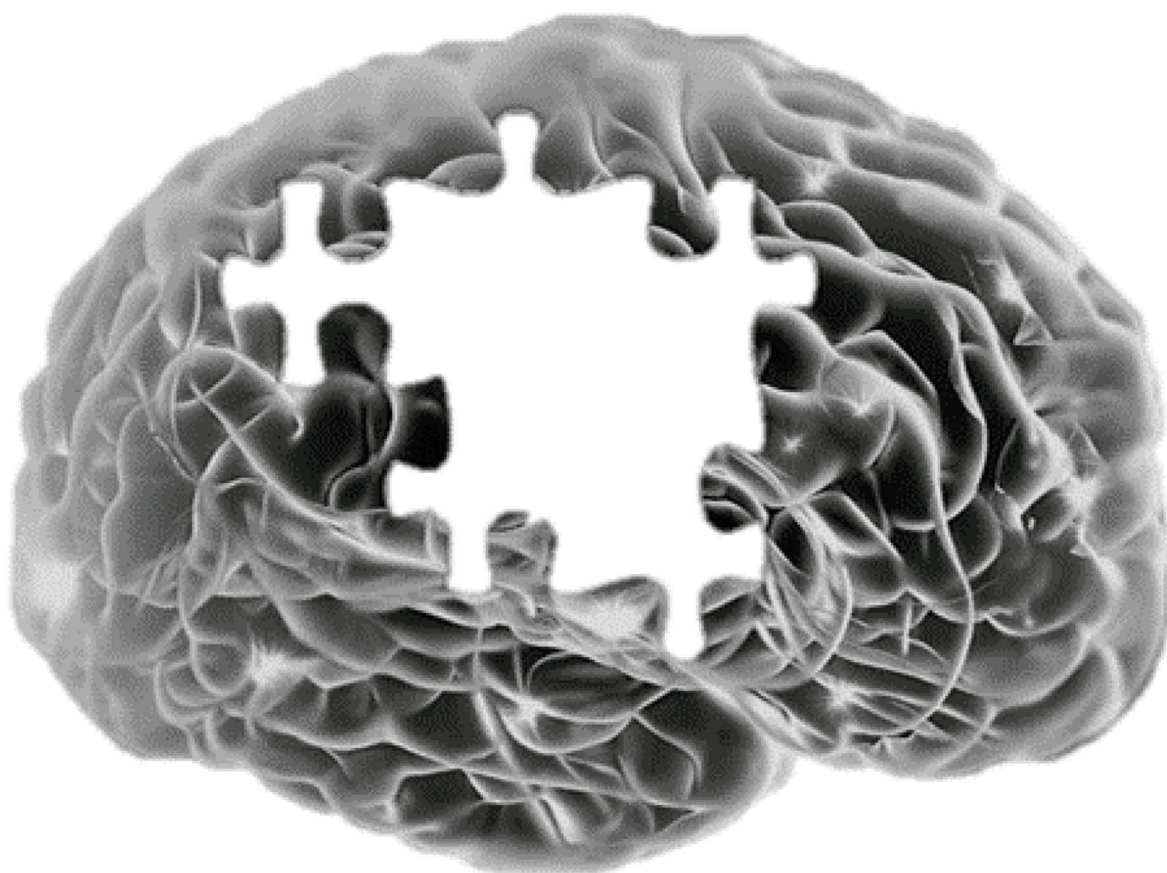

Daniela de Oliveira Gonçalves

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Tese de Doutoramento em Ciências Farmacêuticas, especialidade de Farmacologia e Farmacoterapia, orientada pelo Professor Doutor Amílcar Celta Falcão Ramos Ferreira e pelo Professor Doutor Gilberto Lourenço Alves, apresentada à Faculdade de Farmácia da Universidade de Coimbra.

Março 2017

UNIVERSIDADE DE COIMBRA

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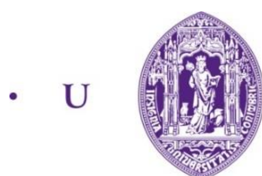
• U  C •

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UNIVERSIDADE DE COIMBRA

The experimental work presented in this thesis was performed under the scientific supervision of Professor Amílcar Celta Falcão Ramos Ferreira and Professor Gilberto Lourenço Alves at the Laboratory of Pharmacology, Faculty of Pharmacy, University of Coimbra and at the Health Sciences Research Centre, Faculty of Health Sciences, University of Beira Interior.

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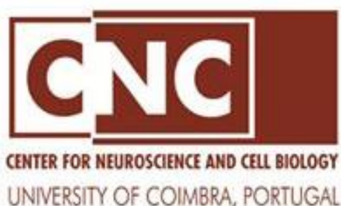

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LIST OF ABBREVIATIONS

AADC	Aromatic *L*-amino acid decarboxylase
AUC	Area under the concentration-time curve
AUC_{extrap} (%)	Percentage of AUC extrapolated from t_{last} to infinity
AUC_{τ}	AUC within the dosing interval
$AUC_{0\text{-}inf}$	AUC from time zero to infinity
$AUC_{0\text{-}t}$	AUC from time zero to the time of the last measurable drug concentration
$AUC_{0\text{-}24h}$	AUC from time zero to 24 hours
AUEC	Area under the effect-time curve
$AUEC_{0\text{-}24h}$	AUEC from time zero to 24 h
$AUEC_{0\text{-}72h}$	AUEC from time zero to 72 h
BBB	Blood-brain barrier
Bias	Deviation from nominal values
C_{last}	Last measurable drug concentration
C_{max}	Maximum concentration
C_{min}	Minimum concentration
COMT	Catechol-*O*-methyltransferase
CV	Coefficient of variation
CYP	Cytochrome P450
DAD	Diode array detection
***DL*-DOPA**	*DL*-3,4-dihydroxyphenylalanine
DMSO	Dimethyl sulfoxide
EGTA	Ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid
EMA	European Medicines Agency
E_{max}	Maximum inhibition of catechol-*O*-methyltransferase activity
FDA	U.S. Food and Drug Administration
HPLC	High performance liquid chromatography
HPMC	Hydroxypropyl methylcellulose
IC_{50}	Half maximal inhibitory concentration
i.p.	Intraperitoneal
IS	Internal standard
k	Kinetic recovery constant
k_{el}	Apparent plasma elimination rate constant
k_{tis}	Apparent tissues elimination rate constant
LC	Liquid chromatography

LID	Levodopa-induced dyskinesia
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LOD	Limit of detection
MAO	Monoamine oxidase
MB-COMT	Membrane-bound isoform of catechol-*O*-methyltransferase
MDS	International Parkinson and Movement Disorder Society
Met	Methionine
MRT	Mean residence time
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
OATP	Organic anion transporting polypeptide
PD	Parkinson's disease
QC	Quality control
$\mathbf{QC_{DIL}}$	Quality control of dilution
$\mathbf{QC_{LLOQ}}$	Quality control at the lower limit of quantification
$\mathbf{R_{ac}}$	Observed accumulation ratio
REM	Rapid eye movement
$\mathbf{R_0}$	Theoretical accumulation ratio
r^2	Coefficient of determination
SAM	*S*-adenosyl-*L*-methionine
S-COMT	Soluble isoform of catechol-*O*-methyltransferase
SEM	Standard error of the mean
SNpc	*Substantia nigra* pars compact
t_{Emax}	Time to achieve the maximum inhibition of catechol-*O*-methyltransferase activity
t_{last}	Time of the last measurable drug concentration
t_{max}	Time to reach the maximum concentration
$t_{1/2el}$	Plasma elimination half-life
$t_{1/2tis}$	Tissues elimination half-life
Val	Valine
τ	Dosing interval
3-*O*-MD	3-*O*-methyldopa

PUBLICATIONS

PUBLICATIONS

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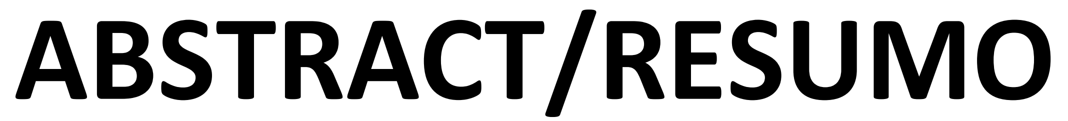
ABSTRACT/RESUMO



ABSTRACT

Parkinson's disease (PD) is a chronic and progressive neurodegenerative disorder with an increasing worldwide incidence. Despite the progress in understanding the pathogenesis of the disease and in its therapy over the last years, no treatments are currently available to cure or modify the neurodegenerative process. Therefore, PD treatment remains symptomatic. Curiously, almost 50 years after the clinical introduction of levodopa, this drug continues to be considered the most effective therapy for the management of motor symptoms of PD and, when patients develop motor fluctuations, a catechol-*O*-methyltransferase (COMT) inhibitor is widely recommended to be associated with the levodopa/aromatic *L*-amino acid decarboxylase (AADC) inhibitor therapy. However, the well-known COMT inhibitors (tolcapone and entacapone) exhibit relevant drawbacks that have restricted their clinical success, demanding the development of new COMT inhibitors with better therapeutic profiles. In this context, opicapone has emerged as an attempt to fulfil this unmet therapeutic need.

At the onset of the project underlying the present PhD thesis, opicapone was in clinical development and little information was available in literature about this new and promising COMT inhibitor. Thus, in order to deepen the non-clinical pharmacological knowledge about opicapone and complement the data generated during its clinical development program, the present work was focused on providing non-clinical information on systemic and tissue pharmacokinetics of opicapone and its active metabolite (BIA 9-1079), as well as their effect on erythrocyte, liver and kidney COMT activity after single and multiple (once-daily for seven consecutive days) oral administrations of opicapone to Wistar rats.

Owing to the lack of an available analytical technique to support the execution of the planned pharmacokinetic studies, the present project began with the development and full validation of a suitable and reliable bioanalytical method. Hence, a high performance liquid chromatography method coupled with a diode array detector was adequately validated for the quantification of opicapone and BIA 9-1079 in rat plasma, liver and kidney matrices. In addition, a similar method developed in human plasma revealed to be an appropriate bioanalytical tool to support future clinical pharmacokinetic studies involving opicapone.

The obtained results suggest that the gastrointestinal absorption of opicapone was relatively rapid as well as its elimination from systemic circulation following both single- and multiple-dose administrations. BIA 9-1079 was also quickly eliminated and represented a high relative percentage of the systemic exposure of opicapone, suggesting that this

active metabolite may contribute to the pharmacological activity of opicapone in rats. Interestingly, although opicapone apparently exhibited an approximately dose-proportional increase in its extent of systemic exposure following a single oral administration in the tested dose range (30–90 mg/kg), the systemic exposure to BIA 9-1079 was similar after the administration of 60 and 90 mg/kg of opicapone, suggesting a saturation of this metabolic pathway at the higher tested doses.

The systemic accumulation of opicapone and BIA 9-1079 seems to be negligible or, perhaps, non-existent at the evaluated multiple-dose regimen (30 mg/kg once-daily for seven consecutive days), with no hepatic or renal accumulation of both compounds, except for BIA 9-1079 in liver. In addition, the tissue-systemic exposure relationships to opicapone indicated a low exposure of the liver and kidneys to the drug.

Following the single and multiple oral administration of 30 mg/kg opicapone, the COMT inhibition profiles were reasonably comparable in all the assessed biological matrices (erythrocytes, liver and kidneys). Furthermore, in spite of its poor exposure in tissues and rapid elimination, opicapone showed a strong and long-lasting pharmacological effect independently of the dosage regimen. Nevertheless, a slightly higher extent of COMT inhibition was observed after repeated administrations.

In conclusion, the experimental work herein described contributed to enhance the current non-clinical pharmacological knowledge of a new drug recently approved by European Medicines Agency to be used as adjunct to levodopa/AADC inhibitor therapy in PD patients with motor fluctuations. Moreover, according to our results, it seems that no pharmacokinetic or pharmacodynamic concerns related to the physiological restoration of COMT activity are expected when opicapone is administered once-daily.

Keywords: BIA 9-1079, bioanalysis, catechol-*O*-methyltransferase inhibitors, high performance liquid chromatography, *in vivo* studies, opicapone, Parkinson´s disease – therapy, pharmacodynamics, pharmacokinetics, rat

RESUMO

A doença de Parkinson (DP) é uma doença neurodegenerativa crónica e progressiva com uma incidência crescente a nível mundial. Apesar do progresso observado nos últimos anos na compreensão da patogénese da doença e na sua terapêutica, nenhum tratamento parece ser capaz de curar ou modificar o processo neurodegenerativo. Desta forma, o tratamento da DP permanece sintomático. Curiosamente, quase 50 anos após a introdução clínica da levodopa, esta continua a ser considerada a terapia mais eficaz no controlo dos sintomas motores da DP, sendo amplamente recomendado adicionar-se um inibidor da catecol-*O*-metiltransferase (COMT) à terapia combinada levodopa/inibidor da descarboxilase dos *L*-aminoácidos aromáticos (DCAA) em doentes que desenvolvem flutuações motoras. Contudo, os inibidores da COMT de referência (tolcapone e entacapone) apresentam inconvenientes clinicamente relevantes que têm vindo a limitar o seu sucesso, permanecendo assim a necessidade de desenvolver novos inibidores com um melhor perfil terapêutico. Neste contexto, o opicapone surgiu como uma tentativa de colmatar esta necessidade terapêutica.

