. In addition, the consumption is not recommended before bedtime since it may cause sleep disturbances (EMA 2013). Additionally, *P. cupana* consumption is contraindicated in cases of hypersensitivity to the active substance, gastric and duodenal ulcers, hyperthyroidism and cardiovascular disorders such as hypertension and arrhythmias. Patients under monoamine oxidase inhibitors therapy should also be monitored. It is important to refer that caffeine-containing preparations reduce sedative effects and increase side effects caused by sympathomimetic drugs (EMA 2013).

Adverse reactions were associated with *P. cupana* ingestion in seven cases involving multi-ingredient plant-food supplements, and two of them related to severe symptoms. For instance, a 30-year-old man taking a supplement containing *P. cupana, C. aurantium*, *Camellia sinensis* and *Coleus forskohlii*, in combination with a product containing *Rhodiola rosea* to lose weight suffered a myocardial infarction. In another case, a 40-year-old patient ingested a supplement containing *P. cupana, Panax ginseng, Ilex paraguariensis*, *Lepidium meyenii*, *Turnera diffusa*, *Avena sativa* and *Capsicum* sp. and suffered a transient ischemic attack (Lüde et al. 2016). Also, other twenty-eight cases of abuse or misuse involving *P. cupana* were reported in adolescents, who presented some clinical effects like vomiting and agitation/irritability (Biggs et al. 2017).

Interactions between caffeine and other components of caffeine-containing products can also increase the risk of adverse effects. In fact, 90% of caffeine is primary cleared by liver through CYP1A2 and hepatic disease like cirrhosis and hepatitis can reduce caffeine clearance. The severity of the adverse effects of caffeine ingestion usually is dose-dependent and the consumption of 200 mg or less of caffeine is not usually associated with toxic effects. The European Food Safety Authority has recommended 400 mg/day as the maximum safe amount of caffeine for healthy non-pregnant adults, 200 mg/day for healthy pregnant women, and 3 mg/kg body weight per day for children (EFSA 2015). A dose higher than 300 mg of caffeine taken at once can indeed result in caffeine intoxication and the ingestion of 1–2 g of caffeine has been already related to seizures or arrhythmias (Musgrave et al. 2016).

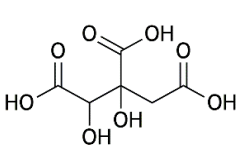
Considering that weight loss caffeine-containing products, like *P. cupana* extracts, may have a typical caffeine content of 6-200 mg per dose, and that an instant coffee has about 65 mg of caffeine and tea may have 50-80 mg of caffeine, the recommended caffeine threshold per day can be easily reached if concomitant consumption of different caffeine-containing products occurs (Musgrave et al. 2016).

Actually, several case reports of seizures ascribed to caffeine have been described and the caffeine intake should be a factor to consider in achieving and maintaining seizure control in epilepsy (van Koert et al. 2018).

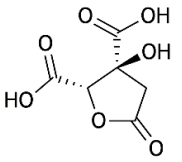
I.3.2. *Garcinia cambogia*

G. cambogia or *Garcinia gummi-gutta* is a native plant of the South-eastern Asia (India, Nepal and Sri Lanka), and it is also found in subtropical regions, including Malaysia, Philippines and China. From a medicinal perspective, *G. cambogia* is one of the most important members of the *Clusiaceae* family (Semwal et al. 2015). The fruits of *G. cambogia,* known as Malabar tamarind, are edible but very acidic. Due to its sweet and sour taste, *G. cambogia* fruit pure compounds or fruit extracts are commonly used in cooking as appetite moderators, as flavouring and food preservative agent (Sripradha et al. 2016; Yu 2017). Tamarind extracts have been used to enhance the flavour of food such as meat, shellfish, and some beverages. Traditionally the fruit extracts have been used to achieve a feeling of satiety after eating (Márquez et al. 2012). They have an economically value as flavour condiment, especially in fish curries, and also as a polishing and varnish. In terms of therapeutic properties, the fruit rind has been used to treat rheumatism and bowel complaints and is also used as a purgative, hydragogue, anthelmintic and emetic. It has also been used in veterinary medicine to treat mouth diseases in cattle (Semwal et al. 2015).

G. cambogia fruits contain several secondary metabolites like organic acids, such as hydroxycitric acid (HCA), xanthones (e.g. oxy-guttiferones I, K, K2 and M), amino acids (like glutamine, glycine and γ-aminobutyric acid) and benzophenones (guttiferones I, N, J, K and M). HCA is the *G. cambogia* fruit's major bioactive phytochemical (**Figure I.5**) (Sripradha et al. 2016).



Hydroxycitric acid



Hydroxycitric acid lactone

Figure I.5. Chemical structures of hydroxycitric acid and hydroxycitric acid lactone present in *Garcinia cambogia* (Semwal et al. 2015).

In the bark of *G. cambogia* other phytochemicals are found namely the rheediaxanthone A (a xanthone), and garcinol (camboginol or guttiferone E) and isogarcinol (cambogin) as benzophenones. Garbogiol (another xanthone) can be found in the roots of *G. cambogia* (Semwal et al. 2015).

The stereoisomer (-)-HCA is particularly found in *Garcinia* species (*G. cambogia, Garcinia atroviridis* and *Garcinia indica)* instead of the (+)-HCA isomer, which is found in *Hibiscus* species (Chuah et al. 2013). HCA can be found in *Garcinia* fruits in approximately 10 to 30% and can be isolated in the free form as a mineral salt or as a lactone (**Figure I.5**) (Márquez et al. 2012; Semwal et al. 2015; Yu et al. 2017).

The free acid form of HCA is considered to be biologically active, but as the free acid is unstable it is converted to its more stable lactone form. In commercially available food supplements, HCA salts with sodium, calcium–potassium, or magnesium are usually used in order to increase the stability of the acid and prevent it from being converted into its lactone form, which is thought to be less active (Bakhiya et al. 2017).

Various extracts, as well as pure compounds obtained mainly from *G. cambogia* fruit, have shown bioactivity in both *in vitro* and *in vivo* models, as appetite-suppressant and anti-obesity agents; other properties such as hypolipidemic, antidiabetic, anti-inflammatory, antioxidant, hepatoprotective, anticancer, anti-ulcer, anticholinesterase, antimicrobial, anthelmintic and diuretic, as well as on fertility have also been described for *G. cambogia*-based products. *In vivo* studies have confirmed that *G. cambogia* or the HCA itself stimulate fat oxidation, increasing serotonin release in brain cortex and normalising lipid profiles in humans. In opposition, *in vitro* anticancer and antimicrobial activities need to be confirmed in *in vivo* assays (Semwal et al. 2015). In rodents, *G. cambogia* fruit extracts have also been associated with weight loss and appetite suppression activity, probably due to the increase in brain serotonin levels, the reduction in plasma insulin levels and the inhibition of the enteral absorption of glucose (Hayamizu et al. 2003; Ohia et al. 2001; Wielinga et al. 2005).

G. cambogia supplements and extracts, with 50 to 60% of HCA (Bakhiya et al. 2017; Márquez et al. 2012) are indeed gaining popularity for weight loss and weight management mainly due to the claimed appetite-suppressant, anti-obesity and hypolipidemic activities (Lopez et al. 2014; Semwal et al. 2015; Yu et al. 2017). The potent inhibitory effect of HCA isolated from *G.*

cambogia on the adenosine triphosphate (ATP) citrate lyase, a key enzyme in the biosynthesis of fatty acids, has been reported to have important effects on lipogenesis (Jena et al. 2002; Márquez et al. 2012; Vasques et al. 2013). Additionally, decreased levels of serum triglycerides and cholesterol as well as enhanced gluconeogenesis and glycogenesis have also been ascribed to HCA (Bakhiya et al. 2017; Esteghamati et al. 2015; Mopuri and Islam 2017).

Preclinical studies confirmed the body weight reduction, appetite suppression, and subsequently food intake reduction effects of HCA in rats (Chuah et al. 2013). In humans, data on weight management and hypolipidemic activity of HCA seem to be somewhat controversial. In a study the daily administration of 300 mg of HCA during 14 days was found to reduce the body weight and the 24-hour energy intake. In another investigation, doses of 2800 mg/day and 5600 mg/day, in obese individuals were found to be safe for appetite suppression and weight management. However, other authors found no significant changes in body weight, including in a randomised clinical study that involved the administration of 1500 mg of HCA per day for 12 weeks. Another clinical trial conducted in obese women, who received orally 800 mg three times daily of *G. cambogia* extract with 50% of HCA for 60 days, revealed a reduction in triglycerides levels. It seems that in obese individuals the use of *G. cambogia* extracts has more benefits than in healthy subjects (Chuah et al. 2013; Semwal et al. 2015). Nevertheless, considering that some *Garcinia* or HCA-containing supplements marketed for weight management are indeed a combination of herbal substances and other active ingredients, the specific effects of *Garcinia* or HCA are difficult to be evaluated (Chuah et al. 2013).

The safety of *G. cambogia* supplements for weight control has also been questioned in several reports, although most of them revealed that *G. cambogia* did not have significant toxic effects. Chuah *et al.* (2012) reviewed the results of seventeen clinical studies in which the safety of HCA and related supplements for human consumption was demonstrated. Indeed, the no observed adverse effect level (NOAEL) of *G. cambogia* up to 2800 mg/day suggests that it is safe for human use. Hayamizu *et al.* (2008) investigated the effect of *G. cambogia* extract (1667.3 mg/day corresponding to 1000 mg HCA/day), administered during twelve weeks, on serum sex hormones in overweight subjects and they found no significant changes in the sex hormones and blood parameters.

However, formulations containing *G. cambogia* as a key ingredient in addition to other ingredients exhibited various toxic effects (Semwal et al. 2015). Hydroxycut®, for example, a dietary supplement constituted by *G. cambogia*, *Cissus quadrangularis*, caffeine, ephedra and green tea, among others, was related to liver injury and high levels of transaminases (Garcia-Cortes et al. 2016; Lunsford et al. 2016; Stickel and Shouval 2015). Despite the controversial hepatotoxic potential, *G. cambogia* has been linked to hepatic fibrosis, inflammation, and oxidative stress (Zheng and Navarro 2015). Additionally, toxic effects on testis have been reported related to high doses of *G. cambogia* (Yu et al. 2017). Lopez and collaborators (2014) reported a case of suspected serotonin toxicity related to the simultaneous administration of selective serotonin reuptake inhibitors with a nutritional supplement containing *G. cambogia* and HCA. In addition, several case-reports of (hypo)mania and/or psychosis following the

administration of *G. cambogia*-containing products have also been published (Cotovio and Oliveira-Maia 2016; Nguyen et al. 2017).

Recently, Yu and collaborators (2017) investigated the potential role of *G. cambogia* extract on CYP enzymes in *in vitro* conditions and the extract had significant inhibitory effects on CYP2B6 activity in a concentration-dependent manner. Additionally, HCA as the major constituent of *G. cambogia* extract also was tested; HCA showed inhibitory effects on several CYP isoenzymes other than CYP2B6, suggesting that the inhibition of CYPs mediated by HCA is not specific. Hence, the authors suggested that components other than HCA may be responsible for the inhibitory activity of *G. cambogia* extract against CYP2B6.

I.3.3. *Citrus aurantium*

Bitter, sour or Seville orange (*C. aurantium*) is a flowering, fruit-bearing evergreen tree native to tropical Asia widely cultivated in the Mediterranean and in other tropical and subtropical countries (Gamboa-Gómez et al. 2015; Yuan et al. 2016). Bitter orange is also known as Seville orange because this plant has been grown in Seville Spain for over 800 years where it is widely used in different food products including marmalades, syrups and juices (Stohs and Preuss 2012; Stohs et al. 2011). *C. aurantium* belongs to the *Rutaceae* family of fruit trees that yield bitter "brigarade" oranges (*Fructus Aurantii*) (Jiang et al. 2014). Due to its aromatic essential oil *C. aurantium* has a rich history of uses in food, cosmetics, and medicine (Koncic and Tomczyk 2013). In traditional Chinese medicine *C. aurantium* has been used for hundreds of years for indigestion, diarrhoea and dysentery, constipation and as expectorant. In South American folk medicine, *C. aurantium* was used to treat insomnia, anxiety and epilepsy (Shara et al. 2018; Stohs 2017). Bitter orange extracts have been used in the last two decades for weight loss and weight management, in sports performance, in appetite control and mental focus and cognition (Shara et al. 2018; Stohs 2017).

Herbal extracts containing *p*-synephrine (6-10%) are obtained from the dried immature fruits of *Citrus* species, particularly from *C. aurantium* (Bakhiya et al. 2017); however, *p*-synephrine can also be found in the fruits of other *Citrus* species, such as mandarin oranges (*Citrus reticulata*) and Marrs sweet oranges (*Citrus sinensis)* (Stohs and Badmaev 2016).

The *p*-synephrine (also known as oxedrine) is the primary protoalkaloid found in *C. aurantium*, but other protoalkaloids can be found in small amounts, such as octopamine, hordenine, tyramine and *N*-methyltyramine. Although structurally related to ephedrine, norepinephrine and epinephrine (**Figure I.6**), *p*-synephrine is an example of a non-stimulatory thermogenic substance, which preferentially activates ß-3 adrenergic receptors, resulting in an increased lipolytic activity and an enhanced thermogenesis (Bakhiya et al. 2017; Stohs and Badmaev 2016).

Due to the structural similarities between *p*-synephrine and ephedrine (**Figure I.6**), it is frequently assumed that *p*-synephrine exhibits similar cardiovascular and stimulant effects.

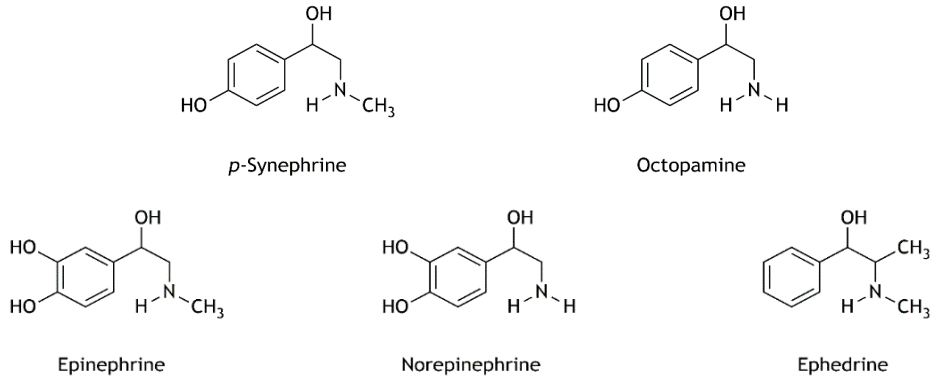


Figure I.6. Chemical structures of *p*-synephrine and octopamine present in *Citrus aurantium* and related compounds (Bakhiya et al. 2017; Stohs 2017).

However, *p*-synephrine is a phenylethylamine derivative with a hydroxyl group in the *para*-position on the benzene ring of the molecule, and ephedrine is a phenylpropylamine derivative that lacks the hydroxyl group in the *para*-position. So, as a consequence of these structural differences, *p*-synephrine has a proper stereochemistry and specific adrenergic receptor binding characteristics and also distinct pharmacokinetic properties (Shara et al. 2018; Stohs and Badmaev 2016).

Among other phytochemical substances, such as monoterpenes (like limonene), coumarins (umbelliferone, 6,7-dimethoxycoumarin, 6,7-dihydroxybergamottin and bergapten), pigments, mineral salts and vitamins, *Citrus* flavonoids can also play an important role in metabolic regulation and can prevent hepatic steatosis and dyslipidaemia (Stohs and Badmaev 2016). So, herbal extracts containing the combination of flavonoids, such as naringin and hesperidin, present in *Citrus* fruits, can enhance the non-stimulant thermogenic effect of *p*-synephrine (Ríos-Hoyo and Gutiérrez-Salmeán 2016; Stohs and Badmaev 2016).

Regarding pharmacokinetics, *p*-synephrine appears in blood 2 h after a 49-mg oral dose. The half-life of *p*-synephrine is estimated to be about 2–3 h and it is rapidly extracted from the blood by the liver. In terms of metabolic pathways, *p*-synephrine undergoes rapid *N*-demethylation to *p*-octopamine; however, even after the administration of oral doses up to 150 mg of *p*-synephrine, *p*-octopamine is not detected in urine since it also undergoes rapid oxidative deamination (Stohs 2017).

The cardiovascular effects observed in some animal studies at very high doses can be somehow explained due to the fact that *p*-synephrine binds up to 10 times more readily to adrenergic receptors in rodents than in humans, which can explain the lower cardiovascular effects in man. In humans, *p*-synephrine exhibits little affinity to α-1, α-2, ß-1, and ß-2 adrenergic receptors, binding preferably to ß-3 adrenergic receptors that regulate lipid and carbohydrate metabolism. Hence, *p*-synephrine exerts metabolic enhancement without acting as a CNS or cardiovascular stimulant. At commonly used doses, *p*-synephrine is not expected to increase heart rate or blood pressure, as well as haematological or other cardiovascular

changes. As *p*-synephrine exhibits greater adrenergic receptor binding in rodents than humans, data from animals cannot be directly extrapolated to humans (Stohs 2017).

Several studies have also addressed the safety and efficacy of *C. aurantium* extracts (Stohs 2017; Suntar et al. 2018). For instance, in a placebo-controlled, double-blinded clinical study, Kaats *et al.* (2013) found no adverse effects after the administration of a bitter orange extract twice daily (98 mg *p*-synephrine/day) during 60 days, in the absence and presence of naringin and hesperidin. Stohs (2017) has recently reviewed data of human, animal and *in vitro* studies involving *C. aurantium* extracts and *p*-synephrine and found that in about thirty human studies *p*-synephrine and bitter orange extracts did not result in cardiovascular effects and did not act as stimulants at commonly used doses. More recently, Shara *et al.* (2018) evaluated the hemodynamic and cardiovascular effects after the daily administration of a bitter orange extract (49 mg of *p*-synephrine) to healthy human subjects for 15 days; the results showed no significant differences in the systolic or diastolic blood pressure values, in heart rate and in the electrocardiogram between the experimental and control groups. Also no differences were detected on serum electrolytes, glucose, lipids or proteins, on liver and kidney function, or on blood cell counts. Indeed, studies in humans indicate that *p*-synephrine has a wide margin of safety, and in rats the oral median lethal dose (LD_{50}) is greater than 2500 mg/kg (Deshmukh et al. 2017).

The potential of Seville orange juices to produce drug interactions have been focused in some studies mainly due to the presence of furanocoumarins and flavonoids. But bitter orange extracts usually have small doses of both furanocoumarins and flavonoids and no significant effects were observed in human CYP isoforms. Additionally, no studies have shown potential teratogenic or mutagenic effects (Stohs 2017).

I.3.4. *Fucus vesiculosus*

Bladderwrack (*F. vesiculosus*) is a small edible brown seaweed (from *Fucaceae* family) that grows on hard substrate such as stone and pebble in shallow water down to a depth of less than 10 m (Winde et al. 2017). *Fucaceae* is a family of brown algae containing the five subordinate taxa *Ascophyllum*, *Fucus*, *Pelvetia*, *Pelvetiopsis* and *Silvetia*, being *Fucus* the most prominent genus from this family and *F. vesiculosus* the well-known species (Catarino et al. 2017).

The most popular application of *Fucus* spp. is for the treatment of goitre and thyroid-related complications caused by iodine deficits. Iodine deficiency can cause hypothyroidism, while iodine excess uptake can induce either hyper or hypothyroidism. As iodine is essential for the production of thyroid hormones, which in turn are responsible for the increase of metabolism in most tissues (Catarino et al. 2017; Wells et al. 2017).

Indeed, *F. vesiculosus* is a natural iodine source constituted by other phytochemicals with biological activity such as laminarin, alginate and fucoidan (as polysaccharides) (**Figure I.7**), phlorotannin, fucols and fucophlorethols (as polyphenols), fucosterol and ß-sitosterol (as sterols), pigments, vitamins and other minerals (like bromide, sodium, potassium, calcium,

magnesium, iron, phosphor, sulphates, copper, chrome, chloride, zinc, manganese, silicon and selenium) (Chater et al. 2016; EMA 2014c; Raposo 2016).

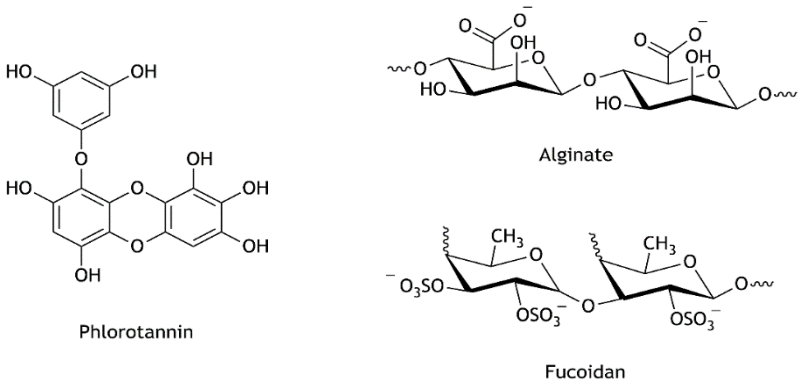


Figure I.7. Chemical structures of alginate, fucoidan and phlorotannin present in *Fucus vesiculosus* (Hussain et al. 2016).

Besides the nutritional benefits, *F. vesiculosus* supplements are commonly used not only for goitre treatment, but also for obesity. The traditional and medicinal use of *F. vesiculosus* is well-established as an adjuvant to reduced calorie ingestion, in order to help weight loss in overweight adults (EMA 2014b). Other effects related to seaweeds consumption are the anticoagulant, antithrombotic, antiviral, antitumor, anti-inflammatory, antioxidant and antidiabetic effects (Catarino et al. 2017; Liu et al. 2016; Park et al. 2011). *F. vesiculosus* has also been commonly used for the treatment of rheumatoid arthritis, asthma, atherosclerosis, psoriasis and skin diseases (Catarino et al. 2017).

Algae extracts are considered a good source of digestive enzyme inhibitors (Cardoso et al. 2015) and recent investigations suggest that *Fucus* polysaccharides and polyphenols act as potential modulators of enzyme activity. Indeed, alginate inhibits pepsin, a proteolytic enzyme, and also inhibits pancreatic lipase (Chater et al. 2016; Wan-Loy 2016). Additionally, phlorotannins are inhibitors of glucosidase, a key enzyme for starch breakdown and absorption (Gabbia et al. 2017). Several research groups have also reported effects of fucoidans in adipogenesis inhibition, glucose homeostasis regulation in mice, and lipid modulation in rats, as well as α-amylase and α-glucosidase inhibition. Fucoidans (**Figure I.7**) are a family of sulphated heteropolysaccharides extracted from brown algae that have been reported in several experimental models to possess anticoagulant, anti-inflammatory, antioxidant, anti-tumoral, and immuno-modulatory and anti-complement properties (EMA 2014a; Myers et al. 2016; Park et al. 2011; Shan et al. 2016). Additionally, fucoidans may be candidates for neurodegenerative disease therapies, as referred by Nelson *et al*. (2012), protecting cells from apoptosis and brain damage associated with ischemic stroke. Despite the high carbohydrate contents in marine algae (25–75% of the dry weight), most of them are not digested in the human gastrointestinal tract. Therefore, they act as dietary fibres, some being soluble fibres (50-85%), such as alginates and fucoidans, which are not completely fermented by colonic

microbiota to short-chain fatty acids. Additionally, since soluble fibres pass along the gastrointestinal tract without being completely metabolized, they slow down digestion accompanied by the decrease of nutrient absorption, since minerals and other nutrients may adhere to the fibres by chelating with them. Insoluble fibres, like cellulose and lignin, may interfere with mineral and protein absorption, decreasing transit time and increasing the faecal stool bulk due to their capacity to hold water (Raposo et al. 2016).

Consumption of *F. vesiculosus* should be monitored and precautions should be taken in patients with hypertension, kidney diseases and anaemia (fucoidan may lead to reduced gastrointestinal absorption of iron). *F. vesiculosus* consumption is contraindicated in cases of hyperthyroidism, Graves or Basedow disease, Hashimoto thyroiditis, after partial resection of the thyroid gland, excess of iodine, pregnancy or lactation, children under five years, hypersensitivity to halogens, malicious diseases and tuberculosis. Moreover, interactions can occur with lithium carbonate, thyroid medications, antihypertensive drugs, blood-diluting agents and iodine-containing drugs (EMA 2014c).

I.4. Epilepsy and pharmacotherapeutic approaches

Epilepsy affects people of all ages and results in social, behavioural, health and economic consequences to the patients and their families. It is estimated that more than 50 million people worldwide are affected by this chronic neurological disorder and 0.3% of all deaths are caused by epilepsy. Approximately 80% of people with epilepsy live in low- to medium-income countries and about 75% of patients in low-income countries do not receive or receive inadequate treatment (Guerreiro 2016).

Epilepsy definition and classification of the epilepsies have been changed over time. 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 neurobiologic, cognitive, psychological, and social consequences of this condition”*, which requires 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 et al. 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. 2017).

The ILAE also presented recently (2017) an updated classification of the epilepsies (**Figure I.8**). 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 aetiology 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, and communication around the world (Scheffer et al. 2017).

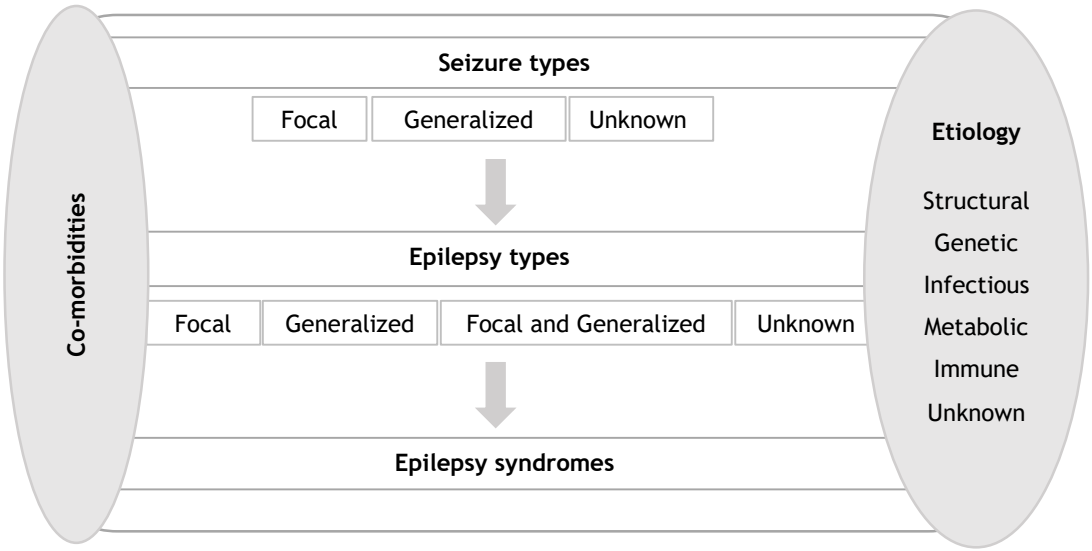


Figure I.8. Classification of epilepsies (Scheffer et al. 2017).

I.4.1. Obesity and its association with epilepsy

Obesity and overweight are recognized as modifiers of therapy and prognosis of several chronic conditions. Indeed, obesity is a biomarker for the cardiovascular risk factors and diseases that have a direct impact on cognitive function, including diabetes and insulin resistance, elevated triglyceride levels, white matter disease, hypertension, and hypercholesterolemia (Baxendale et al. 2015). On the other hand, several biological factors have been implicated in the pathogenesis of obesity. For instance, a complex interaction between environmental factors, CNS, neurotransmitters (serotonin, glutamate, GABA and others), neurotransmitter receptors, peripheral endocrine systems and some circadian rhythms influence food energy intake and energy expenditure (Chukwu et al. 2014).

Notably, the brain is a highly metabolic organ and the master regulator of energy homeostasis, monitoring short-term energy intake and long-term energy stores in order to modulate both energy intake and energy expenditure. There are multiple CNS-based humoral (by hormones secreted by the pancreas, adipose tissue and the gastrointestinal tract) and neural (regulation by autonomic nervous system) mechanisms that regulate energy homeostasis. In addition to the autonomic nervous system, CNS also regulates appetite, satiety, motivation, feeding behaviour and exercise behaviour. Changes in metabolism due to obesity may adversely affect the brain.

Additionally to metabolic changes, hormonal changes are also likely to alter CNS structure and function. Chronic activation of inflammatory pathways may also potentially affect the CNS (Lee and Mattson 2014). Obesity can change levels of peptides secreted by adipose tissue, which commonly lead to systemic inflammation, and so changing seizure susceptibility and severity. Seizure occurrence by itself can induce downstream inflammatory cascade, aggravating pre-existing neuronal excitability that can lead to the development of an epileptic brain (Hafizi et al. 2017).

Comorbidity of obesity and epilepsy has been recently reported with a high prevalence in children and adults (Arya et al. 2016; Janousek et al. 2013). Although the increasing childhood obesity all over the world, obesity and epilepsy in children and adolescents are of particular concerning given the adverse weight effects and endocrine changes associated with many commonly used AEDs (Daniels et al. 2009). Arya *et al.* (2016) stated that obesity was a common comorbidity in children with newly diagnosed untreated epilepsy. It was also found that patients with symptomatic epilepsy had a lower frequency of obesity compared to patients with cryptogenic or idiopathic epilepsy (Daniels et al. 2009). Epilepsy and overweight have also an additive effect on caesarean section, excessive bleeding during delivery and transfer to a neonatal ward. Thus, overweight or obese women with epilepsy should be considered a high-risk subgroup for pregnancy and delivery complications. Kolstad and collaborators found that women receiving LTG were especially at risk for complications such as caesarean section and severe peripartum depression and anxiety if they were overweight (Kolstad et al. 2016).

Other investigations have also focused the association between overweight or obesity and epilepsy. Ladino *et al.* (2014) found that 72% of adult patients with epilepsy were overweight (34%), had obesity (25%) or even morbid obesity (13%). Another study referred to that 55.2% of patients with epilepsy were overweight or obese (Janousek et al. 2013). There is also evidence that obesity is more common in patients with refractory epilepsy and in those treated with polytherapy regimens (Baxendale et al. 2015; Chukwu et al. 2014; Janousek et al. 2013). Janousek *et al.* (2013) found that overweight and obesity rates were higher in patients with refractory than non-refractory epilepsy, and that obesity was more frequent in patients under polytherapy than those under monotherapy. However, other authors found no correlation between obesity and drug-resistant epilepsy (Ladino et al. 2014).

Obesity rates in patients with seizure disorders are assumed to be secondary to medication-related changes in metabolism or disability-associated decreases in physical activity. Indeed,

many effective AEDs are known to alter metabolic pathways and can be associated with either an increase or reduction in body weight, although most AEDs are weight-neutral. Carbamazepine, retigabine, gabapentin, perampanel, pregabalin and valproate are more likely to cause weight gain or obesity (Chukwu et al. 2014; Hamed 2015; Lee and Mattson 2014). Otherwise, AEDs closely associated with weight loss are felbamate, topiramate, and zonisamide. LTG, levetiracetam, and phenytoin are believed to be weight-neutral (Hamed 2015). In spite of the limited data supporting the role of obesity in seizure severity, obesity can play a central role in the aggravation of this neurological disorder (Hafizi et al. 2017). Therefore, epilepsy patients' treatment must take into account comorbid conditions that may compromise the efficacy and safety of AEDs. Obesity status should also be considered as an important factor in AED selection for initial monotherapy (Daniels et al. 2009). Hence, weight gain induced by AEDs constitutes a serious problem in the management of people with epilepsy since excessive weight gain can lead to non-compliance with treatment and also to an exacerbation of obesity-related conditions.

I.4.2. Pharmacotherapy in epilepsy

The ancient treatments for epilepsy included rituals like punishment, incantations, amulets, and also the use of mineral, animal and plant products until the introduction of phenobarbital, a synthetic AED, in 1912 (Sucher and Carles 2015). Plants and herbal remedies for epilepsy therapy have been used in a centuries-old practiced medical form in diversified cultures. *Cannabis sativa, Ginseng, Lavandula officinalis* and *stoechas, Passiflora invarnate, Pimpinella anisum, Salvia miltiorrhiza, Viscum album* and *Zingiber officinale* were some of the plants used in epilepsy patients (Liu et al. 2017). Adams and collaborators reviewed the herbal medicines to treat epilepsy documented in nine original herbals from the Swiss Pharmaceutical Museum (in Basel) that contained the most important herbals of the 16th and 17th century (Adams et al. 2012). They have identified 221 plants from 53 families as remedies for treating epilepsy and found 24 plants in common with Jager *et al*. (2006) who have done the largest *in vitro* study of the anticonvulsant European plants. Those common plants were *Pimpinella anisum, Hedera helix, Hieracium pilosella, Buxus sempervirens, Stellaria media, Bryonia alba, Betonica officinalis, Melissa officinalis, Origanum vulgare, Rosmarinus officinalis, Thymus vulgaris, Convallaria majalis, Viscum album, Malva sylvestris, Paeonia* sp., *Primula elatior, Primula veris, Helleborus* sp., *Ruta graveolens, Tilia europaea, Valeriana officinalis, Verbena officinalis, Viola odorata* and *Viola tricolor* (Adams et al. 2012; Zhu et al. 2014). Another 25 plants were identified from important books in Iranian traditional medicine to treat epilepsy between the 10th and 18th centuries (Sahranavard et al. 2014). Tagarelli and collaborators (2013) reviewed the prophylactic and therapeutic remedies used by folk medicine to cure epilepsy in Italy during the 19th and 20th centuries. Of the 78 heterogeneous healing methods, which included 16 magical, 20 religious and 42 natural remedies, 17 were plant-based remedies. These remedies were mainly used as decoctions of *Matricaria chamomilla*, *Papaver*

somniferum, *Digitalis purpurea*, *Valeriana officinalis*, *Hypericum perforatum*, *Tilia* spp., *Paeonia* spp., *Viscum album*, *Ruta* spp. and *Melissa officinalis*. The juice of *Sempervivum tectorum*, the infusion of *Galium verum*, the water of *Petroselinum crispum* and well-cooked flowers of *Rosmarinus officinalis* were also herbal preparations used to treat epilepsy (Tagarelli et al. 2013).

Traditional communities in Africa and Latin America, as well as Ayurvedic medicine and traditional Chinese medicine still use herbal medicines to treat epilepsy (Kakooza-Mwesige 2015; Sriranjini et al. 2015; Xiao et al. 2015; Zhu et al. 2014). Unfortunately, there is a lack of documentation of this ancestral knowledge in some traditional societies. One of the barriers to the use of botanicals in epilepsy is the incomplete definition and composition of extracts used ancestrally. Indeed, thousands of studies reported that herbal medicines can be used for epilepsy. Extracts of hundreds of plants have been reported to exhibit anticonvulsant activity in phenotypic screens in experimental animals and dozens of plant-derived chemical compounds have similarly been shown to act as anticonvulsants in various *in vivo* and *in vitro* assays. In these studies, it was clearly identified that the anticonvulsant effects of plant extracts were attributed to the secondary metabolites as alkaloids, flavonoids, coumarins, saponins, and some monoterpenes and phenylpropanoids found in essential oils. The alkaloids aconitine (diterpene alkaloid), berberine, montanine, and tetrahydropalmatine (isoquinoline alkaloids), ibogaine (indole alkaloids), piperine (piperidine alkaloids), piplartine (amide alkaloids), rhynchophylline and isorhynchophylline (tetracyclic oxindole alkaloids), nantenine (aporphine alkaloids), raubasine (monoterpenoid indole alkaloid) have shown antiepileptic and/or anticonvulsant activities in animal models (Zhu et al. 2014). Flavonoids (as apigenin, baicalin, chrysin, fisetin, rutin, vitexin and wogonin) and coumarins (as esculetin, bergapten, imperatorin, osthole, xanthotoxin, heraclenin and oxypeucedanin) have been shown to interact with the benzodiazepine site of the gamma-aminobutyric acid type A ($GABA_A$) receptor and various voltage-gated ion channels. Also terpenoids have shown anticonvulsant activities in animal models such as borneol, citronellol, carvone, carvacrol, eugenol, isopulegol, linalool, safranal and terpineol (as monoterpenes), bilobalide (as sesquiterpene) abietic acid, delta-8-tetrahydrocannabinol (Δ8-THC), delta-9-tetrahydrocannabinol (Δ9-THC), and cannabidiol (from *Cannabis sativa*) (as diterpenes), baccoside A and ursolic acid (as triterpenes) and otophylloside A and B and saikosaponin (as saponins) (Zhu et al. 2014). Many anticonvulsant complex extracts and single plant-derived compounds have also exhibited additional anti-inflammatory, neuroprotective and cognition-enhancing activities that may be beneficial in the treatment of epilepsy (Sucher and Carles 2015; Zhu et al. 2014).

Although herbal medicine is accepted worldwide and extensively used as antiepileptic treatment, there is a lack of robust evidence for efficacy and safety of most herbs (Liu et al. 2017). Moreover, some plants or plant-derived products may have epileptogenic or neurotoxic components (Ephedra, Evening primrose oil, *Artemisia absinthium)* and some case reports have described herbal induced seizures caused mainly by Ephedra, Eucalyptus, *Ginkgo biloba* and Pennyroyal (Pearl et al. 2011; Samuels et al. 2008).

Currently, AEDs are the mainstream form of therapy in epilepsy. During the last decades, the development and launch of several novel AEDs increased the pharmacological options for the therapeutic management of epilepsy (Potschka 2013). In the past fifty years the application of pharmacokinetic principles in therapeutics, in association with a better drug assessment and understanding of the therapeutic outcomes brought important advances in epilepsy treatment. Before 1989, carbamazepine, ethosuximide, phenobarbital, phenytoin, primidone, valproic acid and the benzodiazepines were discovered and introduced in therapeutics. Since 1989, more seventeen new drugs have been licensed and marketed, which undoubtedly improved the treatment and prognosis of epilepsy (Brodie 2010; Burakgazi and French 2016; Campos et al. 2018; Shorvon 2009).

AEDs are commonly divided in three generations. The first-generation includes phenobarbital, primidone, phenytoin, ethosuximide, valproic acid, and carbamazepine; the second-generation includes felbamate, gabapentin, LTG, levetiracetam, oxcarbazepine, tiagabine, topiramate, pregabalin, and zonisamide; and the third one includes lacosamide, eslicarbazepine acetate, rufinamide, brivaracetam, perampanel, vigabatrin, clobazam, and retigabine (also called ezogabine). Some well-known disadvantages of first-generation AEDs are the non-linear kinetics of phenytoin, auto- and heteroinduction of metabolism associated with carbamazepine, high protein binding of phenytoin and valproic acid, metabolism through major CYP isoenzymes, and anticonvulsant hypersensitivity syndrome. Although second-generation AEDs have been developed to improve efficacy and tolerability, some limitations have also been reported and include cognitive impairment with topiramate, Steven-Johnson syndrome with LTG, kidney stones with topiramate and zonisamide, encephalopathy and non-convulsive status epilepticus with tiagabine. AEDs from the third-generation share good bioavailability with a relatively low plasma protein binding (except for clobazam, retigabine, and perampanel), and usually are not metabolized via major CYP isoenzymes (except for clobazam, perampanel, and eslicarbazepine acetate) and so presenting a more favourable drug interaction profile (LaPenna and Tormoehlen 2017). Some of these AEDs were developed as result of the great advances in neurochemistry and neurobiology, and particularly after the recognition of γ-aminobutyric acid (GABA) as the major inhibitory neurotransmitter in the brain (Brodie 2010; Shorvon 2009).

The primary drug targets and mechanisms of action by which the currently available AEDs stop or control seizures involve GABA and glutamate receptors and neuronal ion channels, including voltage-gated sodium and calcium channels (Kambli et al. 2017; Potschka 2013). Blockade of potassium channels, gap junctions, synaptic vesicle proteins, and neuronal adenosine, nicotinic acetylcholine, and serotonin receptors are other targets for AEDs therapy (Shorvon 2009).

Carbamazepine, eslicarbazepine acetate, phenytoin and oxcarbazepine block voltage-gated sodium channels promoting fast inactivation and increasing the number of channels in the inactivated state. On the contrary, lacosamide promotes a slow inactivation of these voltage-gated channels. Voltage-gated sodium channels, as well as voltage-gated calcium channels, significantly contribute to the action potential in neuronal excitability and neurotransmitter

release (Potschka 2013). Felbamate, LTG, topiramate and zonisamide also inhibit these voltage-gated sodium channels but are also involved in more complex actions (Brodie et al. 2011; Potschka 2013). Felbamate, LTG and topiramate also stimulate high voltage-activated calcium channels, unlike zonisamide that stimulates the low voltage-activated (T-type) calcium channels. T-type calcium channels are considered an important target for therapy of absence seizures (Brodie 2017; Potschka 2013). Still considering the drug action on calcium voltage-gated channels, ethosuximide interacts with the T-type calcium channels; gabapentin and pregabalin interact on the alpha-2-delta (α2δ) auxiliary subunit of high voltage-activated calcium channels, and levetiracetam and phenobarbital interact on the high voltage-activated calcium channels. However, other anion and cation channels can be activated by AEDs. LTG seems to interact within hyperpolarization-activated cyclic nucleotide-gated ion channels, in addition to its interaction with voltage-gated sodium and calcium channels, and retigabine interacts with Kv7 potassium channel subtype (Brodie et al. 2011; Manford 2017; Potschka 2013).

In what concerns the targeting of GABAergic neurotransmission, modulation of GABA receptors is one of the oldest mechanisms in pharmacotherapy of epilepsy due to inhibitory action of GABA neurotransmitter. In the brain, GABA acts at two different receptors, post-synaptic $GABA_A$ receptors and pre- and post-synaptic $GABA_B$ receptors. Activation of ionotropic $GABA_A$ receptors mediates chloride influx into the neuron resulting in hyperpolarization and reduced excitability. $GABA_B$ receptors constitute metabotropic G-protein coupled receptors and their activation can result in inhibition of adenylyl cyclase, inhibition of voltage-gated calcium channels and activation of G-protein-linked inwardly rectifying potassium channels (Potschka 2013). Phenobarbital, felbamate, retigabine and topiramate bind to specific sites on the $GABA_A$ receptor complex and potentiate GABA responses. Felbamate, topiramate and zonisamide modulate $GABA_A$-receptor mediated chloride currents. Vigabatrin is an irreversible inhibitor of the enzyme GABA transaminase, which degrades the neurotransmitter in pre-synaptic neurons and glial cells. Tiagabine inhibits the re-uptake of GABA in pre-synaptic neurons and potentiates post-synaptic GABAergic potentials. Gabapentin and valproic acid increase GABA turnover and levetiracetam modulates the $GABA_A$ receptor (Brodie 2010; Potschka 2013). Benzodiazepine drugs activity is a function of its allosteric effect on the $GABA_A$ receptor, thus potentiating the GABAergic neurotransmission (Greenfield Jr 2013).

Glutamatergic neurotransmission is related to the effects of glutamate, which is the most important excitatory neurotransmitter in the CNS. This neurotransmitter significantly contributes to fast excitatory neurotransmission by activation of ionotropic glutamate receptors. Ligand-mediated activation of these receptors enhances cation fluxes into post-synaptic cells resulting in depolarization of the post-synaptic membrane and enhanced neuronal excitability. Felbamate inhibits the *N*-methyl-D-aspartate (NMDA) subtype of glutamate receptors, while topiramate and perampanel interact with non-NMDA receptors such as kainate and α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (Potschka 2013).

Overall, AEDs having additional mechanisms of action normally have a wider range of efficacy and this is what happens, for instance, with LTG, levetiracetam and topiramate (Brodie 2017; Hanaya and Arita 2016; Potschka 2013); while other AEDs have a narrower spectrum of efficacy, such as carbamazepine, eslicarbazepine, gabapentin, lacosamide, oxcarbazepine, phenytoin, pregabalin, retigabine, tiagabine and vigabatrin (Burakgazi and French 2016).

I.4.2.1. Selecting the best therapeutic option

Although AED therapy is the conventional form of epilepsy treatment, it is also true that the selection of the best therapeutic approaches in epilepsy can be somehow complex and challenging. In addition to AEDs, hormonal therapies, diet, surgery, neurostimulation, and behavioural modification techniques can also be used to optimize seizure control. The treatment of epilepsy patients should also be a dynamic process considering patient's medical, social and occupational conditions (Burakgazi and French 2016). Hence, the choice of an AED should be tailored to each patient considering the seizure and epilepsy type, as well as the epilepsy syndrome (**Table I.1** and **Table I.2**).