No início dos trabalhos de investigação subjacentes ao desenvolvimento da presente tese de doutoramento, o opicapone encontrava-se em fase de desenvolvimento clínico e pouca informação estava disponível na literatura sobre este novo e promissor inibidor da COMT. Assim, a fim de aprofundar o conhecimento farmacológico não-clínico sobre o opicapone e complementar os dados gerados durante o seu desenvolvimento clínico, o trabalho aqui apresentado visou obter informações não-clínicas sobre a farmacocinética sistémica e tecidular do opicapone e do seu metabolito ativo (BIA 9-1079), bem como sobre o seu efeito na atividade da COMT eritrocitária, hepática e renal após administrações orais em dose única e múltipla (uma vez por dia durante sete dias consecutivos) do opicapone a ratos Wistar.

Devido à não existência de uma técnica analítica disponível para suportar a execução dos estudos farmacocinéticos planeados, o início deste projeto foi dedicado ao desenvolvimento e validação completa de um método bioanalítico adequado e fiável. Assim, foi adequadamente validado um método de cromatografia líquida de alta pressão acoplada a um detetor de fotodíodos para a quantificação do opicapone e do BIA 9-1079 em matrizes de plasma, fígado e rim de rato. Em adição, um método similar foi desenvolvido em plasma humano e revelou ser uma ferramenta bioanalítica apropriada para suportar futuros estudos de farmacocinética clínica que envolvam o opicapone.

Os resultados obtidos sugerem que a absorção gastrointestinal do opicapone foi relativamente rápida, bem como a sua eliminação da circulação sistémica após cada um

dos regimes posológicos instituídos (dose única e dose múltipla). O BIA 9-1079 foi também rapidamente eliminado e representou uma elevada percentagem relativa da exposição sistémica ao opicapone, sugerindo que este metabolito ativo pode contribuir para a atividade farmacológica do opicapone no rato. Curiosamente, embora o opicapone aparentemente tenha exibido um aumento na sua extensão de exposição sistémica aproximadamente proporcional à dose após a administração oral de uma dose única na gama testada (30–90 mg/kg), a exposição sistémica ao BIA 9-1079 foi similar após a administração de 60 e 90 mg/kg de opicapone, apontando para uma saturação desta via metabólica nas doses mais elevadas.

A acumulação sistémica do opicapone e do BIA 9-1079 parece ser negligenciável, ou mesmo inexistente, no regime de dose múltipla avaliado (30 mg/kg uma vez por dia durante sete dias consecutivos), não tendo sido detetada acumulação hepática ou renal para os compostos, exceto para o BIA 9-1079 no fígado. Além disso, as relações de exposição tecido-plasma ao opicapone indicaram uma baixa exposição do fígado e dos rins ao fármaco.

Após a administração oral única e múltipla de 30 mg/kg de opicapone, os perfis de inibição da COMT foram razoavelmente comparáveis entre as matrizes biológicas avaliadas (eritrócitos, fígado e rins). Ademais, apesar da sua fraca exposição tecidular e rápida eliminação, o opicapone exibiu um efeito farmacológico forte e duradouro independentemente do regime de administração considerado. No entanto, observou-se uma extensão de inibição da COMT ligeiramente superior após a administração repetida.

Em conclusão, o trabalho experimental aqui descrito contribui para pormenorizar o conhecimento farmacológico não-clínico atual de um novo fármaco que foi recentemente aprovado pela Agência Europeia de Medicamentos para ser utilizado como adjuvante da terapia combinada levodopa/inibidor da DCAA em doentes de Parkinson com flutuações motoras. Mais ainda, de acordo com os nossos resultados, não são esperadas preocupações do ponto de vista farmacocinético ou farmacodinâmico relacionadas com a restauração fisiológica da atividade da COMT quando o opicapone é administrado uma vez por dia.

Palavras-chave: BIA 9-1079, bioanálise, cromatografia líquida de alta pressão, doença de Parkinson – terapia, estudos *in vivo*, farmacodinâmica, farmacocinética, inibidores da catecol-*O*-metiltransferase, opicapone, rato

CHAPTER I

GENERAL INTRODUCTION

CHAPTER I

I.1. Parkinson’s Disease and its Pharmacological Treatment

I.1.1. A Bit of History

Since ancient times, symptoms resembling those of Parkinson's disease (PD) were described in Egyptian, Indian and Chinese texts and even in the Bible [1,2]. An Egyptian papyrus of the nineteenth dynasty (ca. 1350–1200 B.C.) mentions the sialorrhea of a king, as follows: "divine old age had slackened his mouth. He cast his spittle upon the ground and spit it out" [2]. Other early descriptions of this disease are also found in the oldest medicine system in the world, the Ayurveda (ancient Indian medical system). In Ayurvedic treatises from approximately 1000 B.C. was described a disease named "kampavata", which description includes tremors, difficulty of movement, sialorrhea, love of solitude, somnolence and stare. Interestingly, it was treated with preparations containing the plant *Mucuna puriens* from which levodopa was isolated three millenniums later [3]. Some references to tremor can also be seen in two books of the Old Testament, Job and Ecclesiastes [4]. Moreover, in the Traditional Chinese Medicine literature, the first descriptions of tremor and stiffness date back to 425 B.C. and, probably, the first clinical case of PD was reported by Zhang Zihe during the Jin dynasty (1151–1231 A.D.). The Traditional Chinese Medicine also provided an "antitremor pill" which was a mixture of herbs containing anticholinergic, antioxidant and monoamine oxidase (MAO)-B inhibitor compounds [5].

Galen (2nd century), Sylvius de la Boë (1680), Gaubius (1758), Boissier de Sauvages (1768), Johannes Sagar (1776) and Hunter (1776) were examples of physicians that also reported some observations likely related to the PD [2,6]. Nevertheless, the first comprehensive clinical description of PD was written by James Parkinson in 1817. James Parkinson, in his famous monograph "An Essay on the Shaking Palsy", reported six cases of the malady that he termed *Paralysis Agitans*, defining it by the presence of resting tremor, lessened muscular power, and propensity for flexed posture and festination [7]. A few decades later, Jean-Martin Charcot, the father of neurology, distinguished bradykinesia as a cardinal symptom, separate from rigidity, and employed for the first time the term "Parkinson's disease" referring to the disorder [1,2]. Charcot and his students also differentiated this disease from other tremulous disorders, specifically multiple sclerosis, and they identified variants of the classical PD, which are probable examples of disorders later grouped under the term Parkinsonism-plus syndromes [1]. In 1895, Brissaud was the first to suggest that lesions of the *substantia nigra* could be on the basis of PD and Fritz Lewy (1912/1913) discovered inclusion bodies in the brain of PD patients [8,9]. Some years later, Trétiakoff established the presence of these bodies in the *substantia nigra* as a pathologic hallmark of the malady and designated them as "Lewy bodies". However, the most comprehensive pathological analysis of PD was provided only in 1953 by Greenfield

and Bosanquet, who confirmed the selective loss of the ventrolateral cell groups within the *substantia nigra* pars compact (SNpc) and highlighted the nigral lesion and the Lewy bodies as characteristic features of PD [8].

Immediately after the recognition of PD as a medical condition by James Parkinson, no effective therapy was available, but some references stated the use of different agents like metals and herbal constituents [10]. Fifty years later, the pharmacological treatment of PD began to be based on the use of alkaloids derived from plants. Initially, Charcot and his student Leopold Ordenstein (1867) recommended the use of hyoscyamine and then Wilhelm Erb (1887) introduced scopolamine, both central active anticholinergic (antimuscarinic) alkaloids. The use of similar plant derived treatments remained until 1950s, when synthetic anticholinergic (antimuscarinic) and antihistaminergic drugs with better adverse effect profiles replaced them [8,10,11].

In the late 1950s, important neurochemical breakthroughs were achieved, including: the recognition that dopamine was present in the mammalian brain; the demonstration by Carlsson and colleagues (1957–1958) that *DL*-3,4-dihydroxyphenylalanine (*DL*-DOPA) reversed the sedative and bradykinetic effects of reserpine in animals, which was later observed in humans (1960) following the administration of *L*-DOPA (levodopa); and the discovery by Hornykiewicz and Ehringer of a marked striatal depletion of dopamine in PD and postencephalitic parkinsonian brains [12–14]. Hence, bearing in mind the new knowledge, Hornykiewicz started to consider levodopa as a possible treatment for PD and persuaded the neurologist Walther Birkmayer to test the effect of levodopa in parkinsonian patients. Agreeing, Birkmayer administered intravenously levodopa to patients with PD, which successfully controlled the symptoms of the disease [10,12,15]. However, in some subsequent trials no benefits of levodopa intake were observed, until the trial performed by George Cotzias in 1967. In this trial, *DL*-DOPA was administered to parkinsonian patients at progressively higher dosages showing benefits in reversing the symptoms even though hematologic adverse effects were registered. Right away after these encouraging results, Cotzias and colleagues tested solely levodopa (the active isomer) by increasing dosages in the same way (slowly and steadily). They attained the same benefits of the former study with half the dosage and without the hematologic problems, recognizing that *D*-DOPA is the inactive isomer and responsible for some of the observed toxicity. These findings with levodopa were rapidly confirmed in a double-blind trial performed by Melvin Yahr, and levodopa quickly became the first well-established drug to treat the symptoms of PD [1,8,12,14]. In point of fact, the clinical introduction of levodopa [approved by U.S. Food and Drug Administration (FDA) in 1970]

revolutionized the treatment of PD and it remains nowadays as the most efficacious symptomatic treatment and the mainstay of PD therapy [10,11,13,16].

Although tremendous progress has been achieved over the last few decades in the pathogenesis of PD, as well as improvements in its therapy, the aetiology of PD is still mysterious and it remains incurable [16–18].

I.1.2. Parkinson's Disease

I.1.2.1. Epidemiology

Only surpassed by Alzheimer's disease, PD is the second most common neurodegenerative condition worldwide, affecting about 1–2% of the population over 60 years of age [19,20]. PD frequency increases with age, being rare before 50 years old [21]. Indeed, its incidence and prevalence increase sharply and progressively after age 60 years, although the incidence seems to stabilize in the highest age group (over age 80) [22,23].

Overall, incidence rates for PD in epidemiological studies including all age groups ranged from 1.5 to 22 per 100 000 person-years, while studies restricted to the older populations (above 55 or 65 years old) reported overall incidence rates between 410 and 529 per 100 000 person-years [24]. Regarding overall prevalence, in door-to-door studies, it varied between 167 and 5 703 per 100 000, with those studying an elderly population (above 60 or 65 years of age) reporting the highest values [19]. However, in studies using case-finding strategies, such as registries, the figures are lower with most studies reporting a prevalence between 100 and 300 per 100 000 [24]. In addition, owing to the longer lifespan and the general ageing of the population, if no cure arises in a near future, the number of individuals with PD is expected to rise substantially. In fact, according to a study based on prevalence data for Western Europe's five most and world's ten most populous nations, it is expected that the number of individuals over 50 years old with PD will double between 2005 and 2030, increasing from 4.1–4.6 million in 2005 to 8.7–9.3 million people in 2030 [25].

Even though several studies reported lower frequency of PD in Africa, Asia, and South America in relation to Europe, others reported similar statistics [19,21]. Therefore, although there are some indications of geographical and ethnic variations in PD frequency, it is unclear if they result from differences in the used methodologies across studies, demography, healthcare or true ethnic and geographic differences related to genetic or environmental susceptibilities to PD [21,24]. PD is usually more common in men than

women probably due to the more frequent occupational exposures in men, neuroprotective effects of oestrogens in women and X-linked genetic factors [21–23].

Approximately 10–15% of patients with PD have the familial form of the disorder, reporting a positive family history. This form of disease is caused by a gene mutation (a monogenic cause) that triggers the pathology [26,27]. Nowadays, seven genes are unequivocally implicated in monogenic PD (Table I.1.1). Among them, four (*SNCA*, *LRRK2*, *VPS35* and *EIF4G1*) have been associated with the autosomal dominant PD and three (*Parkin*, *PINK1* and *DJ-1*) have been linked to the autosomal recessive PD [28,29]. More recently, at least other two genes (*DNAJC13* and *CHCHD2*; Table I.1.1) were related to autosomal dominant PD, even though their contribution awaits confirmation [30–32].

Table I.1.1 Familial (monogenic) forms of Parkinson's disease [29,30,32,33].

Gene	Protein encoded
Autosomal dominant form	
SNCA	**α-synuclein** - major component of Lewy bodies and neurites
LRRK2	**Leucine-rich repeat kinase 2** (or dardarin) - multiple domain protein involved in several processes, such as neuronal dendrite formation and growth, dopamine neurotransmission, protein recycling and inflammation response
VPS35	**Vacuolar protein sorting 35 homolog** - involved in endosomal trafficking and protein recycling
EIF4G1	**Eukaryotic translation initiation factor 4-γ 1** - involved in mRNA translation processes
DNAJC13	**Receptor-mediated endocytosis 8** (RME-8) - involved in endosomal trafficking and protein recycling
CHCHD2	**Coiled-coil-helix-coiled-coil-helix domain containing 2** - mitochondrial protein
Autosomal recessive form	
Parkin	**Parkin** - implicated in mitochondrial functions
PINK1	**PTEN-induced kinase 1** - implicated in mitochondrial functions
DJ-1	**Daisuke-Junko-1** - implicated in mitochondrial functions

However, the majority of PD cases have unknown aetiology and occur in people with no apparent family history of the disorder. These cases are classified as sporadic form of PD and, despite their cause remains unknown, they probably result from a complex interplay of genetic and non-genetic factors [20,34]. Besides the gene mutations that cause the monogenic PD, there are specific alterations in certain genes that do not cause the disease *per se*, but they may be associated with higher susceptibility to develop it. Additionally, there are also other mutations that reduce the risk of developing PD. To date, more than 20 independent *loci* were nominated as PD susceptibility loci, including genes linked to familial forms of PD (*LRRK2* and *SNCA*) and, for example, specific mutations in the susceptibility genes *GBA* and *SMPD1* increase the risk to develop PD in 5-fold and 9-fold, respectively [29,34].

Regarding the non-genetic factors, the advancing age is definitely a risk factor to develop PD, but there are also environmental factors involved [35]. These modifiable factors may have a beneficial (protective factors) or detrimental (risk factors) effect. Tobacco smoking and coffee and tea drinking have been included among the protective factors, as well as high serum uric acid levels, physical activity and alcohol consumption. In opposition, pesticide exposure, prior brain injury and dairy products intake are examples of factors that have been associated with a detrimental effect [19,21,24,36]. Interestingly, according to a very recent umbrella review of systematic reviews and meta-analyses, many environmental factors have substantial evidence of association with PD, but several, perhaps most of them, may reflect reverse causation, residual confounding, information bias, sponsor conflicts or other caveats [37]. Therefore, data from more studies are needed to better understand the relation between these factors and PD.

I.1.2.2. Pathology and Pathogenesis

Nowadays, PD is regarded as a slowly progressive neurodegenerative disease that begins years before of its diagnosis and involves several brain areas. Pathologically, PD is characterized by two hallmarks: the *loss of neuromelanin-pigmented dopaminergic neurons within the SNpc* and the *Lewy pathology*, both associated with a chronic *neuroinflammation* state [38].

Loss of dopaminergic neurons within the SNpc

The PD-vulnerable SNpc neurons project to the striatum (putamen and caudate nucleus), but the neuronal loss is uneven, being most marked in the ventrolateral tier which projects primarily to putamen [39]. The loss of these neurons (neuromelanin-pigmented

dopaminergic neurons) leads to depigmentation of the SNpc in patients with PD and to striatal dopamine deficiency, which is on the basis of the motor symptoms of the disorder [40]. At the onset of motor symptoms, approximately 60% of dopaminergic SNpc neurons are lost and a depletion around 80% of striatal dopamine is observed [41]. However, the neuronal loss in PD is not restricted to dopaminergic neurons, involving also noradrenergic, serotonergic and cholinergic neurons, and occurs in many other brain regions, including the amygdala, nucleus basalis of Meynert, locus ceruleus, raphe nucleus, pedunculopontine nucleus, dorsal motor nucleus of vagus and hypothalamus [38,42].

Lewy pathology

The Lewy pathology is characterized by the presence of intracellular inclusions which result from abnormally folded proteins aggregation being the α-synuclein the most abundant one. α-Synuclein is a neuronal cytosolic protein and, usually, concentrates in the presynaptic terminals of mature neurons, where it may be involved in neurotransmission (especially dopamine release) and synaptic homeostasis [38,43]. It is natively unfolded and soluble, but in a misfolded state it becomes insoluble and aggregates to form different types of insoluble inclusions. The formation of spherical and eosinophilic inclusions, named Lewy bodies, in the cell body of neurons characterizes in general the Lewy pathology. Nevertheless, it is also associated with the formation of elongated spindle-shaped or thread-like inclusions in neuronal cell axons and dendrites (Lewy neurites), dot-like structures and axonal spheroids, as well as diffuse, granular or pleomorphic neuronal perikaryal structures [38,44].

The Lewy pathology can be observed in especially vulnerable cell types (projection neurons with a long, thin, and weakly myelinated or non-myelinated axon) of central, peripheral, and enteric nervous systems [43]. According to Braak and colleagues, the progression of Lewy pathology in sporadic PD has a pattern of regional spread affecting first the peripheral nervous systems and progressively the central nervous system in a caudal-to-rostral direction [45,46]. Hence, six stages have been proposed for the disease progression in order to explain its clinical evolution (Figure I.1.1). In the stages 1 and 2 (prodromal stages), the Lewy pathology affects the peripheral nervous system, medulla oblongata and olfactory system, originating autonomic and olfactory dysfunctions. In the stages 3 and 4, the pathology advances to the midbrain, being the SNpc one of the areas involved, as well as the forebrain. At some point during these stages, the majority of individuals most likely cross the threshold to manifest the motor symptoms of the illness. In the end-stages 5 and 6, multiple neocortical regions are compromised and patients manifest all clinical dimensions of PD [46,47]. In spite of the lack of a clear connectivity

between some of the neuronal populations vulnerable to develop Lewy pathology and the existence of some PD cases which do not fit the staging pattern, the majority of PD patients have a pathology pattern that corresponds to the one proposed by Braak and colleagues [44].

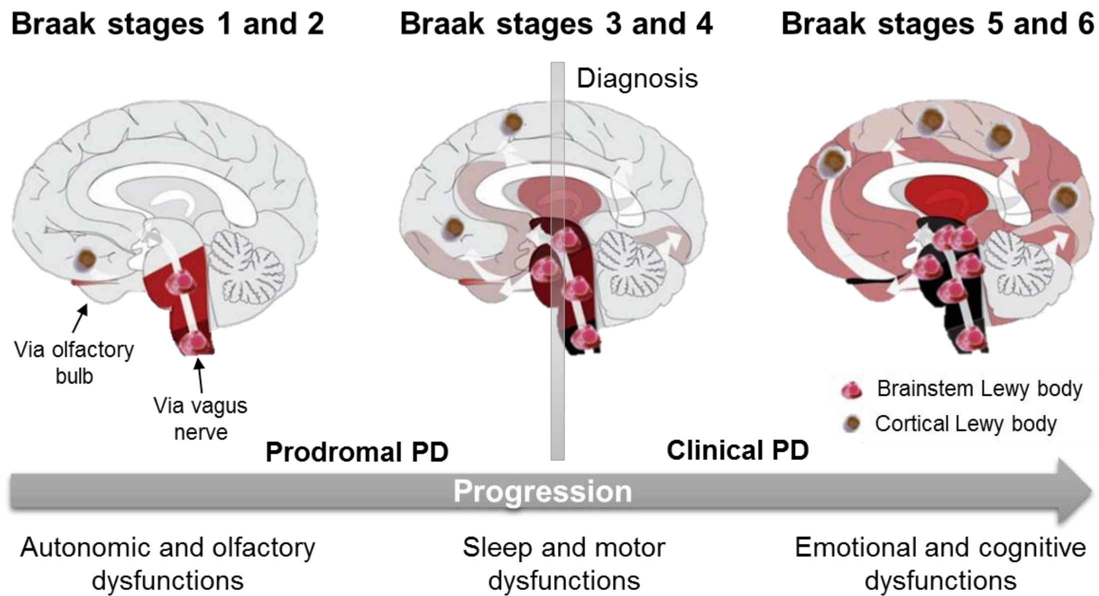


Figure I.1.1 The Braak staging for Parkinson's disease (PD) showing the initiation sites in the medulla oblongata and olfactory bulb through to the later infiltration of Lewy pathology into the neocortical regions (adapted from [47]).

Owing to the co-incidence of both neuron loss and Lewy bodies, it is often proposed that these inclusions have a causal role in neurodegeneration. However, pertinent findings achieved in the past years, such as the formation of not only Lewy bodies but also other aggregate structures of α-synuclein, the presence of inclusions containing other types of proteins (β-amyloid and Tau) in the brain of some PD patients and the occurrence of some familial forms of PD without Lewy pathology, have indicated that the PD pathology is more complex than neurodegeneration due to Lewy pathology alone [48,49].

Neuroinflammation

The neuroinflammation in PD is mainly mediated by microglia and astrocytes, though peripheral immune system is also implicated [50]. Currently, there is evidence of high concentrations of activated microglia in SNpc of early PD, leading to some discussion whether microglia activation are secondary to neurodegeneration or a primary inducer of

the disease [44,51]. Importantly, even if it is not a primary cause of the disease, neuroinflammation is undoubtedly involved in the progression of neurodegeneration since, throughout the inflammatory processes, pro-inflammatory cytokines and reactive oxygen and nitrogen species are released, exacerbating the dopamine neurons degeneration. Then, the neuronal death further activates inflammatory mechanisms, resulting in a vicious cycle of inflammation and neuronal death [50–52].

The pathogenesis of PD is still largely uncertain although several advances in its understanding have resulted from pathological observations, epidemiological findings and genetic mutations involved in familial and sporadic PD. Over the years, a variety of possible processes/mechanisms have been proposed, including α-synuclein aggregation, impairment of mitochondrial function, disruption of calcium homeostasis, excessive release of oxygen free radicals during enzymatic dopamine breakdown, loss of trophic support, abnormal kinase activity, proteasomal and lysosomal system dysfunction and neuroinflammation [49,50,53]. Hence, multiple cellular processes are variably involved in neurodegeneration in PD.

I.1.2.3. Clinical Features and Diagnosis

Clinically, PD has been regarded as a predominantly motor disorder although it is nowadays broadly accepted that a wide range of non-motor symptoms is also an integral part of the illness [54].

From the motor viewpoint, most PD patients manifest the cardinal symptoms of parkinsonism, which are bradykinesia, rigidity, rest tremor, and postural instability (Table I.1.2). Importantly, the postural instability occurs only in later stages of PD, otherwise it suggests other diagnosis rather than PD [55]. Hence, it has been suggested the omission of this item from the list of cardinal motor features of PD [56]. The cardinal symptoms have an insidious, progressive, and characteristically asymmetric onset. Interestingly, because of their gradual onset, the earliest symptoms might be unnoticed or misinterpreted for a long time and, consequently, some subtle motor features can be present before diagnosis [57,58]. Therefore, nowadays, it is not appropriate to use the term "premotor" for a PD stage that precedes the clinical diagnosis. Currently, according to the recommendations of the International Parkinson and Movement Disorder Society (MDS), the PD should be divided into three stages: *preclinical PD*, characterized by a neurodegenerative process without clinical symptoms; *prodromal PD*, presence of early symptoms and signs before PD diagnosis is possible; and *clinical PD*, diagnosis of PD has been made based on the presence of cardinal motor signs [59,60]. PD also presents

secondary motor symptoms (Table I.1.2) and some of them occur as a result of the combination of specific cardinal motor symptoms and/or of their worsening [61–63].

Table I.1.2 Motor and non-motor symptoms of Parkinson's disease [54,61,62,64–67].