Table I.1. Antiepileptic drugs (AEDs) therapeutic indications according to seizure types *(adapted from NICE guideline CG137)* (NICE 2018).

Seizure types	First-line AEDs	Alternative first-line therapy	Adjunctive therapy
Generalized tonic-clonic seizures	Valproic acid **Lamotrigine**	Carbamazepine Oxcarbazepine	Clobazam **Lamotrigine** Levetiracetam Valproic acid Topiramate
Focal seizures	Carbamazepine **Lamotrigine**	Levetiracetam Oxcarbazepine Valproic acid	Carbamazepine Clobazam Gabapentin **Lamotrigine** Levetiracetam Oxcarbazepine Valproic acid Topiramate
Absence seizures	Valproic acid Ethosuximide	**Lamotrigine**	Clobazam Clonazepam Levetiracetam Topiramate Zonisamide
Myoclonic seizures	Valproic acid	Levetiracetam Topiramate	Clobazam Clonazepam Topiramate
Tonic or atonic seizures	Valproic acid		**Lamotrigine**

Table I.2. Antiepileptic drugs (AEDs) therapeutic indications according to epilepsy syndromes *(adapted from NICE guideline CG137)* (NICE 2018).

Epilepsy syndromes	First-line AEDs	Adjunctive therapy
Childhood and juvenile absence epilepsy	Ethosuximide **Lamotrigine** Valproic acid	Ethosuximide **Lamotrigine** Valproic acid
Juvenile myoclonic epilepsy	**Lamotrigine** Levetiracetam Valproic acid Topiramate	**Lamotrigine** Levetiracetam Valproic acid Topiramate
Epilepsy with generalised tonic-clonic seizures only	Carbamazepine **Lamotrigine** Oxcarbazepine Valproic acid	Clobazam **Lamotrigine** Levetiracetam Valproic acid Topiramate
Idiopathic generalised epilepsy	**Lamotrigine** Valproic acid Topiramate	**Lamotrigine** Levetiracetam Valproic acid Topiramate
Benign epilepsy with centrotemporal spikes	Carbamazepine **Lamotrigine** Levetiracetam Oxcarbazepine Valproic acid	Carbamazepine Clobazam Gabapentin **Lamotrigine** Levetiracetam Oxcarbazepine Valproic acid Topiramate
Late-onset childhood occipital epilepsy (Gastaut type)	Carbamazepine **Lamotrigine** Levetiracetam Oxcarbazepine Valproic acid	Carbamazepine Clobazam Gabapentin **Lamotrigine** Levetiracetam Oxcarbazepine Valproic acid Topiramate
Lennox-Gastaut syndrome	Valproic acid	**Lamotrigine**
Dravet syndrome	Valproic acid Topiramate	Clobazam Stiripentol
Panayiotopoulos syndrome	Carbamazepine **Lamotrigine** Levetiracetam Oxcarbazepine Valproic acid	Carbamazepine Clobazam Gabapentin **Lamotrigine** Levetiracetam Oxcarbazepine Valproic acid Topiramate

It is also important to consider for each AED its efficacy, tolerability, the adverse effects profile with no long-term safety issues (as teratogenicity, hypersensitivity reactions, or organ toxicity) provided by solid evidence from well-designed randomized clinical trials.

The pharmacokinetic and pharmacodynamic profile of each AED should be also analysed (**Table I.3**), taking into consideration the age, gender, concomitant medications, presence of comorbid conditions and cost (Burakgazi and French 2016; Perucca and Tomson 2011; Schmidt 2016).

Table I.3. Pharmacokinetic parameters and reference therapeutic ranges of major antiepileptic drugs (AEDs) (Chong and Lerman 2016; Jacob and Nair 2016; Landmark et al. 2012; Patsalos et al. 2008; Verrotti et al. 2014).

AEDs	F_{Oral} (%)	Protein binding (%)	T_{max} (h)	Time to steady-state (days)	Half-life (h)	Half-life with enzyme inducers (h)	Range (mg/L)
CBZ	≤ 85	75	2-9	2-4	8-20	5-12	4-12
CLB	≥ 95	85	1-3	7-10	10-30	NE	0.03-0.3
CLZ	≥ 95	85	1-4	3-10	17-56	11-35	0.02-0.07
ESL	≥ 80	35	1-4	4-5	20-40	9-20	10-35
ESM	≥ 90	0	1-4	7-10	40-60	20-40	40-100
FBM	> 90	25	2-6	3-4	16-22	10-18	30-60
GBP	< 60	0	2-3	1-2	5-9	5-9	2-20
LCM	≥ 95	15	0.5-4	2-4	13	12-16	5-10
LTG	≥ 95	55	1-3	3-6 (+VPA: 5-15)	15-35 (+VPA: 30-90)	8-20 (+VPA: 15-35)	2.5-15
LEV	≥ 95	0	1	1-2	6-8	5-7	12-46
OXC	90	40	3-6	2-3	8-15	7-12	3-35
PER	≥ 95	96	0.5-1.5	14-21	70-120	NE	200-1000
PB	≥ 95	55	0.5-4	12-24	70-140	70-140	10-40
PHT	≥ 80	90	1-12	5-17	30-100	30-100	10-20
PGB	≥90	0	1-2	1-2	5-7	5-7	2-5
PRM	≥ 90	10	2-5	2-4	7-22	3-12	5-10
RTG	60	80	0.5-2	1-2	8	6-10	NE
RFM	> 85	26-35	5-6	2	6-10	6-9	5-30
STP	> 90	99	1-2	1-2	2-13	4-13	4-22
TGB	≥ 90	96	0.5-2	1-2	5-9	2-4	0.02-0.2
TPM	≥ 80	15	2-4	4-5	20-30	10-15	5-20
VPA	≥ 90	90	3-6	2-4	11-20	6-12	50-100
VGB	≥ 60	0	1-2	1-2	5-8	5-8	0.8-36
ZNS	≥ 65	50	2-5	9-12	50-70	25-35	10-40

CBZ, Carbamazepine; CLB, Clobazam; CLZ, Clonazepam; ESL, Eslicarbazepine; ESM, Ethosuximide; FBM, Felbamate; F_{oral}, Oral bioavailability; GBP, Gabapentin; LCM, Lacosamide; LTG, Lamotrigine; LEV, Levetiracetam; NE, not established; OXC, Oxcarbazepine; PER, Perampanel; PB, Phenobarbital; PHT, Phenytoin; PGB, Pregabalin; PRM, Primidone; RTG, Retigabine; RFM, Rufinamide; STP, Stiripentol; TGB, Tiagabine; t_{max}, time to reach peak plasma concentration; TPM, Topiramate; VPA, Valproic acid; VGB, Vigabatrin; ZNS, Zonisamide.

Comorbidities must be taken into account not only because of potential interactions of drugs used concomitantly, but also because some AEDs may adversely or positively affect comorbid disorders (Perucca and Tomson 2011). Long-term use of AEDs was already associated with cognitive impairment, and idiosyncratic and chronic effects such as weight changes, increased risk of teratogenicity, and endocrine effects on reproductive, adrenal and thyroid systems (Adhimoolam and Arulmozhi 2017; Hamed 2015).

The ultimate goal of epilepsy treatment targets monotherapy or at least the use of the smallest possible number of AEDs, along with the smallest doses, in order to provide seizure freedom with optimal tolerability and minimal side effects (Burakgazi and French 2016; Zaccara et al. 2017). It is important to focus that the treatment with AEDs may require dose adjustment and therapeutic drug monitoring (TDM). It is known that short-term adverse effects, which may lead to drug withdrawal, are critically influenced by dose. In addition, for most AEDs, a gradual dose titration can improve CNS tolerability and reduce the risk of idiosyncratic adverse reactions (Zaccara et al. 2017).

Despite the reference therapeutic ranges of either old or new AEDs are often associated with an optimal response (Perucca and Tomson 2011), unfortunately, in some patients, it is difficult to achieve a complete absence of seizures and control the adverse effects. For example, elderly patients are more susceptible to the adverse effects of AEDs (Perucca and Tomson 2011). Add-on and polytherapy are often required to achieve seizure freedom or at least to reduce seizure frequency and severity (Santulli et al. 2016). AED combination may be safer than AED substitution, especially in patients with frequent or severe seizures. Evidence from experimental studies in animal models indicates that the concomitant administration of two AEDs may result in antagonistic (or infra-additive), additive, or supra-additive (synergistic) anticonvulsant or toxic effects (Brigo et al. 2013). Combinations of AEDs with different mechanisms of action can usually be advantageous and offer increased benefits in some patients (Perucca and Tomson 2011). One of the best examples of human evidence for synergism in terms of anticonvulsant efficacy is the combination of LTG and valproic acid, although there is also evidence that this combination is associated with a pharmacodynamic interaction that brings adverse effects, like tremor. Additionally, as LTG metabolism is inhibited by valproic acid, skin rash can occur specially associated with high doses of LTG. Data also indicate that there is a possible favourable pharmacodynamic interaction between lacosamide and levetiracetam, due to the distinct and non-overlapping mechanisms of action of both AEDs (Brigo et al. 2013).

Uncontrolled seizures are intricately related with an increased risk of pharmacoresistant epilepsy. It has been seen that up to 8–40% of epileptic patients show resistance to antiepileptic medications (Kambli et al. 2017) and this is a major health problem nowadays concerning epilepsy treatment. Pharmacoresistant epilepsy or also called drug resistant epilepsy may be defined as the failure of adequate trials of two tolerated and appropriately chosen and used AEDs (whether as monotherapy or in combination) to achieve sustained seizure freedom (Kwan et al. 2010).

About 60% of patients with focal epilepsy manifest pharmacoresistance (Alexopoulos 2013). Despite the availability of several AEDs, the problem of pharmacoresistance persists. Several causes are underlying the phenomena of pharmacoresistance, which may be abnormalities in brain maturation, severe brain injuries with resultant irreversible changes of cerebral neuroglia organization and inhibitory neuron function, kindling phenomenon, seizure-induced disturbances of oxygen supply, as well as acquired (or hereditary) changes in protein receptors (as GABA receptors), in voltage-gated calcium and sodium channels, or in efflux transporter systems (as P-gp) (Sharma et al. 2015).

In addition to the pharmacoresistance phenomena, it is important to consider that several adverse effects of AED therapy (in monotherapy or in add-on therapy) may be caused by either pharmacokinetic or pharmacodynamic drug interactions (Perucca and Tomson 2011). Adverse effects related to enzyme induction may include impaired bone health, endocrine dysfunction, and, possibly, changes in cholesterol levels and other markers of vascular risks. The use of enzyme inducers such as carbamazepine, phenytoin, or phenobarbital as first-line drugs may therefore be questioned, particularly after the introduction of alternative AEDs that are devoid of enzyme-inducing activity (like levetiracetam) or that have a reduced potential for interactions (like LTG and oxcarbazepine) (Perucca and Tomson 2011).

Drug interactions involving AEDs can occur between AEDs themselves or between AEDs and other drugs that are concomitantly administrated, like analgesics, antidepressants, antipsychotics, anticoagulants, antimicrobials, antiretrovirals, antineoplastic agents, immunossuppressants, oral contraceptives and steroids (Patsalos 2013b; Zaccara and Perucca 2014). Some AEDs have an overall substantial propensity to interact either with other AEDs or with other drugs, considering their metabolic characteristics and the number of pharmacokinetic and pharmacodynamic interactions. When compared with the first-generation AEDs (carbamazepine, phenytoin, phenobarbital, primidone and valproate), the new AEDs are clearly associated with fewer pharmacokinetic and pharmacodynamic interactions. Of the newer AEDs, felbamate, LTG, oxcarbazepine and rufinamide are those with the great number of pharmacokinetic interactions described. Until 2013, a total of thirty-nine interactions involving LTG and other AEDs were reported, seventeen of them with pharmacokinetic basis and five with pharmacodynamic basis (Patsalos 2013a).

I.4.2.2. Enzyme induction

As aforementioned, carbamazepine, phenytoin, phenobarbital and primidone are broad-spectrum enzyme inducers that can stimulate the activity of many CYP and UGTs enzymes, reducing the serum concentrations of other concurrently administered AEDs. Oxcarbazepine, eslicarbazepine acetate, felbamate, rufinamide, topiramate and perampanel are weaker enzyme inducers, but they can also decrease the serum concentrations of some concomitantly administered AEDs. Dosage adjustments are needed for valproic acid, LTG, and tiagabine in patients taking concomitantly carbamazepine, barbiturates and phenytoin. The clearance of

perampanel can also be increased threefold and twofold by carbamazepine and phenytoin, respectively (Zaccara and Perucca 2014). It is important to reinforce the need of a proper monitoring and dose adjustment when an enzyme-inducing AED is discontinued, or substituted with a drug that does not have enzyme-inducing effects, since the serum concentrations of the affected drugs may increase, and even raise to potentially toxic concentrations (Zaccara and Perucca 2014).

I.4.2.3. Enzyme inhibition

Like enzyme induction, enzyme inhibition can be predicted by knowing which isoenzymes are involved in the metabolism of drugs, including AEDs (**Table I.4**). Valproic acid is a broad-spectrum enzyme inhibitor of UGT enzymes (UGT1A4 and UGT2B7), as well as a CYP2C9 inhibitor; but it is a weak inhibitor of CYP2C19 and CYP3A4. These effects can be seen when valproic acid is concomitantly used with LTG and phenobarbital. One interesting point related to the interaction between valproic acid and LTG is the bidirectional character of this drug interaction. Valproic acid also increases serum rufinamide concentrations (Zaccara and Perucca 2014).

Weak enzyme inhibitors of CYP2C19 include oxcarbazepine, eslicarbazepine, and topiramate. Felbamate inhibits CYP2C19 and can cause an increase in the plasma concentrations of phenobarbital, phenytoin and valproic acid. Similarly, stiripentol potently inhibits CYP3A4, CYP1A2, CYP2D6, and CYP2C19 and may cause the increase of plasma concentrations of clobazam, *N*-desmethyl-clobazam, valproic acid, phenytoin, carbamazepine, and phenobarbital (Patsalos 2013a; Zaccara and Perucca 2014).

I.4.2.4. Herb-drug interactions involving antiepileptic drugs

Globally, HDIs involving AEDs and herbal medicines are still scarcely reported in the literature. Interactions between AEDs or with other drugs indicated for non-epilepsy comorbidities are, on the contrary, numerous and common (Johannessen Landmark and Patsalos 2008).

One explanation concerning the scarce number of interactions described between AEDs and herbal medicines is the fact that epilepsy patients may be unaware of the potential for this kind of interactions, and so they do not have the awareness to inform their doctors about the concomitant consumption of herbal-based medicinal products and conventional medicines. Nevertheless, these potential interactions may have clinical consequences such as lack of efficacy, toxicity, unexpected adverse effects, and non-compliance (Johannessen Landmark and Patsalos 2008).

Table I.4. Elimination routes of antiepileptic drugs (AEDs) (Patsalos 2013a; Patsalos 2013b; Zaccara and Perucca 2014).

AEDs	Elimination routes	Enzyme inducer	Enzyme inhibitor
CBZ	Oxidation (CYP3A4); *other routes*: oxidation (CYP2C8, CYP1A2) and UGT conjugation (UGT2B7)	yes	yes
CLB	Oxidation (CYP3A4 and CYP2C19); *other routes*: oxidation (CYP2C18, CYP2B6)	no	no
CLZ	Oxidation (CYP3A4)	no	no
ESL	UGT conjugation (UGT1A4, UGT1A9, UGT2B4, UGT2B7, UGT2B17)	yes	yes
ESM	Oxidation (CYP3A4); *other* routes: oxidation (CYP2E1)	no	no
FBM	Oxidation (CYP3A4) (> 50%); *other routes:* oxidation (CYP2E1) and UGT	yes	yes
GBP	Renal excretion	no	no
LCM	Demethylation (CYP3A4, CYP2C9, CYP2C19)	no	no
LTG	UGT conjugation (UGT1A4)	yes	no
LEV	Renal excretion (75%) and hydrolysis (25%)	no	no
OXC	UGT conjugation (> 50%) and renal excretion (< 30%)	yes	yes
PER	Oxidation (CYP3A4); *other routes:* UGT conjugation	yes	no
PB	Oxidation (CYP2C9) and *N*-glucosidation; *other routes*: oxidation (CYP2C19, CYP2E1) and renal excretion (25%)	yes	no
PHT	Oxidation (CYP2C9 and CYP2C19); *other routes:* oxidation (CYP2C18, CYP3A4)	yes	no
PGB	Renal excretion	no	no
PRM	Oxidation (CYP2C9); *other routes:* renal excretion, oxidation (CYP2C19, CYP2E1), *N*-glucosidation	yes	no
RTG	*N*-acetylation; *other routes:* UGT conjugation (UGT1A1, UGT1A3, UGT1A4, UGT1A9)	no	no
RFM	Hydrolysis (carboxylesterases); *other routes:* UGT conjugation	yes	yes
STP	Oxidation (CYP1A2, CYP2C19, CYP3A4); *other routes:* UGT conjugation	no	yes
TGB	Oxidation (CYP3A4)	no	no
TPM	Renal excretion (40–80%); *other routes:* oxidation (inducible CYP isoforms: 20–60%)	yes	yes
VPA	Oxidation (CYP2C9 and other CYPs: > 50%) and glucuronide conjugation (several UGTs: 30–40%)	no	yes
VGB	Renal excretion	no	no
ZNS	Oxidation (CYP3A4), reduction and *N*-acetylation (> 50%); *other routes:* renal excretion (30%)	no	no

CBZ, Carbamazepine; CLB, Clobazam; CLZ, Clonazepam; ESL, Eslicarbazepine; ESM, Ethosuximide; FBM, Felbamate; GBP, Gabapentin; LCM, Lacosamide; LTG, Lamotrigine; LEV, Levetiracetam; OXC, Oxcarbazepine; PER, Perampanel; PB, Phenobarbital; PHT, Phenytoin; PGB, Pregabalin; PRM, Primidone; RTG, Retigabine; RFM, Rufinamide; STP, Stiripentol; TGB, Tiagabine; TPM, Topiramate; VPA, Valproic acid; VGB, Vigabatrin; ZNS, Zonisamide.

Herbs and supplements may have variable effects on the absorption and disposition of AEDs and so they may alter the effectiveness of the medications and may also directly affect the seizure threshold (Pearl et al. 2011). In a study involving 92 patients with epilepsy, it was found that 24% were using complementary and alternative therapies, of which 41% were using herbs and supplements and, in most cases, it was not of the doctor's knowledge (Peebles et al. 2000). In a more recent study conducted by Eyal *et al.* (2014) in adult patients with epilepsy, 48% of them took dietary supplements simultaneously with AEDs and patient awareness for potential drug interactions involving AEDs was very limited.

However, severe adverse drug reactions have been associated with HDIs in patients taking herbs and prescribed medications (Awortwe et al. 2018). Noni juice caused a reduction of the blood levels of phenytoin in a 49-years-old man, probably due to the CYP2C9 induction. In a 55-year-old man, a Ginkgo supplement administrated with valproic acid and phenytoin may have precipitated an episode of seizures, leading to death while swimming (Awortwe et al. 2018). Also, the administration of a *Ginkgo biloba* extract in volunteers treated with midazolam (a benzodiazepine used for status epilepticus treatment) caused an increase of the plasma concentrations of midazolam, with an increase of $AUC_{0-\infty}$ (25%) and a decrease of oral clearance (26%) (Uchida et al. 2006).

In addition, Fong *et al.* (2013) performed a systematic review on the interaction of herbs, dietary supplements, and food with carbamazepine, and identified thirty-three herbal products/dietary supplement/food interacting with carbamazepine and 80% of them had a pharmacokinetic basis. For example, grapefruit juice significantly increased the oral bioavailability of carbamazepine (Garg et al. 1998) and diazepam (Ozdemir et al. 1998). The metabolism of carbamazepine and phenytoin may be decreased by St. John's wort, which may reduce the efficacy of these AEDs with possible loss of seizure control (Patsalos et al. 2002). An HDI between Ginseng and LTG was also reported in a 44-year-old white man with generalized tonic–clonic seizures, which caused an adverse drug reaction with eosinophilia and systemic symptoms syndrome, with a pruritic rash on more than 50% of his body, eosinophilia, myalgias, and elevated liver enzymes, probably due to the inhibition UGT2B7 (Awortwe et al. 2018; Myers et al. 2015a).

I.5. Lamotrigine

LTG was developed by Wellcome Research Laboratories (Beckenham, Kent, England) in the early 1980s and then approved in Ireland in 1991 for use in adult patients. It was later approved by the FDA in 1994, and in France in 1995 (Yasam et al. 2016). LTG is an anticonvulsant drug used in the treatment of epilepsy, bipolar disorders and also as a mood stabilizer (Brodie 2017; Poureshghi et al. 2017).

In epilepsy, LTG is used to treat focal seizures, primary and secondary tonic-clonic seizures, and seizures associated with Lennox-Gastaut syndrome, a severe age-dependent epileptic encephalopathy occurring between 1 and 8 years of age (Mastrangelo 2017; Poureshghi et al. 2017). LTG has a broad spectrum of activity against various seizures and epilepsy types like absence seizures, childhood and juvenile absence epilepsy and juvenile myoclonic epilepsies, as well as in idiopathic generalized epilepsy (Yasam et al. 2016). Despite its safe use in children, there is also a high-level of evidence on the efficacy of LTG in the elderly (Perucca and Tomson 2011). In pregnant women, LTG has an excellent tolerability and an acceptable safety profile with minimal effects on foetus and foetal malformations (Yasam et al. 2016).

Additionally to its action on voltage-gated ion channels, LTG also acts on the serotonergic pathway causing reuptake inhibition that may explain its antidepressant properties (Alabi et al. 2016; Izadpanah et al. 2017). It is possible that LTG has benefits in the control of affective instability and impulsivity in patients with borderline personality disorder (Alabi et al. 2016). Furthermore, LTG seems to be transported into human brain endothelial cells by the organic cation influx transporters (OCT1), which explains the LTG penetration in the brain reaching higher concentrations there than would be expected from its physicochemical properties (Dickens et al. 2012).

LTG is well tolerated in the usual therapeutic doses when it is slowly introduced. The usual initial dosage in adults is 12.5-25 mg/day (Ghaffarpour et al. 2013). For patients taking concomitantly valproic acid the initial dose of LTG should be 25 mg every other day for two weeks, followed by an increase to 25 mg/day for two weeks (Yasam et al. 2016). The dose can then be increased by 25 to 50 mg up to a maintenance dose of 100 to 400 mg/day in two divided doses. However, target dosages of 600-1000 mg/day of LTG may be required to achieve the therapeutic levels when the drug is used together with enzyme inducers. In children, LTG dose can be gradually increased to 15 mg/kg/day, but when combined with valproate the LTG dose should be titrated slowly up to 5 mg/kg/day. LTG is available only for oral administration in 25, 50, 100 and 200 mg tablets, and in 2, 5, 25, 50, 100 and 200 mg chewable tablets (INFARMED 2018; Yasam et al. 2016). LTG can be used either in monotherapy or in combination, and a single morning dose is often administrated due to its long half-life (Goldenberg 2010; Perucca and Tomson 2011; Tsao 2009).

Among others, adverse effects observed after LTG administration include dizziness, somnolence, nausea, asthenia and headaches in 8–20% of patients (Bloom and Amber 2017). Rash can be developed in about 12% of patients and a severe form of skin rash (i.e. Stevens-Johnson syndrome) has been described to occur in 1/1000 adults and 1/100 children treated with LTG (Alabi et al. 2016; Grosso et al. 2017). Some cases of anticonvulsant hypersensitivity syndrome, a potentially fatal drug-induced idiosyncratic immunologic reaction involving multiple organs, have been also associated with LTG therapy (Wang et al. 2012). There is also evidence of clinically important toxicity induced by LTG overdose (Alabi et al. 2016).

Post-mortem investigation of LTG concentrations in blood, serum, liver, bile, urine, vitreous

humour and stomach content revealed supra-therapeutic concentrations of LTG (more than 15 µg/mL) in four of the eight cases studied. LTG concentrations were 20-39 µg/mL in blood, 15-62 µg/mL in serum, 110-420 µg/mL in bile, 6.7-14 µg/mL in vitreous humour, 26-59 µg/mL in urine and 92-290 mg in stomach contents. In the liver, the supra-therapeutic concentrations ranged from 53 to 350 mg/kg. In all of these cases studied were detected other concomitant drugs, particularly AEDs and benzodiazepines (Levine et al. 2000).

I.5.1. Physicochemical properties

Structurally, LTG ($C_9H_7Cl_2N_5$) is a 3,5-diamine-6-(2,3-dichlorophenyl)-1,2,4-triazine (**Figure I.9**) with a molecular weight of 256.09 g/mol (Alabi et al. 2016; Goldenberg 2010; Yasam et al. 2016). LTG is a white to pale cream-colored powder slightly soluble in water (0.17 mg/mL at 25°C) and slightly soluble in 0.1 M hydrochloric acid (4.1 mg/mL at 25°C) (GlaxoSmithKline 2016). Additionally, LTG is a lipophilic weak base with a pKa of 5.7 (Yasam et al. 2016). It has strong chromophores responsible for the two absorption maxima in the UV spectrum: the weaker one at 312 nm and the stronger one at 200 nm. The 2,3-dichlorophenyl ring constitutes the most hydrophobic moiety of the LTG; while the nitrogen atoms of the diaminotriazine substituent act as electron donors and as hydrogen bond donors and acceptors (McEvoy 2008) (blue circle in **Figure I.9**).

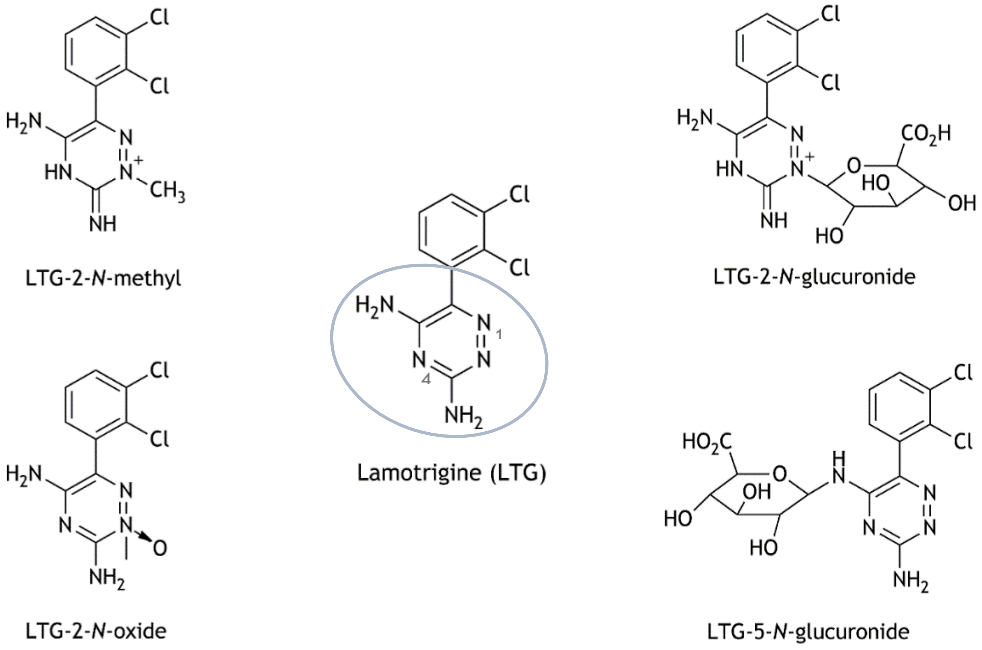


Figure I.9. Lamotrigine (LTG) and its metabolites chemical structures. LTG-2-*N*-glucuronide is the major metabolite in humans; LTG-2-*N*-methyl is mostly found in dogs and LTG-2-*N*-oxide in rats (Chen et al. 2010; Maggs et al. 2000).

I.5.2. Pharmacokinetic and pharmacodynamic properties

After oral administration, with or without meals, LTG is completely absorbed from the human gastrointestinal tract, with a bioavailability superior to 95% (Bialer et al. 2007; Yasam et al. 2016). LTG is not highly bound to plasma proteins (55%) and this percentage of binding is not affected by the presence of other AEDs like phenytoin, phenobarbital or valproate (Alabi et al. 2016; Bialer et al. 2007). LTG has a linear pharmacokinetics and minimal effects on the pharmacokinetics of other drugs (Brzakovic et al. 2012). The intestinal absorption is rapid and almost complete without important first-pass metabolism. The time to reach the peak concentration is about 1 to 3 h (t_{max}) and the therapeutic reference range in serum is 3-15 µg/mL (10–59 mmol/L) (Patsalos 2013a; Patsalos et al. 2017). The incidence of toxic effects ascribed to LTG is significantly increased when serum or plasma concentrations exceed 15 µg/mL (Jacob and Nair 2016).

LTG has a uniform distribution and the apparent volume of distribution (V_d) following oral administration ranges from 0.9 to 1.3 L/kg, with a reference value of 1.2 L/kg (Patsalos 2013a; Yasam et al. 2016); V_d is independent of the dose and is similar following single and multiple doses in both patients with epilepsy and healthy volunteers (Biton 2006). LTG distributes into saliva, and salivary concentrations are approximately 40–50% of serum/plasma concentrations (Krasowski 2010; Krasowski and McMillin 2014). Serum and salivary concentrations of LTG in paediatric and adult patients have demonstrated a good saliva/serum correlation with LTG concentration ratios ranging from 0.40 to 1.19 (Ryan et al. 2003). Mallayasamy and collaborators (2010) also reported a 0.683 ratio for salivary to serum LTG concentrations. In human brain, LTG has been shown to accumulate by a factor of 2.8 compared to blood (Dickens et al. 2012). LTG is also distributed into breast milk (Vajda et al. 2013). Indeed, a highly significant correlation was found between maternal and umbilical cord serum LTG levels in patients under LTG monotherapy and in those receiving combination therapy with LTG and valproate (Kacirova et al. 2010b).

The serum half-life of LTG is about 15-35 h when the drug is used in monotherapy. In the presence of enzyme inducers, LTG serum half-life is about 8-20 h and may increase up to 60 h when used together with valproic acid, showing that the systemic elimination of LTG is significantly affected. The half-life of LTG may also increase to 50 h in patients with severe renal failure. Plasma concentrations of LTG decline in pregnancy due to an enhanced elimination rate by induction of the LTG glucuronidation (Alabi et al. 2016; Kacirova et al. 2010a; Ohman et al. 2008a; Ohman et al. 2008b; Vajda et al. 2013). In elderly, a mean half-life of 31.2 h was achieved after the administration of a single-dose of LTG (150 mg) (Biton 2006). In general, to achieve equivalent therapeutic blood concentrations, children require higher doses than adults (Yamamoto et al. 2015). LTG undergoes hepatic metabolism through glucuronidation (**Figure I.9**), which is primarily mediated by the UGT1A4; however, UGT1A3 and UGT2B7 are also involved in LTG glucuronidation (Zhou et al. 2015).

Glucuronidation generally involves the covalent linkage of the glucuronic acid to a substrate bearing a nucleophilic functional group. In humans, 70% of an oral dose of LTG was recovered in urine as LTG-2-*N*-glucuronide (Yasam et al. 2016). As minor metabolites were found the LTG-5-*N*-glucuronide and the LTG-2-*N*-methyl (**Figure I.9**). The metabolism of LTG has also been studied in several animal species like dogs, guinea pigs, rats, rabbits and monkeys. The *N*-glucuronide metabolites have been found in large amounts in humans, rabbits and guinea pigs, but not in dogs and rats. In dogs, the major metabolite was the methylated form in a percentage of about 45% (**Figure I.9**) (Chen et al. 2010; Maggs et al. 2000). Rats, lacking significant glucuronidation and methylation pathways eliminate LTG mostly unchanged. Thus, LTG-2-*N*-oxide seems to be the primary urinary metabolite in the rat and LTG can also be slowly metabolized in this species to a reactive arene oxide intermediate, which is found in bile as an unstable glutathione adduct (Chen et al. 2010).

Despite the metabolic differences between rats and humans, the rat is a commonly used non-clinical *in vivo* model to assess the pharmacokinetics of LTG. Hence, pharmacokinetic studies performed in rats have demonstrated a good oral bioavailability of LTG, similarly to what happens in humans. Moreover, the time to reach the peak concentration is about 3-5 h and there is a linear pharmacokinetics with LTG doses of 2.5 to 10 mg/kg (Yamashita et al. 1997). In rats, LTG has also a good distribution in tissues and organs, with a particular affinity to melanin-containing tissues (i.e. eyes and pigmented skin), and accumulate in kidneys (Castel-Branco et al. 2004; GlaxoSmithKline 2016). Castel-Branco and collaborators (2003) reported a linear relationship between LTG concentrations in the rat plasma and brain. Similarly to what happens in plasma, LTG rapidly appears in rat brain with a peak value achieved between 0.5 to 2 hours after an intraperitoneal dose of 10 mg/kg (Castel-Branco et al. 2003; Walker et al. 2000). The good distribution of LTG observed in the brain may probably be due to its basic and lipophilic properties.

Epilepsy is treated by chronic administration of AEDs. Therefore, it is important to know the pharmacokinetics of AEDs in steady-state conditions. Regarding the LTG, steady-state serum concentrations increase linearly with the dose. The time to reach steady-state is about 3-6 days, but women taking oral contraceptives may have larger fluctuations of the LTG serum concentrations in steady-state. The time to reach the LTG serum concentrations at steady-state is increased up to 5-15 days in patients co-medicated with valproic acid (Aldaz et al. 2011; Patsalos et al. 2008). LTG is also susceptible of autoinduction; for most patients, autoinduction is complete within two weeks, with a 20% reduction in steady-state serum/plasma concentrations if the dose is not changed (Krasowski 2010). However, the autoinduction of LTG is not considered to be clinically relevant (Biton 2006).

LTG is eliminated mostly by kidneys with a minor faecal elimination contribution. After an oral administration of 240 mg of radiolabelled LTG to six healthy volunteers, 94% of the dose was recovered in the urine and 2% was recovered in the faeces. In urine, 10% of LTG was detected unchanged, 76% as LTG-2-*N*- glucuronide, 10% as LTG-5-*N*-glucuronide, 0.14% as LTG-2-*N*-methyl metabolite and 4% of other unidentified minor metabolites (GlaxoSmithKline 2016).

LTG clearance is higher in children than in adults and moderately reduced in elderly (Jacob and Nair 2016). Additionally, LTG clearance seems to decrease as children grow older. In pregnant women LTG clearance is also increased (Burakgazi and French 2016). In rats, after an oral LTG dose of 10 mg/kg the elimination half-life reported was 25 h (Yamashita et al. 1997); a similar elimination half-life value (27.7 h) was estimated after an intraperitoneal LTG administration at the same dose (10 mg/kg) (Castel-Branco et al. 2005a).

Differences in gender, age and ethnic groups have also been implicated in the LTG elimination, particularly at the metabolism level. The differences found in the pharmacokinetics of LTG between ethnic groups may be due to genetic variations in drug-metabolizing enzymes. Mallaysamy *et al.* (2013) studied the population pharmacokinetics of LTG in Indian epilepsy patients, revealing negligible pharmacokinetic differences in comparison with Caucasian patients. Gulcebi *et al.* (2011) detected a decrease in LTG serum levels in patients with polymorphisms of the UGT1A4 enzyme. Milosheska *et al.* (2016) also found that patients carrying the UGT2B7–161TT genotype had 20.4% lower clearance when compared with patients with CC genotype. In patients carrying the UGT2B7 372GG genotype the clearance was higher by 117% compared to patients with the UGT2B7 372AA genotype. Since these polymorphisms may affect the clearance of LTG and its concentrations in plasma and brain, it is important to adjust the therapeutic doses of the drug in order to ensure its efficacy and safety.

Some AEDs and other drugs have also significant effects on LTG serum levels and on its clearance. Carbamazepine, oxcarbazepine, phenobarbital, phenytoin and primidone induce the metabolism of LTG, increase LTG clearance, and reduce LTG serum levels by 34–52%. Valproic acid inhibits LTG metabolism, so that LTG clearance is decreased and its serum levels are increased by twofold. Sertraline and fluoxetine can also increase LTG serum levels by 100 and 50%, respectively, while acetaminophen, olanzapine, rifampicin and ritonavir can increase LTG clearance and decrease LTG serum levels by 20–44% (Aldaz et al. 2011; Krasowski 2010; Landmark and Patsalos 2010; Patsalos et al. 2008). Oral contraceptives induce the glucuronidation and reduce LTG serum concentrations by more than 50% (Johannessen and Landmark 2010). LTG concomitantly administrated with clonazepam, levetiracetam, retigabine and valproic acid may also induce changes in serum levels of these AEDs (Patsalos 2013a).

The enzymatic competition of different substrates for the main glucuronidation pathway may be responsible for the inhibition of the LTG-*N*-glucuronidation, leading to accumulation of toxic concentrations of LTG. Inhibition or saturation of LTG-*N*-glucuronosyltransferase may be correlated to the bioactivation of LTG and, consequently, with the increased risk of skin reactions. Idiosyncratic reactions resulting from LTG therapy, with serious skin rash, agranulocytosis and lymphadenopathy, are presumably associated with the formation of reactive metabolites (Maggs et al. 2000).

In fact, the presence of an aromatic ring in its chemical structure is highly correlated with skin reactions and with the formation of toxic reactive metabolites (Wang et al. 2012).

I.5.3. Therapeutic drug monitoring of lamotrigine

TDM of AEDs in serum or plasma is a well-established practice to optimize epilepsy therapy. AEDs having a significant interindividual variability in the pharmacokinetics and a narrow therapeutic index should be closely monitored based on the measurement of drug concentration levels in a patient's biosample (usually serum or plasma). TDM of AEDs can be challenging since seizures can occur irregularly and unpredictably, often with long periods in between episodes (Patsalos et al. 2008). The persistence or incidence of new seizures with the use of an apparently adequate dosage of an AED are also good indicators to justify TDM. Additionally, TDM allows the evaluation of therapeutic failure caused either due to pharmacokinetic or pharmacodynamic phenomena, as well as the evaluation of toxic effects and interactions with other drugs (Krasowski 2010; Patsalos et al. 2008).

Alternatively to serum and plasma samples, other matrices can been employed for TDM, like dried blood spots, cerebrospinal fluid, hair, tears and saliva. The use of saliva in TDM is emerging, although it is still less used than plasma or serum in the routine clinical practice. The use of saliva has several advantages over blood or serum/plasma in what concerns to the collection and storage, but its major advantage for TDM is that the saliva reflects the free non-protein bound drug concentration in blood (Aps and Martens 2005; Chiappin et al. 2007).

Besides therapeutic reference ranges in serum/plasma have been reported for most AEDs, it is still crucial to target clinical efficacy since most AEDs can be administered in long-term therapy and dose can be adjusted when a particular AED is used in combination with other AEDs or when dosing requirements can change with age, pregnancy and clinical status (Krasowski and McMillin 2014).

For LTG the therapeutic reference range in serum is well defined: 3-15 μg/mL (10–59 mmol/L) (Patsalos 2013a; Patsalos et al. 2017); thus, the incidence of toxic effects is significantly increased when serum or plasma concentrations exceed 15 μg/mL (Jacob and Nair 2016). Nevertheless, LTG has a wide interindividual variability in its pharmacokinetics at any given doses, particularly as a result of pharmacokinetic interactions with concurrently prescribed AEDs, specially phenytoin, carbamazepine and valproic acid. Thus, LTG concentrations should be monitored during concomitant use with drugs that are enzyme inhibitors or inducers, in severe renal failure or in haemodialysis (Krasowski and McMillin 2014; Yasam 2016). Secondly, LTG clearance changes substantially during pregnancy and across different age groups (Dickens et al. 2012; Jacob and Nair 2016). LTG serum quantification is also important to access patients' compliance to therapeutics and to optimize dose regimens in pregnancy, children and elderly. Another important reason for the LTG TDM is the minimization of the risk of drug immunologic hypersensitivity reactions, which are rare but serious events that have been reported in some cases (Jacob and Nair 2016; Krasowski 2010; Wang et al. 2012).

Several bioanalytical methods have been validated and reported for TDM of LTG, mostly based on immunoassays and chromatographic methods (Jacob and Nair 2016; Krasowski 2010; Krasowski and McMillin 2014). Saliva/serum LTG ratio was firstly reported to be 0.46 in healthy

subjects receiving a single-dose and 0.56 in patients receiving adjunctive therapy (Hutchinson et al. 2018). This good correlation between serum and saliva LTG concentrations in these early studies propelled and paved the way to further studies in this scope (Patsalos and Berry 2013). Tsiropoulos and collaborators (2000) compared both stimulated and unstimulated saliva from the same patients and demonstrated a good correlation with LTG serum concentration for both collection procedures (r^2 = 0.85 for unstimulated saliva and r^2 = 0.94 for stimulated saliva). Ryan *et al*. (2003) have also studied the relationship between serum and salivary concentrations of LTG in both paediatric and adult epilepsy patients and also reported good correlations (r^2 = 0.81–0.84). In another study, Malone and his collaborators (2006) also measured LTG concentrations in both stimulated and unstimulated saliva and they found a close correlation in each individual volunteer. More recently, Mallayasamy *et al*. (2010) reported a correlation between salivary and serum LTG concentrations of 0.683. Having in mind the available evidence for LTG regarding the good correlation between salivary and plasma/serum concentrations, it is believed that the use of saliva in routine clinical practice will be a viable alternative to serum or plasma samples for TDM of LTG (Patsalos and Berry 2013).

I.6. Aims of this thesis

Considering that obesity is a common comorbid condition in patients with epilepsy, and being LTG a broad-spectrum AED with a large interindividual variability in its pharmacokinetics and a propensity to interact with other drugs, the main objective of this thesis was the non-clinical assessment of the potential for HDIs between herbal extracts often present in weight loss supplements and LTG.

In the context of the present work four standardized weight loss herbal extracts from *P. cupana*, *G. cambogia*, *C. aurantium* and *F. vesiculosus* were selected. To evaluate the occurrence of potential interactions between these weight loss herbal extracts and LTG a set of experimental studies was planned using male Wistar rats as animal model.

However, to make these goals achievable, appropriate bioanalytical methods must always be conveniently developed and validated in order to obtain accurate and reliable quantitative data in the biological matrices of interest (rat plasma and brain in this case). Moreover, assuming that LTG concentration correlates better with therapeutic and/or toxic effects than the dose, TDM is recommended for the pharmacological treatment optimisation in patients under LTG therapy. Therefore, the availability of a simple bioanalytical tool for the determination of LTG in human plasma and saliva that could be easily adopted by hospitals would certainly be useful from a clinical point of view.

The specific aims outlined for the implementation of the work underlying this thesis were as follows:

- Development and validation of an analytical high-performance liquid chromatography with diode array detection (HPLC-DAD) method to quantify LTG in human plasma and saliva using the microextraction by packed sorbent (MEPS) as sample preparation procedure. This bioanalytical technique aims to be an innovative and alternative tool for supporting the TDM of LTG, even using saliva.

- Development and validation of an analytical HPLC-DAD method to quantify LTG in rat brain and plasma using the MEPS as sample preparation technique. This bioanalytical assay in the rat matrices aims to support the subsequent pharmacokinetic-based studies.

- Conduction of pharmacokinetic studies to investigate potential interactions between each weight loss herbal extract (*P. cupana*, *G. cambogia*, *C. aurantium and F. vesiculosus*) and LTG in rats.

- Evaluation of the effects of herbal extracts on rats' body weight and, whenever possible, on selected biochemical parameters after a 14-day treatment period with each herbal extract (*P. cupana, G. cambogia, C. aurantium and F. vesiculosus*).

Chapter II.

Bioanalysis of lamotrigine

II.1. Bioanalytical methods for lamotrigine quantification

Bioanalysis is well-established in modern laboratories to support toxicokinetic, pharmacokinetic and pharmacodynamic evaluations in preclinical and clinical studies (Moein et al. 2017; Pandey et al. 2010). Usually, bioanalysis involves an analytical process for the quantification of one or more than one analyte of interest (drugs, metabolites, biomarkers) in biological matrices such as plasma, serum, whole blood, urine, saliva and tissues (Moein et al. 2017).