Motor symptoms	Non-motor symptoms
Cardinal motor symptoms	*Autonomic symptoms*
Bradykinesia Slowness of voluntary movements with a progressive loss of amplitude or speed **Rigidity** Resistance to the passive movement occurring in both flexor and extensor muscles throughout the range of motion of a joint **Rest tremor** (4–6Hz frequency) Rhythmic oscillatory involuntary movement that arises when the affected body part is relaxed and supported by a surface **Postural instability**[a] Loss of postural reflexes	**Gastrointestinal** (constipation, dysphagia[b], sialorrhea[b], reflux) **Urinary** (bladder urgency and frequency, nocturia) **Orthostatic hypotension** **Sexual dysfunction** (loss of libido, erectile dysfunction, premature ejaculation, vaginal dryness, failure of orgasm) **Hyperhidrosis, seborrhoea**
	Neuropsychiatric symptoms
Secondary motor symptoms	**Depression, anxiety, apathy** **Psychosis** (hallucinations, illusions, delusions) **Impulse control disorders** (hypersexuality, pathological gambling, compulsive eating and shopping) **Mild cognitive impairment, dementia** **Anhedonia** (i.e., loss of the capacity to experience pleasure)
Hypomimia (i.e., decreased facial expression and eye blinking) **Hypophonia** (i.e., soft voice) **Dysarthria** (i.e., difficulty in articulating language) **Dysphagia**[b] (i.e., difficulty in swallowing) **Sialorrhea**[b] (i.e., drooling) **Micrographia** (i.e., small handwriting) **Slow gait** **Eye movement abnormalities** **Freezing of gait** **Flexed posture** **Camptocormia** (i.e., extreme flexion of the thoracolumbar spine) **Dystonia** (i.e., sustained muscle contractions) **Festination** (i.e., involuntary quickening of gait) **Falls**	*Sleep disorders* **Insomnia** **REM sleep behaviour disorder** **Excessive daytime sleepiness** **Restless legs syndrome** **Sleep apnoea syndrome** *Sensory dysfunctions* **Hyposmia** (i.e., loss of sense of smell) **Ageusia** (i.e., loss of sense of taste) **Visual disturbances** **Paraesthesia** (i.e., abnormal sensations) **Pain** *Other symptoms* **Fatigue, weight loss**

[a]Occurs only in later stages of Parkinson's disease (PD), thus, in the recently published International Parkinson and Movement Disorder Society clinical diagnostic criteria, it is not considered as a cardinal feature of parkinsonism due to PD. [b]Mixed symptoms. REM, rapid eye movement.

PD is also associated with several non-motor symptoms (Table I.1.2), including autonomic and neuropsychiatric symptoms, sleep disorders, sensory dysfunctions and others that significantly impair the quality of patient's lives [54,64]. Neuropathologically, the extranigral distribution of Lewy pathology may explain many of these symptoms. However, to date, it has not yet been proven that α-synuclein accumulation causes those manifestations [68]. Although the non-motor symptoms were frequently related to late or advanced stages of PD, it is presently well-known that they can occur at any stage of the disorder; actually, some of the non-motor symptoms may appear years or decades before the appearance of the cardinal motor symptoms [64,69]. Indeed, hyposmia, rapid eye movement (REM) sleep behaviour disorder, constipation, excessive daytime sleepiness, depression, and anxiety are non-motor symptoms that may precede de PD diagnosis (prodromal symptoms), which is consistent with Braak hypothesis discussed in *section I.1.2.2.* of this thesis [70,71]. Among them, hyposmia is the most prevalent, affecting 36% of individuals prior to diagnosis. Nevertheless, the difference of prevalence of these prodromal symptoms is generally low among cases and controls and they are relatively common in the ageing population [70]. Hence, excluding REM sleep behaviour disorder, which seems to be relatively specific of neurodegeneration, the other symptoms are not specific to predict future PD development [70,72]. Notwithstanding, the predictive value to develop PD might increase when multiple prodromal symptoms are present in the same individual [71].

Initially, motor symptoms can be managed with symptomatic therapies, being the dopaminergic drugs the most effective ones [16]. However, as the disease progresses, the motor and non-motor symptoms worsen and the majority of patients develop a range of complications, including motor and non-motor fluctuations and dyskinesia, which are related to chronic levodopa treatment and, albeit to a lesser extent, to chronic dopamine agonist therapy [73,74].

The motor fluctuations are characterized by alternations between periods when patients experience a good response to medication having a good motor symptoms control (on-period) and periods when parkinsonian motor symptoms re-emerge (off-period) [73]. These fluctuations can be categorized into [74,75]:

- "wearing-off phenomena or end-of-dose", when the motor symptoms appear before the next dose of levodopa as a result of reduction in duration of motor benefit following each dose;
- "delayed-on" and "no-on response", when some doses of levodopa can take more time to become effective or its benefit is absent;

- "freezing", acute freezing of motor behaviour that could occur during on- or off-periods;
- "unpredictable on-off fluctuations", when the motor benefit of drug intake is lost, resulting in sudden and unpredictable shifts between on-periods and off-periods.

The non-motor fluctuations consist of alternations between periods of good and reduced control of non-motor symptoms. Interestingly, many of non-motor symptoms also fluctuate in response to levodopa (i.e., worsening during off-periods and improving during on-periods with levodopa or other dopaminergic drugs administration), but some of them (e.g., psychosis, agitation) seem to be only related to elevated levels of levodopa, appearing during on-period [73].

Dyskinesia occurs mainly in patients with motor fluctuations, and consists of involuntary choreiform, ballistic and/or dystonic movements [73,76]. Based on temporal relation to levodopa dosing, the levodopa-induced dyskinesia (LID) can be classified into [74,76]:

- "peak-dose dyskinesia", the most common type of LID which occurs at maximum plasma levodopa levels;
- "off-period dystonia", when dyskinesia emerges during off-period and presents predominantly dystonic movements;
- "diphasic dyskinesia", the less common form of LID that appears at the beginning and at the end of the clinical efficacy of levodopa.

To date, apart from genetic testing for people with family members with a known monogenic form of PD, there is no effective tests or validated biomarkers available to confirm the PD diagnosis during patient life [61,77]. Consequently, the diagnosis remains merely clinical and it becomes definitive only after patient death with the presence of SNpc degeneration and Lewy pathology on autopsy [49]. In addition, it can only be made when the cardinal symptoms of motor parkinsonism emerge. Up to now, the UK Parkinson's Disease Society Brain Bank criteria (Table I.1.3) are those most commonly used to perform the clinical diagnosis of PD [56,78]. However, in the last two decades huge advances in research are changing profoundly our understanding of PD, thus some aspects of those diagnostic criteria needed to be reconsidered. In this context, the MDS created a task force to develop revised diagnostic criteria for PD [56,71], and recently published the MDS clinical diagnostic criteria for PD, which are under validation [78,79]. Moreover, it was also developed and proposed the first research criteria for prodromal PD (MDS research criteria for prodromal PD) [80].

Table I.1.3 The UK Parkinson's Disease Society Brain Bank clinical diagnostic criteria [49,81].

Step 1: Diagnosis of parkinsonian syndrome
Bradykinesia (slowness of initiation of voluntary movement with progressive reduction in speed and amplitude or repetitive actions) and at least one of the following features:
▪ Muscular rigidity
▪ 4–6 Hz rest tremor
▪ Postural instability not caused by primary visual, vestibular, cerebellar, or proprioceptive dysfunction
Step 2: Exclusion criteria
History of
repeated strokes with stepwise progression of parkinsonian features
repeated head injury
definite encephalitis
Oculogyric crises
Neuroleptic treatment at onset of symptoms
More than one affected relative[a]
Sustained remission
Strictly unilateral features after 3 years
Supranuclear gaze palsy
Cerebellar signs
Early severe autonomic involvement
Early severe dementia with disturbances of memory, language, and praxis
Babinski sign
Presence of a cerebral tumour or communicating hydrocephalus on CT scan or MRI
Negative response to large doses of levodopa (if malabsorption excluded)
MPTP exposure
Step 3: Supportive positive criteria (three or more required for diagnosis of definite PD)
Unilateral onset
Rest tremor present
Progressive disorder
Persistent asymmetry affecting the side onset most
Excellent response to levodopa (70%–100%)
Severe levodopa-induced chorea
Levodopa response for 5 years or more
Clinical course of 10 years or more

[a]This criterion is generally no longer used. CT, computerized tomography; MRI, magnetic resonance imaging; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PD, Parkinson's disease.

The MDS clinical diagnostic criteria for PD were projected specifically for use in research, but they can also be used to guide clinical diagnosis. The purely clinical diagnosis only confirmed on autopsy (pathologic confirmation) remains. Nevertheless, it was created an exception category which includes genetic cases that meet the clinical PD criteria but do not have Lewy pathology on autopsy [60]. Overall, in these new criteria (Table I.1.4) the postural instability as a criterion for parkinsonism caused by PD was not preserved; dementia was removed from the exclusion criteria, even if it starts before the second year of parkinsonism; non-motor symptoms were incorporated since it has become clear that non-motor manifestations often occur before motor symptoms emerge [59,60,78].

In contrast to the MDS clinical diagnostic criteria for PD, the MDS research criteria for prodromal PD were only developed for research purposes and their use in clinical practice is not recommended since no treatment for PD has yet been found to be conclusively neuroprotective/disease-modifying [80]. The prodromal stage of PD provides a potential temporal window during which a neuroprotective/disease-modifying therapy might be administered in order to prevent or delay the development or progression of disorder [49]. Therefore, a primary use of these criteria will be for enrolling patients in disease modification/neuroprotective trials. These criteria are based upon the likelihood of a patient presenting prodromal PD. To diagnosis a probable prodromal PD is required that the individual's final probability of prodromal disease is ≥ 80%, which is estimated using a statistical method, the Bayesian naïve classifier. Concisely, a previous probability of prodromal PD is outlined based on age (prior probability) and a total likelihood ratio is estimated based on diagnostic information, including environmental risk variables, genetic risk variables, prodromal signs and symptoms, or biomarker testing. Then, the individual's final probability of prodromal PD is calculated considering both prior probability and total likelihood ratio [60,80].

Table I.1.4 The International Parkinson and Movement Disorder Society clinical diagnostic criteria for Parkinson's disease [78].

<table>
<tr><th colspan="2">Step 1: Diagnosis of parkinsonian syndrome</th></tr>
<tr><td colspan="2">Bradykinesia [slowness of movement and decrement in amplitude or speed (or progressive hesitation/halts) as movements are continued] and at least one of the following features:
▪ Rigidity
▪ 4–6 Hz rest tremor</td></tr>
<tr><td rowspan="2">Step 2: Diagnosis of PD</td><td>Diagnosis of clinically established PD requires:
- Absence of absolute exclusion criteria
- At least two supportive criteria
- No red flags</td></tr>
<tr><td>Diagnosis of clinically probable PD requires:
- Absence of absolute exclusion criteria
- Presence of red flags counterbalanced by supportive criteria:
If 1 red flag is present, there must also be at least 1 supportive criterion
If 2 red flags, at least 2 supportive criteria are needed
No more than 2 red flags are allowed for this category</td></tr>
<tr><td colspan="2">Supportive criteria</td></tr>
<tr><td colspan="2">1. Clear and dramatic beneficial response to dopaminergic therapy
2. Presence of levodopa-induced dyskinesia
3. Rest tremor of a limb, documented on clinical examination (in past, or on current examination)
4. The presence of either olfactory loss or cardiac sympathetic denervation on MIBG scintigraphy</td></tr>
<tr><td colspan="2">Absolute exclusion criteria (the presence of any of these features rules out PD)</td></tr>
<tr><td colspan="2">1. Unequivocal cerebellar abnormalities, such as cerebellar gait, limb ataxia, or cerebellar oculomotor abnormalities
2. Downward vertical supranuclear gaze palsy, or selective slowing of downward vertical saccades
3. Diagnosis of probable behavioural variant frontotemporal dementia or primary progressive aphasia within the first 5 years of disease
4. Parkinsonian features restricted to the lower limbs for more than 3 years
5. Treatment with a dopamine receptor blocker or a dopamine-depleting agent in a dose and time-course consistent with drug-induced parkinsonism
6. Absence of observable response to high-dose levodopa despite at least moderate severity of disease
7. Unequivocal cortical sensory loss, clear limb ideomotor apraxia, or progressive aphasia
8. Normal functional neuroimaging of the presynaptic dopaminergic system[a]
9. Documentation of an alternative condition known to produce parkinsonism and plausibly connected to the patient's symptoms, or, the expert evaluating physician, based on the full diagnostic assessment feels that an alternative syndrome is more likely than PD[b]</td></tr>
</table>

Table I.1.4 *(Continued)*

Red flags (potential signs of alternate pathology)
1. Rapid progression of gait impairment requiring regular use of wheelchair within 5 years of onset
2. A complete absence of progression of motor symptoms or signs over 5 or more years unless stability is related to treatment
3. Early bulbar dysfunction: severe dysphonia or dysarthria or severe dysphagia within first 5 years
4. Inspiratory respiratory dysfunction: either diurnal or nocturnal inspiratory stridor or frequent inspiratory sighs
5. Severe autonomic failure in the first 5 years of disease. This can include: - Orthostatic hypotension (orthostatic decrease of blood pressure within 3 min of standing by at least 30 mm Hg systolic or 15 mm Hg diastolic, in the absence of dehydration, medication, or other diseases that could plausibly explain autonomic dysfunction), or - Severe urinary retention or urinary incontinence in the first 5 years of disease (excluding long-standing or small amount stress incontinence in women), that is not simply functional incontinence. In men, urinary retention must not be attributable to prostate disease, and must be associated with erectile dysfunction
6. Recurrent (>1/year) falls because of impaired balance within 3 years of onset
7. Disproportionate anterocollis (dystonic) or contractures of hand or feet within the first 10 years
8. Absence of any of the common non-motor features of disease despite 5 years disease duration, including sleep dysfunction (sleep-maintenance insomnia, excessive daytime somnolence, symptoms of REM sleep behaviour disorder), autonomic dysfunction (constipation, daytime urinary urgency, symptomatic orthostasis), hyposmia, or psychiatric dysfunction (depression, anxiety, or hallucinations)
9. Otherwise-unexplained pyramidal tract signs, defined as pyramidal weakness or clear pathologic hyperreflexia (excluding mild reflex asymmetry and isolated extensor plantar response)
10. Bilateral symmetric parkinsonism. The patient or caregiver reports bilateral symptom onset with no side predominance, and no side predominance is observed on objective examination

[a]If no imaging has been performed, this criterion does not apply. [b]Dementia with Lewy bodies is not considered an alternative parkinsonian syndrome according to this criterion. MIBG, metaiodobenzylguanidine; PD, Parkinson's disease; REM, rapid eye movement.

I.1.3. Pharmacotherapy of Parkinson's Disease

In spite of the huge effort in developing effective treatments for PD along the last few years, it remains incurable. In fact, although new drugs have been developed, a clear protective/disease-modifying therapy that retards or stops the progression of PD is an unmet therapeutic need. Different compounds, such as creatine, coenzyme Q10, pramipexole, vitamin E, selegiline and rasagiline, and gene and cell-based therapies were tested in neuroprotective trials, but all of them failed to demonstrate neuroprotective effect [17,82,83]. Therefore, even though other neuroprotective trials with promising compounds are ongoing, the current therapeutic options available to treat PD only offer symptomatic relief [84–86]. In the absence of cure, the focus of care for patients with PD is the improvement of disease-related motor and non-motor symptoms in order to maintain independence, as well as the best quality of life possible. Specifically, maintenance of function and ability to execute daily-life activities, control of motor and non-motor symptoms, and minimization of adverse effects and treatment complications are aspects taken into account when the treatment strategy is selected [87].

The therapeutic options available to treat PD include, besides the classic pharmacological therapy, surgical procedures (considered only in advanced PD patients) and non-pharmacological therapies [88]. PD is a progressive and debilitating disorder with considerable physical, psychological and social repercussions. Hence, in addition to pharmacological interventions, the PD patients should also have access to non-pharmacological strategies, such as physical and exercise therapies, speech and language therapy, and occupational therapy. Although some of these non-pharmacological interventions have insufficient evidence of efficacy, they seem to improve mobility, activities of daily living, communication and quality of life [88,89].

For some decades, the motor symptoms relief was the goal of PD treatment being the non-motor features overlooked. Therefore, the conventional treatment of PD is characterized mainly by the use of antiparkinsonian drugs, which fundamentally improve the motor symptoms of disorder. Nevertheless, it is currently known that the wide range of non-motor symptoms (Table I.1.2) causes relevant functional disability and influences negatively the quality of life of PD patients. Therefore, these symptoms must be treated in parallel with the motor symptoms [90–92]. Some of the non-motor symptoms respond to dopaminergic therapy, while others improve with the reduction, substitution or discontinuation of antiparkinsonian medications and/or the administration of other type of drugs, such as antidepressants, cholinesterase inhibitors and atypical antipsychotics [89,93]. However, robust evidence to guide pharmacologic therapy of non-motor symptoms is scarce, being the treatment management often based on clinical experience

and evidence from other disease areas [16,94,95]. Notwithstanding the great importance of treating the PD-related non-motor symptoms, this thesis will focus on the pharmacological treatment of motor symptoms and treatment-related motor complications.

I.1.3.1. Antiparkinsonian Drugs Used to Treat Motor Symptoms and Treatment-related Motor Complications

As discussed in *section I.1.2.2.*, a pathological hallmark of PD is the loss of dopaminergic neurons within the SNpc that leads to a dopaminergic deficit in the striatum of PD patients with consequent dysregulation of motor function and appearance of disorder-related motor symptoms. Therefore, the treatment of the motor symptoms is focused on increasing cerebral dopamine availability or stimulating dopamine receptors in the brain. This restoration of dopaminergic transmission has been achieved by using the precursor of dopamine (levodopa), inhibitors of some enzymes involved in the levodopa and dopamine metabolism, and dopamine receptors agonists [96]. Another approach to treat the motor symptoms occasionally used is the administration of anticholinergic drugs to correct the imbalance between dopamine and acetylcholine neurotransmission observed in the basal ganglia of PD patients [75].

The main pharmacological treatment options for motor symptoms and complications of PD with a summary of their key advantages and disadvantages are listed in Table I.1.5 with a brief contextualization of their use in the PD pharmacotherapy hereafter presented.

Table I.1.5 The main pharmacological treatment options for relief of motor symptoms and complications of Parkinson's disease and their key advantages and disadvantages [49,75,96–98].

Examples of drugs	Advantages	Disadvantages
Dopamine precursor + peripheral AADC inhibitor		
Levodopa + carbidopa Levodopa + benserazide	The most effective antiparkinsonian treatment; improves disability and prolongs the ability to execute daily-life activities.	Development of dopaminergic adverse effects (nausea, vomiting, orthostatic hypotension), sedation, motor complications (dyskinesias and motor fluctuations) and non-motor fluctuations; does not treat the non-dopaminergic symptoms of PD.
Dopamine agonists		
Ergot derived Bromocriptine Dihydroergocryptine Pergolide Cabergoline Lisuride **Non-ergot derived** Pramipexole Ropinirole Rotigotine Piribedil Apomorphine[a]	Can be used as monotherapy in early disease or as adjunct to levodopa to treat motor complications; effective at any stage of PD; lower potential to induce motor complications than levodopa; levodopa-sparing effect (i.e., capacity to delay the need of levodopa institution).	Development of dopaminergic adverse effects, neuropsychiatric adverse effects (psychosis, hallucinations, impulse control disorder), excessive daytime sleepiness and peripheral oedema; do not eliminate the need for levodopa; do not treat the non-dopaminergic symptoms of PD. Ergoline agonists may cause erythromelalgia, and cardiovascular, retroperitoneal and pulmonary fibrosis.
MAO-B inhibitors		
Rasagiline Selegiline	Can be used as monotherapy in early disease or as adjunct to levodopa to treat motor complications; once-daily dosing; well tolerated; levodopa-sparing effect.	Modest antiparkinsonian effect; theoretical risk of serotonin syndrome and "cheese effect". Selegiline in particular develops amphetamine and methamphetamine metabolites-related adverse effects.
COMT inhibitors		
Entacapone Tolcapone	Used as adjunct to levodopa to treat motor complications; no titration.	Both develop dopaminergic adverse effects (related to increase of levodopa delivery), diarrhoea and harmless discoloration of urine. Tolcapone is associated with fatal liver toxicity and explosive diarrhoea.

Table I.1.5 *(Continued)*

Examples of drugs	Advantages	Disadvantages
Anticholinergics		
Trihexyphenidyl Benztropine Biperiden Orphenadrine Procyclidine	Useful for treating patients younger than 60 years old with a predominant tremor and without cognitive impairment.	Limited efficacy for the majority of PD features; troublesome central and peripheral anticholinergic adverse effects (cognitive impairment, constipation, dry mouth, urinary retention); associated with withdrawal effects.
NMDA receptor antagonist		
Amantadine	Can be used as monotherapy in early disease or as adjunct to levodopa to treat dyskinesia.	Modest antiparkinsonian benefit; development of cognitive adverse effects, livedo reticularis, oedema and tolerance; potential for withdrawal effects.

[a]Used only as adjunct to levodopa in advanced disease. AADC, aromatic *L*-amino acid decarboxylase; COMT, catechol-*O*-methyltransferase; MAO-B, monoamine oxidase-B; NMDA, *N*-methyl-*D*-aspartate; PD, Parkinson's disease.

Levodopa and peripheral AADC inhibitors

Almost 50 years after levodopa clinical introduction, it remains the "gold standard" therapy for treatment of PD-related motor symptoms [82]. Remarkably, levodopa is superior to all other antiparkinsonian treatments that have emerged and the drug against which new therapies must be compared [75,99]. Few years after its introduction in the clinical practice, the hypothesis that levodopa may induce some toxicity on dopamine neurons and accelerate the PD progression was raised because of its predisposition to undergo oxidative metabolism and generate reactive oxygen species. Although no robust evidence of levodopa toxicity has emerged to date from preclinical, pathological, clinical and epidemiological studies, the issue of levodopa toxicity cannot be totally discarded [100,101]. Nevertheless, despite this concern, it is globally recommended to use levodopa as the standard of care for PD, but at the lowest dose that provides satisfactory clinical benefit [101].

Levodopa (*L*-DOPA) is the immediate biological precursor of dopamine (Figure I.1.2). It is well succeeded *in vivo* because, in opposition to dopamine, levodopa crosses the blood-brain barrier (BBB) [102]. Structurally, it is an amino acid but it is not a component of structural proteins [103]. Levodopa is mainly absorbed in the duodenum and proximal jejunum by active transport through the saturable *L*-neutral amino acid transport system, competing, thereby, with the *L*-neutral amino acids from the dietary proteins. Likewise,

it also utilizes that transport system to cross the BBB and reach the biophase [96,103]. Hence, in order to avoid food-levodopa interactions, levodopa should be preferably ingested 1 h before or 1 h after eating [75].

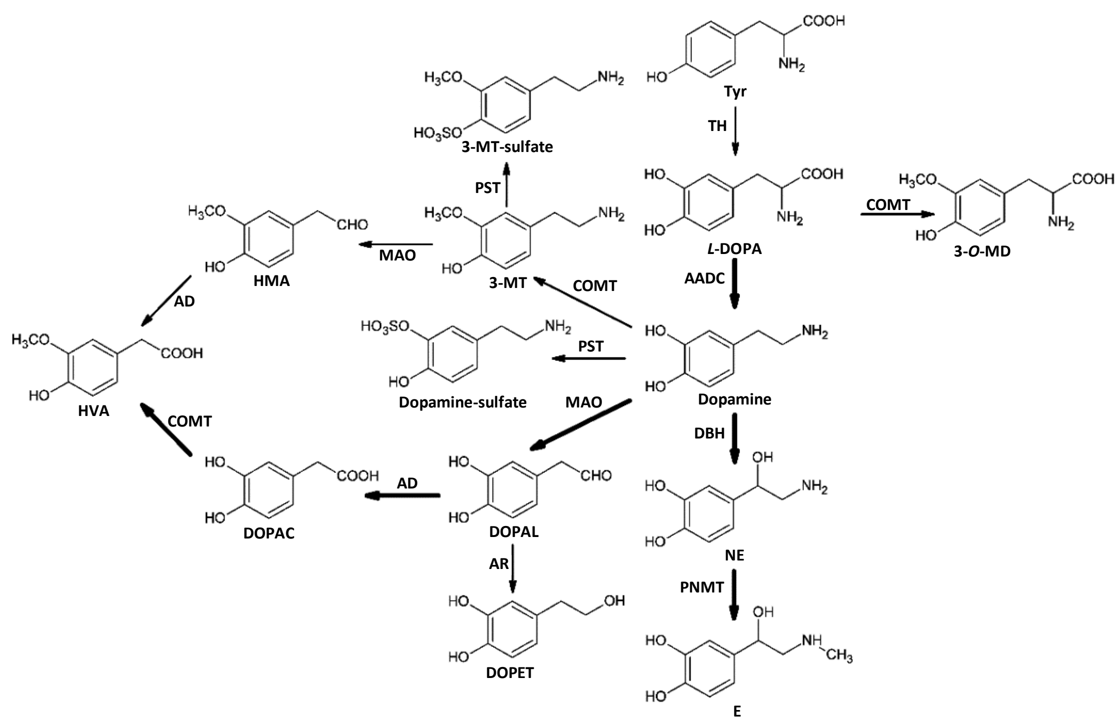


Figure I.1.2 Biosynthesis and metabolism of dopamine (adapted from [104]). AADC, aromatic *L*-amino acid decarboxylase; AD, aldehyde dehydrogenase; AR, aldehyde reductase; COMT, catechol-*O*-methyltransferase; DBH, dopamine-β-hydroxylase; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPAL, 3,4-dihydroxyphenylacetaldehyde; DOPET, 3,4-dihydroxyphenylethanol; E, epinephrine; HMA, 4-hydroxy-3-methoxyphenylacetaldehyde; HVA, homovanillic acid; *L*-DOPA, *L*-3,4-dihydroxyphenylalanine; MAO, monoamine oxidase; NE, norepinephrine; PNMT, phenylethanolamine-*N*-methyltransferase; PST, phenolsulfotransferase; TH, tyrosine hydroxylase; Tyr, tyrosine; 3-*O*-MD, 3-*O*-methyldopa; 3-MT, 3-methoxytyramine.