The development of a bioanalytical method must so define the operating conditions, limitations and suitability of the method for the intended purpose and it should provide accurate and precise results. For each bioanalytical method, it has to be ensured that the development achieved is appropriate before proceeding with the validation procedures. Method validation in bioanalysis is strongly regulated by the EMA and FDA. Both authorities have issued guidelines that address in detail the requirements for bioanalytical method validation (Vlčková et al. 2018).

Validation includes the optimization of bioanalytical parameters such as reference standards, critical reagents, calibration curve, quality control samples, selectivity and specificity, sensitivity, accuracy and precision, recovery and stability of the analyte(s) in the selected matrix. Validated analytical methods for the quantitative evaluation of target analytes are so critical for the successful conduction of nonclinical and clinical pharmacology studies and they provide critical data to support the safety and effectiveness of drugs and biologic products (FDA 2018).

When considering the use and monitoring of drugs in therapy, bioanalytical methods should be available to quantify drugs in the biological samples of interest in order to adjust patient's medication regimen and achieve optimal therapeutic outcomes. Indeed, many AEDs are good candidates for TDM (Aydin et al. 2016) and several methods have been published for AEDs quantification in different matrices and biological fluids. For the particular case of LTG there have been also developed and validated several techniques to quantify this drug in different human matrices (e.g. blood, plasma, serum, urine and saliva), including immunoassay (Biddlecombe et al. 1990; Juenke et al. 2011), electrophoresis (Pucci et al. 2005; Shihabi 1999; Shihabi and Oles 1996; Theurillat et al. 2002; Thormann et al. 2001; Zheng et al. 2004) and chromatographic methods. Indeed, the predominant methodology for LTG bioanalysis is the high-performance liquid chromatography (HPLC) coupled to diode array detection (DAD) (Brunetto et al. 2009; Ferreira et al. 2014; Saracino et al. 2007a; Saracino et al. 2007b; Vermeij and Edelbroek 2007; Zufia et al. 2009), ultraviolet (UV) (Bompadre et al. 2008; Budakova et al. 2008; Cheng et al. 2005; Contin et al. 2005; Contin et al. 2010; Franceschi and Furlanut 2005; Mallayasamy et al. 2010; Morgan et al. 2011; Patil and Bodhankar 2005; Rivas et al. 2010; Serralheiro et al. 2013; Youssef and Taha 2007) or mass spectrometry (MS) detection (Hotha et al. 2012; Kim et al. 2011; Kuhn and Knabbe 2013; Lee et al. 2010).

However, in animal (rat) matrices (e.g. blood, plasma, serum, brain) only a few number of HPLC methods coupled to UV (Castel-Branco et al. 2001b; Liu et al. 2014; Walker et al. 2000; Walton et al. 1996; Yamashita et al. 1997) or MS detection (Yang et al. 2013) have been reported in literature for the quantification of LTG.

II.1.1. Liquid chromatographic methods

Liquid chromatography, particularly HPLC, is a common chromatographic technique used in pharmaceutical laboratories for the qualitative and quantitative analysis of drug substances throughout all the phases of drug development. HPLC indeed emerged as a powerful technique in bioanalysis coupled to different detection systems like UV and DAD or even coupled to MS instrumentation (Moein et al. 2017). HPLC is a versatile separation technique with a wide range of applications but the development of each method is sometimes critical due to the large number of variables associated, which need to be properly adjusted (Sahu et al. 2018).

HPLC operates at a high pressure and separation predominantly depends on the nature of the mobile phase (like polarity, flow rate, pH, composition), properties of sample matrix, type and nature of stationary phase, environmental factors like temperature, and detector type and settings (Sahu et al. 2018). Chromatographic separation of one or more analytes occurs when the sample that contains the analyte or analytes of interest is dragged by the mobile liquid phase that passes through the stationary phase (column) allowing the elution of the analytes according to the affinity and type of interactions established between each of the analytes and the stationary phase.

The selection of the chromatographic column should be made according to the chemistry properties and molecular weight of the analyte(s) and also considering the specificities of the chromatographic system (pressure and temperature). HPLC columns are packed with very fine particles and the separation is achieved due to different intermolecular forces between the solute ant the stationary phases and those between the solute and the mobile phase. The column will retain those substances that interact more strongly with the stationary phase than those that interact more strongly with the mobile phase (Jena 2011).

Octadecylsilica or octylsilica stationary phases are the most popular column packing materials and most of the HPLC procedures for LTG quantification have employed some type of reversed-phase chromatographic column, typically containing an octyl (C_8) or octadecyl (C_{18}) packing, in combination with buffered hydro-organic eluents like water, methanol and acetonitrile (Antonilli et al. 2011; Bompadre et al. 2008; Budakova et al. 2008; Castel-Branco et al. 2005a; Chollet 2002; Contin et al. 2005; Contin et al. 2010; Ferreira et al. 2014; Franceschi and Furlanut 2005; Greiner-Sosanko et al. 2007a; Greiner-Sosanko et al. 2007b; Jebabli et al. 2015; Kim et al. 2011; Kuhn and Knabbe 2013; Martins et al. 2011; Patil and Bodhankar 2005; Peysson and Vulliet 2013; Pucci et al. 2005; Saracino et al. 2007a; Serralheiro et al. 2013; Tai et al. 2011; Vermeij and Edelbroek 2007).

The selection of suitable separation modes, stationary phases, and also the pH of the mobile phase is important to improve method selectivity and sensitivity. Although HPLC enables analysis in various separation modes, including reversed-phase, normal phase, hydrophilic interaction chromatography (HILIC), ion-exchange and mixed mode, most analyses in clinical practice are carried out in reversed-phase mode using the C18 stationary phase, and using an hydrophobic stationary phase and a polar mobile phase (Vlčková et al. 2018; Yabré et al. 2018). Normal phase chromatographic columns have been rarely described for LTG quantification (Chollet 2002). In some LTG methods, it was used a different type of column consisting of a cyano column with bonded ligand that interacts with the polar functional groups, which can be used either in both reversed-phase and normal phase chromatography (AbuRuz et al. 2010; Hotha et al. 2012; Lensmeyer et al. 1997). Heideloff *et al.* (2010) used a monolithic column for the quantification of ten AEDs simultaneously, including LTG, with an increased sensitivity, better resolution, and a shorter analytical time compared with a regular C_{18} column.

In what concerns the mobile phase composition for chromatographic analysis, the main criterion in mobile phase selection and optimization is to achieve optimum separation of the analyte peaks. Indeed, the mobile phase composition plays an important role in analyte peak definition and symmetry. Solvent elution strength in mobile phases has the ability to pull the analytes from the column, and so the composition of the mobile phases should be mostly controlled by the concentration of the solvent with the highest elution strength.

The mobile phase of most reversed-phase HPLC methods is usually a mixture of water (containing additives to adjust pH and ionic strength) and organic solvent, such as acetonitrile and methanol (Yabré et al. 2018). Overall, methanol, acetonitrile and water constitute the major components of mobile phases in LTG analysis, which are present in variable percentages (Antonilli et al. 2011; Bompadre et al. 2008; Brunetto et al. 2009; Budakova et al. 2008; Cantu et al. 2006; Castel-Branco et al. 2005a; Cheng et al. 2005; Chollet 2002; Contin et al. 2005; Contin et al. 2010; Ferreira et al. 2014; Franceschi and Furlanut 2005; Greiner-Sosanko et al. 2007b; Jebabli et al. 2015; Kim et al. 2011; Kuhn and Knabbe 2013; Martins et al. 2011; Morgan et al. 2011; Patil and Bodhankar 2005; Pucci et al. 2005; Saracino et al. 2007a; Shah et al. 2013; Tai et al. 2011; Vermeij and Edelbroek 2007; Zufia et al. 2009). Additionally, in terms of aqueous buffer solutions, the mostly employed has been phosphate buffer (Antonilli et al. 2011; Bompadre et al. 2008; Brunetto et al. 2009; Castel-Branco et al. 2005a; Chollet 2002; Contin et al. 2010; Greiner-Sosanko et al. 2007a; Greiner-Sosanko et al. 2007b; Martins et al. 2011; Patil and Bodhankar 2005; Pucci et al. 2005; Rivas et al. 2010; Saracino et al. 2007a; Shah et al. 2013; Vermeij and Edelbroek 2007; Youssef and Taha 2007; Zufia et al. 2009). The use of amine additives, such as ion-pair or competing base reagents like triethylamine has also been found, in small amounts, in several mobile phases (AbuRuz et al. 2010; Budakova et al. 2008; Castel-Branco et al. 2005a; Cheng et al. 2005; Chollet 2002; Ferreira et al. 2014; Franceschi and Furlanut 2005; Kuhn and Knabbe 2013; Martins et al. 2011; Rivas et al. 2010; Zufia et al. 2009).

II.1.2. Sample preparation procedures

Due to the complex nature of biological matrices, sample preparation steps are the most important integral part of bioanalytical methods. Sample preparation, or sample pre-treatment, is the first relevant analytical step in biopharmaceutical analysis (Nováková and Vlcková 2009). The main objectives of sample preparation are to remove interfering substances (including proteins, salts and lipids), eliminate ion suppression, pre-concentrate the analyte(s) in order to improve sensitivity, purify the sample, and convert the analyte(s) in a suitable form for the selected bioanalytical method (Ashri and Abdel-Rehim 2011). The extent of sample pre-treatment depends on the complexity of the sample and can include one or more steps.

One of the reasons why biological samples are so problematic is mainly due to the irreversible adsorption of proteins in stationary phases, resulting in a substantial loss of column efficiency and an increase in backpressure. Hence, sample preparation must fulfil its main objectives by either conventional or more sophisticated processes. The conventional approach use mostly protein precipitation (PP), liquid–liquid extraction (LLE), solid-phase extraction (SPE) or a combination of two of these methods (Abdel-Rehim 2011). Indeed, PP with acids or water-miscible organic solvents may precede the extraction steps in a LLE or SPE protocol (Moein et al. 2017). Organic solvents, such as methanol, acetonitrile, acetone and ethanol, although having a relatively low efficiency in removing plasma proteins, have been widely used in bioanalysis because of their compatibility with HPLC mobile phases. In chromatographic procedures, proteins can precipitate, denature and adsorb onto the packing material, leading to backpressure build-up, changes in retention time and the decrease of column efficiency and capacity. In PP both acetonitrile and methanol ensure a good recovery, although methanol may be particularly selected because of its safer use and lower potential for drug degradation (Ashri and Abdel-Rehim 2011; Wohlfarth and Weinmann 2010). After the addition of the precipitating agents centrifugation is essential to separate the supernatant, which should be clean and should contain the analyte(s) of interest. Although PP is considered the fastest and the simplest extraction approach for both hydrophilic and hydrophobic compounds, it is somehow time-consuming; thus, other extraction techniques are also commonly used in practice because they show fewer restrictions (Nováková and Vlcková 2009).

LLE was one of the first sample preparation techniques and continues to be widely used in bioanalysis. LLE is a good method that allows direct extraction of the analyte(s), which are isolated by partitioning between the aqueous phase of the sample and the immiscible organic phase formed by a solvent or a mixture of solvents with different polarities. LLE is a simple method, but it uses a large amount of solvent usually in more than one step of extraction, and is almost inadequate for hydrophilic analytes (Ashri and Abdel-Rehim 2011; Moein et al. 2017; Wohlfarth and Weinmann 2010).

In SPE the analytes to be extracted are partitioned between a solid phase and a liquid phase. The analyte is retained on the solid phase formed by a packed sorbent by nonpolar, polar or ionic interactions. SPE has replaced most LLE methods and it is being preferred to extract drugs

and metabolites from biological samples due to its selectivity, high recovery, efficiency and easy automation (Ashri and Abdel-Rehim 2011; Moein et al. 2017).

Until 2002 the extraction techniques used in analytical methods for LTG quantification involved typically LLE (with different solvents or mixtures of solvents, such as dichloromethane, propanol, ethyl acetate, diethyl ether or chloroform), PP (with acetonitrile, methanol, aqueous trichloroacetic acid or zinc sulphate) or SPE protocols (Chollet 2002). Since then miniaturized techniques were introduced such as liquid-liquid microextraction (LLME), solid-phase microextraction (SPME) and microextraction by packed sorbent (MEPS) (Moein et al. 2017).

Actually, the liquid chromatographic methods reported in literature for the determination of LTG in human matrices have involved LLE (Antonilli et al. 2011; Barbosa and Midio 2000; Budakova et al. 2008; Castel-Branco et al. 2001b; Greiner-Sosanko et al. 2007b; Hart et al. 1997; Mashru et al. 2005; Matar et al. 1999; Rivas et al. 2010), PP (Contin et al. 2005; Contin et al. 2010; Kuhn and Knabbe 2013; Lee et al. 2010; Pucci et al. 2005; Ramachandran et al. 1994; Saracino et al. 2007a; Theurillat et al. 2002; Youssef and Taha 2007), SPE (Bompadre et al. 2008; Shah et al. 2013; Tai et al. 2011; Torra et al. 2000; Vermeij and Edelbroek 2007; Yamashita et al. 1995; Zufia et al. 2009), SPME (Cantu et al. 2006) and MEPS procedures as sample preparation approaches (Ferreira et al. 2014). On the other hand, in the reported liquid chromatography methods developed to quantify LTG in animal (rat) matrices the sample preparation has been mainly performed through classic procedures such as LLE (Castel-Branco et al. 2001a; Liu et al. 2014; Walton et al. 1996), PP (Castel-Branco et al. 2001a; Liu et al. 2014; Walker et al. 2000; Yang et al. 2013) and/or SPE (Yamashita et al. 1997).

Among the several microextraction techniques recently developed, MEPS has represented an outstanding approach for sample preparation and pre-concentration of target analytes from biological matrices. Indeed, MEPS has been successfully applied to the qualitative and quantitative determination of a wide variety of drugs and/or metabolites in biological samples, such as plasma, serum, blood, urine, saliva and hair (Alves et al. 2013). Considering the important advantages that have been ascribed to MEPS, which basically consist of a miniaturized version of SPE, this emerging microextraction technique was selected to support the sample preparation procedures required in the bioanalytical methods developed and validated in the context of this thesis.

II.1.2.1. Microextraction by packed sorbent: a brief overview

MEPS was introduced in 2003 as a miniaturized SPE technique and rapidly emerged as a simple, fast, cost-effective, readily automated and green sample preparation method (Moein et al. 2017). MEPS has been successfully applied to the quantitative analysis of several therapeutic agents, namely antibiotics, antihypertensives, antiarrhythmics, antidepressants, antipsychotics, and even AEDs (Alves et al. 2013). As aforementioned, the experimental steps

involved in MEPS protocols (i.e. cartridge activation/conditioning, sample loading, sorbent washing, and elution) are quite similar to those used in conventional SPE; in fact, the nature of the adsorbents used as packing materials is also similar in both techniques MEPS and SPE (Abdel-Rehim 2011).

MEPS solid packing material (solid phase) is either inserted into the barrel of a syringe or between the syringe barrel and the injection needle as a cartridge. The MEPS barrel insert and needle (BIN) assembly contains the stationary phase that can be formed by different sorbents (**Figure II.1**). Sorbents examples include pure silica or silica-based sorbents (C_2, C_8, C_{18}), strong cation exchanger (SCX), restricted access material (RAM), carbon, polystyrene-divinylbenzene copolymer (PS-DVB) or molecularly imprinted polymers (MIP) (Abdel-Rehim 2010; Abdel-Rehim 2011; Alves et al. 2013; Nováková and Vlcková 2009).

Before the use of MEPS sorbent, it should be submitted to a conditioning step and sorbent must be activated by an appropriate solvent like methanol or acetonitrile. The excess of this organic solvent is then removed by passing a more polar solvent like water, buffer solutions (with formic acid or ammonium acetate) or a mixture of solvents such as water/methanol (90:10, v/v) through the solid sorbent, preparing the packed sorbent to receive the aqueous sample (Alves et al. 2013). Sample extraction in MEPS protocols may also involve previous steps of centrifugation and dilution to increase sample fluidity and to avoid obstruction of MEPS sorbent. Blood samples must be diluted 20- to 25-times and plasma samples 4- to 5-times with pure water or 0.1% formic acid in water (dilution, 1:5 for plasma and 1:25 for blood) (Abdel-Rehim 2010). Similarly, PP may also precede MEPS protocols thus increasing the reuse of each MEPS cartridge (Alves et al. 2013).

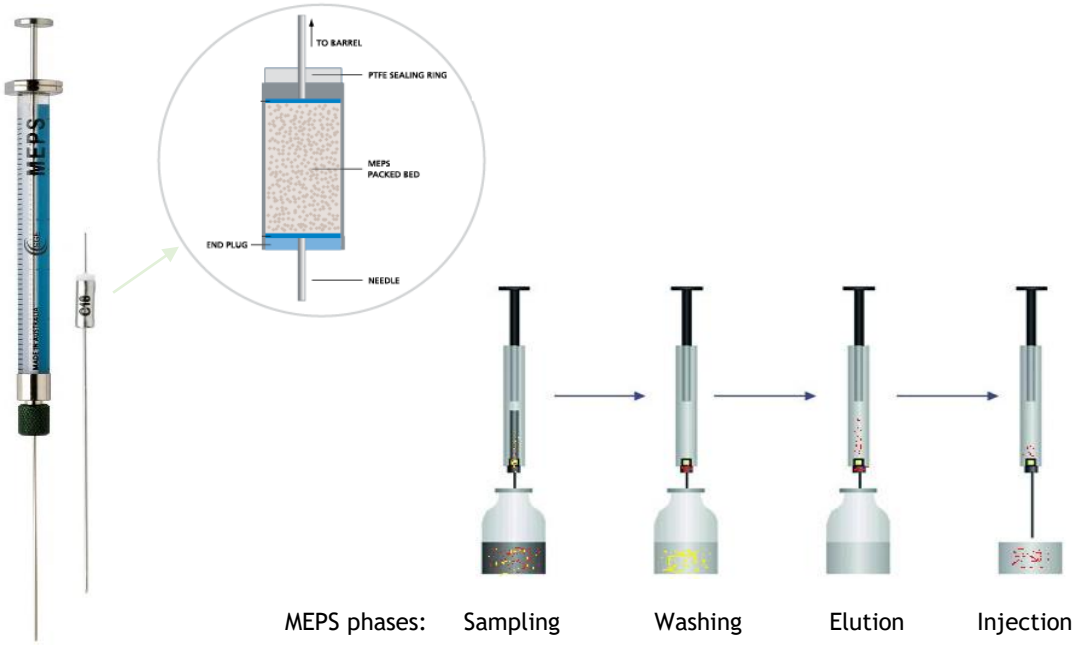


Figure II.1. Schematic representation of the microextraction by packed sorbent (MEPS) procedure (in http://www.sge.com).

When the sample is drawn through the syringe (typically with three to ten draw-eject sampling cycles), the analyte(s) of interest are adsorbed onto the solid phase. Sample aspiration and ejection should be slowly performed (approximately 5–20 µl/s) in order to obtain a good percolation between sample and solid support and, thus, a better interaction between the analytes and the sorbent. The sorbent is then washed mainly with pure water, acidic water (0.1% formic acid), water/methanol (95:5, v/v) or water/methanol (90:10, v/v) and eluted typically with pure methanol, and methanol/water with 0–0.25% ammonium hydroxide (95:5, v/v) (Alves et al. 2013).

A limitation of MEPS is the carry-over effect which is closely related to analyte adsorption. In order to reduce carry-over effects, it is important to apply an appropriate washing solution and an optimal number of rinsing cycles after analytes elution. In most bioanalytical methods involving MEPS, carry-over elimination has been accomplished with three to five washing cycles with elution solution followed by washing cycles with the reconditioning solution (Alves et al. 2013).

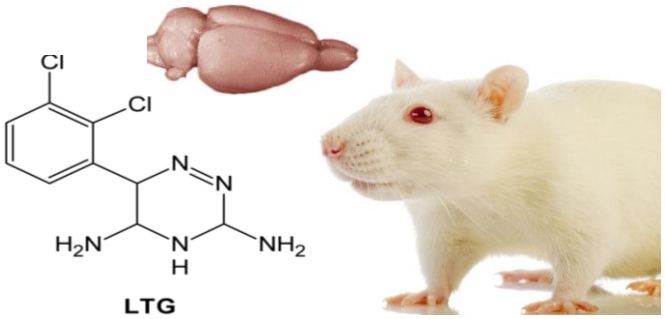
Compared with liquid LLE and SPE, MEPS reduces sample volume and preparation time, organic solvent consumption and allows the reuse of MEPS cartridges for a variable number of extractions (10–300-times) (Alves et al. 2013; Nováková and Vlcková 2009). Additionally, MEPS allows good recovery and acceptable sensitivity when compared to SPE and SPME (Abdel-Rehim 2011).

II.1.3. Validation of bioanalytical methods

The implementation of a bioanalytical method either for clinical and nonclinical studies must follow the principles on good laboratory practices (GLPs). The existence of standard operating procedures from sample collection to data handling is crucial to ensure the quality and reliability of all experimental procedures, including in the validation of a bioanalytical method (Moein et al. 2017). So, the bioanalytical method validation can be seen as a piece of the puzzle in the scope of the good laboratory standards. Validation is a necessary process to demonstrate that an analytical method is suitable and can offer accurate, precise and reproducible results (González et al. 2014). In the development of a bioanalytical method, several parameters must be confirmed in order to meet the acceptance and reliability requirements such as selectivity, sensitivity (limits of quantification and/or detection), calibration curve, accuracy and precision, recovery, and stability of the analyte(s) in the intended biological matrix and in the stock and working solutions over the entire period of storage and under the processing conditions (EMA 2011a; Moein et al. 2017). The validation procedures must reflect the performance characteristics of the selected method in terms of suitability and reliability for the intended purposes. Fundamental validation parameters include selectivity, sensitivity (limits of quantification and/or detection), calibration curve, accuracy and precision, recovery and stability (EMA 2011a; FDA 2013; González et al. 2014).

A bioanalytical method developed for the first time or that includes a new drug or metabolite to be analysed should be fully validated (González et al. 2014). A full validation is also required for the analysis of a new drug entity and for improvement of an existing method aiming the addition of a new analyte (e.g. a metabolite). However, when minor changes are made to an analytical method that has been previously validated, a full validation may not be necessary. A partial validation is often applied, for instance, when a bioanalytical method is transferred from a laboratory to another, when the equipment used is different and when changes occur in the calibration range, in the sample volume, in matrices or species, in the sample processing procedure, in the storage conditions, and also when the anticoagulant is changed. On the other hand, a cross-validation is needed to data comparison when a bioanalytical method is used in different laboratories.

In this context, in order to standardize as much as possible the procedures to be applied in the validation of bioanalytical methods, international guidelines have been issued by important regulatory agencies such as the United States FDA (FDA 2013) and the EMA (EMA 2011a), which were used in the validation of the bioanalytical methods developed in this thesis.



II.2. *Experimental Section*

An easy-to-use liquid chromatography assay for the analysis of lamotrigine in rat plasma and brain samples using microextraction by packed sorbent: *application to a pharmacokinetic study*

II.2.1. Introduction

Lamotrigine (LTG) is a second-generation antiepileptic drug (AED) exhibiting a broad spectrum of efficacy against several types of epilepsy seizures, and it is also effective as a mood stabilizer agent in bipolar syndromes (Krasowski 2010; Landmark and Patsalos 2010; Schiller and Krivoy 2009). LTG has a narrow therapeutic range, a large inter-individual variability in its pharmacokinetics and some side effects are concentration-dependent, justifying therapeutic drug monitoring (TDM) in many clinical circumstances. For instance, LTG undergoes extensive metabolism to an inactive glucuronide metabolite, and its own metabolism is characterized by an autoinduction phenomenon that appears to be complete within 2 weeks, resulting in a 17% reduction in LTG serum concentrations (Patsalos et al. 2008). The biotransformation of LTG is also susceptible to heteroinduction and enzyme inhibition. Indeed, the metabolism of LTG is significantly affected by concomitant use of hepatic enzyme inducers such as classic AEDs (carbamazepine, phenytoin, primidone and phenobarbital) and oxcarbazepine, as well as others drugs such as rifampicin, ritonavir, acetaminophen and olanzapine (Landmark and Patsalos 2010; Patsalos 2013a; Patsalos et al. 2008; Zaccara and Perucca 2014). Contraceptives containing estradiol can also reduce the serum concentration of LTG by 50% and in women on oral contraceptives this interaction results in different steady-state LTG concentrations between the days of pill intake compared with the pill-free interval (Landmark and Patsalos 2010; Patsalos et al. 2008). On the other hand, the LTG metabolism is inhibited by valproic acid and sertraline. In fact, the inhibitory interaction with valproic acid was found to be clinically relevant and smaller doses of LTG as well as a slower titration rate should be used to minimize the risk of side effects (Patsalos et al. 2008). The most serious adverse effect observed within the LTG therapeutic range (2.5-15 μg/mL) is skin rash, probably related to its aromatic ring and the formation of toxic metabolites (Musenga et al. 2009). Indeed, the incidence of toxic effects is significantly increased when serum or plasma concentrations exceed 15 μg/mL (Patsalos et al. 2008).

Over the years, rodents (rats and mice) have been largely employed as whole laboratory animal models to identify new anticonvulsant compounds and to obtain a better understanding of the pharmacokinetics of established AEDs at non-clinical level, and to study their involvement in drug-drug interactions (Galanopoulou et al. 2013; Giblin and Blumenfeld 2010; Guillemain et al. 2012; Harward and McNamara 2014; Loscher 2007; Loscher 2011; Rogawski 2006; Sankaraneni and Lachhwani 2015; White 2003). Due to the fact that rodents eliminate most drugs much more rapidly than humans, anticonvulsant doses of AEDs are usually much higher in rodent models of seizures than effective doses in epilepsy patients. In spite of the pharmacokinetic differences observed between species, the effective plasma levels of AEDs are usually similar among rodents and humans (Castel-Branco et al. 2005a; Loscher 2011). Therefore, rodent models can be used to evaluate and predict plasma levels in humans by

calculating the corresponding doses that will produce a similar anticonvulsant effect (Loscher 2007).

More specifically, LTG efficacy has been extrapolated from pharmacological studies conducted in rats. However, like other AEDs, LTG needs to cross the blood–brain barrier to exert its therapeutic effect. Thus, the determination of LTG levels in plasma and brain tissue is essential to characterise its pharmacokinetic/pharmacodynamic relationship (Castel-Branco et al. 2005a; Castel-Branco et al. 2005b; Castel-Branco et al. 2003). Likewise, information on the LTG concentrations achieved simultaneously in plasma/serum and brain (biophase) is also determinant to predict the impact of drug-drug or herb-drug interactions involving LTG as the victim (object) drug. Hence, the availability of suitable bioanalytical methodologies to support the measurement of LTG concentrations in these particular biological samples is imperative.

To date, only a few number of high performance liquid chromatography (HPLC) methods coupled to UV (Castel-Branco et al. 2001a; Liu et al. 2014; Walker et al. 2000; Walton et al. 1996; Yamashita et al. 1997) or MS (Yang et al. 2013) detection have been reported in literature for the quantification of LTG in rat plasma/serum and brain. However, in those methods, sample preparation has been mainly performed through classic procedures, such as solid-phase extraction (Yamashita et al. 1997), protein precipitation (Castel-Branco et al. 2001b; Liu et al. 2014; Walker et al. 2000; Yang et al. 2013) and/or liquid-liquid extraction (Castel-Branco et al. 2001b; Liu et al. 2014; Walton et al. 1996).

Nevertheless, in recent years several miniaturized sample preparation techniques have been developed whose importance in bioanalysis has been increasingly recognized, among them is microextraction by packed sorbent (MEPS). In fact, MEPS has been successfully applied to the quantitative analysis of several therapeutic agents, namely antibiotics, antihypertensives, antiarrhythmics, antidepressants, antipsychotics, and even antiepileptic drugs including LTG (Alves et al. 2013). Nonetheless, as far as we know, no bioanalytical assay has been developed for the quantification of LTG specifically in rat plasma and brain tissue samples using MEPS. Therefore, the purpose of this work was to develop and validate a novel method for the quantification of LTG in rat plasma and brain homogenate using the innovative MEPS technology in sample preparation.

II.2.2. Material and methods

II.2.2.1. Materials and reagents

LTG was kindly provided by Bluepharma (Coimbra, Portugal). Chloramphenicol, used as internal standard (IS), was purchased from Sigma–Aldrich (St Louis, MO, USA). Methanol and acetonitrile, both of HPLC gradient grade, were purchased from Fisher Scientific (Leicestershire, United Kingdom) and the ultra-pure water (HPLC grade, >18 MΩ.cm) was prepared by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA).

Triethylamine, dihydrogen phosphate dehydrate and di-sodium hydrogen phosphate anhydrous were acquired from Merck KGaA (Darmstadt, Germany) and the 85% *ortho*-phosphoric acid from Fischer Scientific UK. Pentobarbital (Eutasil® 200 mg/mL, Ceva Saúde Animal) used as anaesthetic drug was commercially acquired. MEPS 250 µL syringe and MEPS BIN (barrel insert and needle) containing ~4 mg of solid-phase silica – C_{18} material (SGE Analytical Science, Australia) were supplied by ILC (Porto, Portugal).

II.2.2.2. Blank rat matrices

Healthy adult male Wistar rats (300–380 g, 10–12 weeks old) were obtained from local certified animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal) and were used as source of blank matrices (plasma and brain tissue) required for the validation experiments. For that, rats not subjected to any other treatment were anesthetized with pentobarbital (60 mg/kg) and then decapitated. Blood samples were directly collected into heparinised tubes and after exsanguination the brain was quickly excised. The blood samples were centrifuged at 4000 rpm for 10 min (4 ºC) and then the plasma was immediately separated from the blood cells and stored at –20 ºC until to be used. The brain tissue was weighed and homogenized in 0.1 M sodium phosphate buffer, pH 5.5 (4 mL/g of tissue) using a Ultraturrax® tissue homogenizer. The brain tissue homogenates were centrifuged at 13500 rpm for 10 min (4 ºC) and the supernatants were collected and stored at –20 ºC until used. The animal procedures were conducted in accordance with the European Directive (2010/63/EU).

II.2.2.3. Stock solutions, calibration standards and quality control samples

The LTG stock solution (1 mg/mL) and working solution (100 µg/mL) were prepared in methanol, and then adequately diluted in water-methanol (50:50, v/v) to afford six different spiking solutions at 0.5, 1, 3.5, 15, 62.5 and 100 µg/mL. Each one of these solutions were used daily for spiking aliquots of blank rat samples (plasma and brain homogenate; 20 µL spiking solution to 80 µL of blank sample) in order to prepare the corresponding calibration standards at six different concentrations (0.1, 0.2, 0.7, 3, 12.5 and 20 µg/mL). The stock solution of the IS was also prepared in methanol (1 mg/mL) and the working solution (250 µg/mL) was obtained after diluting an appropriate volume of the stock solution with water-methanol (50:50, v/v). All solutions were stored at 4 ºC and protected from light, except the IS working solution which was daily prepared.

Quality control (QC) samples at four concentration levels were also prepared independently in the same biological matrices, representing the lowest (QC_{LOQ}), low (QC_1), medium (QC_2) and high (QC_3) ranges of the calibration curves. For that purpose, aliquots of blank rat plasma and brain homogenate samples were similarly spiked in order to obtain final LTG concentrations of 0.1, 0.3, 10 and 18 µg/mL.

II.2.2.4. Sample preparation and extraction

The optimal sample preparation and extraction conditions were set as follows. To aliquots (100 µL) of plasma or brain homogenate, spiked with 20 µL of the IS working solution (250 µg/mL), 400 µL of ice-cold acetonitrile were added; the final mixture was vortex-mixed for 30s and centrifuged at 13,500 rpm for 10 min to precipitate proteins in order to minimize sample interferences. The resulting clear supernatant was collected and evaporated under a gentle nitrogen stream at 45 ºC and the dry residue was reconstituted with 200 µL of 0.3% triethylamine-water solution (pH 6.5) and then submitted to MEPS procedure (**Figure II.2**).

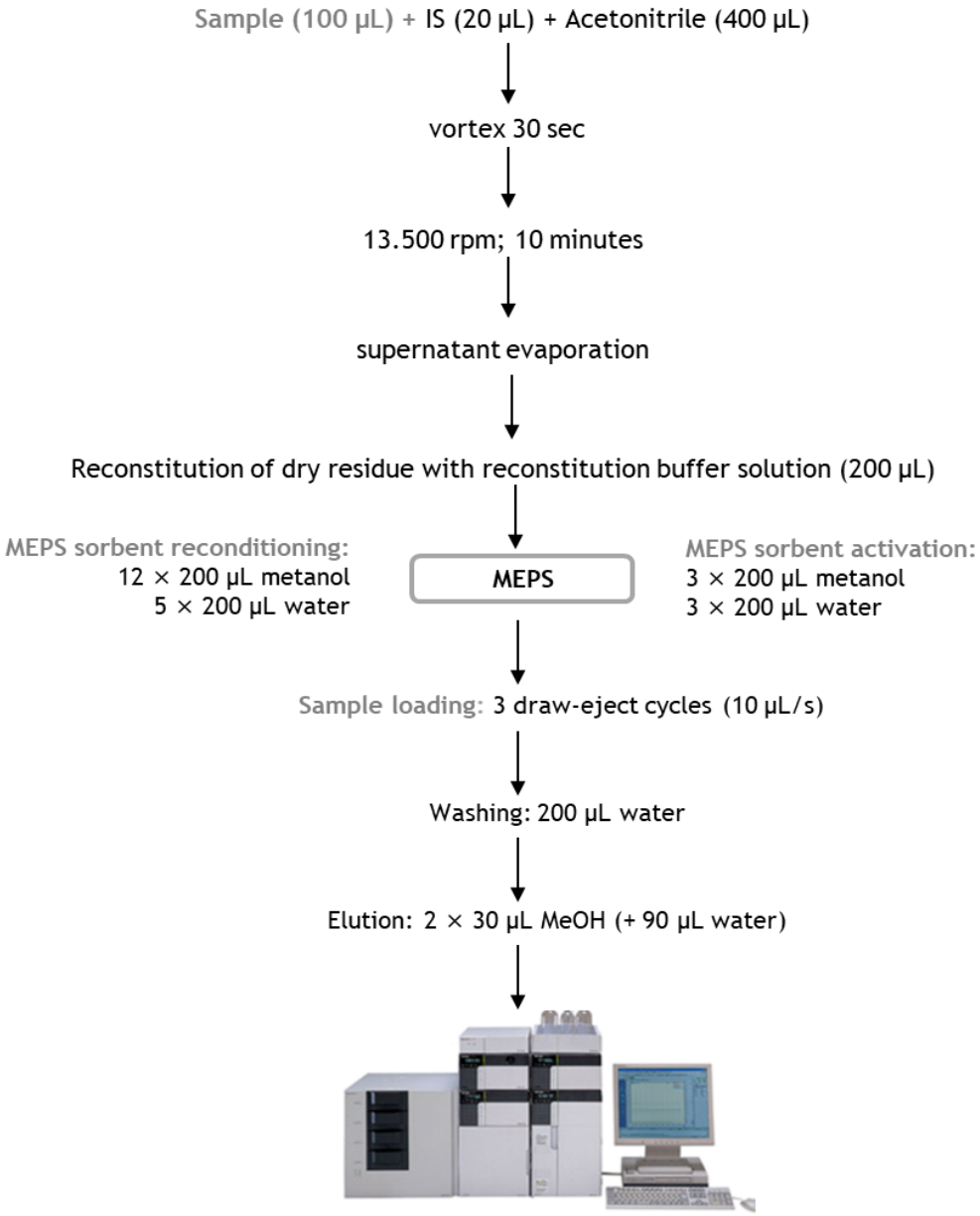


Figure II.2. Schematic representation of lamotrigine sample preparation involving a combination of protein precipitation and microextraction by packed sorbent (MEPS).

The MEPS sorbent (C_{18}) inserted into a 250 µL gas-tight syringe was activated with methanol (3 x 200 µL) and then conditioned with ultra-pure water (3 x 200 µL) before use. Afterwards, the reconstituted sample (200 µL) was drawn up and down through the syringe three times in the same vial, at a flow rate of 10 µL/s. In the next step, the sorbent was washed once with ultra-pure water (200 µL) in order to remove interferences and then the compounds of interest (LTG and IS) were eluted with methanol (2 x 30 µL). The resulting methanolic extract was diluted with 90 µL of ultra-pure water, and 20 µL were injected into the chromatographic system. After each sample extraction, the MEPS sorbent was washed/reconditioned with 12 x 200 µL of methanol followed by 2 x 200 µL of ultra-pure water to avoid carry-over phenomena, and to allow the reutilization of the MEPS cartridge. Applying this protocol, each MEPS cartridge was reused for approximately 200 extractions before it was discarded.

II.2.2.5. Apparatus and chromatographic conditions

The chromatographic analysis was carried out using an HPLC system (Shimadzu LC-2010A HT Liquid Chromatography) coupled with a DAD (Shimadzu SPD-M20A). All instrumental parts were automatically controlled by LC solution software (Shimadzu, Kyoto, Japan). The chromatographic separation of LTG and the IS was carried out at 35 ºC on a reversed-phase LiChroCART® Purospher Star column (C_{18}, 55 mm × 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany). An isocratic elution was applied at a flow rate of 1.0 mL/min with a mobile phase composed of acetonitrile (13%), methanol (13%) and a mixture (74%) of water–triethylamine 0.3%, pH 6.0 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 the wavelength of 215 nm was selected for the detection of both compounds (LTG and IS).

II.2.2.6. Method validation

The method validation procedures were carried out in agreement with the international guidelines on bioanalytical method validation (EMA 2011a; FDA 2013). Several specific validation parameters such as selectivity, linearity, limit of quantification (LOQ), accuracy, precision, recovery and analyte stability were studied and assessed taking into account the corresponding acceptance criteria.

The selectivity of the method was evaluated by analysing blank plasma and brain homogenate samples obtained from six different rats in order to assess the existence of potential interference of endogenous compounds at the same retention times of the analyte (LTG) and IS. Additionally, the potential interference of exogenous compounds such as anaesthetics (pentobarbital, xylazine and ketamine) commonly used in nonclinical *in vivo* studies were also tested, by injecting 20 µL of standard drug solutions with a concentration of 10 µg/mL.

The calibration curves for each biological matrix of interest (rat plasma and brain homogenate) were obtained after processing the six calibration standards, including the LOQ, in the concentration range previously defined, on five different days (*n* = 5), and the LTG/IS peak area ratios obtained were plotted against the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis (Almeida et al. 2002).

The LOQ, defined as the lowest concentration of the calibration curve that can be measured with acceptable intra e interday precision and accuracy, was evaluated by analysing plasma and brain tissue homogenate samples prepared in replicate (*n* = 5). The LOQ for LTG in both matrices was assessed considering as acceptance criteria a coefficient of variation (CV) not exceeding 20% and a deviation from nominal concentration (*bias*) within ±20%.

The interday precision and accuracy were evaluated after processing four QC samples (QC_{LOQ}, QC_1, QC_2, QC_3) prepared in plasma and brain homogenate, which were tested on five consecutive days (*n* = 5), whereas the intraday precision and accuracy were tested by processing five sets of the corresponding QC samples in a single day (*n* = 5). The acceptance of inter and intraday precision criterion was defined by a CV value lower than or equal to 15% (or 20% for the LOQ), and for the intra and interday accuracy a *bias* value lower than or equal to 15% (or ±20% for the LOQ).

The absolute recovery of LTG and the IS from rat plasma and brain homogenate was estimated after the extraction of QC samples at three concentration levels (QC_1, QC_2 and QC_3) in five replicates (*n* = 5) and comparing the resultant peak area with the peak area obtained by the direct injection of the corresponding non-extracted LTG and IS solutions at the same nominal concentrations. The values of absolute recovery for LTG and the IS were then obtained by the ratio of the peak areas of extracted and non-extracted samples.

The stability of LTG in rat plasma and brain homogenate samples was investigated for QC_1 and QC_3 (*n* = 5) in several experimental conditions to simulate the handling and storage of samples. Specifically, the stability of LTG was assessed in processed samples maintained in the autosampler during a period of 12 h; and also, in unprocessed samples simulating the short-term and long-term stability conditions, particularly at room temperature for 4 h, at 4 °C for 24 h and at −20 °C for 30 days (*n* = 5). The stability was assessed by comparing the data of samples analysed before (reference samples) and after being exposed to the conditions for stability assessment (stability samples). A stability/reference samples ratio of 85–115% was accepted as the stability criterion (*n* = 5).

II.2.2.7. Method application and pharmacokinetic analysis

To demonstrate the applicability of the proposed method a pharmacokinetic study was conducted in a group of five Wistar rats (*n* = 5), which received a single oral dose of LTG (10 mg/kg). At several pre-defined post-dose time points (0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h), blood samples (~0.3 mL) were collected into heparinised tubes through a cannula introduced in the tail vein of rats or by decapitation at the end of the experiments. Brain tissue was also

obtained from the same rats at two previously defined endpoints: at 24 h (*n* = 1) and at 72 h (*n* = 4) post-dosing; this procedure was designed to ensure the determination of LTG concentrations above the LOQ in at least one brain sample (at 24 h post-dose), since LTG concentration levels in brain could be below the LOQ of the method (BLQ) at 72 h post-dose. Blood samples and brain tissue were processed and stored until analysis as described in section *II.2.2.2. Blank rat matrices*. The obtained data were submitted to a non-compartmental pharmacokinetic analysis using WinNonlin® version 5.2 (Pharsight Co., Mountain View, CA, USA).

II.2.3. Results and discussion

II.2.3.1. Optimization of chromatographic conditions

The chromatographic conditions were optimized according to the experience of the in-house developed techniques for the determination of AEDs and in order to achieve a symmetric peak shape and a good chromatographic resolution for LTG and the IS, within the shortest running time. Since LTG a UV-absorbing compound with weak basic properties, containing a hydrophobic moiety, the use of reverse-phase liquid chromatography coupled to a DAD detector was considered to be appropriate for the quantification of LTG in both rat plasma and brain samples. Different conditions were tested in order to find the best mobile phase, the most appropriate wavelength values bearing in mind a good relationship between selectivity and sensitivity, and the selection of the IS was also carefully studied. In what concerns the composition of the mobile phase and considering the reversed-phase (C_{18}) retention mechanisms, different percentages of acetonitrile and methanol were tested as organic modifiers and a mobile phase composed by acetonitrile (13%), methanol (13%) and a mixture (74%) of water–triethylamine 0.3% was selected. Although the use of amine additives is not consensual, in this case the addition of a small amount of triethylamine was favourable perhaps due to the saturation of free silanol groups on the stationary phase, reducing the peak asymmetry and peak tailing phenomenon (Li et al. 2010). In addition, the aqueous component of the mobile phase (water-triethylamine 0.3%) was also tested at different pH values. The most favourable retention times and the best peak separation and shapes were achieved with an aqueous component of water-triethylamine 0.3% at pH 6.0, adjusted with 85% *ortho*-phosphoric acid.

Regarding detection conditions, although different wavelengths were tested considering the absorption of the two chromophores that compose LTG, the best compromise in terms of sensitivity and selectivity was achieved at 215 nm. Moreover, in HPLC analysis, the reliable quantification of any analyte requires the use of an adequate IS. The selection of chloramphenicol as IS was made according to reported experiments (Greiner-Sosanko et al. 2007a; Greiner-Sosanko et al. 2007b; Matar et al. 1998) and also due to its favourable behaviour in the selected chromatographic conditions in comparison with other tested compounds (e.g., levamisole). Under these bioanalytical conditions, the LTG and IS peaks showed a symmetric shape and were well separated in a running time shorter than 5 min, enabling a faster

chromatographic analysis than the previously reported methods (Castel-Branco et al. 2001a; Liu et al. 2014; Yang et al. 2013). The chromatographic instrumentation required and the simple bioanalytical conditions established enable the easy implementation of this assay in any analytical laboratory.

II.2.3.2. Development and optimization of sample extraction procedure

Proper sample pre-treatment is a key step and a prerequisite for most bioanalytical procedures. The introduction of MEPS as microextraction procedure brought several advantages in comparison with solid-phase extraction (SPE), enabling a good recovery and sensitivity, using smaller sample and solvent volumes (Alves et al. 2013).