In spite of the great improvement in the management of motor symptoms of PD achieved with the administration of levodopa, it soon became clear that the levodopa given alone had several limitations, such as its very low bioavailability, short plasma elimination half-life ($t_{1/2el}$ ~ 50 min), long titration period, and frequent occurrence of adverse effects. In general, only 30% and 1% of an oral dose of levodopa reaches the systemic circulation and the brain, respectively, even though just 2% of the parent drug is excreted unmodified in the faeces. Hence, those drawbacks were mostly assigned to the decarboxylation of levodopa to dopamine (the main metabolic pathway of levodopa; Figure I.1.2) in the extracerebral tissues by the aromatic *L*-amino acid decarboxylase (AADC), also called dopa

descarboxylase [102,105]. This is an ubiquitous and non-specific enzyme with high activity in the gastrointestinal tract, liver, and in the brain and its capillaries [106]. Indeed, the decarboxylation of levodopa in the gastrointestinal tract and liver is responsible for its extensive first-pass metabolism, rapid plasma clearance, and for the formation of a great amount of peripheral dopamine which consequently triggers the levodopa-related peripheral dopaminergic side effects (e.g., nausea, vomiting, and orthostatic hypotension) [13].

In this context, the first and logic improvement in the levodopa therapy consisted in the association of peripheral AADC inhibitors. This strategy reduces the peripheral metabolism of levodopa, but simultaneously preserves the central AADC activity ensuring the formation of dopamine on the therapeutic target organ [105]. Soon, two AADC inhibitors unable to cross the BBB, carbidopa and benserazide, were studied and introduced into the market in combination with levodopa in 1975 [13]. Carbidopa and the active metabolite of benserazide (trihydroxybenzylhydrazine) inhibit the activity of AADC (a pyridoxal 5'-phosphate-dependent enzyme) by binding to the pyridoxal 5'-phosphate molecule, thus blocking it and inactivating the enzyme [107,108]. Since the introduction of these inhibitors, levodopa has been routinely administered in combination with one of them not only to minimize the characteristic peripheral side effects of the systemic dopamine, but also to improve its pharmacokinetics by extending its $t_{1/2el}$ (prolonged to approximately 90 min) and increasing its bioavailability, allowing a reduction in the required levodopa dosage of about 60–80%. In sum, this combination ameliorates the pharmacokinetics and safety profile of levodopa, as well as its therapeutic efficacy [105,109].

In addition to the aforementioned limitations, the chronic administration of levodopa, even with an AADC inhibitor, was quickly associated with different long-term adverse effects, such as motor complications and non-motor fluctuations. In fact, in the early disease, nigrostriatal system can convert levodopa to dopamine, store it in pre-synaptic vesicles and release it in response to physiological stimuli, and therefore levodopa has a long duration of action with a suitable symptomatic control when administered only three times per day. Nevertheless, this ability is lost through the disease progression, which seems to limit the conversion of levodopa to dopamine, implying its storage in non-neuronal cells with no longer release in response to physiological stimuli [100]. Therefore, the striatal dopamine levels are increasingly dependent on the amount of levodopa that crosses the BBB. Given the short $t_{1/2el}$ of levodopa that entails its administration in repeated doses throughout the day, the stimulation of dopamine receptors becomes intermittent leading to the appearance of motor and non-motor

complications [109]. Hence, in order to avoid or minimize these undesirable adverse effects, new classes of antiparkinsonian drugs were progressively developed, including synthetic dopamine agonists and inhibitors of MAO-B and of catechol-*O*-methyltransferase (COMT), which are involved in the metabolism of levodopa and/or dopamine (Figure I.1.2) [106].

Dopamine agonists

Dopamine agonists are drugs that, by directly stimulating the striatal dopaminergic receptors, treat the motor symptoms of PD. Structurally, they can be divided into the ergot derivatives, the older ones, and the non-ergot derivatives (Table I.1.5). Despite the differences in their chemical structures, all dopamine agonists share the ability to bind and stimulate the dopamine D2-like family of dopamine receptors (D2, D3 and D4) [110,111]. Nonetheless, some of them may also bind to D1 dopamine receptors and mnon-dopaminergic receptors such as serotonin and norepinephrine receptors [111]. Nowadays, the ergoline dopamine agonists are rarely used in clinical practice since they are associated, among other adverse effects, with fibrotic reactions (cardiovascular, retroperitoneal and pulmonary fibrosis) [17,112].

Dopamine agonists can be used as monotherapy in early stages of disease or as adjunct to levodopa therapy to treat motor complications, except apomorphine that is usually reserved for rescue therapy in patients whose PD is particularly advanced and are experiencing severe off-periods [75]. Interestingly, although they do not eliminate the need of levodopa in advanced PD stages, dopamine agonists have been increasingly employed early in the treatment of PD aiming to diminish or delay the emergence of motor complications [112].

Dopamine agonists have some advantages over levodopa: they do not need carrier-mediated transport for gastrointestinal absorption or penetration into the brain, and hence, their bioavailability is not affected by the presence of food or amino acids; they do not require further metabolism within the dopaminergic neurons to exert their pharmacologic effect, acting independently of the progressive neurodegeneration of the dopaminergic neurons, and thus, at any stage of PD; and, they mostly possess a longer $t_{1/2el}$ than levodopa, providing a more sustained stimulation of dopaminergic receptors, and thereby, having lower potential to induce motor complications [75,106,110]. However, these antiparkinsonian drugs are less effective than levodopa in treating motor symptoms and have also some risks related to their use. The acute side effects are comparable to those observed with levodopa (nausea, vomiting, orthostatic hypotension), but dopamine agonists also induce other adverse effects that are more common or specific of their

administration. The occurrence of peripheral oedema (especially in legs), psychosis, hallucinations, impulse control disorder, sleep attacks and excessive daytime sleepiness is more frequent with dopamine agonists than with levodopa, while the development of fibrotic reactions seems to be only related to the use of ergot derivatives [113,114].

MAO-B inhibitors

The introduction of selective MAO-B inhibitors in the treatment of PD symptoms was based on their ability to prolong the activity of both endogenously and exogenously derived dopamine in the striatal synapses by blocking the MAO-B-catalysed oxidation of dopamine, and hence, improving motor symptoms of disease (Figure I.1.2) [75,115]. MAO is an enzyme present in several tissues throughout the body under two isoforms: MAO-A and MAO-B, being the second one the most responsible for dopamine oxidative deamination [105,116,117]. Moreover, besides the predominance of MAO-B in the human brain, the inhibition of MAO-A (predominantly expressed in the gut and involved in the metabolism of serotonin) may induce an acute syndrome, the tyramine-induced hypertension often called "cheese effect", as well as the serotonin syndrome when another serotonergic drug is used at the same time [115]. Therefore, only selective MAO-B inhibitors are indicated for the treatment of PD-related motor symptoms, although there is also a theoretic risk of developing those syndromes, albeit very low [89,98,115].

Until 2015, solely two irreversible MAO-B inhibitors were approved for use in PD, selegiline and rasagiline. At the therapeutic doses, both compounds are selective for MAO-B, but their selectivity is lost at higher doses [105]. Rasagiline is more recent and potent than selegiline and, unlike selegiline, does not suffer conversion to amphetamine metabolites, which are responsible from some adverse effects of selegiline such as insomnia [97,105]. Both drugs are used in monotherapy in early PD patients in spite of their modest symptomatic benefit. Furthermore, they are also used as adjunct to levodopa therapy to treat motor fluctuations, even though only rasagiline demonstrated to reduce significantly off-time (period of time experiencing little to no therapeutic benefit) [16,89]. In 2015, the European Medicines Agency (EMA) approved a new potent, selective, and reversible MAO-B inhibitor, safinamide, to be used in addition to levodopa alone or in combination with other antiparkinsonian drugs, in mid-to-late-stage patients with motor fluctuations [118]. Safinamide has a double mechanism of action: dopaminergic as a result of MAO-B inhibition, leading to the improvement of motor symptoms and fluctuations; and non-dopaminergic owing to inhibition of glutamate release as a result of blockage of sodium channels and modulation of calcium channels, which might ameliorate dyskinesias and non-motor symptoms [117,119]. This new drug seems to be highly selective MAO-B

inhibitor, even at supratherapeutic doses, having a selectivity for MAO-B superior to that of selegiline and rasagiline, and thereby, a lower risk to induce the "cheese effect" and serotonin syndrome [117]. Currently, this novel MAO-B inhibitor can only be use as adjunct to levodopa to treat motor complications, since there is no evidence or insufficient evidence to support its use as monotherapy in early PD or as adjunct to dopamine agonists in patients who are not yet treated with levodopa, respectively [120].

COMT inhibitors

Notwithstanding the improvement of PD treatment with the introduction of dopamine agonists and MAO-B inhibitors, levodopa remained the most effective drug to treat the motor symptoms of PD. Hence, another interesting and promising approach using COMT inhibitors was further developed to enhance the effectiveness and diminish the long-term adverse effects of levodopa. In fact, when an AADC inhibitor is administered in combination with levodopa, the methylation of levodopa to 3-*O*-methyldopa (3-*O*-MD) by COMT (Figure I.1.2) becomes its predominant metabolic pathway, reducing the plasma levodopa available to cross the BBB [121]. Indeed, only 5–10% of an oral dose of levodopa reaches the brain after its co-administration with an AADC inhibitor [13]. Furthermore, the metabolic product 3-*O*-MD has no therapeutic value and presents a long $t_{1/2el}$ (~ 15 h) accumulating in the body during chronic levodopa therapy [122]. Importantly, the 3-*O*-MD is a competitive inhibitor of the active transport of levodopa both at the intestinal mucosa and BBB [103,123], although it has been suggested that this competition is negligible at clinical concentrations [121]. In this context, it was expected that the concomitant blockage of COMT and peripheral AADC would further diminish the metabolism of levodopa, prolonging its $t_{1/2el}$, increasing its bioavailability, and thereby, augmenting its efficacy. These potential benefits were confirmed in practice and this approach became a reality with the introduction of two reversible COMT inhibitors, tolcapone and entacapone, into the market in the late 1990's [13,122]. In fact, in single-dose studies with either of compounds at doses up to 200 mg, the inhibition of COMT increased the $t_{1/2el}$ and area under the concentration-time curve (AUC) of levodopa without leading to a significant alteration in its maximum concentration (C_{max}) and time to reach the C_{max} (t_{max}) in plasma, and decreased the C_{max} and AUC of 3-*O*-MD [122,124]. However, it was observed a delay on the t_{max} of levodopa when tolcapone and entacapone were administered at higher than therapeutic doses (> 200 mg) [122]. A possible explanation that has been considered is the competition between COMT inhibitors and levodopa for gastrointestinal absorption through the saturable *L*-neutral amino acid transport system [121,125,126]. Nevertheless, according to a recent study performed with *Xenopus laevis* oocytes, Madin-Darby canine

kidney cells and *ex vivo* preparations from wild-type and knockout mice, the major *L*-neutral amino acid transporter involved in the intestinal absorption of levodopa was identified ($b^{0,+}$AT-rBAT) and, in contrast to some amino acids, neither entacapone nor AADC inhibitors competed with levodopa for that transporter in the presence of a 1:50 ratio of levodopa to inhibitors [127]. In multiple-dose studies, COMT inhibitors increased the average concentrations and prolonged the $t_{1/2el}$ of circulating levodopa, increased the minimum concentration (C_{min}) of levodopa in plasma between doses more than its C_{max}, and reduced the daily variability of levodopa plasma concentrations [128–132]. These changes in levodopa pharmacokinetics and the more stable levodopa plasma levels can provide a more continuous levodopa delivery to the brain and a more sustained brain dopaminergic stimulation, which results in a good control of motor fluctuations [100]. Currently, the continuous dopaminergic stimulation, contrary to discontinuous or pulsatile stimulation, has been regarded as the ideal therapy to delay or reduce motor complications and, consequently, improve patient's quality of life [133,134].

Tolcapone is a potent COMT inhibitor that inhibits both peripheral and central enzyme, while entacapone is an essentially peripheral inhibitor [106]. Among both compounds, tolcapone demonstrated to be a more potent inhibitor of COMT than entacapone [135]. Consequently, tolcapone increases the levodopa AUC and decreases the 3-*O*-MD AUC more significantly than entacapone in healthy volunteers and parkinsonian patients. For a single-dose of 200 mg, the levodopa AUC increase in healthy volunteers and PD patients was respectively 88% and 58% for tolcapone, whereas entacapone increased the AUC of levodopa in 42% and 48%, respectively. With respect to 3-*O*-MD, the AUC decrease was 64% and 79% for tolcapone in healthy volunteers (single-dose of 200 mg) and parkinsonian patients (multiple-dose of 200 mg), respectively, while it was 46% and 45 to 63% for entacapone, respectively [122]. Thereby, as expected, both compounds are clinically effective in the management of motor fluctuations [122] being tolcapone more effective than entacapone, according to a recent systematic review [136]. Even though no true comparative clinical studies between tolcapone and entacapone are available, a Cochrane meta-analysis of randomized, double-blind, placebo-controlled trials of tolcapone and entacapone [137] indicates that tolcapone increases the on-time (duration of time experiencing symptom relief) and reduces the off-time more than entacapone, in relation to placebo, with an approximately 2-fold higher improvement. In detail, administering 200 mg tolcapone the weighted placebo-adjusted mean difference in on-time increase from baseline was 1.91 h and the weighted placebo-adjusted mean difference in off-time decrease from baseline was 1.63 h, while administering 200 mg entacapone was 1.01 h and 0.68 h, respectively.