The sample extraction steps were optimized from a validated MEPS procedure used in a previous work of the research group (Ferreira et al. 2014) in order to reach suitable MEPS efficiency for the extraction of LTG and the IS in both samples (rat plasma and brain homogenate). Taking into account our practical experience with MEPS protocols (Ferreira et al. 2014; Magalhães et al. 2014; Rodrigues et al. 2013c) and as Abdel-Rehim (2010) also highlighted, the rat samples were deproteinized with acetonitrile before sample loading to avoid the rapid clogging of the MEPS cartridges. Then, due to the high percentage of acetonitrile in the sample supernatant, which strongly impairs the retention of the compounds of interest (LTG and IS) in the MEPS sorbent, the supernatant was collected and evaporated to dryness and the residue was reconstituted in an aqueous buffer before MEPS loading.

Specifically, the pH of the aqueous reconstitution solution (0.3% triethylamine-water) was the first experimental variable to be evaluated during the optimization of the MEPS protocol, and it was assessed in the pH range of 3.5-7.5; considering the similarity in the obtained results concerning the influence of the pH of the reconstitution buffer on the recovery of LTG and the IS (**Figure II.3A**), the pH value of 6.5 was selected. In addition, other MEPS variables such as the number of draw-eject cycles and washing and elution conditions were also investigated.

Considering the overall results of this set of experiments (**Figure II.3B-D**) and in order to streamline the MEPS protocol, three draw-eject cycles were selected in the sample loading stage, 200 µL of water were used in the washing step and the desorption (elution) of the compounds of interest (LTG and IS) was efficiently accomplished with methanol (2 x 30 µL).

Moreover, to ensure the total removal of LTG, the IS and other endogenous compounds from the packed sorbent before the next sample extraction, the MEPS cartridge was cleaned/ reconditioned 12 x 200 µL of methanol and 2 x 200 µL of water between each extraction. All these experiments were carried out with aliquots (100 µL) of rat plasma samples spiked with LTG at 20 µg/mL and added with 20 µL of IS solution at 250 µg/mL.

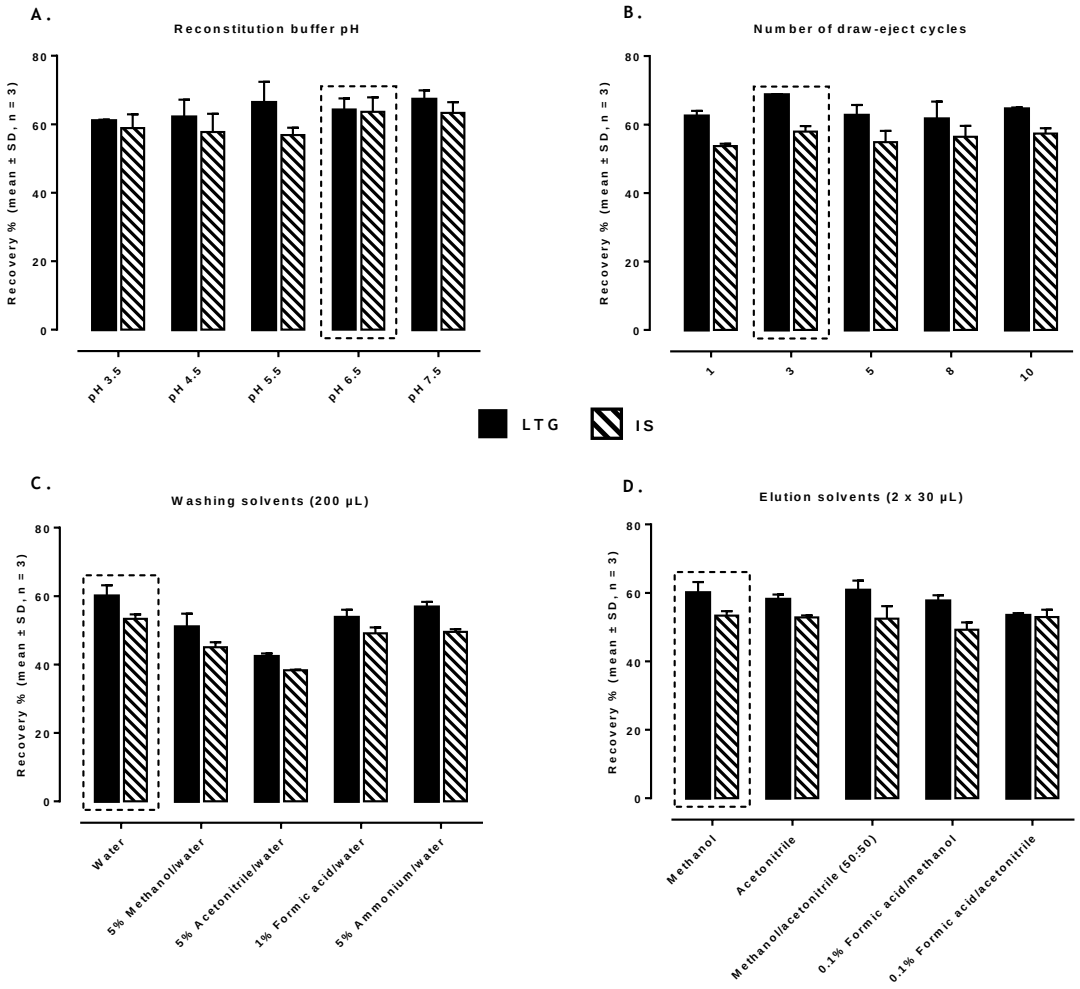


Figure II.3. Effect of different MEPS conditions on the extraction efficiency of lamotrigine (LTG) and internal standard (IS): influence of the reconstitution buffer pH (A), number of draw-eject cycles at pH 6.5 (B), different washing (C) and elution solvents (D).

Indeed, the sample extraction procedure was formally developed and optimized using rat plasma matrix, but in parallel some assays were also conducted using brain homogenate samples in order to anticipate its applicability to both rat matrices.

II.2.3.3. Method validation

II.2.3.3.1. Selectivity

The chromatograms of blank and spiked rat plasma and brain homogenate samples are shown in **Figure II.4**. The analysis of blank rat plasma and brain samples from six rats confirmed the absence of endogenous interferences in the retention times of LTG and the IS, using the established chromatographic and detection conditions.

Furthermore, considering the chromatographic behaviour of the anaesthetic drugs tested as

potentially exogenous interferences, only xylazine was found to interfere in the retention of LTG. Thus, in future pharmacokinetic studies involving the determination of LTG is desirable to avoid anaesthetic procedures with xylazine.

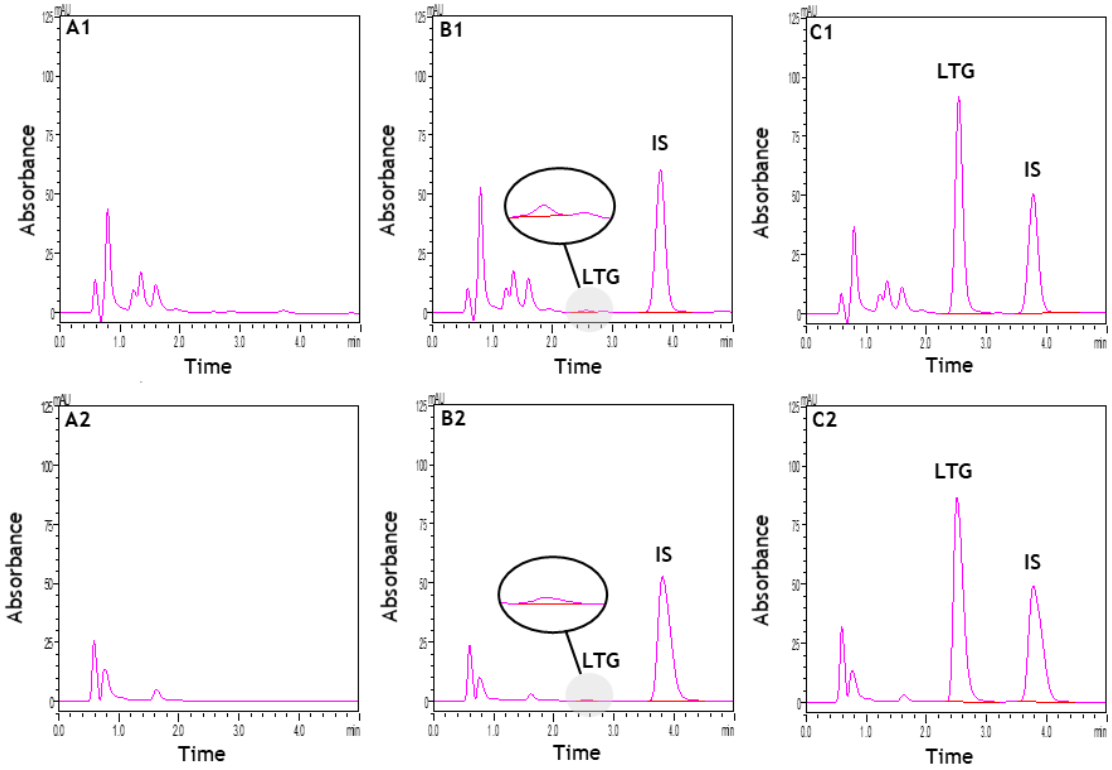


Figure II.4. Typical chromatograms of extracted rat plasma and brain homogenate samples obtained by the method developed: blank plasma (A1) and blank brain homogenate (A2); plasma (B1) and brain homogenate (B2) spiked with the internal standard (IS) and lamotrigine (LTG) at the lower limit of quantification (0.1 µg/mL); and plasma (C1) and brain homogenate (C2) spiked with the IS and LTG at the concentration of the upper limit of calibration range (20 µg/mL).

II.2.3.3.2. Calibration curves and LOQ

The calibration curves obtained in rat plasma and brain homogenates were linear within the concentration ranges previously defined and showed a consistent relationship between analyte-IS peak area ratios and the corresponding nominal concentrations.

A weighted linear regression analysis was used due to the wide calibration range and to compensate for heteroscedasticity. The calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the weighting factor. The regression equations of the calibration curves and the corresponding determination coefficients (r^2) achieved for LTG in rat plasma and brain homogenates were y=0.06499x+0.00272 (r^2 = 0.9947) and y=0.06647x+0.00187 (r^2 = 0.9952), respectively. The LOQs were experimentally defined as 0.1 µg/mL in both rat plasma and brain homogenate with acceptable precision and accuracy (**Table II.1**). In addition, as shown in **Table II.2**, the sensitivity (LOQ) achieved with our method is similar or even better

Table II.1. Intra and interday precision (% CV) and accuracy (% *bias*) values obtained for lamotrigine (LTG) in rat plasma and brain homogenate samples at the lower limit of quantification (QC_{LOQ}), and at low (QC_1), middle (QC_2) and high (QC_3) concentration levels representative of the calibration ranges (n = 5).

Matrix	$C_{nominal}$ (µg/mL)		Interday			Intraday		
			$C_{experimental}$ (Mean ± SD) (µg/mL)	Precision (% CV)	Accuracy (% *bias*)	$C_{experimental}$ (Mean ± SD) (µg/mL)	Precision (% CV)	Accuracy (% *bias*)
Plasma	QC_{LOQ}	0.1	0.106 ± 0.003	1.9	6.0	0.098 ± 0.004	2.6	-2.0
	QC_1	0.3	0.315 ± 0.010	2.8	5.1	0.276 ± 0.028	8.6	-8.1
	QC_2	10	10.451 ± 0.509	4.8	4.5	10.253 ± 0.110	1.1	2.5
	QC_3	18	18.222 ± 0.637	3.5	1.2	17.866 ± 0.196	1.1	-0.7
Brain homogenate	QC_{LOQ}	0.1	0.105 ± 0.008	6.2	5.2	0.104 ± 0.007	5.3	3.9
	QC_1	0.3	0.321 ± 0.017	5.0	6.9	0.341 ± 0.021	5.8	13.5
	QC_2	10	10.117 ± 0.164	1.6	1.2	9.780 ± 0.195	2.0	-2.2
	QC_3	18	17.610 ± 0.275	1.6	-2.2	17.368 ± 0.175	1.0	-3.5

$C_{experimental}$, experimental concentration; $C_{nominal}$, nominal concentration; CV, coefficient of variation; SD, standard deviation.

Table II.2. Comparison of key bioanalytical aspects (sensitivity, extraction efficiency/recovery and run time) between the current method and previous methods used for the bioanalysis of lamotrigine in rat plasma/serum and brain homogenate samples

Matrix	Sample volume	Extraction method	Analytical method	Sensitivity (LOQ)	Recovery (%)	Run time	Reference
Plasma	100 µL	PP + MEPS	HPLC-DAD	0.1 µg/mL	68.0-73.5	5 min	Current method
Brain	100 µL	PP + MEPS	HPLC-DAD	0.1 µg/mL	71.7-86.7	5 min	Current method
Plasma	20 µL	SPE	HPLC-UV	-	-	-	(Yamashita et al. 1997)
Serum	50 µL	LLE	HPLC-UV	-	-	20 min	(Walton et al. 1996)
Brain	100 µL	LLE	HPLC-UV	-	-	20 min	(Walton et al. 1996)
Serum	50 µL	PP	HPLC-UV	-	-	-	(Walker et al. 2000)
Brain	1000 µL	PP + LLE	HPLC-UV	0.1 µg/mL	74.3-98.6	10 min	(Castel-Branco et al. 2001a)
Plasma	100 µL	LLE	HPLC-UV	0.5 µg/mL	82.2-93.1	≈11 min	(Liu et al. 2014)
Brain	100 µL	PP	HPLC-UV	0.25 µg/g	81.3-89.5	≈11 min	(Liu et al. 2014)
Plasma	100 µL	PP	HPLC-MS	0.01 µg/mL	90.4-94.5	12 min	(Yang et al. 2013)

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; PP, protein precipitation; SPE, solid-phase extraction; UV, ultraviolet.

than that obtained by other HPLC-UV techniques reported in the literature and, comparatively, a quicker chromatographic analysis is accomplished.

II.2.3.3.3. Precision and accuracy

The intraday and interday precision and accuracy results obtained in rat plasma and brain homogenates at four different concentration levels (QC_{LOQ}, QC_1, QC_2 and QC_3) are presented in **Table II.1.** In plasma, the intra and interday CV values did not exceed 8.6%, and the intra and interday *bias* values ranged from −8.1 to 6.0%. Likewise, in brain homogenate, the intra and interday CV values did not exceed 6.2%, and the intra and interday *bias* values varied between −3.5 and 13.5%. All results fulfilled the acceptance criteria of the international guidelines; therefore, the developed method is precise and accurate for the quantification of LTG in the rat matrices studied.

II.2.3.3.4. Recovery

The LTG recovery results in both rat plasma and brain homogenates tested at three different concentration levels (QC_1, QC_2 and QC_3) are provided in **Table II.3**. The absolute mean recovery values ranged from 68.0 to 73.5% in rat plasma with CV values equal or lower than 6.3% and ranged from 71.7 to 86.7% in brain homogenates with maximal CV values of 4.8%. **T**he absolute recovery of the IS in rat plasma was 61.0% with a CV value of 3.7% and in brain homogenate was 64.2% with a CV value of 4.5%. The extraction efficiency estimated for the present bioanalytical assay is within the values usually achieved when MEPS is used as a sample preparation procedure.

Table II.3. Recovery (values in percentage) of lamotrigine (LTG) from rat plasma and brain homogenate samples at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration ranges (n = 5).

Matrix	$C_{nominal}$ (μg/mL)		Recovery (%)	
			Mean ± SD (n = 5)	CV (%)
Plasma				
	QC1	0.3	73.5 ± 4.6	6.3
	QC2	10.0	68.0 ± 2.7	4.0
	QC3	18.0	71.4 ± 3.0	4.3
Brain homogenate				
	QC1	0.3	86.7 ± 3.5	4.1
	QC2	10.0	71.7 ± 3.4	4.8
	QC3	18.0	74.6 ± 1.6	2.1

$C_{nominal}$, nominal concentration; CV, coefficient of variation; SD, standard deviation.

II.2.3.3.5. Stability

The results for LTG stability in rat plasma and brain homogenate are shown in **Table II.4.** According to the data obtained, no significant loss of LTG was observed in unprocessed and processed rat plasma and brain homogenate samples in the different handling and storage conditions studied.

Table II.4. Stability (values in percentage) of lamotrigine (LTG) at low (QC_1) and high (QC_3) concentrations of the calibration ranges, in unprocessed rat plasma and brain homogenate samples at room temperature for 4 h, at 4 ºC for 24 h, and at -20 ºC for 30 days; and in processed rat plasma and brain homogenate samples left in the HPLC autosampler for 12 h (*n* = 5).

Analyte	Plasma		Brain homogenate	
	QC_1	QC_3	QC_1	QC_3
$C_{nominal}$ (µg/mL)	0.3	18.0	0.3	18.0
Unprocessed samples				
Room temperature (4 h)	107.7	106.0	110.3	104.7
4 ºC (24 h)	102.0	102.3	88.1	97.0
-20 ºC (30 days)	107.9	99.4	88.0	97.2
Processed samples				
Autosampler (12 h)	97.9	99.4	101.0	100.1

$C_{nominal}$, nominal concentration.

II.2.3.3.6. Method application and pharmacokinetics

The validated MEPS/HPLC-DAD method was applied to the analysis of LTG concentration levels in plasma and brain homogenate samples obtained from Wistar rats (*n* = 5) treated with a single oral dose of LTG (10 mg/kg). Representative chromatograms of the analysis of real samples of rat plasma and brain homogenate are shown in **Figure II.5**. In general, the plasma concentration–time profiles of LTG were obtained over a period of 72 h post-dose (*n* = 4) in order to appropriately characterize the terminal elimination phase of the drug.

In contrast, in one rat (*n* = 1) the LTG plasma concentration–time profile was obtained only up to 24 h post-dose because an early collection of a brain sample was considered to be important to ensure LTG concentration levels above the LOQ of the method. Whenever possible, the corresponding individual pharmacokinetic profiles were analysed and the estimated pharmacokinetic parameters are summarized in **Table II.5**.

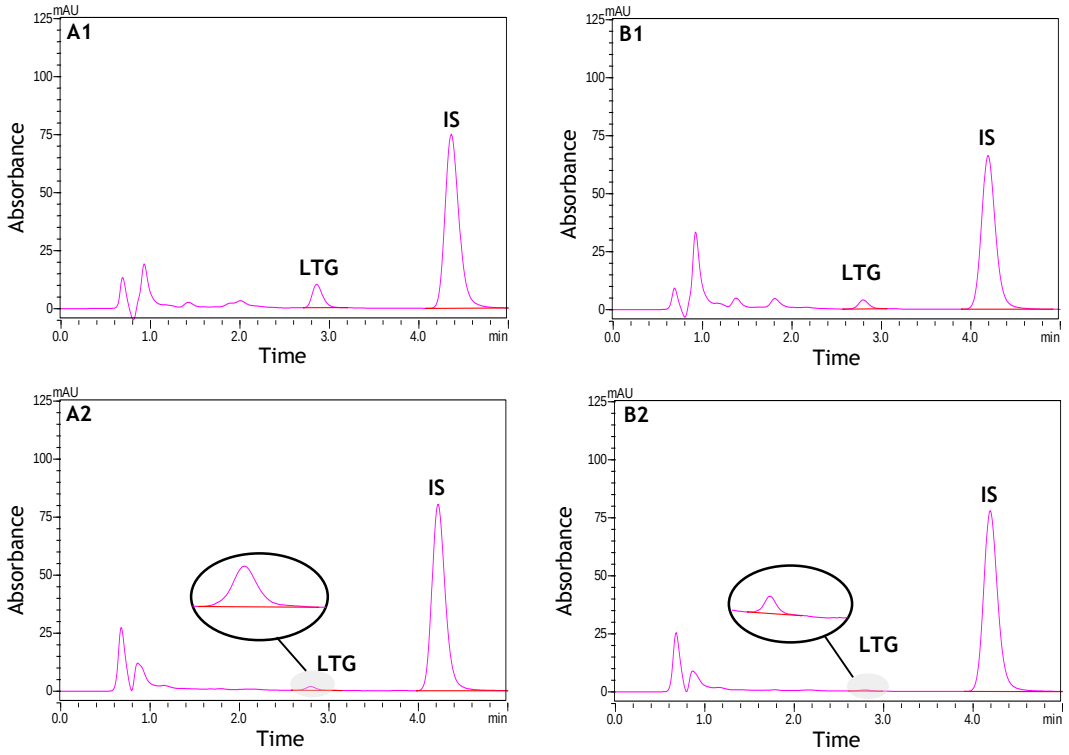


Figure II.5. Representative chromatograms of the analysis of real samples of rat plasma and brain homogenate: at 24 h post-dose in plasma (A1) and brain homogenate (A2), and at 72 h post-dose in plasma (B1) and brain homogenate (B2).

Table II.5. Pharmacokinetic parameters estimated by non-compartmental analysis of the individual plasma concentration-time profiles of lamotrigine (LTG) obtained in rats (*n* = 5) after a single oral dose of LTG (10 mg/kg).

Pharmacokinetic parameters	Rat 1[#]	Rat 2	Rat 3	Rat 4	Rat 5
t_{max} (h)	2.0	24.0	2.0	24.0	2.0
C_{max} (μg/mL)	1.543	3.423	3.719	3.474	4.317
AUC_{0-t} (μg h/mL)	NC	161.00	142.92	149.98	165.02
$AUC_{0-\infty}$ (μg h/mL)	NC	241.67	168.78	171.79	184.14
k_{el} (h^{-1})	NC	0.0179	0.0272	0.0329	0.0329
$t_{1/2el}$ (h)	NC	38.6	25.5	21.1	21.1
MRT (h)	NC	64.5	38.7	37.4	32.8

t_{max}, time to reach peak concentration; C_{max}, peak concentration; AUC_{0-t}, area under the concentration-time curve from time zero to the last sampling time with measurable concentration; $AUC_{0-\infty}$, area under the concentration-time curve from time zero to infinite; k_{el}, apparent terminal elimination rate constant; MRT, mean residence time; NC, not calculated; $t_{1/2el}$, apparent terminal elimination half-life. C_{max} and t_{max} are experimental values; AUC_{0-t}, $AUC_{0-\infty}$, k_{el}, $t_{1/2el}$ and MRT values were calculated by non-compartmental analysis. [#]Rat 1 was sacrificed at 24 h post-dose to collect an earlier brain sample.

The mean plasma concentration-time profile of LTG (*n* = 5, unless otherwise indicated) is depicted in **Figure II.6**, as well as the concentration of LTG quantified in brain homogenate at 24 h post-dose (0.197 µg/mL); as expected, at 72 h post-dose the brain concentrations of LTGwere found at BLQ levels in all rats (*n* = 4). At this point, it is worthy to mention that the low concentrations of LTG measured in brain tissue homogenate do not compromise the application of the method; however, it is suggested that shorter post-dose sampling time points should be considered in future pharmacokinetic studies designed to assess the brain disposition of LTG.

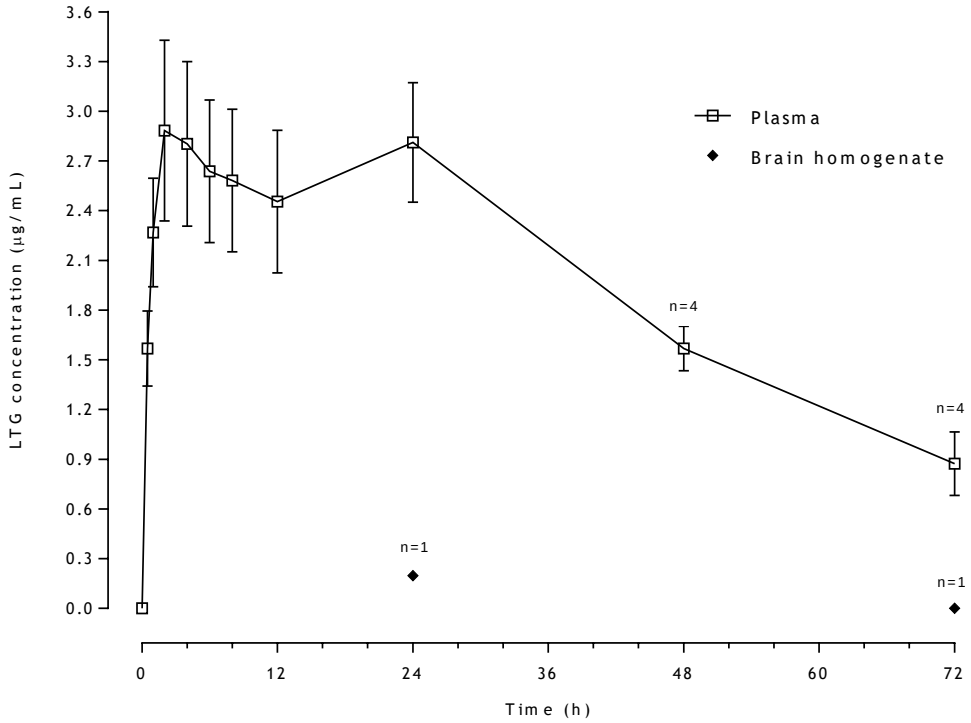


Figure II.6. Mean plasma concentration-time profile of lamotrigine (LTG), over a period of 72 h, obtained from rats treated with a single dose of LTG (10 mg/kg) administered by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of five determinations per time point (*n* = 5, unless otherwise indicated). The concentration of LTG in a brain homogenate sample collected at 24 h post-dose (*n* = 1) is also represented; at 72 h post-dose the brain concentrations of LTG were found at BLQ levels in all rats analysed (*n* = 4).

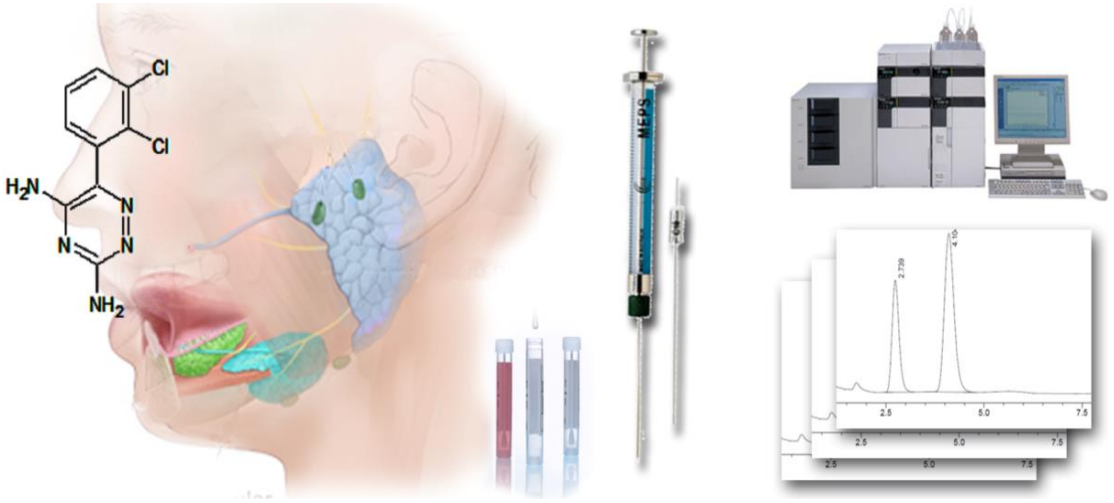
II.2.4. Conclusion

This new MEPS/HPLC-DAD method developed for the quantification of LTG in rat plasma and brain homogenate was successfully validated. The sample microextraction procedure involving MEPS seems to be cost-effective because each MEPS cartridge can be reused for the extraction of a high number of samples before being discarded.

One important aspect that should be emphasised is that MEPS was applied for the determination of LTG in brain tissues. Indeed, MEPS has frequently been applied for the determination of drugs in serum, plasma and other biological fluids but has rarely been applied

in tissues. Another important aspect of this method is the small sample volume (100 µL) required, allowing the collection of several blood samples from the same animal during pharmacokinetic studies and, therefore, reducing the number of animals used.

The reported bioanalytical method was also successfully applied to quantify LTG in real biological samples. Therefore, it can be concluded that this MEPS/HPLC-DAD method is a useful tool to support future pharmacokinetic and biodisposition studies in rats involving LTG administration.

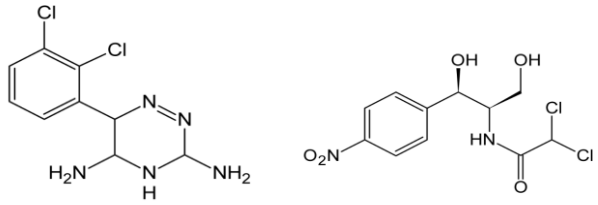


II.3. *Experimental Section*

Determination of lamotrigine in human plasma and saliva using microextraction by packed sorbent and high performance liquid chromatography–diode array detection: an innovative bioanalytical tool for therapeutic drug monitoring

II.3.1. Introduction

Lamotrigine (LTG) is a broad-spectrum antiepileptic drug (AED) used as monotherapy or in add-on therapy regimens in adults and children (Goldenberg 2010; Krasowski 2010; Werz 2008). LTG is also approved for Lennoux-Gastaut, a rare and intractable form of childhood epilepsy, and for bipolar disorders (Bialer et al. 2007; Morris et al. 1998; Perucca and Mula 2013). Structurally, LTG is a 3,5-diamino-6-(2,3-dichlorophenyl)-1,2,4-triazine (**Figure II.7**), belonging to the phenyltriazine class, which is chemically unrelated to other existing AEDs (Mallayasamy et al. 2010; Werz 2008). The physicochemical and pharmacological properties of LTG determine its unique pharmacokinetic and pharmacodynamic profile (Arif et al. 2010).



LTG **CAM**

Figure II.7. Chemical structure of lamotrigine (LTG) and chloramphenicol (CAM) used as internal standard (IS)

Therapeutic drug monitoring (TDM) of LTG is of crucial interest in many clinical circumstances due to the large inter and intraindividual variability of its systemic drug concentrations, including under steady-state conditions, particularly in cotherapy with other AEDs such as phenytoin, carbamazepine and valproate (Ohman et al. 2008a). Indeed, it is well-established that plasma/serum LTG concentrations should be monitored during its concomitant use with other drugs that are enzyme inhibitors (e.g. valproate, sertraline) or inducers (e.g., phenobarbital, phenytoin, carbamazepine, rifampicin, oral contraceptives) (Landmark and Patsalos 2010; Patsalos 2013a; Patsalos et al. 2008; Zaccara and Perucca 2014). Although the therapeutic concentration range for LTG has been progressively modified over the years, the range of 2.5-15 µg/mL has been proposed for seizure control. However, there is a considerable overlap in serum concentrations among responders and nonresponders, and some refractory patients may need higher concentration levels (Patsalos et al. 2008). TDM has also an important clinical value in pregnancy and in children taking LTG, because plasma drug concentrations are reduced throughout gestation and the clearance of LTG is higher in children compared to adults and the elderly (Krasowski 2010). The implementation of TDM for clinical management of LTG therapy requires the availability of suitable bioanalytical methodologies to support the LTG concentration measurements in the biological samples of interest in order to adjust patient's medication regimen and achieve optimal therapeutic outcomes. Therefore, several techniques

have been developed and validated to quantify LTG in different human matrices (e.g. blood, plasma, serum, urine and saliva) through chromatography (Ferreira et al. 2014; Greiner-Sosanko et al. 2007a; Greiner-Sosanko et al. 2007b; Heideloff 2010; Juenke et al. 2011; Kim et al. 2011; Kuhn and Knabbe 2013; Serralheiro et al. 2013; Shah et al. 2013; Shibata et al. 2012; Tai et al. 2011; Zufia et al. 2009) immunoassay (Biddlecombe et al. 1990; Juenke et al. 2011) and electrophoresis methods (Pucci et al. 2005; Shihabi 1999; Shihabi and Oles 1996; Theurillat et al. 2002; Thormann et al. 2001; Zheng et al. 2004). The predominant methodology for LTG bioanalysis is HPLC coupled to DAD or UV detection (Bompadre et al. 2008; Brunetto et al. 2009; Budakova et al. 2008; Cheng et al. 2005; Chollet 2002; Contin et al. 2005; Contin et al. 2010; Ferreira et al. 2014; Franceschi and Furlanut 2005; Heideloff 2010; Hotha et al. 2012; Morgan et al. 2011; Patil and Bodhankar 2005; Rivas et al. 2010; Saracino et al. 2007a; Saracino et al. 2007b; Serralheiro et al. 2013; Shah et al. 2013; Vermeij and Edelbroek 2007; Youssef and Taha 2007; Zufia et al. 2009). On the other hand, considering all of these LC methods, the sample preparation/extraction processes employed involved SPME (Cantu et al. 2006), SPE (Bompadre et al. 2008; Shah et al. 2013; Tai et al. 2011; Torra et al. 2000; Vermeij and Edelbroek 2007; Yamashita et al. 1995; Zufia et al. 2009), protein precipitation (PP) (Contin et al. 2005; Contin et al. 2010; Kuhn and Knabbe 2013; Lee et al. 2010; Pucci et al. 2005; Ramachandran et al. 1994; Saracino et al. 2007a; Theurillat et al. 2002; Youssef and Taha 2007), LLE (Antonilli et al. 2011; Barbosa and Midio 2000; Budakova et al. 2008; Castel-Branco et al. 2001b; Emami et al. 2006; Greiner-Sosanko et al. 2007b; Hart et al. 1997; Mashru et al. 2005; Matar et al. 1999; Rivas et al. 2010) and microextraction by packed sorbent (MEPS) (Ferreira et al. 2014).

MEPS is indeed a novel sample preparation approach in the field of bioanalysis, directed towards miniaturization and automation, and it has been used for qualitative and quantitative bioanalysis of a vast number of drugs and metabolites (Alves et al. 2013; Ferreira et al. 2014; Magalhães et al. 2014; Rodrigues et al. 2013c). Specifically, regarding the application of MEPS in the bioanalysis of LTG, up to date and to the best of our knowledge, no method was developed and validated for human saliva.

Saliva was firstly investigated as an alternative biological fluid for TDM of AEDs in the 1970s (Patsalos and Berry 2013). The use of saliva instead of plasma/serum has several advantages: the collection of saliva is simple and non-invasive, avoiding discomfort or stress in patients, particularly in children and the elderly; in addition, the drug levels in saliva reflect the free non-protein bounded drug concentrations in blood (Patsalos and Berry 2013).

Bearing in mind that some recent reports show a good relationship between salivary concentrations and plasma/serum concentrations, strengthening the idea that saliva represents a viable alternative sample to perform TDM (Incecayir et al. 2011; Krasowski 2010; Mallayasamy et al. 2010; Patsalos and Berry 2013; Ryan et al. 2003) this work was planned to develop and validate a novel HPLC method for the quantification of LTG in human plasma and saliva using the innovative MEPS technology in sample preparation.

II.3.2. Material and methods

II.3.2.1. Materials and reagents

LTG was kindly provided by Bluepharma (Coimbra, Portugal) and chloramphenicol (CAM), used as internal standard (IS), was purchased from Sigma–Aldrich (St Louis, MO, USA). The chemical structures of these compounds are shown in **Figure II.7.**

Methanol and acetonitrile, both of HPLC gradient grade, were purchased from Chem-Lab (Zedelgem, Belgium) and the ultra-pure 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 from Panreac Química SA (Barcelona, Spain).

The MEPS 250 µL syringe and the MEPS BIN (barrel insert and needle) containing ~4 mg of solid-phase silica–C_{18} material (SGE Analytical Science, Australia) were purchased from ILC (Porto, Portugal). Blank human plasma from healthy blood donors was provided by the Portuguese Blood Institute after the written consent of each subject, and saliva was kindly obtained from a set of volunteers.

II.3.2.2. Stock solutions, calibration standards and QC samples

The LTG stock solution (1 mg/mL) and the working solution (100 µg/mL) were properly prepared in methanol and then adequately diluted in water-methanol (50:50, v/v) to afford six spiking solutions with final concentrations of 0.5, 1, 3.5, 15, 62.5 and 100 µg/mL. Each one of these solutions were daily used for spiking aliquots of blank human plasma and saliva in order to prepare six calibration standards in the concentration range of 0.1-20 µg/mL. The stock solution of the IS was also prepared in methanol (1 mg/mL) and the working solution (250 µg/mL) was obtained after diluting an appropriate volume of the stock solution in water-methanol (50:50, v/v). All stock, working and spiking solutions were stored at 4 °C and protected from light, with the exception of the IS working solution which was daily prepared.
QC samples at four concentration levels, representing the lowest (QC_{LOQ}) and the low (QC_1), medium (QC_2) and high (QC_3) ranges of the calibration curve, were also independently prepared. For that purpose, aliquots of blank human plasma and saliva were spiked to obtain final LTG concentrations of 0.1, 0.3, 10 and 18 µg/mL.

II.3.2.3. Apparatus and chromatographic conditions

The chromatographic analysis was carried out using an HPLC system (Shimadzu LC-2010A HT Liquid Chromatography) coupled with a DAD (Shimadzu SPD-M20A). All instrumental parts were automatically controlled by the LC solution software (Shimadzu, Kyoto, Japan).

The chromatographic separation of the analytes was carried out at 35 °C on a reversed-phase LiChroCART® Purospher Star column (C_{18}, 55 mm × 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany).

An isocratic elution was applied at a flow rate of 1.0 mL/min with a mobile phase composed of acetonitrile (13%), methanol (13%) and water–triethylamine 0.3% (74%) at pH 6.0, 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 a wavelength of 215 nm was selected for the detection of all compounds.

II.3.2.4. Sample preparation and extraction

The sample preparation procedure was optimised and the final conditions were as follows. It should be noted that saliva was collected without stimulation and was sonicated prior to sample extraction. Each aliquot (100 µL) of human plasma or saliva was spiked with 20 µL of the IS working solution, and then 400 µL of ice-cold acetonitrile was added for protein precipitation in order to minimize sample interferences in the MEPS step. The mixture was vortex-mixed for 30 seconds and centrifuged at 13,500 rpm for 10 minutes. Afterwards, the resulting supernatant was evaporated under a gentle nitrogen stream at 45 °C and the dry residue was reconstituted with 200 µL of 0.3% triethylamine-water solution (pH 6.0). This reconstituted sample was then submitted to MEPS.

Previous to MEPS procedures, the sorbent (C_{18}) was activated with methanol (3 x 200 µL) and passed through ultra-pure water (3 x 200 µL). Then, the reconstituted sample was drawn through the needle into the syringe and ejected at a flow rate of approximately 10 µL/s and three draw-eject cycles were applied on the same sample aliquot. After discarding the sample, the sorbent was washed with 200 µL of ultra-pure water in order to remove matrix interferences and, at the end, the analytes were eluted with methanol (2 x 30 µL) and diluted with 90 µL of ultra-pure water. An aliquot (20 µL) of the final sample extract was injected into the chromatographic system. After the extraction of each sample, the MEPS device was reconditioned with 12 x 200 µL of methanol followed by 2 x 200 µL of ultra-pure water to avoid transferring the analyte to the next sample (carry-over effect). Each MEPS cartridge was reused for approximately 200 times before being discarded.

II.3.2.5. Method validation

The international guidelines on bioanalytical method validation include several criteria for specific validation parameters that should be considered in the validation of any quantitative method. Such parameters are selectivity, linearity, LOQ, accuracy, precision, recovery and stability (EMA 2011a; FDA 2013).

The selectivity of the method was evaluated by analysing six blank plasma and saliva samples from different subjects to evaluate the existence of matrix endogenous substances in retention

times that could interfere with LTG and the IS. Additionally, the interference of other drugs that can potentially be co-administered with LTG was evaluated, by injecting standard drug solutions at 10 µg/mL. The drugs tested in this selectivity assay included other AEDs (carbamazepine, phenytoin, phenobarbital, fosphenytoin, oxcarbazepine, primidone, valproic acid), analgesics/antipyretics/anti-inflammatory drugs (acetylsalicylic acid, ketoprofen, ibuprofen, acetaminophen, piroxicam), antidepressants (amitriptyline, escitalopram, fluoxetine, mirtazapine, paroxetine, sertraline, trazodone, venlafaxine), antihypertensives (atenolol, furosemide), anxiolytics/sedatives/hypnotics (clorazepate, mexazolam) and many other drugs such as sulpiride, hydrocortisone, omeprazole, caffeine and nicotine.

The calibration curves for each biological matrix (plasma and saliva) were constructed after preparation of six calibration standards, including the LOQ, in the concentration range previously defined, on five distinct days (n = 5), and plotted according to the LTG/IS peak area ratio against the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis (Almeida et al. 2002). The LOQ, defined as the lowest concentration of the calibration curve that can be measured with adequate intra e interday precision and accuracy, was evaluated by analysing plasma and saliva samples prepared in five replicates (n = 5). The LOQ for LTG in both matrices was assessed considering relative standard deviation (RSD) values ≤ 20% and deviation from nominal concentration (*bias*) within ±20%.

The interday precision and accuracy were evaluated after processing four QC samples (QC_{LOQ}, QC_1, QC_2, QC_3) prepared in plasma and saliva, which were tested on five consecutive days (n = 5), whereas the intraday precision and accuracy were tested by processing five sets of the corresponding QC samples in a single day (n = 5). The acceptance criteria for interday and intraday precision is a RSD value lower than or equal to 15% (or 20% in the LOQ) and for accuracy, a *bias* value lower than or equal to 15% (or ±20% in the LOQ).

The absolute recovery of LTG and the IS from human plasma and saliva samples was determined after the extraction of the corresponding QC samples at three concentration levels (QC_1, QC_2 and QC_3) in five replicates (n = 5), and by comparing the resultant peak area with the peak area obtained after the direct injection of non-extracted LTG and IS solutions at the same nominal concentrations, also in five replicates. The values of absolute recovery for LTG and the IS were then obtained by the ratio of the peak areas of extracted and non-extracted samples.

The stability of LTG in human plasma and saliva was investigated for QC_1 and QC_3 (n = 5) in different experimental conditions. On the one hand, in processed samples maintained in the autosampler during a period of 12 h; and on the other hand, in unprocessed samples, simulating the short-term and long-term stability conditions, at room temperature for 4 h, at 4°C for 24 h, and at −20 °C for 30 days (n = 5). Additionally, the effect of three freeze-thaw cycles on the stability of the LTG in human plasma and saliva samples was also studied at −20 °C. For that purpose, aliquots of spiked plasma and saliva samples (QC_1 and QC_3) were stored at −20 °C for 24 h, thawed unassisted at room temperature, and when completely thawed, the samples were frozen again for 24 h under the same conditions until completing the three freeze-thaw cycles.

II.3.2.6. Clinical application

Blood and saliva samples were obtained from two volunteer patients after the written consent of each subject. Plasma and saliva aliquots were analysed to demonstrate the clinical applicability of this bioanalytical method. Four blood and saliva samples were collect from each patient, who were under continuous long-term treatment with LTG, at predefined time-points (2 h, 4 h, 8 h and 12 h) after the first daily dose of the drug. The period between 0-2 h after the oral drug administration was not considered for sampling because some residual drug could be retained in the mouth (saliva) during this period of time (Malone et al. 2006). Therefore, the collection of blood and saliva samples was only initiated at 2 h post-dose in order to obtain more reliable values for the saliva to plasma ratio observed for LTG concentrations.

Blood samples were collected into heparinised tubes, centrifuged at 4000 rpm (4 °C) for 10 minutes and the plasma was transferred to eppendorfs and stored at −20 °C until analysis. After mouth flushing, saliva samples were collected without stimulation into falcon tubes immediately after blood sampling, and stored at −20 °C until analysis. Ingestion of food, coffee and tobacco was not permitted within the two hours preceding saliva collection.

II.3.3. Results and discussion

A set of preliminary studies were carried out to optimize the bioanalytical process in order to validate an efficient method for the quantitative analysis of LTG in both human plasma and saliva. The final chromatographic and sample preparation/extraction conditions established were those previously mentioned in *section II.2.3* and *section II.2.4*, respectively.

Actually, proper sample preparation is a key step and a prerequisite for most bioanalytical procedures. The introduction of MEPS as microextraction procedure brought several advantages namely, good recovery and enough sensitivity, and the use of more reduced sample and solvent volumes when compared to SPE. Under the defined bioanalytical conditions, the LTG and IS peaks showed a symmetric shape and were well separated in a running time shorter than 5 minutes (**Figure II.8**). Hence, the analytical instrumentation required, as well as the simple experimental conditions established, enable the easy implementation of this assay in most hospital settings interested in the TDM of LTG.

II.3.3.1. Method validation

II.3.3.1.1. Selectivity

The chromatograms of blank and spiked human plasma and saliva samples are presented in (**Figure II.8**). The analysis of blank human plasma and saliva samples from six healthy volunteers confirmed the absence of endogenous interferences in the retention times of LTG and the IS. Most of the tested drugs potentially co-administered with the AED under investigation (LTG)

were also not found to interfere using the established chromatographic and detection conditions. However, some drugs such as furosemide, phenobarbital, fosphenytoin and piroxicam eluted around the retention time of LTG and/or IS and, therefore, they can interfere in the quantification of LTG. The retention times observed for the tested drugs that potentially may be prescribed with the LTG are indicated in **Table II.6**.

Table II.6. Retention times (RT) in minutes (min) of tested drugs potentially co-prescribed with lamotrigine (LTG).

Drugs	RT (min)	Drugs	RT (min)	Drugs	RT (min)
LTG	**2.803**	Acetylsalicylic acid	nd	Furosemide	3.381
IS	**4.174**	Ketoprofen	6.530	Escitalopram	18.278
Carbamazepine	12.991	Ibuprofen	23.382	Atenolol	0.839
Phenytoin	12.277	Paracetamol	nd	Mirtazapine	2.056
Phenobarbital	3.784	Piroxicam	3.778	Amitriptyline	nd
Fosphenytoin	3.480	Omeprazole	nd	Sertraline	1.694
Oxcarbazepine	6.521	Hydrocortisone	12.456	Trazadone	2.111
Primidone	2.013	Theophylline	nd	Venlafaxine	4.859
Valproic acid	1.986	Glibenclamide	1.700	Mexazolam	1.995
Valerian	nd	Caffeine	1.116	Nicotine	nd

IS, Internal standard; nd, Not detected in the analytical conditions used.

II.3.3.1.2. Calibration curves and LOQ

The calibration curves obtained for human plasma and saliva were linear within the concentration range previously defined and showed a consistent correlation between the analyte-IS peak area ratios and the corresponding nominal concentrations. A weighted linear regression analysis was performed due to the wide calibration range and to compensate for heteroscedasticity. The calibration curves were subjected to weighted linear regression analysis using $1/x^2$ as the weighting factor.

The regression equations of the calibration curves and the corresponding determination coefficients (r^2) achieved for LTG in human plasma were y=0.05954x-0.00246 (r^2=0.9945) and in human saliva were y=0.05725x-0.00127 (r^2=0.9936). The calibration curves were defined within the range of 0.1-20 µg/mL in order to largely cover the therapeutic range of LTG (2.5-15 µg/mL) (Patsalos et al. 2008).

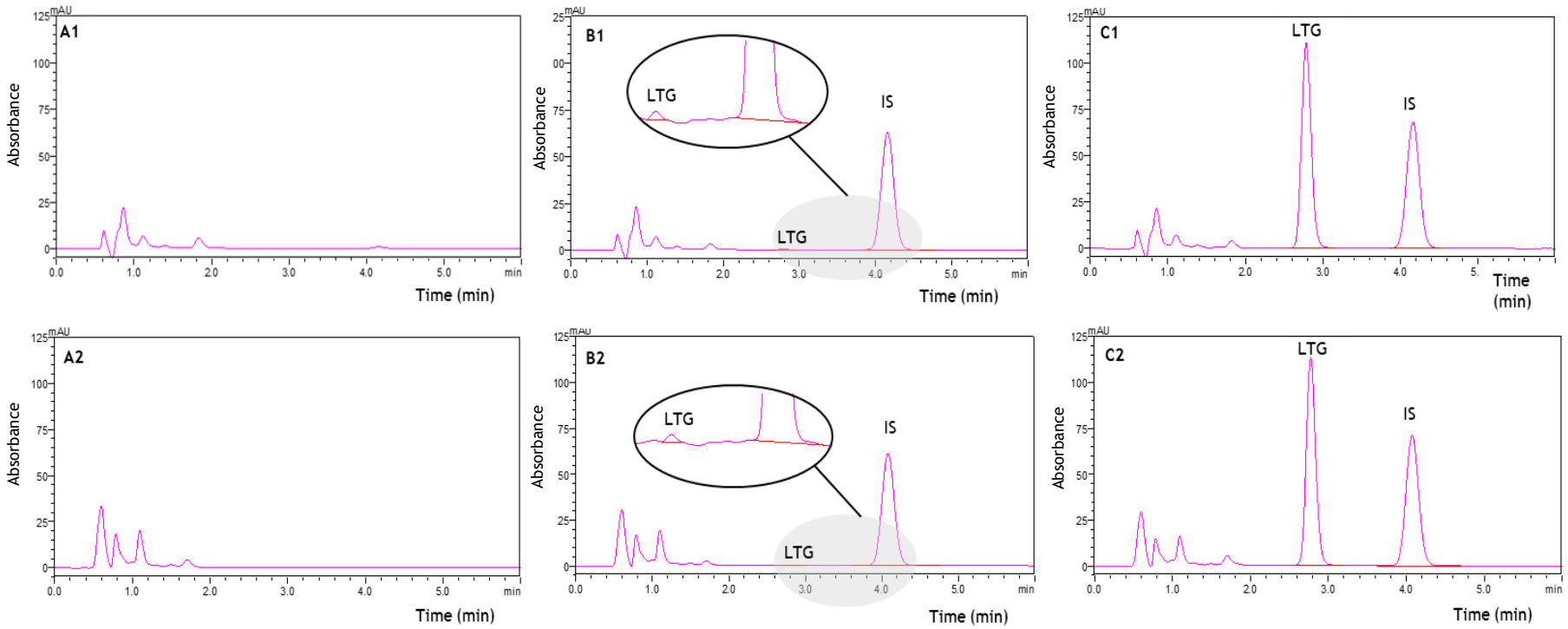


Figure II.8. Typical chromatograms of extracted human plasma and saliva samples obtained by the MEPS/HPLC-DAD method developed: blank plasma (A1) and saliva (A2); plasma (B1) and saliva (B2) spiked with the internal standard (IS) and lamotrigine (LTG) at the LOQ (0.1 µg/mL); and plasma (C1) and saliva (C2) spiked with the IS and LTG at the concentration of the upper limit of the calibration range (20 µg/mL).

The LOQ was experimentally defined as 0.1 μg/mL, in both human plasma and saliva, with acceptable precision and accuracy (**Table II.7**). Of note, the LOQ value obtained in plasma using our method is lower than those achieved by many other HPLC–UV/DAD techniques reported in the literature, even though a smaller volume of plasma is employed herein (Budakova et al. 2008; Franceschi and Furlanut 2005; Greiner-Sosanko et al. 2007b; Patil and Bodhankar 2005).

In the case of human saliva, the LOQ obtained with the current method is similar to those reported in previous works (Incecayir et al. 2011; Mallayasamy et al. 2010; Ryan et al. 2003) (**Table II.8).**

II.3.3.1.3. Precision and accuracy

The results for intra and interday precision and accuracy obtained from QC samples of human plasma and saliva at the four different concentration levels (QC_{LOQ}, QC_1, QC_2 and QC_3) are presented in **Table II.7.** In human plasma, the intra and interday RSD values did not exceed 14.5%, and the intra and interday *bias* values varied between −10.0 and 9.3%. Likewise, in human saliva, the intra and interday RSD values did not exceed 12.7%, and the intra and interday *bias* values varied between −4.5 and 13.4%.

II.3.3.1.4. Recovery

Overall, the results for LTG absolute recovery from both human plasma and saliva samples, tested at three different concentration levels (QC_1, QC_2 and QC_3), ranged from 64.9% to 73.6% with RSD values equal or lower than 7.0%; the detailed data are available in **Table II.9.** The absolute recovery for the IS in human plasma was 65.8% with a RSD value of 6.8% and in human saliva was 63.9% with a RSD value of 14.7%.

II.3.3.1.5. Stability

The results for LTG stability in human plasma and saliva achieved in the different conditions studied are presented in **Table II.10.** According to the results obtained, LTG was stable in unprocessed and processed human plasma and saliva samples in the different handling and storage conditions.

II.3.3.1.6. Clinical application

The plasma and saliva samples from the two volunteer patients were analysed to demonstrate the clinical usefulness of the method validated herein. The patient ID1 was receiving LTG 100 mg (p.o.) once-daily, whereas the other patient (ID2) was taking LTG twice-daily: 150 mg (p.o.) in the morning and 200 mg (p.o.) at night co-administrated with valproic acid.

Table II.7. Intra and interday precision (% RSD) and accuracy (% *bias*) values obtained for lamotrigine (LTG) in human plasma and saliva at the limit of quantification (QC_{LOQ}) concentration and at low (QC_1), medium (QC_2) and high (QC_3) concentrations representative of the calibration ranges (n = 5).

Matrix	$C_{nominal}$ (µg/mL)		Interday			Intraday		
			$C_{experimental}$ (Mean ± SD) (µg/mL)	Precision (% RSD)	Accuracy (% *bias*)	$C_{experimental}$ (Mean ± SD) (µg/mL)	Precision (% RSD)	Accuracy (% *bias*)
Plasma	QC_{LOQ}	0.1	0.109 ± 0.010	14.5	9.3	0.103 ± 0.005	8.8	3.1
	QC_1	0.3	0.294 ± 0.014	5.6	-2.1	0.278 ± 0.014	5.9	-7.5
	QC_2	10	9.357 ± 0.372	4.0	-6.4	9.260 ± 0.536	5.8	-7.4
	QC_3	18	17.208 ± 0.408	2.4	-4.4	16.197 ± 0.713	4.4	-10.0
Saliva	QC_{LOQ}	0.1	0.106 ± 0.005	6.3	6.0	0.106 ± 0.013	12.7	5.6
	QC_1	0.3	0.294 ± 0.011	4.1	-1.9	0.287 ± 0.006	2.2	-4.5
	QC_2	10	10.333 ± 0.231	2.2	3.3	11.236 ± 0.933	8.3	12.4
	QC_3	18	18.863 ± 1.369	7.3	4.8	20.420 ± 0.893	4.4	13.4

$C_{experimental}$, experimental concentration; $C_{nominal}$, nominal concentration

Table II.8. Comparison of determinant bioanalytical aspects between the current method and previous methods used for the bioanalysis of lamotrigine in human plasma and saliva samples.

Sample	Bioanalytical method	Sample volume	Sample extraction	LOQ (µg/mL)	Recovery	Reference
Plasma	**HPLC-DAD**	**100 µL**	**MEPS**	**0.10**	**72%**	**Current method**
	HPLC-UV	50 µL	SPE	0.20	98%	(Bompadre et al. 2008)
	HPLC-DAD	50 µL	SPE	0.25	99%	(Brunetto et al. 2009)
	HPLC-UV	50 µL	LLE	0.50	97%	(Budakova et al. 2008)
	HPLC-UV	1000 µL	LLE	0.10	82%	(Castel-Branco et al. 2001a)
	HPLC-UV	500 µL	PP	0.50	100%	(Contin et al. 2005)
	HPLC-UV	250 µL	LLE	1.0	96%	(Greiner-Sosanko et al. 2007b)
	HPLC-UV	500 µL	PP	0.10	97-98%	(Incecayir et al. 2011)
	HPLC-UV	200 µL	LLE	0.10	95%	(Mallayasamy et al. 2010)
	HPLC-UV	500 µL	LLE	0.02	88%	(Malone et al. 2006)
Saliva	**HPLC-DAD**	**100 µL**	**MEPS**	**0.10**	**71%**	**Current method**
	HPLC-UV	100 µL	PP	0.10	106%	(Mallayasamy et al. 2010)
	HPLC-UV	500 µL	PP	0.10	105%	(Incecayir et al. 2011)
	HPLC-UV	500 µL	LLE	0.01	ND	(Malone et al. 2006)

DAD, Diode array detection; MEPS, Microextraction by packed sorbent; ND, Not determined; PP, Protein precipitation.

Table II.9. Recovery (values in percentage) of lamotrigine (LTG) from human matrices (plasma and saliva) at low (QC_1), medium (QC_2) and high (QC_3) concentrations of the calibration range (n = 5).

Matrix		$C_{nominal}$ (µg/mL)	Recovery (%)	
			Mean ± SD (n = 5)	RSD (%)
Plasma	QC_1	0.3	72.1 ± 5.0	7.0
	QC^2	10.0	73.6 ± 2.4	3.2
	QC_3	18.0	70.3 ± 4.8	6.9
Saliva	QC_1	0.3	70.9 ± 4.0	5.7
	QC_2	10.0	67.8 ± 4.2	6.2
	QC_3	18.0	64.9 ± 3.3	5.0

$C_{nominal}$, nominal concentration

Table II.10. Stability (values in percentage) of lamotrigine (LTG) at low (QC_1) and high (QC_3) concentrations of the calibration range in unprocessed and processed human plasma and saliva samples (n = 5).

	Stability (values in percentage) of LTG			
Analyte	Plasma		Saliva	
	QC_1	QC_3	QC_1	QC_3
$C_{nominal}$ (µg/mL)	**0.3**	**18.0**	**0.3**	**18.0**
Unprocessed samples				
Room temperature (4 h)	106.0	94.4	87.6	114.9
4 ºC (24 h)	105.6	111.5	85.2	106.3
Freeze-thaw (3 cycles; -20 ºC)	89.9	103.2	99.6	98.8
-20 ºC (30 days)	113.3	105.1	112.5	103.2
Processed samples				
Autosampler (12 h)	95.2	102.7	105.6	97.4

$C_{nominal}$, nominal concentration

Given that the use of morning drug levels is a standard practice for TDM of AEDs (Nielsen et al. 2008), this aspect was adopted in the sample collection protocol for these two patients.

The peaks obtained from the patients' processed samples revealed symmetry and good resolution, similarly to those obtained in the analysis of spiked human plasma and saliva samples (**Figure II.8**). The drug concentrations determined in plasma samples were within the therapeutic range (2.5-15 µg/mL) defined for LTG (Patsalos and Berry 2013; Patsalos et al. 2008; Shah et al. 2013) and, as expected, the LTG concentrations measured in saliva (free drug concentration) were lower than those determined in plasma (**Figure II.9**). Since the total LTG daily dose received by patient ID1 was less than one third of the total daily dose taken by patient ID2 (100 mg *versus* 350 mg), as it could be anticipated, the LTG concentration levels were substantially lower in the both samples (plasma and saliva) of patient ID1. Nevertheless, by normalizing the LTG concentrations obtained by the total daily dose administered, a similar proportion was found. In addition, the salivary to plasma concentration ratio was found to be higher in patient ID2 (0.55 *versus* 0.37), which was receiving adjunctive therapy (**Figure II.10**).

These results led us to anticipate the existence of a good relationship between the LTG concentrations in saliva and plasma in both subjects. Some previous studies reported saliva/serum LTG ratios of 0.46 in healthy subjects receiving a single oral dose of LTG, and of 0.64 in patients receiving adjunctive therapy (Malone et al. 2006; Patsalos and Berry 2013). Ryan (2003) also studied the relationship between serum and salivary concentrations of LTG in paediatric and adult patients and demonstrated a good saliva/serum correlation with LTG concentration ratios ranging from 0.40 to 1.19. Mallayasamy (2010) also reported an identical salivary to serum LTG concentration ratio (0.683).

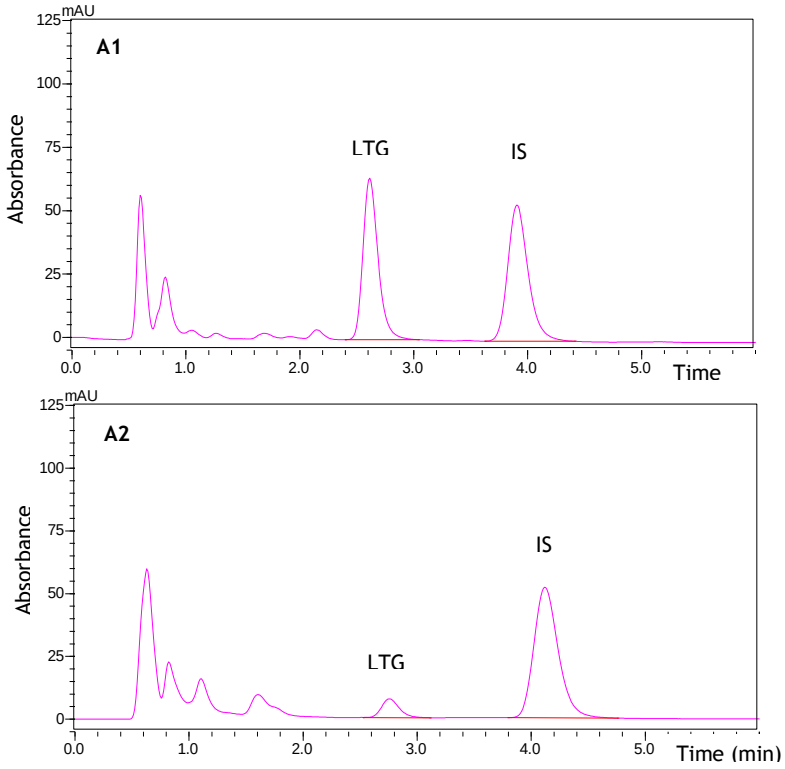


Figure II.9. Representative chromatograms of the analysis of real plasma (A1) and saliva (A2) samples at 2 h post-dose obtained from the patients treated with lamotrigine (LTG). IS, internal standard.

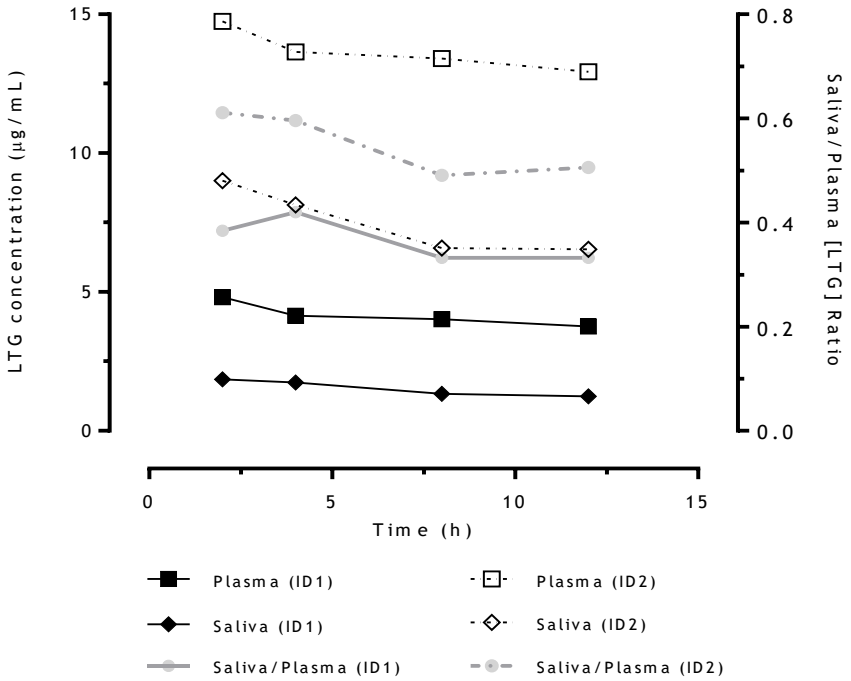


Figure II.10. Concentration-time profiles of lamotrigine (LTG) obtained from plasma and saliva samples collected at 2, 4, 8 and 12 h post-dose (taking as reference the morning dose) in two patients (ID1 and ID2) under oral LTG therapy (ID1, 100 mg once-daily in the morning; ID2, 150 mg in the morning, and 200 mg at night in cotherapy with valproic acid). The corresponding salivary to plasma LTG concentration ratios were also calculated at 2, 4, 8 and 12 h post-dose and graphically represented for both patients.

Moreover, observing the LTG concentration-time profiles (2-12 h) found in plasma and saliva in both subjects, the high stability of drug concentrations over time and the high parallelism between salivary and plasma levels should be highlighted, which clearly supports the use of saliva as a promising and alternative sample for TDM of patients under LTG treatment.

II.3.4. Conclusion

This MEPS/HPLC-DAD method for LTG quantification was successfully validated in both plasma and saliva matrices with high sensitivity, selectivity, precision and accuracy. The small sample volume needed for MEPS processing, the absence of significant chromatographic interferences from the biological matrices, together with the short running time in the LTG analysis and the low LOQ achieved, all enhance the clinical interest of this assay. The use of MEPS as microextraction procedure also has several important advantages, which are usually associated with the miniaturization and automation of bioanalytical procedures.

In spite of the lack of information on a consensual and specific therapeutic range for LTG in saliva, the saliva/plasma correlation achieved in this study indicates a good relationship between salivary and plasma drug concentrations, and it is expected that in the near future the use of saliva samples for TDM of patients under LTG therapy will be a reality in routine clinical practice.

Chapter III.

Effects of *Paullinia cupana* extract on lamotrigine pharmacokinetics in rats:
a herb-drug interaction on the gastrointestinal tract with potential clinical impact

III.1. Introduction

Paullinia cupana, also known as Guarana, is a species that belongs to the *Sapindaceae* family and it is being consumed worldwide in herbal supplements and stimulating drinks (Portella et al. 2013). This native Amazonian plant has been described as having stimulant effects and other medicinal properties (Schimpl et al. 2013), mainly due to the presence of caffeine (2-8%) in the seeds of its fruits. Other methylxanthines, like theophylline and theobromine, are also found in small amounts (< 0.3%) in the seeds, bark, flowers and leaves of *P. cupana* (Ashihara et al. 2008; Schimpl et al. 2013). Among several species of plants that produce caffeine, *P. cupana* has the higher natural content of this compound when compared to coffee (*Coffea arabica),* tea *(Camellia sinensis)* and yerba mate *(Ilex paraguariensis)* (Ashihara and Crozier 2001; Ashihara et al. 2008). In fact, depending on how the extracts are prepared, *P. cupana* extracts may contain caffeine in an amount four times higher than that found in coffee beans (Moustakas et al. 2015). Other constituents that can be found in *P. cupana* seeds are polysaccharides, polyphenols (e.g. catechins, epicatechins and tannins), lipids, saponins, proteins, choline and pigments (Schimpl et al. 2013).

P. cupana has a well-established medicinal use for symptoms of fatigue and feeling of weakness (EMA 2013). However, several other pharmacological effects have been related to *P. cupana* consumption, including antiplatelet aggregation, cardioprotective and chemopreventive effects, and also antioxidant, antidepressant, antimicrobial and anti-obesity properties (Hamerski et al. 2013). Some studies have demonstrated that *P. cupana*-containing products improve lipid metabolism, promote weight loss and increase the basal energy expenditure, acting as thermogenics or metabolic stimulants (Glade 2010; Hamerski et al. 2013; Portella et al. 2013). Indeed, caffeine increases the excitability of adenosine-sensitive sympathetic nervous system, stimulating fat lipolysis (Glade 2010).

Overweight and obesity are widely recognized as modifiers of therapeutic response and prognosis of several chronic health conditions. More specifically, obesity has been commonly reported as a comorbid condition of epilepsy, with a high prevalence in children and adults (Arya et al. 2016; Janousek et al. 2013). Recent studies have focused on the association between overweight or obesity and epilepsy. For instance, Ladino *et al*. (2014) found that 72% of adult patients with epilepsy present overweight, obesity or even morbid obesity, corresponding respectively to 34%, 25% and 13%. Another study referred to that 55.2% of patients with epilepsy were overweight or obese (Janousek et al. 2013). There is also evidence that obesity is more common in patients with refractory epilepsy and in those treated in polytherapy regimens (Baxendale et al. 2015; Chukwu et al. 2014; Janousek et al. 2013). Despite the limited data supporting the role of obesity in seizure severity, obesity may play a central role in the worsening of this neurological disorder (Hafizi et al. 2017).

Taking into account that the use of herbal dietary supplements has increased worldwide at an unprecedented rate, and given the growing prevalence of obesity among patients with

epilepsy, it is expected an increasing consumption of herbal weight loss medicines by this patient subpopulation over the coming years. Moreover, bearing in mind that some constituents of plant extracts have been identified as substrates, inducers and/or inhibitors of transporters and/or enzymes responsible for antiepileptic drugs (AEDs) biodisposition (Oga et al. 2015; Roe et al. 2016; Tarirai et al. 2010; Wu et al. 2015), it is important not to neglect the potential risks associated with the combined use of herbal medicinal products and AEDs, which may compromise the control of seizures and even increase the risk of adverse drug reactions.

As lamotrigine (LTG) is an AED extensively used in the clinical practice, particularly due to its broad spectrum of efficacy in several types of epileptic disorders (Patsalos 2013b), and considering its narrow therapeutic range (3–15 μg/mL) (Patsalos et al. 2017) and its pharmacokinetics variability and propensity to interact with other drugs (Patsalos 2013b), it is fully justified to investigate the effects of *P. cupana* extract on the pharmacokinetics of LTG.

In fact, up to date, to the best of our knowledge, no study was previously conducted to evaluate the potential of interaction between *P. cupana* and LTG. Therefore, this work was planned to investigate whether a commercial standardized *P. cupana* extract may influence the absorption and biodisposition of LTG in rats after their oral co-administration and following a 14-day *P. cupana* pre-treatment period. In addition, the impact of the repeated treatment with *P. cupana* extract on the body weight of rats and in some relevant biochemical parameters was also evaluated.

III.2. Materials and methods

III.2.1. Herbal extract, drugs and materials

P. cupana extract from seeds, containing 12% of caffeine, was purchased from Bio Serae Laboratories (Bram, France) and the corresponding certificate of analysis was received and preserved. LTG dispersible tablets (Lamictal® 25 mg, GSK), chloramphenicol (Sigma–Aldrich, St Louis, USA), used as internal standard (IS), pentobarbital (Eutasil®, 200 mg/ml, Ceva Saúde Animal), sodium chloride 0.9% solution (Labesfal, Portugal), heparin sodium 5000 I.U./mL (B. Braun Medical, Portugal), polyurethane cannula (Introcan® Certo IV indwelling cannula 22G; 0.9 x 2.5 mm; B. Braun Melsungen AG, Germany), disposable cholesterol and triglycerides test strips (Accutrend®, Roche, Germany) and disposable blood glucose test strips (Freestyle Lite, Abbott®) were commercially acquired.

III.2.2. Animals

Thirty-four healthy adult male Wistar rats (247 ± 14 g) were obtained from local certifiedanimal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal) and housed at 12 h light/dark cycle under controlled environmental conditions

(temperature 20 ± 2 °C; relative humidity 55 ± 5%). The animals were allowed free access to a standard rodent diet and water *ad libitum*.

The experimental procedures were approved by the Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção Geral de Alimentação e Veterinária) and all the animal experiments were conducted in accordance with the European Directive (2010/63/EU) for animal experiments.

III.2.3. Preparation of herbal extract and lamotrigine solutions

P. cupana extract solution was daily prepared by dissolving the powdered extract in distilled water. The dose of *P. cupana* administrated to each animal was 821 mg/kg (p.o.), using an administration volume of 10 mL/kg of rat body weight. The selected dose was defined taking into account the human dose recommendation from the extract supplier, which was converted to rat species following a Food and Drug Administration (FDA) Guidance for Industry, which refers to the conversion of animal doses to human equivalent doses based on body surface area (FDA 2005). Furthermore, a 10-fold potentiation of interaction was employed to avoid false negative results.

LTG dispersible tablets were dissolved in a proper volume of distilled water to obtain the LTG solution for rat administration. A LTG dose of 10 mg/kg (p.o.) was administrated taking into consideration an administration volume of 4 mL/kg of rat body weight. LTG dose was selected according to the previous in-house group experience in rat studies, and taking also into account that with this dose, saturation phenomena in the processes of drug absorption and/or elimination are not probable to occur (Avula and Veesam 2015; Ventura et al. 2016; Yamashita et al. 1997).

III.2.4. Systemic pharmacokinetic studies

Twenty-four rats were randomly distributed in four groups, each one containing six animals (n = 6). These studies were designed to investigate the effects of *P. cupana* extract on the bioavailability and plasmatic kinetics of LTG in two independent experimental assays. In the first pharmacokinetic study, rats of the experimental group were concomitantly treated with a single-oral dose of *P. cupana* extract (821 mg/kg, p.o.) and LTG (10 mg/kg, p.o.). In the second study, rats of the experimental group were orally pre-treated during 14 days with *P. cupana extract* (821 mg/kg/day, p.o.) followed by a single dose of LTG (10 mg/kg, p.o.) administrated on the 15th day. A 14-day period of time was considered for the repeated administration of the *P. cupana* extract based on available scientific literature (ICH 2009; Ma and Ma 2016), in which it is described that the repeated administration studies should be conducted during at least 14 days. Rats of the control groups received the corresponding volume of the vehicle of the herbal extract (i.e. water) and were similarly treated with LTG.

In each study, on the night before LTG administration, each animal was anesthetized for insertion of an Introcan® Certo IV indwelling cannula (22G; 0.9 x 2.5 mm) in a lateral tail vein for the subsequent serial blood sampling. Anesthesia was induced with pentobarbital (60 mg/kg) administered intraperitoneally. The rats fully recovered from anesthesia and were fasted before LTG administration, but they were maintained with free access to water. To avoid the food effect on LTG absorption and biodisposition, the fasting period was maintained until 4 h after drug administration.

LTG and *P. cupana extract* (or vehicle, in the control groups) were orally administrated by gavage during the morning in each study. After LTG administration, blood sampling was performed at 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h post-dose. Blood samples of approximately 0.3 mL were collected into EDTA tubes through the cannula inserted in the rat tail vein and then centrifuged at 4000 rpm for 10 min (4 ºC) to separate the plasma which was stored at −20 ºC until analysis.

III.2.5. Plasma-to-brain biodistribution study

To further investigate the potential effects of *P. cupana* on the plasma-to-brain distribution of LTG an independent study was performed. In this study, ten rats were randomly distributed in two groups, each one containing five animals (n = 5). Each animal received by gavage a single oral dose of *P. cupana* extract (821 mg/kg, p.o.) or vehicle (in the control group) co-administrated with a single-oral dose of LTG (10 mg/kg, p.o.). Then, in order to measure the LTG concentrations achieved in plasma and brain at 6 h post-dose, rats were anaesthetized and sacrificed by decapitation. Blood samples were collected and centrifuged as previously described, and the resulting plasma was stored at −20 ºC until analysis. Brain tissue was quickly excised after exsanguination, weighed and homogenized in 0.1 M sodium phosphate buffer at pH 5.5 (4 mL per gram of tissue) using an Ultra-Turrax® tissue homogenizer. The brain tissue homogenates were centrifuged at 13500 rpm for 10 min (4 ºC) and the supernatants were collected and stored at –20 ºC until use.

III.2.6. Liquid chromatography analysis

LTG concentrations in plasma and brain homogenate samples were determined using a microextraction by packed sorbent (MEPS) procedure combined with a high-performance liquid chromatography–diode array detection (HPLC-DAD) method previously developed and validated (Ventura et al. 2016). Briefly, to each aliquot (100 µL) of plasma or brain homogenate supernatant, spiked with 20 µL of the IS working solution (250 µg/mL), was added 400 µL of ice-cold acetonitrile (precipitating agent). The mixture was vortex-mixed for 30s and centrifuged at 13500 rpm for 10 min to precipitate proteins. The clear supernatant was collected and evaporated to dryness under a gentle nitrogen stream at 45 ºC. The dry residue was reconstituted with 200 µL of 0.3% triethylamine-water solution (pH 6.5). Each reconstituted

sample was extracted in three draw-eject cycles through the MEPS syringe, at a flow rate of 10 µL/s. The sorbent was then washed once with 200 µL ultra-pure water and, after that, LTG and IS were eluted with methanol (2 × 30 µL). This methanolic extract was diluted with 90 µL of ultra-pure water and 20 µL were injected into the chromatographic system. The lower limit of quantification was established at 0.1 µg/mL for LTG in plasma and in brain tissue homogenate.

III.2.7. Pharmacokinetic analysis

The peak plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) of LTG were obtained directly from the experimental data. The remaining pharmacokinetic parameters were estimated from the individual plasma concentration-time profiles by non-compartmental pharmacokinetic analysis using WinNonlin version 5.2 (Pharsight Co, Mountain View, CA, USA). For each animal, the estimated pharmacokinetic parameters included the truncated area under the concentration-time curve (AUC) from time zero to 24 h (AUC_{0-24}), AUC from time zero to the last measurable concentration (AUC_{0-t}), which were calculated by the linear trapezoidal rule; and the AUC from time zero to infinite ($AUC_{0-\infty}$), which was determined from AUC_{0-t} + (C_{last}/k_{el}), where C_{last} is the quantifiable concentration at the time of the last measurable drug concentration (t_{last}) and k_{el} is the apparent elimination rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile. The apparent terminal elimination half-life ($t_{1/2el}$) and the mean residence time (MRT) were also estimated. The drug concentrations below the lower limit of quantification of the assay were taken as zero for all calculations.

III.2.8. Effects of repeated-dose administration of *P. cupana* extract on biochemical parameters

To assess the effects of repeated treatment with *P. cupana* extract on biochemical parameters, the blood levels of glucose, total cholesterol and triglycerides were evaluated in all rats of the experimental (*P. cupana*) and control (vehicle) groups on the 14th day of the *P. cupana* pretreatment study and compared. The blood determination of these three biochemical parameters was performed using appropriate medical devices (Accutrend® Plus, Roche, for cholesterol and triglycerides analysis; and Freestyle Freedom Lite, Abbott®, for glucose analysis) and the corresponding disposable test strips.

III.2.9. Effects of repeated-dose administration of *P. cupana* extract on body weight

To evaluate the effects of *P. cupana* extract on rats' body weight over the 14 days of treatment, the body weight of the animals of both experimental (*P. cupana*) and control

(vehicle) groups was determined in the first and last day (14th) of the *P. cupana* pretreatment study, and then compared.

III.2.10. Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM), except for t_{max}. As t_{max} is a categorical variable in the performed pharmacokinetic studies, which can only take values based on planned sampling schedule, t_{max} values were expressed as median and range. Non-parametric Mann-Whitney test was used to compare the t_{max} values from two different groups. The statistical comparisons of the other pharmacokinetic parameters, body weight and biochemical markers between two groups were performed using unpaired two-tailed Student's *t*-test; in addition, for comparisons of body weight changes within the same group a paired Student's *t*-test was employed. A difference was considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

III.3. Results

III.3.1. Effects of *P. cupana* extract on LTG pharmacokinetics after co-administration

The mean plasma concentration-time profiles ($n = 6$) of LTG obtained in rats after the simultaneous administration of a single-oral dose of *P. cupana* extract (821 mg/kg) or vehicle and the drug itself (10 mg/kg) are represented in **Figure III.1**, and the corresponding pharmacokinetic parameters estimated by applying non-compartmental analysis to each individual concentration-time profile are summarized in **Table III.1**. From the observation of the mean plasma pharmacokinetic profiles (**Figure III.1**), it is evident the occurrence of important differences in the extent of systemic exposure to LTG, which was considerably reduced in the presence of *P. cupana* extract. A statistically significant decrease in LTG plasma concentrations was observed in the experimental group, between 0.5 h and 8 h, when compared to the control group ($p < 0.05$). The effect of *P. cupana* extract was found to be particularly marked on the LTG C_{max} and truncated AUC_{0-24}, which were reduced by 32.6% and 36.6%, respectively ($p < 0.05$) (**Table III.1**).

Nevertheless, only a slight reduction was observed in the extent of total systemic drug exposure (as assessed by AUC_{0-t} and $AUC_{0-\infty}$). Despite the statistically significant differences found in the extent of systemic drug exposure achieved up to 24 h post-dose, the time to reach the peak plasma concentration of LTG is similar in both experimental (*P. cupana*) and control (vehicle) groups. Specifically, as shown in **Table III.1**, the median values for t_{max} were 4 h in both experimental and control groups, with mean values of 8 h and 5.58 h for *P. cupana* extract group and control group, respectively.

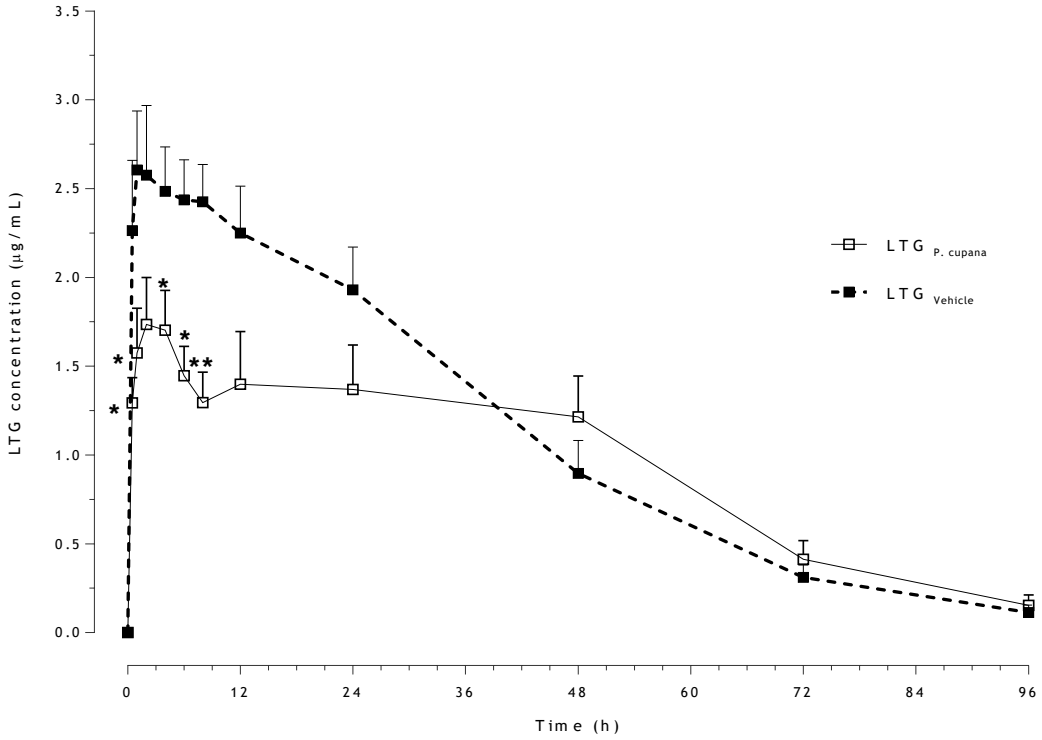


Figure III.1. Mean plasma concentration-time profiles of lamotrigine (LTG) obtained, over a period of 96 h, from rats co-administered with a single-dose of *Paullinia cupana* extract (821 mg/kg, p.o.) or vehicle of the extract (water) and LTG (10 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6). *p < 0.05 and **p < 0.005 compared to control (vehicle).

Table III.1. Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of lamotrigine (LTG) obtained in rats after the co-administration with a single-dose of *Paullinia cupana* extract (821 mg/kg, p.o.) or vehicle of the extract (water) and LTG (10 mg/kg, p.o.) by oral gavage (n = 6). Data are presented as mean values ± standard error of the mean (SEM), except for t_{max} that is expressed as median values (range).

Parameter	Experimental Group LTG *P. cupana*	Control Group LTG Vehicle
C_{max} (µg/mL)	2.022 ± 0.222*	3.00 ± 0.284
t_{max} (h)	4.0 (2.0-24.0)	4.0 (0.5-12.0)
AUC_{0-24} (µg.h/mL)	34.028 ± 4.229*	53.642 ± 4.990
AUC_{0-t} (µg.h/mL)	90.383 ± 13.242	106.488 ± 11.692
$AUC_{0-\infty}$ (µg.h/mL)	96.842 ± 13.890	110.660 ± 12.103
k_{el} (1/h)	0.0386 ± 0.0030	0.0415 ± 0.0020
$t_{1/2el}$ (h)	18.5 ± 1.5	16.9 ± 0.8
MRT (h)	38.6 ± 3.1*	29.1 ± 2.2

AUC, area under the concentration-time curve; AUC_{0-24}, AUC from time zero to 24 h; AUC_{0-t}, AUC from time zero to the last measurable concentration; $AUC_{0-\infty}$, AUC from time zero to infinite; C_{max}, peak concentration; k_{el}, apparent elimination rate constant; MRT, mean residence time; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach peak concentration. *****p < 0.05, significantly different from the control (vehicle) group.

Additionally, a statistically significant increase of the MRT value of LTG was observed in the experimental group when compared to the control group ($p < 0.05$). On the other hand, the mean values estimated for the elimination pharmacokinetic parameters (k_{el} and $t_{1/2el}$) of LTG are similar in both groups (*P. cupana* extract *versus* vehicle) (**Table III.1**).

III.3.2. Effects of repeated-dose pretreatment with *P. cupana* extract on LTG pharmacokinetics

The mean plasma pharmacokinetic profiles of LTG following a single-oral administration of 10 mg/kg of the drug (at 15th day) to rats previously submitted to a 14-day treatment period with *P. cupana* extract (821 mg/kg/day) or vehicle are depicted in **Figure III.2.**

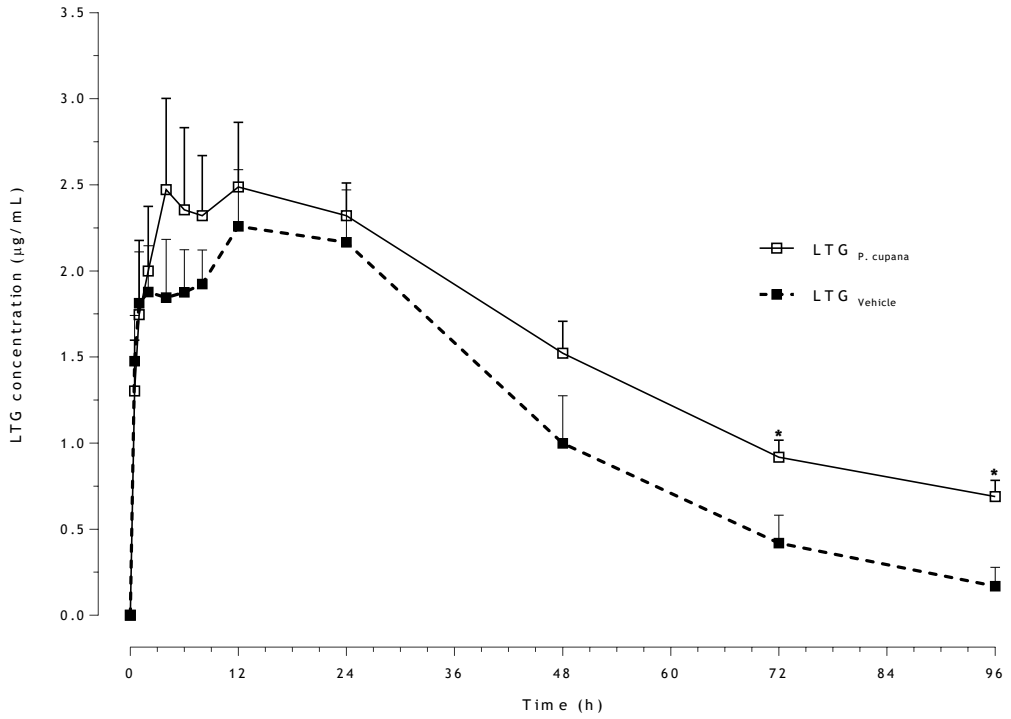


Figure III.2. Mean plasma concentration-time profiles of lamotrigine (LTG) obtained, over a period of 96 h, from rats submitted to a 14-day pre-treatment period with *Paullinia cupana* extract (821 mg/kg/day, p.o.) or vehicle of the extract (water), and treated on the 15th day with a single-dose of LTG (10 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point ($n = 6$). ***$p < 0.05$ compared to control (vehicle).

In addition, the respective mean (or median) pharmacokinetic parameters are presented in **Table III.2**. A similar pattern of the plasma concentration-time curves was observed in both experimental (*P. cupana*) and control (vehicle) groups, although slightly higher LTG concentrations were obtained in the experimental group over most of the study time, presenting statistically significant differences only at 72 h and 96 h post-dose ($p < 0.05$).

Table III.2. Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of lamotrigine (LTG) obtained in rats submitted to a 14-day pre-treatment period with *Paullinia cupana* extract (821 mg/kg, p.o.) or vehicle of the extract (water), and treated on the 15th day with a single-dose of LTG (10 mg/kg, p.o.) by oral gavage (*n* = 6, unless otherwise noted). Data are presented as mean values ± standard error of the mean (SEM), except for t_{max} that is expressed as median values (range).