However, after the introduction of tolcapone in the clinical practice, it was associated with severe hepatotoxicity, highlighting three cases of fatal fulminant hepatic failure [138,139]. Relevantly, although COMT is involved in the metabolism of dopamine into 3-methoxytyramine, the use of COMT inhibitors in monotherapy does not have antiparkinsonian effects [105], even using tolcapone that has central activity and might decrease the central metabolism of endogenous dopamine [140]. Hence, the COMT inhibitors are recommended to be used as adjunct to levodopa/AADC therapy in patients with motor fluctuations to treat wearing-off phenomena [88,89]. Nevertheless, tolcapone should only be administered to patients who are intolerant or who failed to respond to other COMT inhibitors, and under strict hepatic monitoring [141]. Very recently (June 2016), the new potent, reversible, and purely peripheral third-generation COMT inhibitor opicapone (the object of study of the present thesis) was licensed by EMA to be used as adjunct to levodopa/AADC inhibitor therapy in patients with motor fluctuations [142]. More information about COMT inhibitors and in particular about opicapone is reviewed in *section I.2*.

Other antiparkinsonian drugs

In addition to the previously described therapeutic options, other medicines, including anticholinergics and amantadine, are occasionally employed to treat the symptoms of PD.

Historically, the anticholinergics were the first drugs used in the treatment of PD, but with the introduction of levodopa their clinical use declined considerably due to their poor safety profile and less efficacy for treating the majority of PD symptoms [75]. In fact, the anticholinergic drugs are associated with many peripheral anticholinergic side effects (constipation, blurred vision, nausea, dry mouth, urinary retention), as well as limiting central anticholinergic effects, such as delirium, hallucinations and, particularly worrying, cognitive impairment more probable in older patients [98]. However, the treatment of tremor with dopamine replacement therapy is sometimes inconsistent in contrast to the remaining cardinal motor symptoms of the disease [143]. Therefore, the anticholinergic drugs are basically recommended for young patients (< 60 years) with severe resting tremor and without cognitive impairment as monotherapy or as adjunct therapy [16,75].

Amantadine inhibits non-selectively the *N*-methyl-*D*-aspartate glutamate receptors, but it may also have anticholinergic properties and enhance the dopaminergic transmission [89]. Although it improves the motor symptoms of PD when used as monotherapy in early stages, amantadine should not be the first option of treatment in that situation. Nonetheless, it has a relevant role as adjunct to levodopa therapy in patients with motor

complications for reducing dyskinesia [88,89]. In point of fact, it is considered the most effective drug for the treatment of LID currently available [76].

In the last years, research has been dedicated in part to find compounds with novel non-dopaminergic mechanisms of action, in an attempt to control motor complications with fewer dopaminergic adverse reactions. As a result, in 2013, the first adenosine A2A receptor antagonist, istradefylline, was approved in Japan as an antiparkinsonian medicine to be used as an adjunctive treatment in PD patients with wearing-off fluctuations [144].

Over the last decades, a considerable number of new antiparkinsonian drugs have emerged, but PD stays incurable and the "(g)old standard" therapy (levodopa) remains the most effective for symptomatic management of the disorder. However, during the therapy, levodopa benefits usually diminish or become less consistent. Therefore, besides the intense focus on developing protective or disease-modifying therapies, scientific community has been striving to improve the PD symptomatic therapy by trying to develop new treatments with novel therapeutic targets and better dopaminergic drugs to optimize the outcomes of levodopa therapy. In this context, the need of developing a novel COMT inhibitor with a better pharmacological profile was identified, and therefore, opicapone emerged.

Opicapone is the object of study of the project underlying this PhD dissertation, which was carried out to deepen the non-clinical pharmacological knowledge about this novel COMT inhibitor. In the next section, an overview about COMT enzyme, its inhibitors and the necessity of developing new COMT inhibitors will be given. In addition, a brief summary of the information that has been published about opicapone during the course of the present work will be also presented.

CHAPTER I

I.2. COMT Enzyme and its Inhibitors in Parkinson's Disease

I.2. COMT Enzyme and its Inhibitors in Parkinson's Disease

COMT inhibitors are drugs frequently employed in the treatment of PD as adjunct to levodopa therapy, but they could be also used in other central or peripheral nervous system disorder (e.g., restless leg syndrome, schizophrenia, depression, gastrointestinal disturbances, cardiovascular disorders) [145]. Although the clinical introduction of this therapeutic class in the late 1990s did not revolutionize the therapy of PD as did levodopa, the use of COMT inhibitors helps the PD patients that are experiencing the levodopa therapy-related motor fluctuations. The rational of the COMT inhibitors institution in the PD pharmacotherapy was explained in *section I.1.3.1.*, being directly linked to the levodopa metabolism. Succinctly, levodopa is quickly and extensively metabolised in the extracerebral tissues predominantly to dopamine by AADC. Nevertheless, this main metabolic pathway is routinely inhibited in the treatment of PD by using peripheral AADC inhibitors in combination with levodopa. Hence, the metabolism of the dopamine precursor (levodopa) shifts mostly to *O*-methylation by COMT. Therefore, the concomitant inhibition of both metabolic pathways potentiates the action of levodopa since the level of its unmetabolized form is increased (Figure I.2.1).

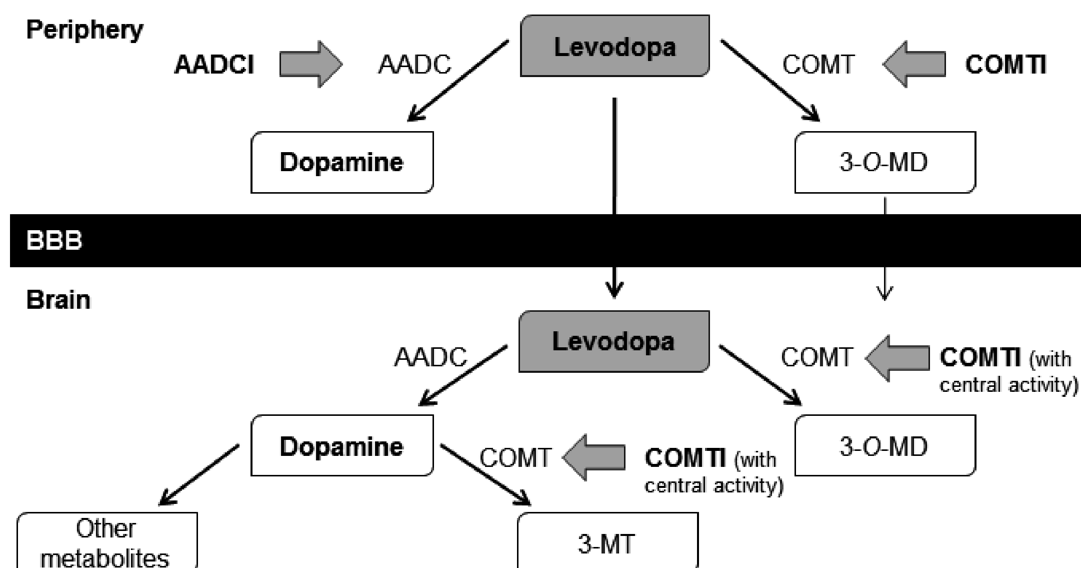


Figure I.2.1 Metabolism of levodopa by aromatic *L*-amino acid decarboxylase (AADC) and catechol-*O*-methyltransferase (COMT). AADCI, AADC inhibitors; COMTI, COMT inhibitors; 3-MT, 3-methoxytyramine; 3-*O*-MD, 3-*O*-methyldopa (adapted from [146]).

Currently, several in deep reviews are available in the literature regarding COMT enzyme, COMT inhibitors and their use in PD [122,124,126,145,147–156]. Hence, in this

thesis only a brief overview of those aspects for contextualization will be presented, focusing on the need of new COMT inhibitors and the new COMT inhibitor herein exploited, opicapone.

I.2.1. COMT Enzyme

In 1958, COMT enzyme (EC 2.1.1.6) was described and partially characterized for the first time by Julius Axelrod and Robert Tomchick [157]. It catalyses the *O*-methylation, a reaction of phase II metabolism, of catechol-containing compounds by transferring a methyl group from the coenzyme *S*-adenosyl-*L*-methionine (SAM) in the presence of magnesium ions (Mg^{2+}), originating a mono-*O*-methylated catechol and *S*-adenosyl-*L*-homocysteine [147].

The main physiological role of COMT is the inactivation and/or elimination of a wide variety of biologically active or toxic endogenous and exogenous catechols. Endogenous substrates of COMT include catecholamines (dopamine, epinephrine, and norepinephrine), their hydroxylates metabolites (e.g., 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid), levodopa, catechol oestrogens (e.g., 2-hydroxyoestradiol, 4-hydroxyoestradiol, 2-hidroxyoestrogen, 2-hydroxyoestron), and dihydroxyindolic intermediates of melanin [145]. In this context, COMT appears to play an indirect role in modulating dopaminergic functions in some peripheral organs, such as kidney and intestine, but it may also regulate the amounts of dopamine and norepinephrine in different areas of the brain and thereby its activity may be related to mood and other mental processes [148]. Interestingly, COMT has been associated with a particularly relevant role in the regulation of dopamine flux in the prefrontal cortex. In opposition to striatum, prefrontal cortex has very low levels of dopamine transporters, which have 1000-fold higher affinity for dopamine than COMT [158,159]. Therefore, COMT activity may be involved in cognitive functions [160]. Additionally, several dietary phytochemicals and therapeutic compounds are substrates of COMT, which hence acts as an enzymatic defence in blood and other tissues against xenobiotics [148]. Those exogenous substrates comprise AADC inhibitors (carbidopa and benserazide), dopamine agonists (dobutamine, apomorphine and fenoldopam), the antihypertensive α-methyldopa, the β-blocker nadolol, bronchodilating compounds (isoprenaline and rimiterol), the prodrug droxidopa, tea polyphenols, vitamin C, and many other compounds [161]. Of particular relevance is the methylation of levodopa in PD patients treated with levodopa therapy.

COMT enzyme is found in very different organisms ranging from bacteria, yeast and plants to animals, including vertebrates and invertebrates [126,147]. In mammals, COMT is expressed in two distinct intracellular isoforms: the soluble-COMT (S-COMT), essentially present in cytoplasm of cells but also in their nucleus; and, the membrane-bound isoform (MB-COMT), which is bound to intracellular membranes (predominantly to the membrane of rough endoplasmic reticulum) and oriented towards the cytoplasm [162,163].

Regarding body distribution, COMT is an ubiquitous enzyme that is distributed throughout the brain and the peripheral tissues, but its activity is significantly higher in peripheral tissues [164,165]. In brain, the protein amount and COMT activity is quite similar among the various areas, whereas in the peripheral tissues considerable differences in COMT activity and quantity were found between the diverse organs [163,164]. Liver is, by far, the tissue with the highest COMT activity followed by kidneys and gastrointestinal tract [148]. Nevertheless, COMT is also expressed in other tissues including spleen, lungs, heart, adrenals, adipocyte tissue, various glands, reproductive organs, muscles, skin, eyes and erythrocytes [147]. Although COMT presents a low activity in erythrocytes, the produced effect upon COMT activity by a compound in clinical trials is habitually evaluated by determining the erythrocyte S-COMT activity. The choice of this enzyme source is based not only on its easier and less evasive collection from the body, but also on the good correlation of COMT activity among erythrocytes and other organs, such as liver, lungs and kidneys [149,166]. Overall, in human, rat and mouse, the S-COMT is the predominant isoform in the peripheral tissues, while the MB-COMT is dominant in the brain, with the exception of rat brain [163,167–169]. In human and mouse brain, the MB-COMT represents around 70% [167] and 60% [163] of the all COMT proteins, respectively, whereas in rat it is present in a less proportion around 20–30% [170].

Even though there is two isoforms of COMT enzyme in mammals, they are coded by a single gene located on chromosome 22 band q11.21 in humans and on chromosome 11 band q23 in rats [126]. The structure of *COMT* gene in human and rat was solved some years ago, showing an overall genomic organization similar between both species [167,171]. The human gene is composed of six exons of which two are non-coding, while rat gene is composed by five exons being the first one non-coding. In both species, there are two promoters (P1 and P2) that control transcription of two different mRNAs from the same gene. The P1 promoter regulates the expression of the shorter transcript and the P2 promoter is responsible for the expression of the longer transcript. In its turn, the shorter transcript produces only S-COMT, whereas the longer transcript produces MB-COMT but also S-COMT [167,171].