Parameter	Experimental Group LTG *P. cupana*	Control Group LTG Vehicle
C_{max} (µg/mL)	3.165 ± 0.358	2.706 ± 0.237
t_{max} (h)	9.0 (4.0-24.0)	12.0 (4.0-24.0)
AUC_{0-24} (µg.h/mL)	55.398 ± 6.973	49.195 ± 5.578
AUC_{0-t} (µg.h/mL)	150.108 ± 11.737	109.770 ± 15.950
$AUC_{0-\infty}$ (µg.h/mL)	157.212 ± 26.779[a]	118.699 ± 18.679
k_{el} (1/h)	0.0229 ± 0.0041[a]	0.0419 ± 0.0050
$t_{1/2el}$ (h)	31.2 ± 5.6[a]	18.1 ± 2.5
MRT (h)	45.7 ± 10.3[a]	32.8 ± 5.2

AUC, area under the concentration-time curve; AUC_{0-24}, AUC from time zero to 24 h; AUC_{0-t}, AUC from time zero to the last measurable concentration; $AUC_{0-\infty}$, AUC from time zero to infinite; C_{max}, peak concentration; k_{el}, apparent elimination rate constant; MRT, mean residence time; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach peak concentration. [a]n = 2.

C_{max} was slightly higher in the experimental group (16.9%) compared to the control group. The median LTG t_{max} was 9 h in the experimental group and 12 h in the control group, ranging the t_{max} values from 4 to 24 h (**Table III.2**). Also worthy of note are the values estimated for the truncated AUC_{0-24}, which were very similar in both groups. Regarding the AUC_{0-t} parameter no statistically significant differences ($p > 0.05$) were detected, but considering the mean values, there was a trend towards a higher systemic exposure (36.7%) in the group of rats treated with *P. cupana* extract.

Otherwise, despite the 96-h sampling period established for this study, it was not possible to appropriately characterize the terminal elimination phase of the pharmacokinetic profile of LTG in some rats of the experimental group; thus, in this case, no reliable conclusions can be drawn by the comparison of the average values calculated for secondary pharmacokinetic parameters, which are highly influenced by the measurements in the terminal elimination phase of the concentration-time curve (k_{el}, $t_{1/2el}$, MRT and $AUC_{0-\infty}$).

III.3.3. Effects of *P. cupana* extract on the LTG plasma-to-brain biodistribution after co-administration

As LTG needs to cross the blood-brain barrier to achieve its biophase, and considering the pharmacokinetic herb-drug interaction evidenced systemically after the co-administration of

P. cupana extract and LTG, this additional study was designed to evaluate the impact of such herb-drug interaction on the LTG plasma-to-brain biodistribution, employing the same dosing regimen (i.e. a single-oral dose of 10 mg/kg of LTG and 821 mg/kg of *P. cupana* extract). For this purpose, LTG concentrations were measured in plasma and brain tissue of rats sacrificed at 6 h post-dose. This time-point was selected because, among the serial sampling time-points defined in the systemic pharmacokinetic study described in *section 3.1*, the 6 h represent a post-dose time-point that is very close to the median t_{max} value estimated (6.5 h) considering together the t_{max} data (n = 12) of both groups (experimental and control groups).

The results obtained are shown in **Figure III.3**. Analyzing and comparing the data obtained in this study, it is evident that statistically significant differences were found between plasma concentrations of LTG measured in the groups of rats that received *P. cupana* extract and vehicle (1.926 ± 0.226 µg/mL *versus* 3.683 ± 0.239 µg/mL, $p < 0.005$). On the other hand, although the mean concentrations of LTG achieved in brain tissue were lower in the experimental (*P. cupana)* group (1.389 ± 0.217 µg/g) than in the control (vehicle) group (1.900 ± 0.256 µg/g), no statistically significant differences were found at this single point of sampling ($p > 0.05$).

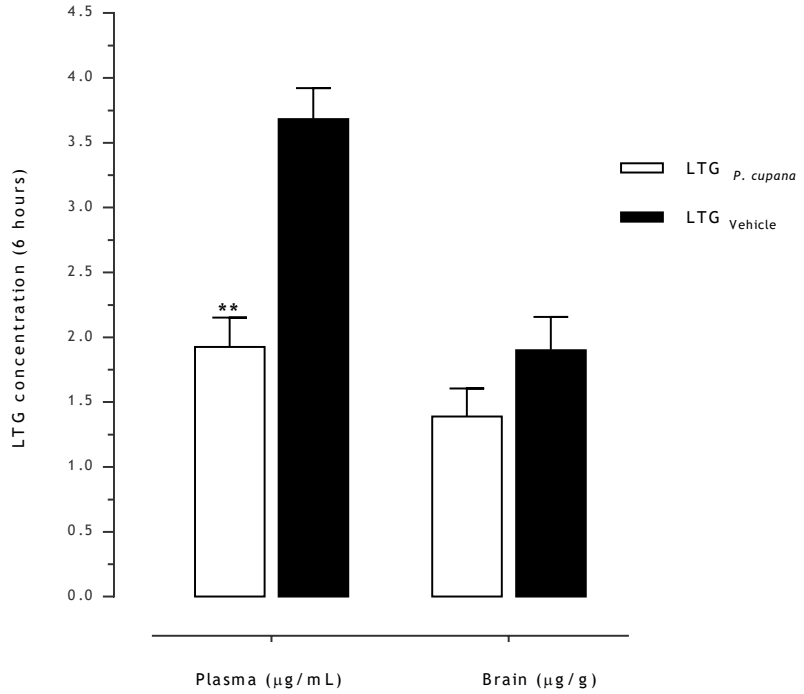


Figure III.3. Mean plasma and brain tissue concentrations of lamotrigine (LTG), obtained at 6 h post-dose, from rats co-administrated with a single-dose of *Paullinia cupana* extract (821 mg/kg, p.o.) or vehicle (water) and LTG (10 mg/kg, p.o.) by oral gavage. Data are presented as the mean values ± standard error of the mean (SEM) of five determinations (n = 5). **$p < 0.005$ compared to control (vehicle).

III.3.4. Effects of repeated-dose administration of *P. cupana* extract on biochemical parameters

The blood levels of glucose, total cholesterol and triglycerides determined in rats treated repeatedly with *P. cupana* extract (experimental group) or vehicle (control group), over a period of 14 days, are shown in **Figure III.4**. Statistically significant differences were detected between experimental and control groups for glucose ($p < 0.005$) and triglycerides ($p < 0.05$) blood levels. The mean glucose levels measured in the rats of experimental (*P. cupana*) group were higher than those found in rats of control (vehicle) group, which were 74.2 ± 2.8 mg/dL and 56.0 ± 2.0 mg/dL, respectively. On the contrary, the mean triglyceride levels determined in the rats treated with *P. cupana* extract were lower than those measured in the rats that received the vehicle of the extract (i.e. water), being 79.2 ± 2.1 mg/dL and 94.5 ± 5.5 mg/dL, respectively. Regarding the total cholesterol levels, the values obtained were very similar in both experimental and control groups, with mean concentrations of 156.2 ± 1.4 mg/dL and 158.7 ± 2.1 mg/dL, respectively.

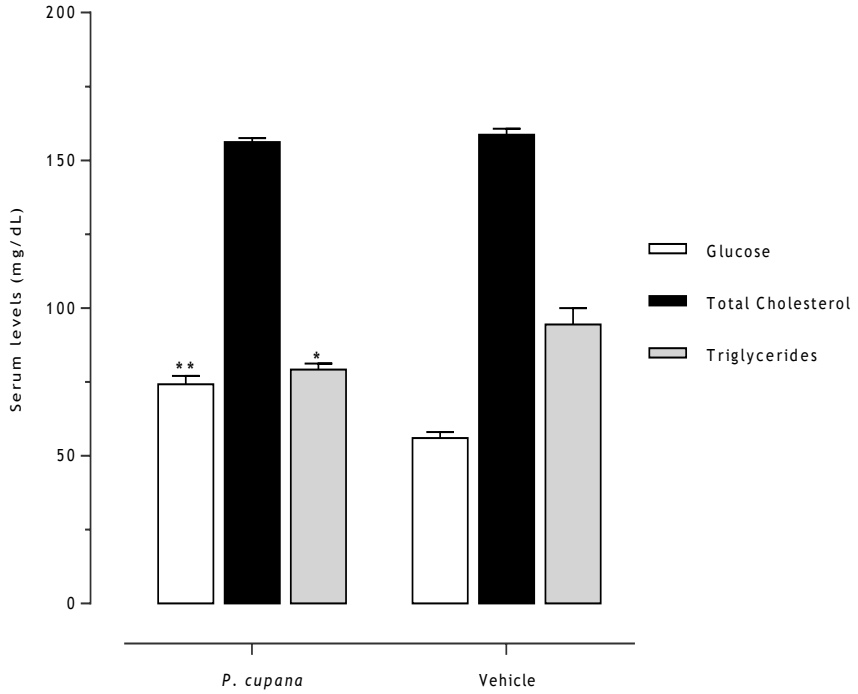


Figure III.4. Effects of *Paullinia cupana* extract on biochemical parameters (blood glucose, total cholesterol and triglycerides) after a 14-day treatment period with *Paullinia cupana* extract (821 mg/kg/day, p.o.) or vehicle (water) by oral gavage. Data are presented as the mean values ± standard error of the mean (SEM) of six determinations ($n = 6$). ***$p < 0.05$ and ****$p < 0.005$ compared to control (vehicle).

III.3.5. Effects of repeated-dose administration of *P. cupana* extract on body weight

The data regarding body weight of rats treated with *P. cupana* extract (821 mg/kg/day, p.o) or vehicle during 14 consecutive days are shown in **Figure III.5**. The rats of both control and experimental groups had a similar body weight at the beginning of the study (day 1), with mean values of 250.7± 6.0 g and 243.3 ± 5.4 g, respectively. From the results obtained, a statistically significant increase in the body weight of rats was observed between day 1 and day 14 in both experimental (*P. cupana*) and control (vehicle) groups ($p < 0.005$). When comparing the body weight gains of the rats during the period of the study, a trend towards a lower weight increase was observed in the rats that received *P. cupana* extract, however, such difference was not found to be statistically significant ($p = 0.06$).

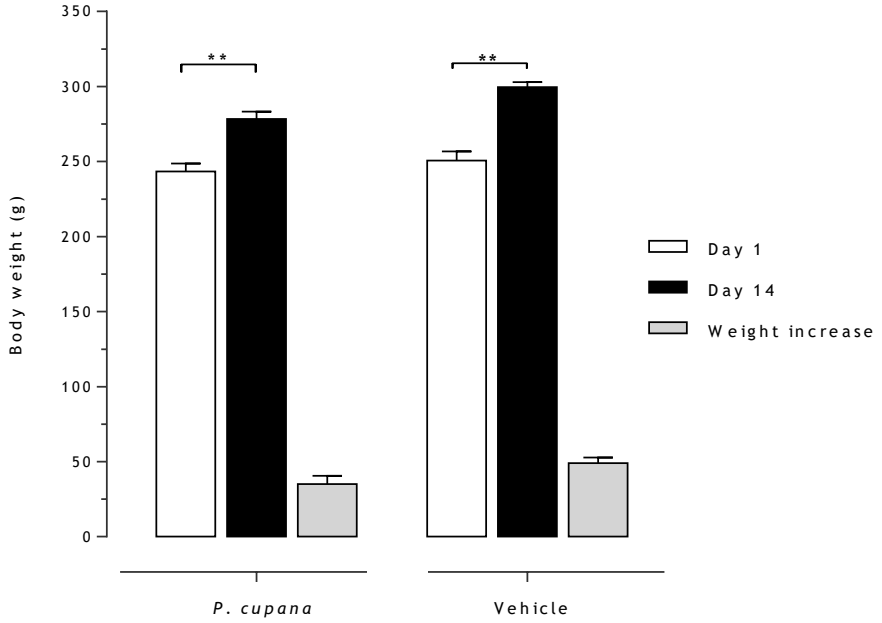


Figure III.5. Effects of *Paullinia cupana* extract on the body weight of rats after a 14-day treatment period with *Paullinia cupana* extract (821 mg/kg/day, p.o.) or vehicle (water) by oral gavage. Data are presented as the mean values ± standard error of the mean (SEM) of six determinations ($n = 6$). **$p < 0.005$, day 1 *versus* day 14.

III.4. Discussion

The goal of AED therapy is to control seizures and improve the patient's quality of life. Drug-related problems, including those resulting from interactions between herbal substances and AEDs can influence the efficacy, safety and adherence to AED therapy. Also of concern is the fact that many patients believe in the safety of herbal medicines and therefore do not reportits use to the physician. In a study involving 92 patients with epilepsy, it was found that 24% were

using complementary and alternative therapies, of which 41% were using herbs and supplements and, in most cases, it was not of the doctor's knowledge (Peebles et al. 2000). In a more recent study conducted by Eyal et al. (2014), in adult patients with epilepsy, the results showed that 48% of them took dietary supplements simultaneously with AEDs and patient awareness for potential drug interactions involving AEDs was very limited.

The pharmacokinetic studies herein reported were designed to assess the potential of interaction between *P. cupana* extract and LTG *in vivo* in Wistar rats. Considering that no interaction has been previously reported among these components, our starting point for a preliminary preclinical risk assessment was to evaluate the effects of *P. cupana* extract on the LTG bioavailability after their simultaneous administration, aiming at investigating a possible interference of *P. cupana* extract on the gastrointestinal absorption of LTG. In these experimental conditions, the obtained pharmacokinetic results clearly evidenced a decrease in absorption rate of LTG from the gastrointestinal tract of rats (as denoted by C_{max} and AUC_{0-24}), even though no important differences were detected in the extent of total systemic drug exposure (as assessed by AUC_{0-t} and $AUC_{0-\infty}$). These findings support that *P. cupana* extract, or some of its constituents, interact in some way with LTG in the gastrointestinal tract of rats, delaying the drug absorption. These data converge with results previously reported by our research group in a similar study involving *P. cupana* extract and amiodarone, in which a significant reduction in the peak plasma concentration (73.2%) and in the extent of systemic exposure (57.8%) to amiodarone were found (Rodrigues et al. 2012). The simultaneous co-administration of a *Fucus vesiculosus* extract and amiodarone also resulted in a significant decrease (55.4%) of the peak plasma concentration and in a reduction of approximately 30% in the extent of systemic exposure to amiodarone (Rodrigues et al. 2013a). Moreover, similar results were found *in vivo* after oral pretreatment of rats with green tea extract (175 mg/kg/day) for 4 days followed by a single-dose administration of clozapine 20 mg/kg, which resulted in a significant decrease of C_{max} and $AUC_{0-\infty}$. The authors suggested that green tea extract delayed the gastric emptying of clozapine, reducing the rate and amount of clozapine absorbed (Jang et al. 2005).

Moreover, having in mind the central role that induction of enzymes and transporters plays on drug-drug and herb-drug interactions, and knowing that the induction mechanisms are time-dependent, a second study was delineated to evaluate the effects of the repeated administration of *P. cupana* extract on the pharmacokinetics of LTG. The *P. cupana* extract pretreatment for 14 days resulted in a slightly higher systemic exposure to LTG, however, no important differences were found in comparison with the control group. These results suggest that *P. cupana* extract can interact with LTG disposition, but the similarity observed in the extent of systemic drug exposure in the rats of both experimental and control groups excludes the impact of *P. cupana*-induced metabolism on the bioavailability of LTG. Therefore, by combining the results of the two pharmacokinetic studies, it can be inferred that the herb-drug interaction between *P. cupana* extract and LTG found in the co-administration study occurred

mainly at the absorption level, being unlikely the involvement of metabolism-based mechanisms. Indeed, as LTG has an oral bioavailability of approximately 100% and its absorption is not affected by food (Garnett 1997), and taking also into account the available evidence that LTG permeates through biological mucosa mainly via the non-storable transcellular passive diffusion (Mashru et al. 2005), there is a negligible probability of this reported herb-drug interaction being the result of competition mechanisms by the same carrier. Thus, considering all the results presented herein, it is plausible to hypothesize that a physical-chemical interaction occurred between *P. cupana* extract, or its constituents, and LTG in the gastrointestinal tract of rats, which may explain the decrease in the rate of systemic exposure to LTG after its simultaneous co-administration with *P. cupana* extract. Thus, we presume that the effect of *P. cupana* extract could be related to the adsorption of lamotrigine in an identical manner to the effect caused by charcoal on lamotrigine (Keränen et al. 2011). Nevertheless, further studies are needed to better understand the mechanism associated with this herb-drug interaction (*P. cupana* extract/LTG), which is herein reported for the first time. Although there are pharmacokinetic differences between species, the effective plasma levels of AEDs are usually quite similar among rodents and humans (Castel-Branco et al. 2005a; Loscher 2011); hence, the clinical relevance of this interaction must be further investigated in order to understand the therapeutic impact of a lower systemic incorporation rate of LTG.

The repeated administration of *P. cupana* extract for 14 days had effects on glucose and triglyceride levels, increasing the glycaemia and reducing the blood levels of triglycerides. Another study identified similar results in rats treated with *P. cupana* extract, showing an increase in the glycaemia and a decrease in blood triglyceride levels in the experimental groups (Antonelli-Ushirobira et al. 2010). The reduction in blood triglyceride levels after *P. cupana* intake was also observed in human studies (Krewer et al. 2011; Portella et al. 2013; Suleiman et al. 2016). Indeed, caffeine, a methylxanthine abundantly present in *P. cupana* extract, has already been related to inhibitory effects on pancreatic lipase, a key enzyme in the dietary absorption of triacylglycerols (Yun 2010). Portella et al. (2013) also demonstrated that *P. cupana* extract has peroxyl radical scavenger activity and inhibits lipid peroxidation, which may explain the impact of the extract on the lipid metabolism. Although no statistically significant difference was observed in cholesterol levels, methylxanthines have been related to the control of the transcription of genes for 3-hydroxy-3-methylglutaryl coenzyme A reductase and low density lipoprotein receptor (Ruchel et al. 2017). Likewise, the 14-day treatment period with *P. cupana* extract did not have a strong effect on the body weight of rats; however, according to the available data, there appears to be a tendency for a slower weight gain in the rats of the experimental (*P. cupana*) group. Antonelli-Ushirobira *et al.* also found no statistically significant differences in rats' body weight after 14 days of administration of *P. cupana* extract; nevertheless, when the animals were treated for a longer period of time (90 days) a slower increase in the body weight gain was observed (2010). These effects of *P. cupana* extract intake on the the body weight of rats and in the measured biochemical parameters reinforce the potential benefits of this extract in weight management and in lipid metabolism.

III.5. Conclusion

This work is the first report documenting the occurrence of an herb-drug interaction between *P. cupana* and LTG after their simultaneous co-administration, which led to a significant reduction in the rate and extent of systemic exposure to LTG. On the other hand, the repeat pretreatment with *P. cupana* did not have a significant impact on LTG concentrations when the drug was administered 24 h after the last administration of the extract. Thus, bearing in mind the effects of *P. cupana* extract on the systemic absorption of LTG, it is prudent to advise patients on therapy with LTG to avoid the simultaneous ingestion of *P. cupana*-containing products, thus minimizing the risks of occurrence of interaction. However, if the treatment with *P. cupana*-containing products and LTG is required at the same time in a patient, then they should be administered separately on the day (one in the morning and the other in the evening).

Chapter IV.

Administration of *Garcinia cambogia* and lamotrigine: safety evidence from non-clinical pharmacokinetic studies in Wistar rats

IV.1. Introduction

Garcinia cambogia, also known as Malabar tamarind, has been traditionally used in rheumatic and bowel complaints and is now popularly used as an ingredient of dietary supplements for weight loss (Márquez et al. 2012; Semwal et al. 2015). Biological effects of *G. cambogia* are closely related to its phytochemical constituents. The fruits of *G. cambogia* contain organic acids, such as hydroxycitric acid (HCA), along with xanthones (e.g. oxy-guttiferones I, K, K2 and M), benzophenones (guttiferones I, N, J, K and M) and amino acids as glutamine, glycine and γ-aminobutyric acid (Semwal et al. 2015). In fact, the major bioactive constituent of *G. cambogia* fruits is the stereoisomer (-)-HCA, which is present in amounts of 10-30% in the free form and/or as a mineral salt or a stable lactone form (Márquez et al. 2012).

Marketed supplements of *G. cambogia* extract usually contain until 50-60% of (-)-HCA (Bakhiya et al. 2017; Márquez et al. 2012), which are widely used for weight loss and obesity management mainly due to appetite-suppressant, anti-obesity and hypolipidemic activities (Fassina et al. 2015; Semwal et al. 2015). Indeed, several studies have reported potent inhibitory effects of HCA isolated from *G. cambogia* on lipogenesis and on the adenosine triphosphate (ATP) citrate lyase, a key enzyme in the biosynthesis of fatty acids (Jena et al. 2002; Márquez et al. 2012). Additionally, decreased levels of serum triglycerides and cholesterol as well as enhanced gluconeogenesis and glycogenesis have also been ascribed to HCA (Bakhiya et al. 2017; Esteghamati et al. 2015; Mopuri and Islam 2017). Moreover, in some non-clinical studies in rodents, *G. cambogia* fruit extracts have also been associated with weight loss and appetite suppression activity, probably as a result of the increase in brain serotonin levels, reduction in plasma insulin levels and inhibition of the enteral absorption of glucose (Hayamizu et al. 2003; Ohia et al. 2001; Wielinga et al. 2005).

Natural food supplements are gaining popularity as an attractive alternative to counteract obesity, preventing obesity-related physio-pathologic events. Since obesity and obesity-related chronic diseases are growing at an alarming rate (Esteghamati et al. 2015), conventional pharmacological approaches for the treatment of obesity seem to be overtaken by the use of herbal bioactive components with anti-obesity properties. Actually, epilepsy patients present a growing risk of developing obesity in comparison with general population (Arya et al. 2016; Janousek et al. 2013; Ladino et al. 2014). In particular, a higher prevalence of obesity has been observed in patients with refractory epilepsy and in those treated with antiepileptic drugs (AEDs) polytherapy regimens (Baxendale et al. 2015; Chukwu et al. 2014; Janousek et al. 2013). The long-term use of AEDs has already been associated with changes in some metabolic pathways, thus determining changes in body weight (Hamed 2015). On the other hand, evidence from some experimental studies has suggested that peripheral hormones, such as leptin, ghrelin and adiponectin, which are altered in obesity state, may modulate seizure threshold, epilepsy and/or seizure-related damage (Lee and Mattson 2014). Hence, considering the increasing use of weight-loss herbal medicines and supplements worldwide, including among patients with

epilepsy, it is essential to ensure the absence of important herb-drug interactions between herbal preparations and AEDs to avoid potential deleterious effects in terms of efficacy and safety. Actually, as some constituents of herbal extracts can be substrates, inducers and/or inhibitors of transporters and/or enzymes responsible for AEDs biodisposition (Oga et al. 2015; Roe et al. 2016; Tarirai et al. 2010; Wu et al. 2015), it is urgent to evaluate the potential risk for herb-drug interactions between weight-loss herbal extracts and AEDs.

Bearing in mind that lamotrigine (LTG) is a commonly prescribed AED with unique pharmacokinetic and pharmacodynamic properties, which make it a first-line option for several types of epileptic seizures and also in bipolar disorder (Nevitt et al. 2017; Vajda et al. 2013), it is fully justified to assess the effects of *G. cambogia* extract on the pharmacokinetics of LTG. Indeed, despite its the broad spectrum of efficacy, LTG presents some pharmacological disadvantages such as a narrow therapeutic range (3–15 μg/mL) and a considerable interindividual variability in its pharmacokinetics and some propensity to interact with other drugs (Patsalos 2013b; Patsalos et al. 2017), which raises additional concerns that support the need to investigate the potential for pharmacokinetic-based interactions between *G. cambogia* extract and LTG in *in vivo* conditions.

IV.2. Materials and methods

IV.2.1. *G. cambogia* extract and drugs

The extract of *G. cambogia* containing 60% of HCA, was purchased from Bio Serae Laboratories (Bram, France). The certificate of analysis of the extract was received and preserved. LTG dispersible tablets (Lamictal® 25 mg, GSK), pentobarbital (Eutasil®, 200 mg/ml, Ceva Saúde Animal), sodium chloride 0.9% solution (Labesfal, Portugal), heparin sodium 5000 I.U./mL (B. Braun Medical, Portugal) were commercially acquired from referenced laboratories.

IV.2.2. Animals

Adult male Wistar rats weighing 220 ± 22 g were obtained from local certified animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). Animals were housed at 12 h light/dark cycle under controlled environmental conditions (temperature 20 ± 2 °C; relative humidity 55 ± 5%) and were allowed free access to a standard rodent diet and water *ad libitum*.

The experimental procedures were approved by the Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção Geral de Alimentação e Veterinária) and all the animal experiments were conducted in accordance with the European Directive (2010/63/EU) for animal experiments.

IV.2.3. Preparation of *G. cambogia* extract and LTG solutions

The solution of *G. cambogia* extract was daily prepared by dissolution of the powdered extract in distilled water to be administrated at a dose of 821 mg/kg (p.o.) considering the administration volume of 10 mL/kg of rat body weight. This dose was defined based on the human dose recommendation from the extract supplier, which was converted to rat species following a Food and Drug Administration (FDA) Guidance for Industry; this FDA guidance allows the conversion of animal doses to human equivalent doses based on body surface area (FDA 2005). Additionally, a 10-fold potentiation factor of interaction was employed to avoid false negative results.

The LTG solution was obtained after dissolution of the dispersible tablets in the proper volume of distilled water to obtain the required drug solution to be administrated to animals. Each animal received a LTG dose of 10 mg/kg (p.o.) administered in a volume of 4 mL/kg of rat body weight. The LTG dose employed in these studies was defined based on previous experiments performed in the rat (Ventura et al. 2018; Ventura et al. 2016).

IV.2.4. Pharmacokinetic studies

Two independent pharmacokinetic studies were designed to investigate the potential of interaction between *G. cambogia* extract and LTG in Wistar rats. Twelve animals were used in each pharmacokinetic study, which were balanced and randomly allocated to the control and experimental groups. In the first pharmacokinetic study, rats of the experimental group (n = 6) were concomitantly treated with a single-oral dose of *G. cambogia* extract (821 mg/kg, p.o.) and LTG (10 mg/kg, p.o.). In the second study, rats of the experimental group (n = 6) were orally pre-treated during 14 days with *G. cambogia* extract (821 mg/kg/day, p.o.) followed by a single dose of LTG (10 mg/kg, p.o.) administrated on the 15th day. A 14-day period of time was considered for the repeated administration of *G. cambogia* extract according to the international guidelines and scientific data available on this scope (ICH 2009; Ma and Ma 2016). Rats of each control group (n = 6) received the corresponding volume of the vehicle of the herbal extract (water) and were similarly treated with LTG.

Briefly, each animal of both experimental and control groups was anesthetized on the night before LTG administration for insertion of a polyurethane cannula in a lateral tail vein (Introcan® Certo IV indwelling cannula 22G, 0.9 x 2.5 mm; B. Braun Melsungen AG, Germany) to be used for serial blood sampling. Anesthesia was performed by intraperitoneal injection of pentobarbital (60 mg/kg). Rats completely recovered from anesthesia, and they were submitted to an overnight fasting period, with free access to water, before LTG administration. To avoid the effect of food on LTG absorption and disposition, the fasting period was also maintained for 4 h after drug administration.

In each study, LTG and *G. cambogia* extract (or vehicle, in the control groups) were orally administrated by gavage in the morning. After treatment with LTG, blood samples were

obtained from each animal at 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h post-dose. Each blood sample (approximately 0.3 mL) was collected into EDTA tubes and then centrifuged at 4000 rpm for 10 min (4 ºC) to separate the plasma which was stored at −20 ºC until analysis.

IV.2.5. LTG quantification

The quantification of LTG in each plasma sample was achieved using a microextraction by packed sorbent (MEPS) technique coupled to a high-performance liquid chromatography–diode array detection (HPLC-DAD) method, previously developed and validated (Ventura et al. 2016).

IV.2.6. Pharmacokinetic analysis

The peak plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were directly obtained from the experimental data. The individual plasma concentration-time profiles were submitted to a non-compartmental pharmacokinetic analysis using WinNonlin version 5.2 (Pharsight Co, Mountain View, CA, USA) to estimate a set of relevant pharmacokinetic parameters, including the truncated area under the concentration-time curve (AUC) from time zero to 24 h (AUC_{0-24}); the AUC from time zero to last measurable concentration (AUC_{0-t}), which was calculated by the linear trapezoidal rule; the AUC from time zero to infinite ($AUC_{0-\infty}$), which was determined from $AUC_{0-t} + (C_{last}/k_{el})$, where C_{last} is the quantifiable concentration at the time of the last measurable drug concentration (t_{last}) and k_{el} is the apparent elimination rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile; the apparent terminal elimination half-life ($t_{1/2el}$); and the mean residence time (MRT). The drug concentrations below the lower limit of quantification of the assay were taken as zero for all calculations.

IV.2.7. Effects of repeated-dose administration of *G. cambogia* extract on body weight

In addition to the pharmacokinetic studies, the effects of the repeated administration of *G. cambogia* extract on the body weight of rats, over the 14-day treatment period, were also investigated. So, the body weight of the animals of the experimental (*G. cambogia*) and control (vehicle) groups was evaluated and then compared between the first and the last day (14^{th}) of the *G. cambogia* pre-treatment study.

IV.2.8. *Statistical analysis*

The results are presented as the mean ± standard error of the mean (SEM), except for t_{max} whose values were expressed as median and range since it is a categorical variable in the

pharmacokinetic studies. The non-parametric Mann-Whitney test was used to compare the t_{max} values from two different groups. Statistical analyses and comparisons of the other pharmacokinetic parameters and body weight between two groups were performed using unpaired two-tailed Student's *t*-test; in addition, for comparisons of body weight changes within the same group a paired Student's *t*-test was employed. A difference was considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

IV.3. Results

IV.3.1. Effects of *G. cambogia* extract on LTG pharmacokinetics after co-administration

The mean plasma concentration-time profiles of LTG obtained in rats ($n = 6$) following the simultaneous administration of a single-oral dose of *G. cambogia* extract (821 mg/kg) or vehicle and LTG (10 mg/kg) are shown in **Figure IV.1**.

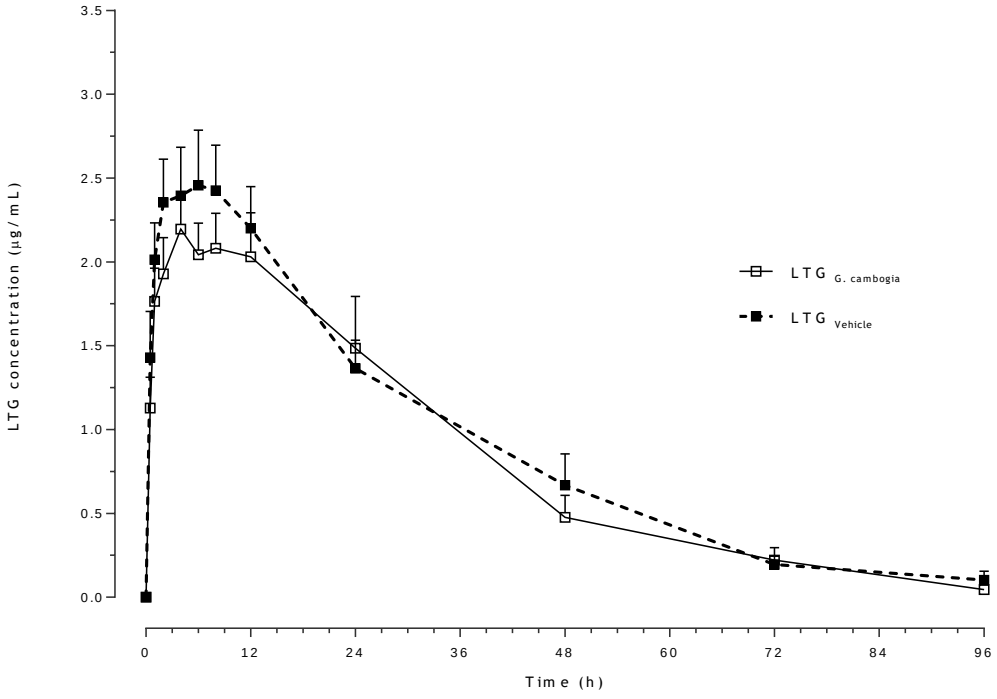


Figure IV.1. Mean plasma concentration-time profiles of lamotrigine (LTG) obtained, over a period of 96 h, from rats co-administered with a single-dose of *Garcinia cambogia* extract (821 mg/kg, p.o.) or vehicle of the extract (water) and LTG (10 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point ($n = 6$).

The corresponding pharmacokinetic parameters directly obtained from experimental data and estimated by non-compartmental analysis are summarized in **Table IV.1**.

Table IV.1. Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of lamotrigine (LTG) obtained in rats after the co-administration with a single-dose of *G. cambogia* extract (821 mg/kg, p.o.) or vehicle of the extract (water) and LTG (10 mg/kg, p.o.) by oral gavage (n = 6). Data are presented as mean values ± standard error of the mean (SEM), except for t_{max} that is expressed as median values (range).

Parameter	Experimental Group *LTG G. cambogia*	Control Group *LTG Vehicle*
C_{max} (µg/mL)	2.363 ± 0.230	2.790 ± 0.236
t_{max} (h)	10.0 (4.0-24.0)	5.0 (1.0-8.0)
AUC_{0-24} (µg.h/mL)	44.647 ± 4.927	48.532 ± 4.776
AUC_{0-t} (µg.h/mL)	80.364 ± 10.433	86.109 ± 8.840
$AUC_{0-\infty}$ (µg.h/mL)	87.641 ± 10.488	90.628 ± 9.568
k_{el} (1/h)	0.0377 ± 0.005	0.0424 ± 0.004
$t_{1/2el}$ (h)	20.0 ± 2.5	17.4 ± 2.2
MRT (h)	30.6 ± 3.4	28.0 ± 3.4

AUC, area under the concentration-time curve; AUC_{0-24}, AUC from time zero to 24 hours; AUC_{0-t}, AUC from time zero to the last measurable concentration; $AUC_{0-\infty}$, AUC from time zero to infinite; C_{max}, peak concentration; k_{el}, apparent elimination rate constant; MRT, mean residence time; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach C_{max}.

As observed, a similar pattern of plasma concentration-time profiles is observed in both experimental (*G. cambogia*) and control (vehicle) groups. Although a slight trend towards lower LTG concentrations is observed in the experimental group (*G. cambogia*) between 1.0 and 12.0 h post-dose, no statistically significant differences were found ($p > 0.05$) between both groups (**Figure IV.1**).

Mean C_{max} was also slightly lower in the experimental group (15.3%) compared to the control group, but without statistical significance ($p > 0.05$). The median LTG t_{max} was 10.0 h in the experimental group and 5.0 h in the control group. Despite the longer median t_{max} value estimated for LTG in the experimental group no statistically significant differences were detected (**Table IV.1**).

Regarding the extent of systemic exposure of LTG (as assessed by AUC values) quite similar values were obtained in both experimental and control groups. In addition, as expected, the mean values estimated for the elimination pharmacokinetic parameters (k_{el} and $t_{1/2el}$) and MRT of LTG were also similar in both groups (*G. cambogia* extract *versus* vehicle).

IV.3.2. Effects of repeated-dose pre-treatment with *G. cambogia* extract on LTG pharmacokinetics

The effects of the repeated administration of *G. cambogia* extract (821 mg/kg) during 14 days followed by a single-oral administration of LTG (10 mg/kg) on the 15th day can be observed from the mean plasma concentration-time profiles obtained in rats (n = 6), which are depicted in **Figure IV.2**. The corresponding pharmacokinetic parameters either directly obtained from the experimental data or estimated by non-compartmental analysis are shown in **Table IV.2**.

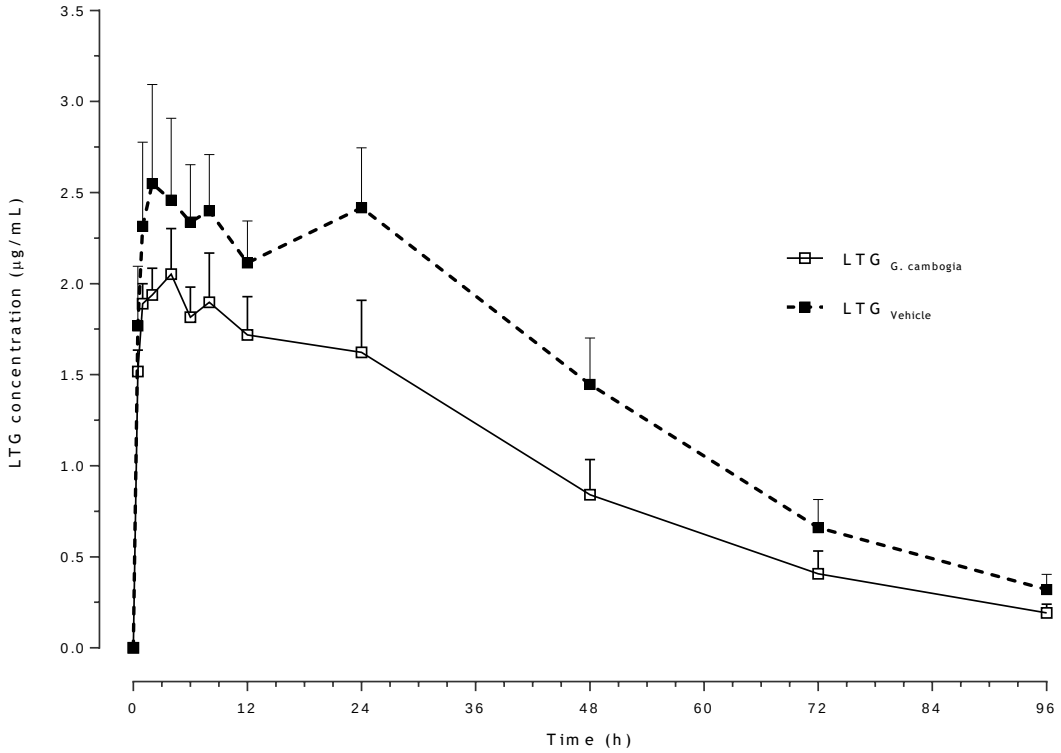


Figure IV.2. Mean plasma concentration-time profiles of lamotrigine (LTG) obtained, over a period of 96 h, from rats submitted to a 14-day pre-treatment period with *Garcinia cambogia* extract (821 mg/kg/day, p.o.) or vehicle of the extract (water) and treated on the 15th day with a single-dose of LTG (10 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6).

From the mean plasma concentration-time profiles, it is clear that substantially lower concentrations of LTG were obtained in the group of rats pre-treated with *G. cambogia* (experimental group) compared with the vehicle (control) group; nevertheless, the differences observed over time in the pharmacokinetic profiles of LTG were not statistically significant at any time point ($p > 0.05$) (**Figure IV.2**). Analyzing the pharmacokinetic parameters, it is evident that the repeated administration of *G. cambogia* extract produced a marked reduction of the C_{max} of LTG, which was reduced by 34.0% ($p < 0.05$); differences were also found in the median t_{max} values estimated for the experimental (3.0 h) and control groups (3.0 h *versus* 16.0 h, respectively) but without statistical significance (**Table IV.2**). The extent of systemic exposure

of LTG was slightly diminished by 23.1% (AUC_{0-24}), 31.5% (AUC_{0-t}) and 31.6% ($AUC_{0-\infty}$) in the group of rats pre-treated with *G. cambogia*; however, no statistical differences were detected between the experimental and control groups ($p > 0.05$). Despite the significant lower C_{max} of LTG and the slightly lower extent of drug systemic exposure in the group of rats subjected to the pre-treatment with *G. cambogia* extract, the elimination phase was not significantly altered as the k_{el}, $t_{1/2el}$ and MRT pharmacokinetic parameters were quite similar between both groups.

Table IV.2. Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of lamotrigine (LTG) obtained in rats submitted to a 14-day pre-treatment period with *G. cambogia* extract (821 mg/kg, p.o.) or vehicle of the extract (water) and treated on the 15th day with a single-dose of LTG (10 mg/kg, p.o.) by oral gavage ($n = 6$). Data are presented as mean values ± standard error of the mean (SEM), except for t_{max} that is expressed as median values (range).

Parameter	Experimental Group *LTG G. cambogia*	Control Group *LTG Vehicle*
C_{max} (µg/mL)	2.256 ± 0.219*	3.416 ± 0.318
t_{max} (h)	3.0 (1.0-8.0)	16.0 (2.0-24.0)
AUC_{0-24} (µg.h/mL)	41.988 ± 5.094	54.635 ± 4.143
AUC_{0-t} (µg.h/mL)	94.465 ± 14.530	137.820 ± 15.164
$AUC_{0-\infty}$ (µg.h/mL)	102.102 ± 14.207	149.164 ± 17.803
k_{el} (1/h)	0.0330 ± 0.004	0.0355 ± 0.005
$t_{1/2el}$ (h)	22.8 ± 3.0	21.3 ± 2.5
MRT (h)	38.9 ± 4.8	38.3 ± 3.5

AUC, area under the concentration-time curve; AUC_{0-24}, AUC from time zero to 24 h; AUC_{0-t}, AUC from time zero to the last measurable concentration; $AUC_{0-\infty}$, AUC from time zero to infinite; C_{max}, peak concentration; k_{el}, apparent elimination rate constant; MRT, mean residence time; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach C_{max}. *$p < 0.05$, significantly different from the control (vehicle) group.

IV.3.3. Effects of repeated-dose administration of *G. cambogia* extract on body weight

The effects of *G. cambogia* extract on the body weight of rats treated during 14 consecutive days are presented in **Figure IV.3**. The rats of both control and experimental groups had a similar body weight at the beginning of the study (day 1). From the analysis of the results, it was observed a statistically significant increase in the body weight of the rats between day 1 and day 14 in both experimental (*G. cambogia*) and control (vehicle) groups ($p < 0.005$); however, there were no statistically significant differences in the body weight gain between both groups.

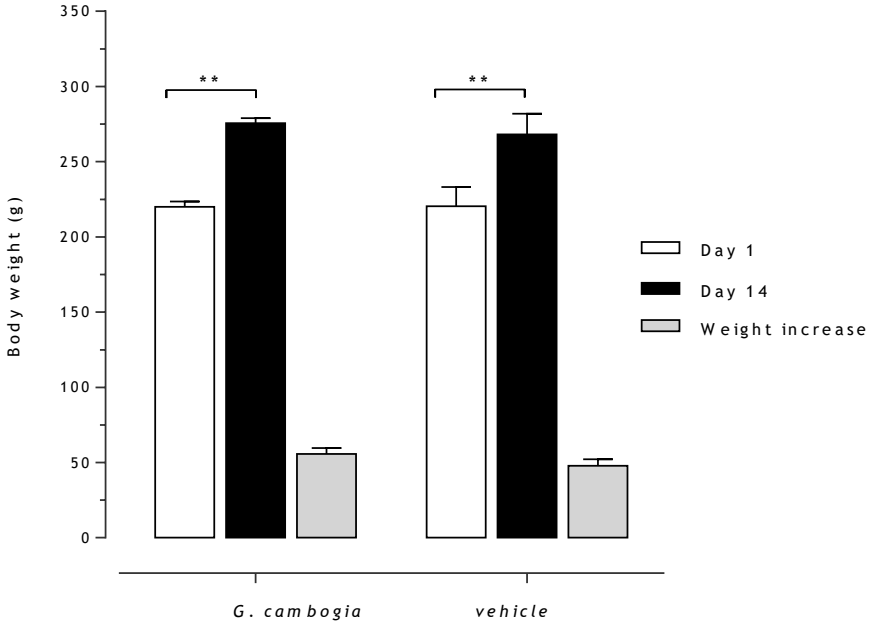


Figure IV.3. Effects of *Garcinia cambogia* extract on the body weight of rats after a 14-day treatment period with *Garcinia cambogia* extract (821 mg/kg/day, p.o.) or vehicle (water) by oral gavage. Data are presented as the mean values ± standard error of the mean (SEM) of six determinations (n = 6). **p < 0.005, day 1 versus day 14.

IV.4. Discussion

Safety assessment of herbal supplements is of utmost importance particularly when such supplements are administrated with conventional drugs. In fact, some authors argue that herb-drug interactions are theoretically more prone to occur than drug-drug interactions due to its more complex phytochemical composition (Izzo et al. 2016). However, the lack of rigorous scientific information available regarding the clinical significance of herb-drug interactions entails difficulties for health professionals and consumers in making rational decisions about the safety of the combination of herbal medicinal supplements and drugs (Zhang et al. 2017). In the particular case of *G. cambogia* supplements, data on their safety have been controversial. On the one hand, there are several case reports in literature describing episodes of severe toxicity associated with the consumption of *G. cambogia* supplements. For instance, Lopez *et al.* (2014) reported a case of suspected serotonin toxicity in a patient under stable therapeutic dosing of escitalopram (a serotonin reuptake inhibitor) after the addition of a nutritional supplement containing *G. cambogia* (Lopez et al. 2014). In addition, several case-reports of (hypo)mania and/or psychosis following the administration of *G. cambogia*-containing products have been published (Cotovio and Oliveira-Maia 2016; Nguyen et al. 2017). On the other hand, Chuah *et al.* (2012) reviewed the results of seventeen clinical studies in which the safety of HCA and related supplements for human consumption was demonstrated, inclusively no adverse effects were observed at levels superior to 2800 mg/day of HCA.

LTG interactions with other AEDs or/and co-prescribed drugs have been documented (Johannessen and Landmark 2010; Patsalos 2013a; Patsalos 2013b; Zaccara and Perucca 2014). However, scarce information is available about LTG interactions with herbs. In particular, an herb-drug interaction between ginseng and LTG was reported, suggesting that the inhibition of UGT2B7 by ginseng constituents predisposed the patient to a drug hypersensitivity reaction (Myers et al. 2015b). Another herb-drug interaction involving LTG was recently identified by our research group, where the simultaneous co-administration of *Paullinia cupana* and LTG resulted in a significant decrease of C_{max} and AUC_{0-24} of LTG (Ventura et al. 2018).

Overall, the data obtained in the current work regarding the effects of *G. cambogia* extract on the pharmacokinetics of LTG did not raise major concerns related to the occurrence of important herb-drug interactions. Indeed, we have designed the co-administration study to investigate the potential effects of *G. cambogia* extract on the gastrointestinal absorption and consequently on the extent of systemic bioavailability of drug, and no statistically significant differences were observed. Despite this, the co-administration of *G. cambogia* and LTG showed a slight tendency for a decrease of the C_{max} values and for a delay in the t_{max}, which are not expected to compromise the efficacy of LTG, and so, the co-administration *G. cambogia* extract and LTG is unlikely to be clinically relevant. Additionally, no significant changes have been observed in the extent of systemic exposure (as assessed by AUCs) and in the elimination pharmacokinetic parameters. On the other hand, the results of the study of the repeated administration of *G. cambogia* extract for 14 days showed a higher impact on mean C_{max} values of LTG, which were lower in the experimental group comparatively to the control group. Additionally, although a decrease in the extent of systemic drug exposure had been observed following the repeated treatment with *G. cambogia* extract, no statistically significant differences were found between both groups. Also, no differences were observed in the elimination pharmacokinetic parameters. Considering that LTG undergoes hepatic elimination susceptible to enzyme modulation and knowing that induction mechanisms are time-dependent, the results observed in this specific study suggest that *G. cambogia* has no marked inducing effects on the LTG metabolism.

The 14-day treatment period with *G. cambogia* extract did not show a significant effect on the body weight of rats, which was somewhat unexpected given the uses claimed for *G. cambogia*-containing supplements. However, other non-clinical studies conducted in mice also found no significant effects on the body weight of animals after *G. cambogia* administration for four (Hayamizu et al. 2003) and sixteen weeks (Kim et al. 2013). On the contrary, in the study of Sripradha *et al.* (2015), *G. cambogia* administered at 400 mg/kg during ten weeks significantly decreased the body weight gain in male Wistar rats fed with high-fat diet. Similarly, some clinical studies have demonstrated that *G. cambogia* has significant effects on body weight management when administered for periods longer than two weeks (Chuah et al. 2013).

IV.5. Conclusion

Based on the findings achieved in this non-clinical work, in which no important changes were observed on the pharmacokinetics of LTG in Wistar rats after the co-administration or pre-treatment with *G. cambogia* extract, it can be concluded that no clinically relevant pharmacokinetic-based herb-drug interactions are expected following the administration of the herbal extract and LTG. Thus, taking together into account the results of this work and those recently published by Ventura *el al*. (2018), if there is a need to administer herbal supplements for weight loss in epilepsy patients under LTG therapy, it may be safer the use of herbal supplements containing *G. cambogia* than those containing *Paulinia cupana*. Nevertheless, in order to generate more robust and reliable clinical evidence, it would be useful to perform a clinical trial specifically designed to assess the safety of the administration of *G. cambogia* extract and LTG.

Chapter V.

Evaluation of the effects of *Citrus aurantium* (bitter orange) extract on lamotrigine pharmacokinetics: insights from *in vivo* studies in rats

V.1. Introduction

Citrus aurantium extracts are being consumed as dietary supplements for at least two decades for weight loss/weight management, sports performance, as well as for appetite control (Koncic and Tomczyk 2013; Shara et al. 2018; Stohs 2017). The fruits of *C. aurantium*, also known as bitter, sour or Seville orange, have been used for hundreds of years in traditional Chinese medicine to treat indigestion, diarrhea, dysentery and constipation, and in South American folk medicine to treat insomnia, anxiety and epilepsy (Shara et al. 2018; Stohs 2017). Although many *Citrus* species contain *p*-synephrine, it is the most important protoalkaloid in *C. aurantium* fruit extracts; in fact, *p*-synephrine comprises approximately 90% or more of the total content in protoalkaloids and so it is the phytochemical compound used for standardization of *C. aurantium* extracts (Bakhiya et al. 2017; Stohs 2017; Stohs and Badmaev 2016). Actually, extracts prepared from the fruit rinds of *C. aurantium* have a *p*-synephrine content of 6-10% (Bakhiya et al. 2017; Stohs 2017).

As *p*-synephrine exhibits some structural similarities to ephedrine, the safety of *C. aurantium* extract (*p*-synephrine) was frequently questioned, assuming that *p*-synephrine had cardiovascular and stimulant effects similar to ephedrine and other structurally related biogenic amines as epinephrine and norepinephrine (Shara et al. 2018; Stohs 2017). However, contrary to what would be expected, a well-designed clinical trial recently conducted by Shara *et al*. (2018) concluded that the daily oral consumption of *C. aurantium* extract containing 49 mg *p*-synephrine for a 15-day period was not associated with significant cardiovascular (stimulant) and hemodynamic effects; hence, *C. aurantium* extract and *p*-synephrine seem to be safe at the dose tested. Therefore, small structural modifications in the chemical structure of these compounds change their stereochemistry, pharmacokinetic properties and adrenergic receptor binding characteristics (Shara et al. 2018; Stohs 2017). Indeed, *p*-synephrine binds several orders of magnitude more poorly to α-, β_1- and β_2-adrenergic receptors than other adrenergic agonists as epinephrine, norepinephrine and ephedrine. In turn, *p*-synephrine selectively activates β_3-adrenergic receptors, thus promoting thermogenesis and lipolysis without unwanted cardiovascular effects. Thus, the claimed medicinal effect of *p*-synephrine as a weight-loss stimulant is attributed, at least in part, to activation of β_3-adrenergic receptors (Bakhiya et al. 2017; Stohs and Badmaev 2016).

Among other phytochemical compounds present in *Citrus* species, flavonoids play an important role in the regulation of carbohydrate and lipid metabolism and in the prevention of hepatic steatosis, dyslipidemia and insulin sensitivity by the inhibition of hepatic fatty acid synthesis, thus increasing fatty acid oxidation (Stohs and Badmaev 2016). So, herbal extracts containing *p*-synephrine in combination with flavonoids, particularly naringin and hesperetin, can potentiate the non-stimulant thermogenic effect of *p*-synephrine (Ríos-Hoyo and Gutiérrez-Salmeán 2016; Stohs and Badmaev 2016). Obesity and overweight are major public health concerns and their prevalence is increasing worldwide. At the same time, obesity and

overweight have been recognized as important conditioning factors in treatment and prognosis of several chronic disorders, including epilepsy (Arya et al. 2016; Ladino et al. 2014). Indeed, epilepsy patients are at increased risk of being overweight or obese because some antiepileptic drugs (AEDs) can change weight homeostasis-regulating processes and metabolic pathways (Hamed 2015). It has been also reported a higher prevalence of obesity in patients treated with more than one AED and also in those who have refractory epilepsy (Baxendale et al. 2015; Chukwu et al. 2014; Janousek et al. 2013). Thus, according to this body of evidence and due to the high probability of epilepsy patients to consume weight-loss herbal supplements, there is a great interest in assessing the potential of herb-drug interactions in order to ensure the safety use of these supplements in patients under AED therapy.

Recognizing that lamotrigine (LTG) is the most widely used second-generation AED to treat both focal and generalized epileptic seizures and other disorders as bipolar syndromes, schizophrenia and neuropathic pain (Nevitt et al. 2017; Patsalos 2013a), and knowing that LTG is a narrow therapeutic index drug that presents high inter-individual variability in its pharmacokinetics, the primary aim of the current study was to evaluate the effects of *C. aurantium* extract on LTG pharmacokinetics in rats.

V.2. Materials and methods

V.2.1. *C. aurantium* extract, drugs and materials

The hidroalcoholic extract of *C. aurantium* fruit, containing 10% of *p*-synephrine, was purchased from Bio Serae Laboratories (Bram, France) and the certificate of analysis of the extract was preserved. LTG dispersible tablets (Lamictal® 25 mg, GSK), pentobarbital (Eutasil®, 200 mg/ml, Ceva Saúde Animal), sodium chloride 0.9% solution (Labesfal, Portugal) and sodium heparin 5000 I.U./mL (B. Braun Medical, Portugal) were acquired in referenced suppliers. Polyurethane cannula (Introcan® Certo IV indwelling cannula 22G; 0.9x2.5 mm; B. Braun Melsungen AG, Germany), disposable cholesterol and triglycerides test strips (Accutrend®, Roche, Germany) and disposable blood glucose test strips (Freestyle Lite, Abbott®) were also commercially acquired in referenced laboratories.

V.2.2. *C. aurantium* extract and lamotrigine solutions

The aqueous solution of *C. aurantium* extract was daily prepared by dissolving an appropriate amount of the herbal extract in distilled water. The dose of 164 mg/kg (p.o.) of *C. aurantium* extract was administered to each animal in a volume of 10 mL/kg body weight. This dose was defined based on the recommended human dose, which was converted from man to rat species following a specific Guidance for Industry of the Food and Drug Administration (FDA); this FDA guidance allows the conversion of animal doses to human equivalent doses based on

body surface area (FDA 2005). Additionally, a 10-fold potentiation factor of interaction was applied to avoid false negative results.

Regarding LTG, a dose of 10 mg/kg (p.o., 4 mL/kg body weight) was selected for these pharmacokinetic studies because no toxic effects were observed in similar non-clinical studies (Ventura et al. 2018; Ventura et al. 2016). For that, dispersible tablets of LTG were dissolved in a proper volume of distilled water to obtain the required drug solution for administration to animals.

V.2.3. Animal experiments

The experimental procedures to which the animals were subjected were previously approved by the Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção Geral de Alimentação e Veterinária) and were conducted in accordance with the European Directive (2010/63/EU) for animal experiments.

Twenty-four adult male Wistar rats were obtained from local certified animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal) and were housed at 12 h light/dark cycle under controlled environmental conditions (temperature 20 ± 2 °C; relative humidity 55 ± 5%). The animals were allowed free access to a standard rodent diet and water *ad libitum*.

V.2.4. Pharmacokinetic studies

The pharmacokinetic studies were designed to investigate the effects of *C. aurantium* on LTG pharmacokinetics. So, two independent studies were planned to evaluate the impact of the herbal extract either in the systemic absorption and/or elimination of LTG. Twelve rats were used in each study, which were balanced and randomly allocated to control and experimental groups. In the first pharmacokinetic study a single-dose of *C. aurantium* extract (164 mg/kg, p.o.) followed by a single-dose of LTG (10 mg/kg, p.o.) were administered by oral gavage to the rats of the experimental group (n = 6) (co-administration study). Rats of the control group (n = 6) received the corresponding volume of the vehicle of the herbal extract (water) and were equally treated with LTG. In the second pharmacokinetic study, a single-dose of *C. aurantium* extract (164 mg/kg, p.o.) was daily administered by oral gavage to the rats of the experimental group (n = 6) for 14 consecutive days, whereas the rats of the control group (n = 6) received water as vehicle for the same period of time (i.e. 14 days) (pre-treatment study). Then, on the 15th day, a single-dose of LTG (10 mg/kg, p.o.) was administered to all animals of experimental and control groups.

Briefly, each animal of both experimental and control groups was anesthetized on the night before LTG administration for insertion of a polyurethane cannula in a lateral tail vein (Introcan® Certo IV indwelling cannula 22G, 0.9 x 2.5 mm) for subsequent blood collection. Anesthesia was performed by intraperitoneal injection using pentobarbital (60 mg/kg, i.p.).

Rats completely recovered from anesthesia and were fasted overnight, with free access to water, before LTG administration. To avoid the effect of food on LTG absorption and disposition, the fasting period was maintained for 4 h after LTG administration.

C. aurantium extract (or vehicle, in the control groups) and LTG were orally administrated by gavage in the morning period. The blood sampling was performed at several pre-defined time-points after LTG administration: 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h. Blood samples of approximately 0.3 mL were collected into EDTA tubes and then centrifuged at 4000 rpm for 10 min (4 ºC) to separate the plasma which was stored at −20 ºC until analysis.

V.2.5. Lamotrigine analysis

LTG concentrations in plasma samples were determined using a previously developed and validated method that involves microextraction by packed sorbent (MEPS) coupled to high-performance liquid chromatography–diode array detection (HPLC/DAD) (Ventura et al. 2016).

V.2.6. Pharmacokinetic analysis

The peak plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were directly obtained from the experimental data.

The individual plasma concentration-time profiles were submitted to a non-compartmental pharmacokinetic analysis using WinNonlin version 5.2 (Pharsight Co, Mountain View, CA, USA) to estimate a set of relevant pharmacokinetic parameters, including the truncated area under the concentration-time curve (AUC) from time zero to 24 h (AUC_{0-24}); the AUC from time zero to last measurable concentration (AUC_{0-t}), which was calculated by the linear trapezoidal rule; the AUC from time zero to infinite ($AUC_{0-\infty}$), which was determined from $AUC_{0-t} + (C_{last}/k_{el})$, where C_{last} is the quantifiable concentration at the time of the last measurable drug concentration (t_{last}) and k_{el} is the apparent elimination rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile; the apparent terminal elimination half-life ($t_{1/2el}$); and the mean residence time (MRT). The drug concentrations below the lower limit of quantification of the assay were taken as zero for all calculations.

V.2.7. Evaluation of repeated-dose administration of *C. aurantium* extract on biochemical parameters

To evaluate the impact of the repeated treatment with *C. aurantium* extract on the blood levels of glucose, total cholesterol and triglycerides, these biochemical parameters were measured in all rats of the experimental (*C. aurantium*) and control (vehicle) groups on the 14th day of the pre-treatment study. The determination of these biochemical parameters was

carried out using appropriate medical devices for glucose (Freestyle Freedom Lite, Abbott®) and for cholesterol and triglycerides (Accutrend® Plus, Roche).

V.2.8. Evaluation of repeated-dose administration of *C. aurantium* extract on body weight

The effects of *C. aurantium* extract on rats' body weight were evaluated by comparing the body weight of the animals of the experimental (*C. aurantium*) and control (vehicle) groups between the first and the 14th day of the pre-treatment study.

V.2.9. Statistical analysis

The results obtained were presented as the mean ± standard error of the mean (SEM), except for t_{max} whose values were expressed as median and range since t_{max} is a categorical variable in the performed pharmacokinetic studies. Non-parametric Mann-Whitney test was used to compare the t_{max} values of two different groups. Statistical analyses and comparisons of the other pharmacokinetic parameters, biochemical markers and body weight between the experimental (*C. aurantium*) and control (vehicle) groups were performed using unpaired two-tailed Student's *t*-test. To compare the body weight changes within the same group a paired Student's *t*-test was employed. A difference was considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

V.3. Results

V.3.1. Effects of *C. aurantium* extract on LTG pharmacokinetics after co-administration

The mean plasma concentration-time profiles of LTG obtained in rats after the concurrent administration of a single-oral dose of *C. aurantium* extract (164 mg/kg, p.o.) or vehicle and LTG (10 mg/kg, p.o.) are depicted in **Figure V.1**. From the direct analysis of **Figure V.1**, the plasma pharmacokinetic profiles of LTG were found to be very similar in both experimental (*C. aurantium*) and control (vehicle) groups. This observation is corroborated by the values obtained for the main pharmacokinetic parameters, which are summarized in **Table V.1**; indeed, it is evident the lack of statistically significant differences for all pharmacokinetic parameters between the experimental (*C. aurantium*) and control (vehicle) groups. These data support the absence of important pharmacokinetic-based herb-drug interactions between the *C. aurantium* extract and LTG in the experimental conditions tested.

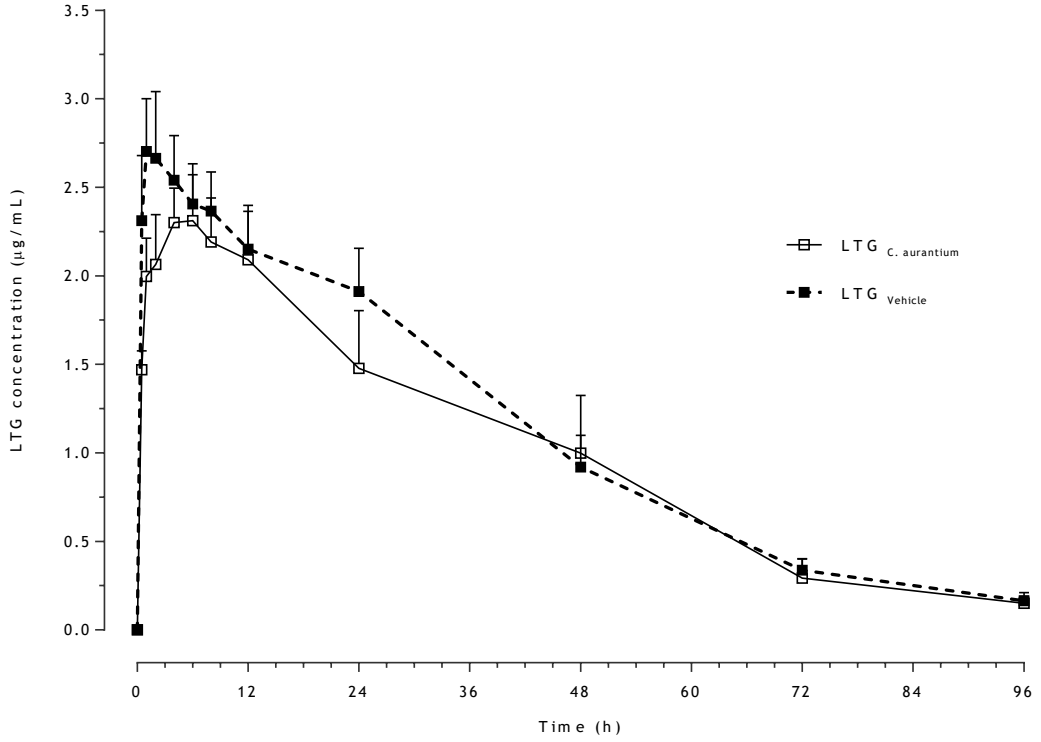


Figure V.1. Mean plasma concentration-time profiles of lamotrigine (LTG) obtained, over a period of 96 h, from rats co-administered with a single-dose of *Citrus aurantium* extract (164 mg/kg, p.o.) or vehicle of the extract (water) and LTG (10 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (*n* = 6).

Table V.1. Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of lamotrigine (LTG) obtained in rats after the co-administration with a single-dose of *Citrus aurantium* extract (164 mg/kg, p.o.) or vehicle of the extract (water) and LTG (10 mg/kg, p.o.) by oral gavage (*n* = 6). Data are presented as mean values ± standard error of the mean (SEM), except for t_{max} that is expressed as median values (range).

Parameter	Experimental Group *LTG C. aurantium*	Control Group *LTG Vehicle*
C_{max} (µg/mL)	2.734 ± 0.248	2.995 ± 0.285
t_{max} (h)	7.0 (2.0-12.0)	3.0 (0.5-12.0)
AUC_{0-24} (µg.h/mL)	46.720 ± 4.443	52.842 ± 5.035
AUC_{0-t} (µg.h/mL)	96.858 ± 14.101	107.632 ± 11.443
$AUC_{0-\infty}$ (µg.h/mL)	101.772 ± 14.596	113.315 ± 11.673
k_{el} (1/h)	0.0391 ± 0.004	0.0374 ± 0.003
$t_{1/2el}$ (h)	18.6 ± 1.8	19.2 ± 1.8
MRT (h)	31.5 ± 2.6	31.8 ± 2.6

AUC, area under the concentration-time curve; AUC_{0-24}, AUC from time zero to 24 hours; AUC_{0-t}, AUC from time zero to the last measurable concentration; $AUC_{0-\infty}$, AUC from time zero to infinite; C_{max}, peak concentration; k_{el}, apparent elimination rate constant; MRT, mean residence time; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach C_{max}.

V.3.2. Effects of repeated-dose pretreatment with *C. aurantium* extract on LTG pharmacokinetics

The mean plasma concentration-time profiles of LTG obtained from the rats that were treated with a single-dose of LTG (10 mg/kg, p.o.) on the 15th day, and previously treated within a 14-day period with a single daily dose of *C. aurantium* extract (164 mg/kg, p.o.) or vehicle are shown in **Figure V.2**.

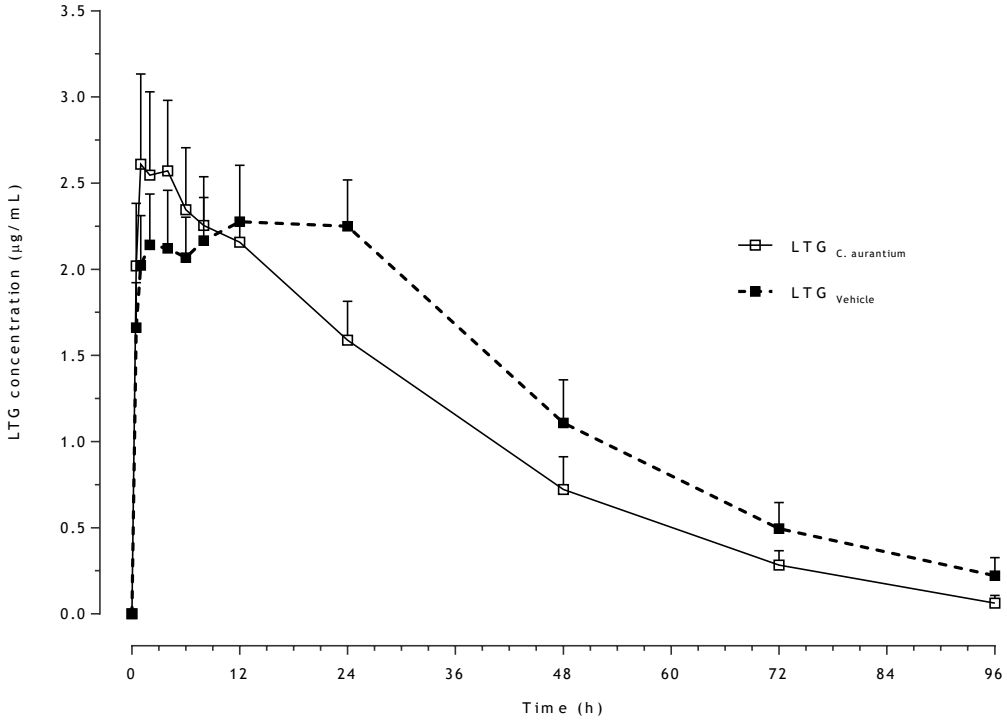


Figure V.2. Mean plasma concentration-time profiles of lamotrigine (LTG) obtained, over a period of 96 h, from rats submitted to a 14-day pre-treatment period with *Citrus aurantium* extract (164 mg/kg, p.o.) or vehicle of the extract (water) and treated on the 15th day with a single-dose of LTG (10 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (n = 6).

After the respective pharmacokinetic analysis, the parameters obtained were summarized in **Table V.2**. Upon observation of the plasma pharmacokinetic profiles, it is clear that substantially lower mean concentrations of LTG were obtained from the 24-hour post-dose in the group of rats pre-treated with *C. aurantium* (experimental group); nevertheless, the differences observed over time between the experimental (*C. aurantium*) and control (vehicle) groups were not found to be statistically significant at any specific time point ($p > 0.05$) (**Figure V.2**). Even so, these data show a trend to a reduction in the extent of systemic exposure to LTG in the experimental group; in fact, in the experimental (*C. aurantium*) group, the mean values estimated for the AUC_{0-t} and $AUC_{0-\infty}$ parameters were reduced by 22.0% and 25.2%, respectively.

Table V.2. Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of lamotrigine (LTG) obtained in rats submitted to a 14-day pre-treatment period with *Citrus aurantium* extract (164 mg/kg/day, p.o.) or vehicle of the extract (water) and treated on the 15th day with a single-dose of LTG (10 mg/kg, p.o.) by oral gavage (n = 6). Data are presented as mean values ± standard error of the mean (SEM), except for t_{max} that is expressed as median values (range).

Parameter	Experimental Group *LTG C. aurantium*	Control Group *LTG Vehicle*
C_{max} (µg/mL)	2.994 ± 0.418	2.872 ± 0.197
t_{max} (h)	3.0 (0.5-12.0)*****	12.0 (4.0-24.0)
AUC_{0-24} (µg.h/mL)	50.167 ± 5.324	52.150 ± 5.071
AUC_{0-t} (µg.h/mL)	92.845 ± 13.673	119.075 ± 13.085
$AUC_{0-\infty}$ (µg.h/mL)	97.027 ± 14.324	129.667 ± 15.475
k_{el} (1/h)	0.0447 ± 0.003	0.0376 ± 0.006
$t_{1/2el}$ (h)	15.9 ± 1.1	20.4 ± 2.7
MRT (h)	26.9 ± 2.3	35.5 ± 5.0

AUC, area under the concentration-time curve; AUC_{0-24}, AUC from time zero to 24 h; AUC_{0-t}, AUC from time zero to the last measurable concentration; $AUC_{0-\infty}$, AUC from time zero to infinite; C_{max}, peak concentration; k_{el}, apparent elimination rate constant; MRT, mean residence time; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach C_{max}. *****p < 0.05, significantly different from the control (vehicle) group.

Regarding the C_{max} values, there is a great similarity between the experimental and control groups (2.994 ± 0.418 µg/mL *versus* 2.872 ± 0.197 µg/mL). However, statistically significant differences were found for t_{max} (p = 0.0455), with a median value of 3 h in the experimental group compared with 12 h in the control group. Additionally, the values estimated for the pharmacokinetic parameters k_{el}, $t_{1/2el}$ and MRT indicate a slight tendency towards an increased elimination rate of LTG in the rats pre-treated with *C. aurantium* extract.

V.3.3. Evaluation of repeated-dose administration of *C. aurantium* extract on biochemical parameters

The effects of the repeated-dose administration of *C. aurantium* extract over a period of 14 days on the blood levels of glucose, total cholesterol and triglycerides are presented in **Figure V.3**. Considering together the three biochemical parameters evaluated, it is evident that very similar values were found between the experimental and control groups and thus no statistically significant differences were detected (p > 0.05).

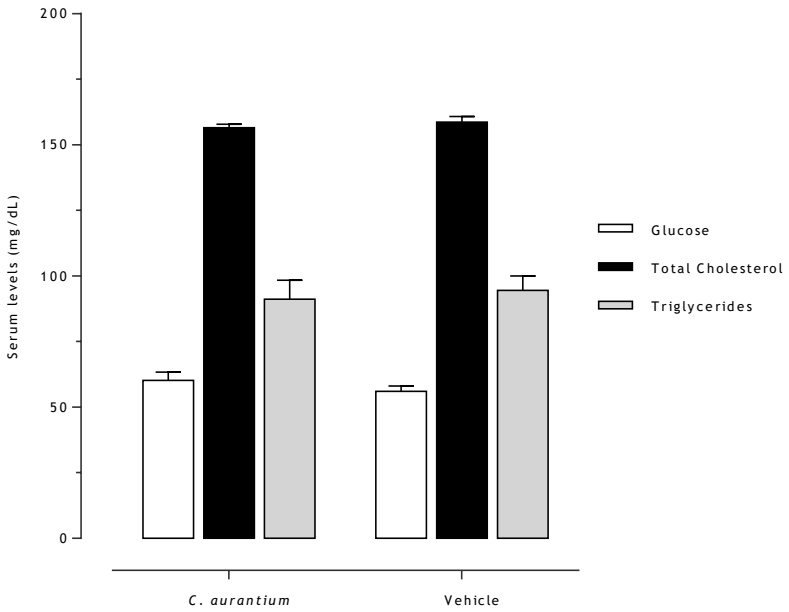


Figure V.3. Effects of *Citrus aurantium* extract on biochemical parameters (blood glucose, total cholesterol and triglycerides) of rats after a 14-day treatment period with *Citrus aurantium* extract (164 mg/kg, p.o.) or vehicle (water) by oral gavage. Data are presented as the mean values ± standard error of the mean (SEM) of six determinations (n = 6).

V.3.4. Evaluation of repeated-dose administration of *C. aurantium* extract on body weight

The effects of *C. aurantium* extract on the body weight of rats daily treated for a 14-day period are shown in **Figure V.4**. By analyzing the **Figure V.4**, a statistically significant increase in rats' body weight was observed between day 1 and day 14 in both experimental (*C. aurantium*) and control (vehicle) groups ($p < 0.005$). However, there were no statistically significant differences in the body weight gains between both groups; the weight increased on average 51.0 ± 3.7 g in the experimental group and 46.0 ± 3.5 g in control group.

V.4. Discussion

Over the years, the need for a rigorous risk-benefit evaluation of herbal medicinal products or dietary supplements has been neglected, probably due to the natural origin of its constituents. Nevertheless, there is a growing consensus among the scientific community that the safety assessment of these products is essential to protect their users from health hazards. Therefore, it is of the utmost importance the conduction of well-designed non-clinical and clinical studies to investigate the potential safety risks of the herbal products themselves, as well as the risk of interacting with conventional drugs, particularly those with a narrow therapeutic index (Agbabiaka et al. 2017; Awortwe et al. 2018; Hermann and von Richter 2012; Rahman et al. 2017; Werba et al. 2018; Zhang et al. 2017).

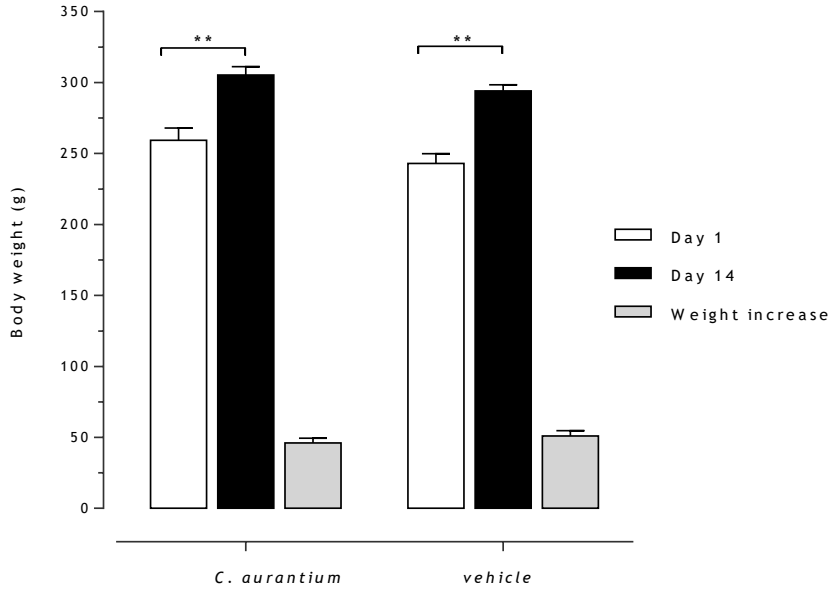


Figure V.4. Effects of *Citrus aurantium* extract on the body weight of rats after a 14-day treatment period with *Citrus aurantium* extract (164 mg/kg, p.o.) or vehicle (water) by oral gavage. Data are presented as the mean values ± standard error of the mean (SEM) of six determinations (n = 6). **p < 0.005, day 1 *versus* day 14.

With regard to safety concerns intrinsic to the *C. aurantium* extract and *p*-synephrine itself, the available clinical data, although somewhat contradictory, have concluded globally that this herbal extract and *p*-synephrine are safe for use in dietary supplements and foods at the commonly used doses (Stohs 2017). Indeed, very recent data obtained from a placebo-controlled, crossover, double-blinded clinical study indicate that the daily oral consumption of *C. aurantium* extract with 49 mg *p*-synephrine is devoid of significant cardiovascular (stimulant) and hemodynamic effects (Shara et al. 2018; Stohs 2017). Moreover, some studies have focused the wide safety margin of *p*-synephrine, with a LD_{50} greater than 2500 mg/kg in rats when administered by oral route (Deshmukh et al. 2017).

On the other hand, several interactions have been documented involving various *C. aurantium* products (e.g. fruit extracts or juices) as perpetrators of herb-drug interactions. For instance, Rodrigues *et al*. (2013b) found that *C. aurantium* fruit extract significantly increased the peak plasma concentration of amiodarone in rats that received the extract during 14 consecutive days. Wason *et al*. (2012) also found that bitter orange juice reduced the C_{max} (24%), AUC (20%) and delayed the t_{max} of colchicine.

Also a set of interactions involving LTG and other drugs, including AEDs, are quite well documented. In addition, an herb-drug interaction was reported between *Panax ginseng* and LTG, probably due to the inhibition of UGT2B7 by ginseng constituents (Myers et al. 2015b). Recently, we have also reported an herb-drug interaction between *Paullinia cupana* extract and LTG in rats (Ventura et al. 2018).

Hence, the pharmacokinetic studies carried out in the present work aimed to evaluate the impact of the *C. aurantium* extract on LTG pharmacokinetics using a whole rat model. Although rodents have some distinct metabolic pathways from humans, they are good animal models in this context because the effective plasma levels of AEDs in rodents and in humans are usually similar (Loscher 2007).

Overall, considering the pharmacokinetic data obtained in the current work, no major concern was identified regarding the effects of *C. aurantium* extract on the pharmacokinetics of LTG; therefore, no clinically relevant risks are anticipated related to the occurrence of interactions between *C. aurantium* and LTG. Bearing in mind the findings obtained in the co-administration study, which was planned to investigate the potential impact of *C. aurantium* extract on the gastrointestinal absorption and consequently on the extent of systemic bioavailability of LTG, it was evident the lack of important interactions at the gastrointestinal level. Indeed, the pharmacokinetic profiles of LTG were essentially overlapping among the experimental and control groups, and the mean values found for the corresponding pharmacokinetic parameters were very similar. In addition, taking also into account the results achieved from the pre-treatment study, which involved the repeated daily administration of *C. aurantium* extract (or vehicle) for 14 days and the administration of a single-dose of LTG on the 15th day, only the earlier achievement of the peak drug concentrations (p = 0.0455) and a trend towards a lower extent of systemic exposure to LTG in the rats treated with *C. aurantium* extract deserve to be highlighted. Thus, knowing that LTG undergoes hepatic elimination susceptible to enzyme modulation and being induction mechanisms time-dependent, the results achieved in this specific study indicate that *C. aurantium* has no marked inducer effects on the metabolic pathways of LTG.

The effects of the 14-day pre-treatment period with *C. aurantium* extract resulted in minor changes in the biochemical parameters evaluated (blood glucose, total cholesterol and triglycerides) and in the body weight of rats.

To a certain extent, these results would not be expected because synephrine alkaloids have been related to a reduction in food intake in rodents (Astell 2013). However, other authors reported identical effects of *C. aurantium* extract on the body weight (Arbo et al. 2008; Rodrigues et al. 2013b).

V.5. Conclusion

As far as we know, up to date, this is the first report about the effects of *C. aurantium* extract, an herbal component often incorporated in weight-loss dietary supplements, on the absorption and biodisposition of LTG. Based on the data obtained in these non-clinical pharmacokinetic studies, no clinically relevant herb-drug interaction is expected to occur between *C. aurantium* extract and LTG. Hence, from the pharmacokinetic point of view, *C. aurantium* extract can be considered as a safer option than other herbal extracts, such as *Paullinia cupana* , to be incorporated in new weight-loss herbal supplements. Even so, to obtain

more reliable clinical evidence, it would be useful to carry out a study in humans specifically planned to evaluate the safety of the joint administration of *C. aurantium* extract and LTG.

Chapter VI.

Safety evidence on the administration of *Fucus vesiculosus* L. (bladderwrack) extract and lamotrigine: data from pharmacokinetic studies in the rat

VI.1. Introduction

Fucus vesiculosus L., also known as bladderwrack, are small edible brown seaweed widely found in Atlantic (including Azores and Canary Islands) and Pacific shores, in Greenland, northern Russia and in the Baltic Sea (Pozharitskaya et al. 2018). These seaweeds have been pointed out as promising functional foods due to its richness in iodine and in several bioactive phytochemicals such as laminarin, alginate and fucoidan (as polysaccharides), phlorotannins, fucols and fucophlorethols (as polyphenols), fucosterol and ß-sitosterol (as sterols), pigments, vitamins and high content of minerals (Chater et al. 2016). Particularly, polysaccharides from seaweeds can act as prebiotics since they are not completely digested by human's digestive system (Gabbia et al. 2017). Besides its nutritional benefits, seaweed extracts are considered a good source of digestive enzyme inhibitors, justifying their use in the treatment of overweight and obesity. Alginate has been related to the *in vitro* inhibition of pepsin and pancreatic lipase, a proteolytic enzyme and a lipolytic enzyme respectively (Chater et al. 2016; Wan-Loy and Siew-Moi 2016). Additionally, phlorotannins are known as glucosidase inhibitors (Catarino et al. 2017; Gabbia et al. 2017), and fucoxanthin has been shown to inhibit pancreatic lipase activity in the gastrointestinal lumen of rats (Chater et al. 2016).

The traditional medicinal use of *F. vesiculosus* is well-established as adjuvant in the reduction of calorie intake and improvement of weight loss in overweight adults (EMA 2014b). *F. vesiculosus* supplements have been also used to treat goiter, rheumatoid arthritis, asthma, psoriasis and healing wounds. Indeed, thyroid hormones regulate energy expenditure and appetite, and the iodine content present in *F. vesiculosus* extracts can stimulate the thyroid gland, which plays an important role in metabolism. In particular, the triiodothyronine (T3) hormone controls metabolic and energy homeostasis and can influence body weight, thermogenesis, and lipid metabolism (Witkowska-Sędek et al. 2017).

Chronic epilepsy and long-term use of antiepileptic drugs (AEDs) may be associated with several adverse metabolic and endocrine effects that can lead to an increase in body weight (Adhimoolam and Arulmozhi 2016). Indeed, obesity is a well-known comorbid condition in epilepsy and there is an increasing prevalence of obesity in patients treated with more than one AED and in those with refractory epilepsy (Baxendale et al. 2015; Chukwu et al. 2014; Janousek et al. 2013). Lamotrigine (LTG) is a phenyltriazine AED used as first-line or adjunctive therapy for several epileptic syndromes characterized by focal or generalized seizures, as well as for bipolar syndromes, schizophrenia, and neuropathic pain (Nevitt et al. 2017; Patsalos 2013b). Nevertheless, over the last years, a set of drug-drug interactions involving LTG as object drug and as perpetrator agents some AEDs, other co-prescribed drugs or herbal substances have been identified (Johannessen and Landmark 2010; Myers et al. 2015b; Patsalos 2013a; Patsalos 2013b; Ventura et al. 2018; Zaccara and Perucca 2014). So, considering that some herbal components have potential to interact with LTG, the primary aim of this research work was to

investigate, at a non-clinical level, the potential occurrence of important interactions between *F. vesiculosus* extract and LTG.

VI.2. Materials and Methods

VI.2.1. Herbal extract and drugs

Bladderwrack 0.10% dry aqueous extract, from thallus of *F. vesiculosus L.*, was purchased from EPO Istituto Farmochimico Fitoterapico s.r.l. (Milano, Italy). LTG dispersible tablets (Lamictal® 25 mg, GSK), pentobarbital (Eutasil®, 200 mg/ml, Ceva Saúde Animal), sodium chloride 0.9% solution (Labesfal, Portugal) and sodium heparin 5000 I.U./mL (B. Braun Medical, Portugal) were commercially acquired.

VI.2.2. Herbal extract and lamotrigine solutions

The aqueous solution of *F. vesiculosus* extract was daily prepared by dissolution of the extract in distilled water to obtain a volume of solution suitable to administer to the animals. The dose of *F. vesiculosus* extract to administer to animals was defined on the basis of the recommended dose for humans (EMA 2014b) and applying the Food and Drug Administration (FDA) Guidance for Industry that allows the conversion of animal doses to human equivalent doses based on body surface area (FDA 2005). To avoid false negative results, a 10-fold potentiation factor of interaction was established, being the final dose of extract to administer of 575 mg/kg. Data from other animal studies indicated that the daily dose of 750 mg/kg body weight for 4 weeks did not show relevant signs of toxicity (Zaragozá et al. 2008).

LTG solution was extemporaneously prepared on the day of administration by dissolving dispersible tablets of LTG in a proper volume of distilled water. The size of the LTG dose selected for these studies (10 mg/kg (p.o.) took into account previous experiments made by the in-house group in the rat (Ventura et al. 2018; Ventura et al. 2016).

VI.2.3. Animals

Twenty-four healthy adult male Wistar rats (212 ± 5 g) were obtained from local certified animal facilities (Faculty of Health Sciences of the University of Beira Interior, Covilhã, Portugal). The animals were housed at 12 h light/dark cycle under controlled environmental conditions (temperature 20 ± 2 °C; relative humidity 55 ± 5%), and they were allowed free access to a standard rodent diet and water *ad libitum*.

The experimental procedures were approved by the Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção Geral de Alimentação e

Veterinária). All animal experiments were conducted in accordance with the European Directive (2010/63/EU).

VI.2.4. Pharmacokinetic studies

Two pharmacokinetic studies were planned to assess the effects of *F. vesiculosus* extract on LTG pharmacokinetics, in an attempt to discriminate the impact of the extract on the systemic absorption (co-administration study) and/or elimination (pre-treatment study) of the drug. For this purpose, twelve rats were used in each study, which were randomly allocated to the control and experimental groups.

In the first pharmacokinetic study, rats of the experimental group were concomitantly treated by oral gavage with a single oral dose of *F. vesiculosus* extract (575 mg/kg, p.o.) and LTG (10 mg/kg, p.o.). Rats of the control group received the corresponding volume of the vehicle of the herbal extract (water) and were equally treated with LTG.

In the second study, rats of the experimental group were orally pre-treated during 14 days with *F. vesiculosus extract* (575 mg/kg/day, p.o.), and a single-dose of LTG (10 mg/kg, p.o.) was administrated to each animal on the 15th day. Similarly, rats allocated to control group received water as vehicle during the 14 days, and then a single-dose of LTG (10 mg/kg, p.o.) on the 15th day.

The experimental protocol followed the same design of previous experiments made with rats in order to investigate the potential for herb-drug interactions (Ventura et al. 2018). Each animal of the experimental or control groups was anesthetized, on the night before LTG administration, for insertion of a polyurethane cannula in a lateral tail vein (Introcan® Certo IV indwelling cannula 22G, 0.9 x 2.5 mm) for subsequent blood collection. Anaesthesia was performed by intraperitoneal injection of pentobarbital (60 mg/kg, i.p.). Rats fully recovered from anaesthesia and were fasted overnight, with free access to water, before LTG administration.

To avoid the effect of food on LTG absorption and disposition, the fasting period was still maintained for 4 h after LTG administration. Following the administration of LTG, blood samples were collected at several pre-defined time-points: 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72 and 96 h. Blood samples of approximately 0.3 mL were collected into EDTA tubes and then centrifuged at 4000 rpm for 10 min (4 ºC) to separate the plasma, which was stored at −20 ºC until analysis.

VI.2.5. Lamotrigine quantification

The quantification of LTG in plasma samples was achieved through a bioanalytical method previously validated, which involves microextraction by packed sorbent (MEPS) and high-performance liquid chromatography–diode array detection (HPLC-DAD) (Ventura et al. 2016).

VI.2.6. Pharmacokinetic analysis

The peak plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were directly obtained from the pharmacokinetic profiles. The individual plasma concentration-time curves were submitted to a non-compartmental analysis using WinNonlin version 5.2 (Pharsight Co, Mountain View, CA, USA) in order to estimate a set of relevant pharmacokinetic parameters, including the truncated area under the concentration-time curve (AUC) from time zero to 24 h (AUC_{0-24}); the AUC from time zero to last measurable concentration (AUC_{0-t}), which was calculated by the linear trapezoidal rule; the AUC from time zero to infinite ($AUC_{0-\infty}$), which was determined from AUC_{0-t} + (C_{last}/k_{el}), where C_{last} is the quantifiable concentration at the time of the last measurable drug concentration (t_{last}) and k_{el} is the apparent elimination rate constant calculated by log-linear regression of the terminal segment of the concentration-time profile; the apparent terminal elimination half-life ($t_{1/2el}$); and the mean residence time (MRT). The drug concentrations below the lower limit of quantification of the assay were taken as zero for all calculations.

VI.2.7. Evaluation of repeated-dose administration of *F. vesiculosus* extract on body weight

The effects of *F. vesiculosus* extract on rats' body weight were evaluated in the pre-treatment study by comparing the body weight of the animals between the first and the fourteenth day.

VI.2.8. Statistical analysis

All results were presented as the mean ± standard error of the mean (SEM), except for t_{max} whose values were expressed as median and range because it is a categorical variable in the performed pharmacokinetic studies. The non-parametric Mann-Whitney test was used to compare the t_{max} values from two different groups. The statistical analysis and comparison of the other pharmacokinetic parameters and the body weight between two groups was performed using unpaired two-tailed Student's *t*-test. The non-parametric Mann-Whitney test was used to compare t_{max} values achieved in the experimental and control groups. To compare body weight changes within the same group, either in experimental or control group, a paired Student's *t*-test was employed.

A difference was considered to be statistically significant for a *p*-value lower than 0.05 ($p < 0.05$).

VI.3. Results

VI.3.1. Effects of *F. vesiculosus* extract on LTG pharmacokinetics after co-administration

The mean plasma concentration-time curves of LTG obtained in rats (*n* = 6) after the co-administration of a single-dose of *F. vesiculosus* extract (575 mg/kg, p.o.) or vehicle and LTG (10 mg/kg, p.o.) are represented in **Figure VI.1**. In addition, **Table VI.1** summarizes the corresponding pharmacokinetic parameters obtained by non-compartmental analysis. As it can be observed from **Figure VI.1**, there is a great parallelism between the plasma concentration-time profiles achieved for LTG in the rats of both experimental (*F. vesiculosus*) and control groups, and an almost complete overlap during the first 6 h post-dose. After this time point (6 h) there was a slight trend towards higher LTG concentrations in the experimental group (*F. vesiculosus*), but no statistically significant differences were found (**Figure VI.1**).

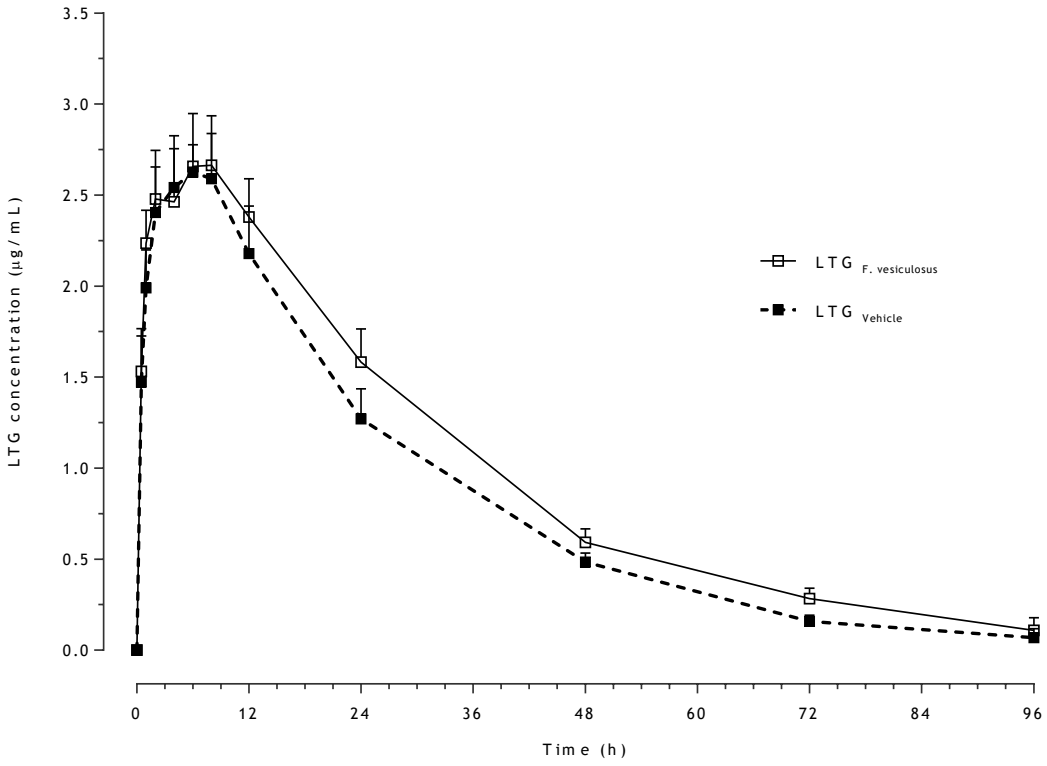


Figure VI.1. Mean plasma concentration-time profiles of lamotrigine (LTG) obtained, over a period of 96 h, from rats co-administered with a single-dose of *Fucus vesiculosus* extract (575 mg/kg, p.o.) or vehicle of the extract (water) and LTG (10 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (*n* = 6).

Table VI.1. Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of lamotrigine (LTG) obtained in rats after the co-administration with a single-dose of *Fucus vesiculosus* extract (575 mg/kg, p.o.) or vehicle of the extract (water) and LTG (10 mg/kg, p.o.) by oral gavage (n = 6). Data are presented as mean values ± standard error of the mean (SEM), except for t_{max} that is expressed as median values (range).

Parameter	Experimental Group LTG *F. vesiculosus*	Control Group LTG Vehicle
C_{max} (µg/mL)	2.974 ± 0.207	2.856 ± 0.233
t_{max} (h)	6.0 (2.0-12.0)	6.0 (4.0-8.0)
AUC_{0-24} (µg.h/mL)	52.908 ± 3.333	48.987 ± 4.667
AUC_{0-t} (µg.h/mL)	92.541 ± 6.253	79.414 ± 7.600
$AUC_{0-\infty}$ (µg.h/mL)	102.085 ± 8.104	83.838 ± 8.429
k_{el} (1/h)	0.0355 ± 0.0055	0.0418 ± 0.0043
$t_{1/2el}$ (h)	23.8 ± 5.6	17.7 ± 2.2
MRT (h)	32.9 ± 5.8	25.9 ± 3.0

AUC, area under the concentration-time curve; AUC_{0-24}, AUC from time zero to 24 h; AUC_{0-t}, AUC from time zero to the last measurable concentration; $AUC_{0-\infty}$, AUC from time zero to infinite; C_{max}, peak concentration; k_{el}, apparent elimination rate constant; MRT, mean residence time; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach C_{max}.

Regarding the pharmacokinetic parameters (**Table VI.1**), as expected according to the mean concentration-time profiles, no statistically significant difference was identified for any of the parameters among the groups (*F. vesiculosus* vs control). These data support the lack of important pharmacokinetic-based herb-drug interactions between the *F. vesiculosus* extract and LTG in the experimental conditions employed.

VI.3.2. Effects of repeated pre-treatment with *F. vesiculosus* extract on LTG pharmacokinetics

The effects of the daily repeated oral administration of *F. vesiculosus* extract (575 mg/kg/day, 14 days) on the mean plasma pharmacokinetic profiles (n = 6) of LTG, given as a single oral dose of 10 mg/kg on the 15th day, are shown in **Figure VI.2**. The pharmacokinetic parameters obtained from experimental data and estimated by non-compartmental analysis are summarized in **Table VI.2**. As observed from **Figure VI.2**, there is a very similar pattern in the plasma concentration-time profiles of LTG achieved in both groups of rats (*F. vesiculosus* extract versus vehicle); however, over the first 24 h post-dose, the average concentrations of LTG tended to be lower in the experimental (*F. vesiculosus*) group comparatively to control (vehicle) group.

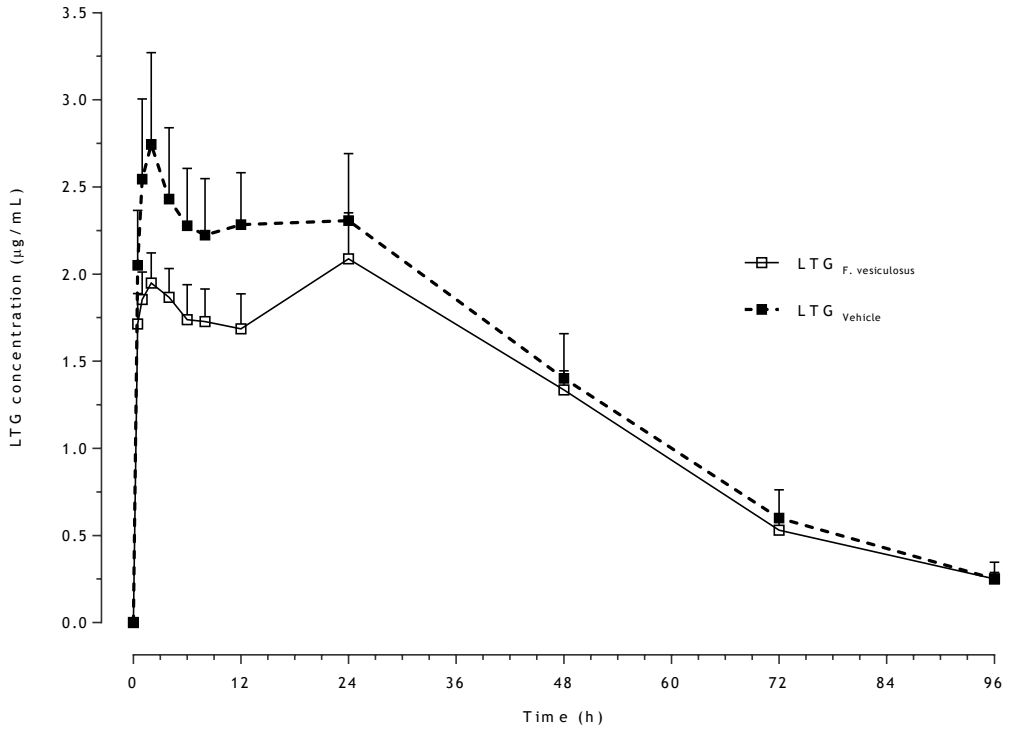


Figure VI.2. Mean plasma concentration-time profiles of lamotrigine (LTG) obtained, over a period of 96 h, from rats submitted to a 14-day pre-treatment period with *Fucus vesiculosus* extract (575 mg/kg, p.o.) or vehicle of the extract (water) and treated on the 15th day with a single-dose of LTG (10 mg/kg, p.o.) by oral gavage. Symbols represent the mean values ± standard error of the mean (SEM) of six determinations per time point (*n* = 6).

Table VI.2. Pharmacokinetic parameters estimated by non-compartmental analysis of the plasma concentration-time profiles of lamotrigine (LTG) obtained in rats submitted to a 14-day pre-treatment period with of *Fucus vesiculosus* extract (575 mg/kg, p.o.) or vehicle of the extract (water) and treated on the 15th day with a single-dose of LTG (10 mg/kg, p.o.) by oral gavage (*n* = 6). Data are presented as mean values ± standard error of the mean (SEM), except for t_{max} that is expressed as median values (range).

Parameter	Experimental Group *LTG F. vesiculosus*	Control Group *LTG Vehicle*
C_{max} (µg/mL)	2.234 ± 0.232*****	3.338 ± 0.374
t_{max} (h)	14.0 (1.0-24.0)	12.0 (2.0-24.0)
AUC_{0-24} (µg.h/mL)	43.573 ± 4.373	55.253 ± 5.376
AUC_{0-t} (µg.h/mL)	116.430 ± 9.629	133.165 ± 16.404
$AUC_{0-\infty}$ (µg.h/mL)	123.778 ± 10.257	143.323 ± 18.593
k_{el} (1/h)	0.0355 ± 0.0016	0.0364 ± 0.0044
$t_{1/2el}$ (h)	19.7 ± 0.9	20.4 ± 2.3
MRT (h)	38.9 ± 1.8	36.5 ± 3.5

AUC, area under the concentration-time curve; AUC_{0-24}, AUC from time zero to 24 h; AUC_{0-t}, AUC from time zero to the last measurable concentration; $AUC_{0-\infty}$, AUC from time zero to infinite; C_{max}, peak concentration; k_{el}, apparent elimination rate constant; MRT, mean residence time; $t_{1/2el}$, apparent terminal elimination half-life; t_{max}, time to reach C_{max}. * $p < 0.05$, significantly different from the control (vehicle) group.

Considering the pharmacokinetic parameters (**Table VI.2**), despite the similarity found in the median t_{max} values for LTG in the experimental group (14.0 h) and control group (12.0 h), the C_{max} was significantly lower in the experimental group (33.1%, $p < 0.05$).

The extent of the systemic exposure to LTG was also slightly decreased in the experimental group as denoted by AUC, but no statistically significant differences were noted ($p > 0.05$). In addition, the mean values estimated for the elimination pharmacokinetic parameters (k_{el} and $t_{1/2el}$) and MRT of LTG were also similar in both groups (*F. vesiculosus* extract versus vehicle).

VI.3.3. Effects of repeated-dose administration of *F. vesiculosus* extract on body weight

The effects of *F. vesiculosus* extract administration during 14 days on rats' body weight are presented in **Figure VI.3**. As observed, a statistically significant increase on rats' body weight was observed between day 1 and day 14 in both experimental and control groups ($p < 0.005$). Despite this, no statistically significant differences were identified in the body weight gain of the rats between the two groups. The body weight of rats increased on average 55.5 ± 4.1 g and 50.0 ± 3.8 g in the experimental (*F. vesiculosus*) group and control (vehicle) group, respectively.

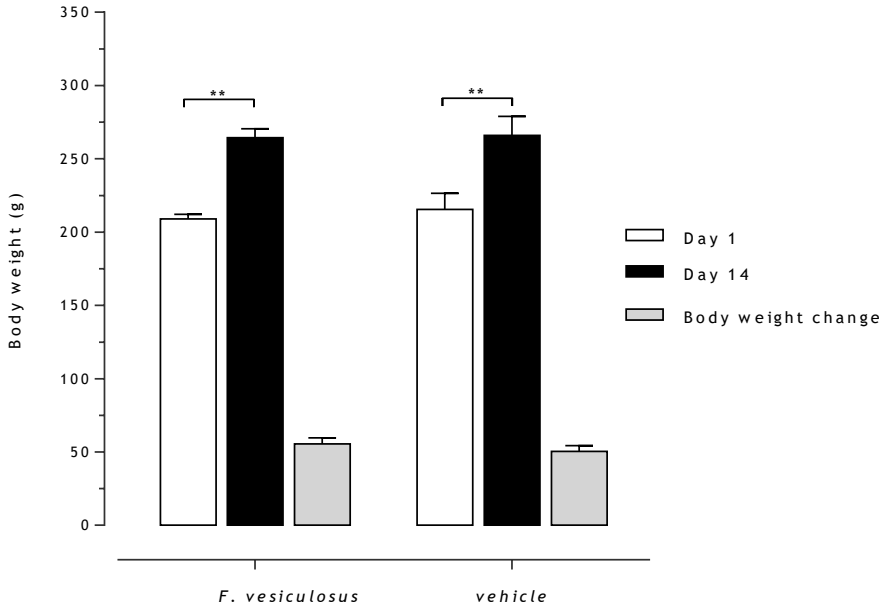


Figure VI.3. Effects of *Fucus vesiculosus* extract on the body weight of rats after a 14-day treatment period with *Fucus vesiculosus* extract (575 mg/kg, p.o.) or vehicle (water) by oral gavage. Data are presented as the mean values ± standard error of the mean (SEM) of six determinations ($n = 6$). **$p <$ 0.005, day 1 *versus* day 14.

VI.4. Discussion

Marine algae are emergent sources of prebiotics and anti-obesity agents. Due to its composition in bioactive compounds, marine algae can play an important role in modulating chronic diseases. In spite of its emergent use as functional food, *F. vesiculosus* consumption should be limited to ensure safe levels of iodine intake (Myers et al. 2016). European regulations fixed the 200 µg/day as the maximum iodine level allowed in food supplements to be administrated to adult humans (Richardson 2014). Also, the stimulating action promoted by iodine on the thyroid gland may be compromised by the concomitant use of AEDs, since chronic therapy with some of these drugs is associated with changes in homeostasis of thyroid hormones. Iodine uptake inhibition by the thyroid gland may be one of the mechanisms by which carbamazepine can induce thyroid dysfunction. So, the evaluation of thyroid hormone levels is needed in epilepsy patients (Adhimoolam and Arulmozhi 2016).

Some years ago, a significant herb-drug interaction between *F. vesiculosus* extract and amiodarone was reported by Rodrigues *et al*. (2013a) after the co-administration of the extract and the drug, which resulted in a remarkable decrease in the rate and extent of systemic drug exposure. On the other hand, interactions between herbal components and LTG have also been reported in literature. For instance, Myers *et al*. (2015b) reported an herb-drug interaction between ginseng and LTG that probably predisposed a patient to a drug hypersensitivity reaction. In addition, the simultaneous administration of *Paullinia cupana* and LTG in rats also resulted in a relevant pharmacokinetic-based herb-drug interaction with potential clinical impact (Ventura et al. 2018).

Considering that similar herb-drug interactions may occur in the pharmacokinetics of LTG when *F. vesiculosus* supplements are administrated together with this drug, in the current work we aimed to investigate the potential of this interaction in *in vivo* conditions in rats. As *in vitro* data are usually not directly extrapolated to humans, the results of studies performed in whole rodents can help to anticipate potential effects in humans (Castel-Branco et al. 2005a; Loscher 2011). To achieve our goals, two experimental studies were designed to evaluate the impact of *F. vesiculosus* on LTG pharmacokinetics *in vivo*. The first study was designed to investigate the effects of *F. vesiculosus* extract on the gastrointestinal absorption and also to evaluate the potential inhibitory effects of this extract on LTG metabolism. Overall, no important differences were observed in the rate and extent of systemic exposure to LTG after the co-administration of the extract and the drug (as assessed by C_{max} and AUC) (**Figure VI.1** and **Table VI.1**). These findings suggest that *F. vesiculosus* extract does not interfere with the oral bioavailability of LTG, evidencing the absence of interaction in the gastrointestinal tract. In fact, these data also highlight that the results of herb-drug interactions obtained for a given drug cannot be extrapolated to other drugs; actually, having *F. vesiculosus* as a common denominator, the results herein obtained for LTG are different from those obtained for amiodarone (Rodrigues et al. 2013a). Therefore, it is fully justified to assess the potential of

herb-drug interactions involving specific binary combinations of weight loss herbal extracts and drugs of narrow therapeutic range as LTG (3–15 μg/mL) (Patsalos 2013b; Patsalos et al. 2017). In the second study, we investigate the effects of the *F. vesiculosus* extract, following 14-days repeated oral administration to rats, on the pharmacokinetics of LTG; as induction phenomena are generally time-dependent, this protocol was established with the aim of evaluating potential inducer effects of the *F. vesiculosus* extract on LTG metabolizing enzymes and transporters. Nevertheless, the pharmacokinetic results obtained in this study did not show significant differences in the elimination rate of LTG. The only difference that deserves to be highlighted is the significant decrease of C_{max} ($p < 0.05$) in the group of rats pre-treated with *F. vesiculosus*. Indeed, considering simultaneously all these findings, it is likely that the repeated administration of *F. vesiculosus* may determine some changes in gastrointestinal motility and, consequently, in the rate and extent of systemic absorption of LTG (**Figure VI.2** and **Table VI.2**). Thus, it seems plausible to consider that polysaccharides found in *F. vesiculosus* extract may be responsible by the decreased absorption of LTG since fibres can slow down digestion and absorption of nutrients (Gabbia et al. 2017). Gabbia and its collaborators also found that the use of *F. vesiculosus* with another edible seaweed delayed and reduced the peak of blood glucose ($p < 0.05$) in mice fed with normal diet, without changing the blood glucose area under the curve. Additionally, the effects of the repeated administration (14 days) of *F. vesiculosus* extract on rats' body weight were not relevant (**Figure VI.3**), being these results in accordance with those previously achieved in the study conducted by Rodrigues *et al*. (2013a). Thus, taking into account all the results generated in the current work, at least from the pharmacokinetic perspective, it is expected that the use of *F. vesiculosus* is safe in patients receiving therapy with LTG and that its use could be interesting as functional food or as an ingredient of weight loss dietary supplements. These conclusions are also supported by the minimal toxic effects reported in rats after the administration of *F. vesiculosus* extract in acute toxicity assays and following a 4-week daily treatment (Zaragozá et al. 2008).

VI.5. Conclusion

Up to date, to the best of our knowledge, this is the first report on the effects of *F. vesiculosus* extract on LTG pharmacokinetics. Based on the findings achieved in this non-clinical work, which overall did not show important changes on the pharmacokinetics of LTG in Wistar rats after the co-administration or pre-treatment with *F. vesiculosus* extract, it can be concluded that no clinically significant pharmacokinetic-based herb-drug interactions are expected from the administration of the herbal extract and LTG. Hence, considering together the results herein obtained and those recently reported for *Paulinia cupana* extract (Ventura et al. 2018), if the administration of weight loss herbal supplements is required for patients undergoing LTG therapy it may be safer the use of herbal supplements containing *F. vesiculosus* than those containing other herbal extracts such as *Paulinia cupana*.

Chapter VII.

General discussion

Following the guiding principles of this thesis, it is important to reflect and make an integrated analysis of the results obtained against the proposed objectives. Therefore, this general discussion intends to focus on the overall results, presented in a greater detail in the respective chapters, which will be integrated herein, emphasizing their importance and ultimately to perceive them critically as pieces of a giant puzzle.

Truly, the greatest impetus for the beginning of this work was motivated by the concern that HDIs occur and that they are not perceptible to most healthcare professionals or to individuals who use herbal-based products simultaneously with conventional prescribed medicines without being aware of the associated potential risks and the deleterious effects that may result from these interactions. Hence, from this point of view, there is a problem to be solved and to be recognized as a public health issue since HDIs can ultimately lead to life-threatening adverse drug events, prolonged hospitalization and even death (Awortwe et al. 2018; Trivedi and Salvo 2016).

Among the great diversity of possible clinical entities to be worked on, the focus was placed on epilepsy, a chronic neurological disorder that affects 50 million people worldwide (Guerreiro 2016) and, in particular, on LTG, which is an AED routinely used in clinical practice (Brodie 2017; Guerreiro 2016; Mastrangelo 2017; Nevitt et al. 2017; Yasam et al. 2016). Although this AED is considered to be safe, its unique physicochemical and pharmacologic properties indicate that LTG has an overall propensity to interact with other AEDs and with other commonly prescribed drugs. Indeed, LTG is a broad-spectrum AED with a large interindividual variability in its pharmacokinetics and its metabolism can be affected by drugs that are enzyme inhibitors (e.g. valproate, sertraline) or inducers (e.g., phenobarbital, phenytoin, carbamazepine, rifampicin, oral contraceptives) (Johannessen Landmark and Patsalos 2008; Patsalos 2013a; Patsalos 2013b; Zaccara and Perucca 2014).

Still focusing the problematic issue of HDIs, and considering that herbs and herbal supplements may have variable effects on the absorption and disposition of AEDs, a new question was made: is it possible that herbal supplements for overweight and obesity can affect the pharmacokinetics of LTG and consequently its efficacy and safety?

Actually, to date, the literature that reports HDIs involving AEDs is scarce and, to a certain point of the time, the only reported HDI involving LTG had Ginseng as perpetrator agent (Myers et al. 2015a). However, it was already recognized by the scientific community that obesity and epilepsy are comorbid conditions with a high prevalence in children and adults (Janousek et al. 2013; Ladino et al. 2014). It has been also evidenced that obesity is more common in patients with refractory epilepsy and in those treated with polytherapy regimens (Baxendale et al. 2015; Chukwu et al. 2014; Janousek et al. 2013).

Having in mind these assumptions, the focus was then directed towards the nonclinical assessment of potential HDIs between herbal extracts often present in the composition of weight loss supplements and LTG. Recognizing the limitations of the *in vitro* studies in terms of the extrapolation of results to *in vivo* systems, the commitment was to design

complementary *in vivo* nonclinical studies in order to achieve essential data on the bioavailability and pharmacokinetics of LTG in rats, either in the presence or absence of the selected herbal extracts (Gurley 2012). In the last decades, rats have been largely employed as laboratory models to obtain a better understanding of the pharmacokinetics of established AEDs at nonclinical level and to study their involvement in drug-drug interactions. Despite the existing pharmacokinetic differences between species, the effective plasma levels of AEDs are indeed similar among rodents and humans. So, the rat is considered a whole-animal model suitable for the assessment of HDIs in order to anticipate and prevent the effects of those interactions before the testing in humans (Castel-Branco et al. 2005a; Loscher 2011).

The next steps were focused on the search of information about the herbal supplements marketed and so easily available to be acquired by the general population either on local pharmacies and parapharmacies or available in the free market and internet. Some of the many examples that follow are just a minority of products that are marketed for overweight, obesity or obesity-related problems. Actually, *P. cupana* extracts can be found isolated in different oral formulas (e.g. Guaraná Arkocapsules®, Guarana Fitoway®, Guaraná Maxinutri®, Guaraná Fitoactive®, Guaraná FormaFit®) or in association with other substances (e.g. EasySlim Drena Active®, Depurelina Rapid®, Super Diet Protocolo de Adelgazamiento®, Diet Limão H®, RaspBerry Ketone Plus®, Drenaslim Super Burner®, Drenaslim Hot Extra Burner®, among others). *G. cambogia* extracts are similarly found isolated in different formulas (e.g. *Garcinia Cambogia* from Bauer Nutrition®, Hydroxy Citrate®, *Garcinia cambogia* FormaFit®, *Garcinia cambogia* Slimming Labs®) or in combined formulas (e.g. EasySlim Lipo 3®, Emagril®, Seiva Optima®, BioLimão Gold®, Elegante Extra Plus chromium®, Super Diet Protocolo de Adelgazamiento®, Blocker Extreme®, Depuralina Express®, RaspBerry Ketone Plus®, Melan Line®, Cetona Extreme®, Cinturina IMC®, Seca Barriga®, Garcinia HCA Max+Green Coffee®, *Garcinia Cambogia* Ultimate Kit®, Garcinia+ RaspBerry+ FormaFit®, among others). *C. aurantium* extracts are found in several supplements that are to be administrated orally either in a simple composition formula (e.g. *Citrus aurantium* Arkocapsules®) or combined with other substances (e.g. EasySlim Depur Max®, Diet Linha®, Linha Leve®, Super Diet Protocolo de Adelgazamiento®, RaspBerry Ketone Plus®, Depuralina Gorduras®, Comprimidos Brasileiros Lister®, Drenaslim Mega Burn&Cell®, QuemaGrasas BiForm®, among others). Similarly, *F. vesiculosus* extracts can be also found isolated in different oral formulas (from Alga *Fucus* Biover®, *Fucus vesiculosus* Physalis®, Fucus Chá&Cia, Fucus Bio Nat&Form, Algae Fucus Nutrione®, *Fucus vesiculosus* Herbal Nature®, Bladderwrack Nature's´Way®, Algas Marinhas Aromas D'Aire®, Fucus Arkocapsules®, Algas Line®) or combined with other components (e.g. EasySlim Depur Max®, Nutridril Classics-NutriAlgas®, Linha Leve®, Super Diet Protocolo de Adelgazamiento®, Detoxine Framboesa+Arando®, among others). Therefore, given the abundance of commercially available herbal supplements containing in its composition bitter orange (*C. aurantium L.*), bladderwrack (*F. vesiculosus* L.), guarana (*P. cupana*) and malabar tamarind (*G. cambogia*) extracts, these four herbal extracts were those selected to investigate possible HDIs with LTG.

The experimental work started with the development of suitable bioanalytical tools to support the precise, accurate and reproducible quantification of LTG in the matrices of interest. Several bioanalytical methods had already been validated for LTG quantification in different biological matrices and using different separation and detection systems. At this point, and considering that liquid chromatographic techniques still remain as the primary analytical methodologies employed for drug quantification, HPLC was selected to be used in this case for LTG bioanalysis. Additionally, having in mind that sample preparation is a key factor in determining the success of analysis from complex matrices such as biological samples and that it typically takes 80% of the total analysis time, the aim was to innovate in the sample preparation methodology in order to achieve a simple and reliable procedure capable of being applied to the analysis of LTG in rat and human samples, thus having utility either to support the pharmacokinetic studies to be performed in rats or for the purpose of TDM of LTG in routine clinical practice. Also considering that no bioanalytical assay had been previously developed for the quantification of LTG in rat plasma and brain tissue using MEPS as sample preparation procedure, this emerging miniaturized and environmentally friendly technique was applied. Thus, several experimental conditions of the MEPS extraction protocol were tested and optimized firstly in rat matrices and then the developed MEPS protocol was successfully applied to human samples (plasma and saliva). Overall, the MEPS extraction protocol brought several advantages to the bioanalytical method, when compared to the use of LLE or SPE techniques. For instance, MEPS enabled a reduction in the use of organic solvents and allowed to analyse several samples with the same cartridge.

The chromatographic conditions were also optimized and the final MEPS/HPLC-DAD method presented multiple advantages, enabling a sensitive and fast analysis of LTG, using low amounts of organic solvents, while still allowing good recoveries in the target matrices.

The implementation and validation of any bioanalytical method for clinical or nonclinical pharmacokinetic studies is indeed crucial to ensure the quality and reliability of the results. The guidelines followed in this thesis for the validation of the bioanalytical methods were the international guidelines on bioanalytical method validation from the FDA (FDA 2013) and EMA (EMA 2011a). So, the selectivity, sensitivity, linearity, accuracy and precision, recovery, and stability of the analyte in the biological matrices of interest were studied to confirm the suitability of the bioanalytical method development achieved.

The validated bioanalytical methods were then preliminarily applied to human plasma and saliva samples of patients under LTG therapy (Ventura et al. 2017), and also to an exploratory pharmacokinetic study (Ventura et al. 2016). The results obtained from real human samples suggested a good correlation between saliva and plasma LTG concentrations, reinforcing the possibility of predicting plasma concentrations of LTG by knowledge of salivary concentrations. Therefore, the measurement of LTG concentration levels in saliva may be clinically relevant for TDM in patients, also benefiting from simple and non-invasive harvesting procedures (Ventura et al. 2017). On the other hand, the method developed and validated in rat matrices (plasma and brain tissue) (Ventura et al. 2016) was essential to support the bioanalytical

requirements of the subsequent nonclinical pharmacokinetic-based studies that constituted the core of this thesis.

To investigate the potential HDIs between *P. cupana*, *G. cambogia*, *C. aurantium* or *F. vesiculosus* and LTG, a set of studies were performed using adult male Wistar rats. The use of female rats was also hypothesized during the experimental design of these studies; however, only male Wistar rats were included to avoid the potential interference of menstrual cycle hormones (possible confounding factors). To assess each of the four extract-LTG combinations, at least two different pharmacokinetic studies were planned. The first pharmacokinetic study (called as co-administration study) was designed to investigate the potential effects of each extract on the gastrointestinal absorption and consequently on the systemic bioavailability of LTG. The second pharmacokinetic study (called as pre-treatment study) was designed to investigate the repeated administration of each extract on LTG pharmacokinetics, having in mind that induction mechanisms are time-dependent and recognizing the central role that induction of enzymes and transporters may play in HDIs.

Globally, taking into account the results of the pharmacokinetic studies carried out involving the four herbal extracts aforementioned, it should be highlighted that *P. cupana* extract was the one that caused the most marked interaction with LTG, whereas the extracts tested of *G. cambogia*, *C. aurantium* and *F. vesiculosus* had minor effects on LTG pharmacokinetics.

In the particular case of the *P. cupana* extract a significant decrease in LTG plasma concentrations was observed in the co-administration study between the 0.5 h and 8 h post-dose, having this interaction a higher impact on peak plasma concentration (C_{max}) and area under the concentration-time curve from time zero to 24 h (AUC_{0-24}), which were significantly reduced by 32.6% and 36.6%, respectively ($p < 0.05$). Additionally, a statistically significant increase of the MRT value of LTG was observed in the experimental group ($p < 0.05$). These results clearly evidenced a decrease in the absorption rate of LTG from the gastrointestinal tract of rats treated with *P. cupana* that probably resulted from physicochemical interactions between *P. cupana* extract, or its constituents, and LTG in the gastrointestinal tract of rats, delaying the drug absorption. This effect of *P. cupana* extract seems to be related to the adsorption of LTG in an identical manner to the effect caused by charcoal on LTG (Keränen et al. 2011). In contrast, the effects of the pre-treatment with *P. cupana* extract for 14 days resulted in a slightly higher systemic exposure to LTG. So, considering the HDI evidenced systemically after the co-administration of *P. cupana* extract and LTG, and considering that LTG needs to cross the blood-brain barrier to achieve its biophase, an additional study was designed to evaluate the impact of such interaction on LTG plasma-to-brain biodistribution. For this purpose, LTG concentrations were measured in plasma and brain tissue of rats sacrificed at 6 h post-dose. As expected, statistically significant differences were found in LTG plasma concentrations between the rats of the experimental and control groups, but surprisingly no important differences were reached in brain. The mean concentrations of LTG achieved in brain tissue were indeed lower in the *P. cupana* group than in the control group, but no statistically

significant differences were found at this single sampling point. Finally, the pre-treatment with *P. cupana* extract for 14 days did not reveal a strong effect on the body weight of the rats; however, the pre-treatment with the extract had some effects on blood glucose and triglyceride levels, which resulted in increased glycaemia and reduced blood levels of triglycerides.

The pharmacokinetic data regarding the effects of *G. cambogia* extract on the pharmacokinetics of LTG did not raise major concerns related to the occurrence of important HDIs. The co-administration of *G. cambogia* extract and LTG showed a slight tendency for a decrease of LTG C_{max} values (15.3%) and for a delay in the time to reach C_{max} (t_{max}). Additionally, no significant changes were observed in the extent of systemic drug exposure and in the elimination pharmacokinetic parameters (k_{el}, $t_{1/2el}$ and MRT). On the other hand, the repeated administration of *G. cambogia* extract for 14 days showed a significant decrease on LTG C_{max} values ($p < 0.05$), which were lower in the experimental group (34.0%) compared to the control group. In this case, although a decrease in the extent of systemic drug exposure in the group of rats pre-treated with *G. cambogia* has also been observed (23.1% in AUC_{0-24}, 31.5% in AUC_{0-t}, and 31.6% in $AUC_{0-\infty}$), no statistically significant differences were found between both groups ($p > 0.05$). Also, no differences were observed in the elimination pharmacokinetic parameters. Moreover, the 14-day pre-treatment period with *G. cambogia* extract did not show a significant effect on the body weight of rats. Based on these results, no important HDIs are expected in the clinical practice from the administration of *G. cambogia* with LTG. Nevertheless, in this context, it should be also considered the results of *in vitro* studies performed by Yu and collaborators (2017), which suggest that *G. cambogia* extract has potential to inhibit the CYP2B6 isoenzyme.

From the observed effects of *C. aurantium* extract on the pharmacokinetics of LTG, no clinically relevant risks are anticipated as a result of the administration of *C. aurantium* and LTG. Indeed, the co-administration of *C. aurantium* extract and LTG evidenced the lack of pharmacokinetic-based HDIs at the level of the gastrointestinal tract in the experimental conditions tested. On the other hand, the effects of the 14-day pre-treatment period with *C. aurantium* extract showed only a trend to a reduction in the extent of systemic exposure to LTG in the experimental group as denoted by AUC_{0-t} and $AUC_{0-\infty}$ parameters, which were reduced by 22.0% and 25.2%, respectively. In this case, from a statistical point of view, significant differences were found only for t_{max} ($p = 0.0455$). Additionally, the values estimated for the secondary pharmacokinetic parameters k_{el}, $t_{1/2el}$ and MRT indicated a slight tendency towards an increased elimination rate of LTG in the rats pre-treated with *C. aurantium* extract. Lastly, the effects of the 14-day pre-treatment period with *C. aurantium* extract resulted in minor changes on the biochemical parameters evaluated (blood glucose, total cholesterol and triglycerides) and in the body weight of rats. As mentioned before, based on these findings, the occurrence of relevant interactions between *C. aurantium* extract and LTG is not expected in real-world clinical settings; however, there is already some evidence that *C. aurantium* extract, or its constituents, may have time-dependent inhibitory effects. Indeed, the interaction potential involving *C. aurantium* has been related to the *C. aurantium* extract itself, juices or

flavonoid constituents. Fang *et al*. (2008) studied the effects of several constituents of *Frutus Aurantii Immaturus* on mouse gastrointestinal tract and found that synephrine inhibited the gastrointestinal movement, while hesperidin stimulated it. Rodrigues *et al*. (2013b) found that *C. aurantium* extract significantly increased the peak plasma concentration of amiodarone in rats pre-treated with the extract during 14 days, without other major effects on amiodarone pharmacokinetics. Wason *et al*. (2012) found that bitter orange juice reduced the C_{max} (24%), AUC (20%) and delayed in one hour the t_{max} of colchicine. Bitter orange juice can inhibit selectively the intestinal, but not the hepatic, CYP3A4 isoform. Also, in animal experiments, hesperidin showed a synergistic effect with diazepam, a benzodiazepine drug, suggesting a probable pharmacodynamic interaction (Fernández et al. 2005).

Regarding the studies performed with *F. vesiculosus,* the co-administration of the extract and LTG resulted in minor differences in the rate and extent of systemic drug exposure (as assessed by C_{max} and AUC), evidencing the absence of interaction in the gastrointestinal tract of rats. It was indeed observed a great parallelism between the plasma concentration-time profiles achieved for LTG in the rats of both experimental (*F. vesiculosus*) and control (vehicle) groups, and an almost complete overlap during the first 6 h post-dose. After this time point (6 h) there was a slight trend towards higher LTG plasma concentrations in the experimental (*F. vesiculosus*) group, but with no statistically significant differences. The effects of the 14-day pre-treatment period with *F. vesiculosus* extract showed that over the first 24 h post-dose, the average concentrations of LTG tended to be lower in the *F. vesiculosus* group comparatively to the control group. The most relevant pharmacokinetic result obtained after the 14-day pre-treatment period with the *F. vesiculosus* extract was the statistically significant decrease of C_{max} (33.1%; $p < 0.05$) in the group of rats that received *F. vesiculosus*. Thus, the repeated administration of the *F. vesiculosus* extract may have determined some changes in gastrointestinal motility and, consequently, in the rate of systemic absorption of LTG. Additionally, the effects of the repeated administration of *F. vesiculosus* extract on rats' body weight were not relevant. From literature, in what concerns the interaction potential of *F. vesiculosus*, or its constituents, there is some evidence that fucoidan may have some inhibition potential at the metabolic level. Mathew *et al*. (2017) evaluated the potential of hepatic metabolism-mediated drug interactions with fucoidan (found in *F. vesiculosus* and other seaweeds) in *in vitro* conditions and they found that *F. vesiculosus* caused inhibition of the CYP2C8, CYP2C9, CYP3A4 and CYP2D6 isoenzymes.

From the global analysis of our data only a significant HDI with potential clinical impact was identified between *P. cupana* and LTG based on the performed experimental nonclinical studies. Actually, as far as it was possible to investigate, before the publication of our results, only one HDI between *Panax ginseng* and LTG was reported (Myers et al. 2015a). On the other hand, the administration of *G. cambogia, C. aurantium* and *F. vesiculosus* extracts caused only minor effects on LTG pharmacokinetics, which did not show a high potential for interactions with LTG. Since these three weight loss herbal extracts did not cause significant changes on

the systemic bioavailability and biodisposition of LTG, they can be considered safer options than *P. cupana* extract. Therefore, if patients on LTG therapy need to take herbal medicinal products to aid in the management of overweight and obesity, it is prudent to avoid the consumption of *P. cupana*-containing herbal products.

Chapter VIII.

Conclusion

As a conclusion, and considering that the main outlined goal of this thesis was the nonclinical assessment of the potential for HDIs between herbal extracts often present in weight loss supplements and LTG, the most relevant key findings brought out from all the experimental work carried out under the scope of the present dissertation are succinctly provided below:

- An HPLC-DAD method using MEPS as innovative sample extraction technique was successfully developed and validated, and it was used to quantify LTG in rat matrices (plasma and brain). The method revealed to be sensitive, reliable, accurate and precise, enabling a simple and rapid analysis. The development and validation of this bioanalytical method was essential to support the subsequent analytical requirements to perform the nonclinical pharmacokinetic-based studies that constituted the core of this thesis.

- A MEPS/HPLC-DAD method was also successfully validated and applied to quantify LTG in human plasma and saliva. This new method was applied to human plasma and saliva samples of patients under LTG therapy, and the preliminary results obtained can be interpreted as good indicators for the application of the method in the clinical laboratory routines for TDM of LTG. Due to the good correlation achieved between salivary and plasmatic concentrations, saliva seems to be an attractive and viable alternative biological matrix to plasma for TDM of patients receiving LTG therapy.

- The results of the pharmacokinetic studies designed to investigate the potential for HDIs between the selected herbal extracts and LTG appeared to be adequate for the intended purposes.

- The *P. cupana* extract co-administration had a higher impact on LTG pharmacokinetics with a significant decrease of C_{max} and $AUC_{0\text{-}24}$ ($p < 0.05$), and a significant increase of the MRT. This HDI between *P. cupana* extract and LTG was herein reported for the first time, which led to a significant reduction in the rate and extent of systemic exposure to LTG.

- The repeated administration of *G. cambogia* and *F. vesiculosus* extracts for 14 days caused a significant decrease on LTG C_{max} values ($p < 0.05$) of LTG.

- The repeated administration of *C. aurantium* extract for a 14-day period caused a significant decrease of the LTG t_{max} value ($p = 0.0455$).

- The effects of the herbal extracts on the rats' body weight submitted to a 14-day treatment period were somehow surprising since that all extracts showed to be

ineffective to control body weight gain. This fact can be explained by the short period of time of the studies and perhaps by the fact that the studies have not been performed in obese animals.

- Finally, the effects of the repeated treatment during 14-days with *P. cupana* and *C. aurantium* extracts on the measured biochemical parameters reinforced the potential benefits of *P. cupana* extract in lipid metabolism due to its major effect on reducing the blood levels of triglycerides.

Based on the findings achieved in these nonclinical studies, it can be concluded that no clinically significant pharmacokinetic-based HDIs are expected from the administration of *G. cambogia*, *C. aurantium* or *F. vesiculosus* extracts and LTG. Therefore, if the administration of weight loss herbal supplements is required for patients undergoing LTG therapy it may be safer to use herbal supplements containing these extracts than those containing *P. cupana* extracts. Nevertheless, further studies are needed to better understand the mechanism associated with this HDI and its clinical relevance must be further investigated to be better understand the therapeutic impact of a lower systemic incorporation rate of LTG.

Hence, the nonclinical assessment of HDIs is of utmost importance to predict and evaluate the potential effects of herbal substances in the pharmacokinetics of conventional drug. The findings reported in this thesis reinforce the importance of investigating the impact of herbal preparations on the efficacy and safety of prescribed medicines, particularly when the object drug has a narrow therapeutic range and exhibits a high variability in its pharmacokinetics.

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