Human and rat S-COMT contain 221 amino acids with a sequence identity of 81%, while MB-COMT has an additional peptide in its amino terminal of 50 (human) or 43 (rat) amino acid residues that contains the hydrophobic membrane-anchor region [167,172]. Both isoforms have similar active sites sharing the same kinetic mechanism, with similar affinities for SAM, inhibition by calcium ion, magnesium ion requirement, a similar optimal pH for activity, and recognition by S-COMT antiserum [148]. However, MB-COMT has a significantly higher substrate affinity (10- to 100-fold higher) for catecholamines than S-COMT, and this characteristic seems to be preserved among different species [126]. In contrast, S-COMT has a significantly higher capacity (higher catalytic activity) than MB-COMT [173]. Gathering these kinetic divergences (affinity and capacity) of both isoforms and their differences in central and peripheral tissues distribution, it has been proposed at least in part distinct roles for the two COMT isoforms. MB-COMT is supposed to be primarily involved in the termination of dopaminergic and noradrenergic synaptic neurotransmission at physiologically relevant low concentrations of catecholamines [174]. On the other hand, S-COMT is believed to be mostly responsible for the elimination of biologically active or toxic (particularly exogenous) catechols and some hydroxylated metabolites, thereby acting as an enzymatic detoxifying barrier [164].

Since the purification of COMT, different reaction kinetic mechanisms have been proposed, but soon became clear that the reaction proceeds through a direct nucleophilic attack by one of the hydroxyl groups of substrate (a catechol-contained compound) on the methyl carbon of SAM via a tight S_N2-type transition state without the involvement of a methylated enzyme intermediate (Figure I.2.2) [149,175,176]. Then, a sequential ordered mechanism was suggested based on product inhibition studies, which was revised few years later with the aid of crystallographic studies [176]. As reviewed by Ma *et al.* [177], several crystallographic studies of human and rat COMT have been performed and the obtained three-dimensional crystal structures revealed that the structural fold of COMT is similar in both species and showed the spatial location of the enzyme active site, the binding site of each involved component on the reaction, and the interactions between them. Hence, by conjugating data from crystallographic and enzymatic studies, it was possible to comprehend deeper the mechanism of *O*-methylation and the precise binding order of ligands, which is essential for the catalytic activity of COMT. Interestingly, it was noticed that the active site of COMT comprises two separate regions: the effective catalytic region set in a shallow groove on the outer surface of COMT, where the Mg^{2+} and the catechol substrate bind, and the SAM-binding pocket located deeper inside the protein behind the Mg^{2+}-binding site. Due to this spatial position of the binding sites, only the sequence of binding with SAM binding first to the COMT, followed by Mg^{2+} and, lastly, the

substrate is possible. If this binding order is shift one or both of the remained ligands are unable to access their binding pocket and the enzyme is incapable of performing its function [126,173,178]. The *O*-methylated catechol substrate subsequently dissociates and the product *S*-adenosyl-*L*-homocysteine needs to be exchanged by a new molecule of SAM to allow a new catalytic cycle [172,179].

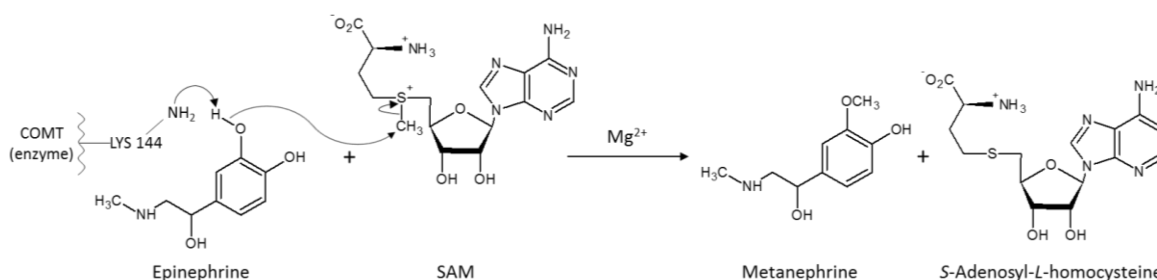


Figure I.2.2 The *O*-methylation reaction of epinephrine to metanephrine catalysed by catechol-*O*-methyltransferase (COMT), as an example (adapted from [126,180]). SAM, *S*-adenosyl-*L*-methionine.

I.2.1.1. *COMT* Gene Polymorphisms

More than 300 polymorphisms have been reported for *COMT* gene despite several of them remain unconfirmed and others occur with very low frequency. Although the vast majority of polymorphic variants are within non-coding sequence and are of unknown function, a small number of single nucleotide polymorphisms and also haplotypes are either of known function or have been connected to COMT mRNA expression [181]. The functional polymorphisms lead to alterations in the enzymatic activity of the encoded COMT enzyme, which may have neurobiological effects since COMT has a crucial role in the regulation of dopamine levels, specifically in the prefrontal cortex [182].

The most widely studied variation of *COMT* gene is the common Val108/158Met single nucleotide polymorphism (rs4680) [181]. It involves a single nucleotide change from guanine to adenine in the gene sequence, causing a valine (Val) to methionine (Met) substitution at position 108 or 158 of the S-COMT or MB-COMT amino acid sequence, respectively. The Met variant has a lower thermostability and therefore a lower COMT activity is observed in Met homozygotes ($Met^{158(108)}/Met^{158(108)}$) compared with Val homozygotes ($Val^{158(108)}/Val^{158(108)}$) [158,173,183]. Since these alleles are codominant, the $Val^{158(108)}/Met^{158(108)}$ heterozygotes have intermediate levels of COMT activity, explaining the observed trimodal distribution of COMT activity in humans. In fact, as result of the existence of this polymorphism, the human COMT activity is characterized as high

(COMT^{HH}), intermediate (COMT^{HL}) and low (COMT^{LL}) [126,181]. This polymorphism presents ethnic differences with the low COMT activity allele being less common in Asians and Africans than in Caucasians [159].

Over the last decades, different genes have been identified and related to PD (*section I.1.2.1.*). Interestingly, the *COMT* polymorphism is also believed to be related to the risk of developing PD. However, according to the results of two recent meta-analyses there is no association between *COMT* gene polymorphisms and the risk of developing PD [183,184]. Nevertheless, another very recent meta-analysis indicates that individuals with the *COMT^{LL}* genotype in combination with the *MAO-B* intron 13 A allele present an increased PD susceptibility in the Asian population, suggesting a coaction of *COMT* polymorphism with other genes to the risk of developing PD [185]. The possible influence of *COMT* genotypes on neuropharmacological variables of PD is controversial, with some studies indicating a relationship between *COMT* genotype and the response to levodopa treatment, the susceptibility to develop motor complications or the effect of COMT inhibitors, while many other studies did not show any relationship [159,184].

I.2.2. COMT Inhibitors

Since the characterization of COMT enzyme, various COMT inhibitors have been developed and studied. Currently, besides the classifications based on their structural core or mechanism of inhibition [145,161], COMT inhibitors have been distributed in four groups: first-generation, second-generation, late atypical inhibitors [126], and third-generation [186].

The first-generation of COMT inhibitors comprises the early compounds identified as having COMT inhibitory properties and a comprehensive review of their pharmacological properties was published by Guldberg and Marsden [147]. Those compounds are typically competitive substrates of COMT and include pyrogallol and its derivatives (e.g., gallic acid), catechols and derivatives (e.g., caffeic acid, 2-hydroxyoestrogens, U-0521, flavonoids), tropolones, 8-hydroxyquinolines, 3-hydroxylated pyrones and pyridones, and iodophenol derivatives [126,147]. However, these first-generation COMT inhibitors were never introduced in clinical practice because of their poor selectivity for COMT, low efficacy *in vivo*, and unacceptable toxicity (mainly central nervous system effects and hepatotoxicity) [148,187,188].

The interest in COMT inhibitors revived only in the late 1980s with the development of the named second-generation COMT inhibitors [122]. All of them share the same nitrocatechol structure, are reversible tight-binding inhibitors, and have improved pharmacological and toxicological profiles comparing to the older inhibitors [159]. They are highly selective to COMT and orally active being mainly peripherally acting, even though some develop central effects [188]. Between the various inhibitors of this class described in the literature, only nitecapone, entacapone, tolcapone and nebicapone were selected for further clinical development. Among these compounds, only tolcapone and entacapone were introduced into the market and are currently available to be use in the clinical practice, even though tolcapone can only be used with some restrictions (*section I.1.3.1.*). It is believed that nitecapone was replaced during clinical trials by entacapone, since the last was evaluated as a better clinical candidate, whereas the clinical development of nebicapone was interrupted due to safety concerns [145]. The recommended posologic regimen of tolcapone is 100 mg three time per day, but it can be increased to 200 mg three times daily if leads to justified clinical benefits [152]; on the other hand, entacapone has a short $t_{1/2el}$ and limited bioavailability, and thus, it is recommended to be co-administered at the dose of 200 mg with levodopa/AADC inhibitor up to ten times per day [122,155].

The search for new COMT inhibitors, especially for PD, has been a constant over the years in order to identify more potent and safer inhibitors in relation to those clinically available. In this context, researches recently developed at BIAL – Portela & Cª S.A.

disclosed new COMT inhibitors that have heterocyclic rings at the *meta*-position relative to the nitro group of nitrocatechol motif [186,189]. Between the developed compound series, opicapone [2,5-dichloro-3-(5-(3,4-dihydroxy-5-nitrophenyl)-1,2,4-oxadiazol-3-yl)-4,6-dimethylpyridine 1-oxide, also known as BIA 9-1067] was the most promising one. In fact, opicapone showed to be an exceptionally potent and purely peripheral COMT inhibitor with an unprecedented duration of action, and to have a reduced toxicity risk. Moreover, it presented a really favourable pharmacodynamic interaction with levodopa providing stable and sustained plasma levodopa levels over a 24 h period [189]. Taking into account these characteristics, a great improvement in the biological properties of the COMT inhibitors was achieved with this new group of compounds over their predecessors [186]. Therefore, opicapone was tested in clinical trials and it was very recently (June 2016) licensed by EMA to be used in the clinical practice as adjunct to levodopa/AADC inhibitor therapy in patients with motor fluctuations. The recommended dosage is 50 mg once a day at bedtime and at least 1 h before or after levodopa/AADC inhibitor therapy [142]. Hence, a new group, third-generation, of COMT inhibitors is emerging [186].

In parallel with second- and third-generation COMT inhibitors, many other compounds with different chemical structures have been also identified as COMT inhibitors and are grouped in the class of the late atypical COMT inhibitors. This class of COMT inhibitors includes the bisubstrate inhibitors, which were developed to target simultaneously the substrate (catechol) and the SAM binding sites, and the bifunctional inhibitors, which have two catecholic structures in the same molecule. Although some of these compounds showed to be potent COMT inhibitors, their *in vivo* efficacy is still to be demonstrated [126,145]. Another late atypical inhibitor is a pyridine derivative, CGP 28014, developed in the late 1980s. It does not inhibit COMT *in vitro* unless at high (millimolar) concentrations, but *in vivo*, it is effective and acts preferably in the brain. Despite having reached phase I clinical trials, CGP 28014 was not further developed for PD or other clinical indications [122,148,190]. Very recently, in order to develop a brain-penetrant MB-COMT inhibitor for the treatment of cognitive symptoms associated with schizophrenia, new hydroxy-pyridone derivatives were synthetized by scientists at Merk and Cerecor, Inc. Those compounds showed to be potent inhibitors of human MB-COMT in *in vitro* assays and, at least one of them, modified the concentration of some dopamine metabolites in the rat cerebrospinal fluid, indicating central COMT inhibition as intended [191,192].

Since the second-generation COMT inhibitors are the currently used in the clinical practice, detailed information about them will be presented in the following subsections in order to highlight their drawbacks and allow the comparison with opicapone.

I.2.2.1. Pharmacokinetics of the Second-generation COMT Inhibitors

The COMT inhibitors with nitrocatechol structure (tolcapone, entacapone, nitecapone and nebicapone) are rapidly absorbed from the gastrointestinal tract after oral administration to humans; depending on the compound and the administration dose, their C_{max} in plasma is attained approximately at 0.5–2.5 h post-dosing. In single-dose studies, tolcapone, entacapone, and nebicapone exhibited a linear pharmacokinetic profile in the ranges of 5–800 mg [193], 5–800 mg [194], and 10–800 mg [195], respectively. Regarding nitecapone, it also presented a systemic exposure dose-proportional in the range of 10–100 mg, when orally administered in combination with levodopa and carbidopa [196]. According to those studies, C_{max} in plasma was specifically reached within 0.8–2 h for tolcapone, 0.4–0.9 h for entacapone, 0.5–0.8 h for nitecapone, and 0.5–2.5 h for nebicapone. On the other hand, these COMT inhibitors are characterized by a fast kinetics of elimination with a $t_{1/2el}$ ranging from 1.7 to 3.4 h for tolcapone, 1.6–3.4 h for entacapone, 0.5–0.8 h for nitecapone, and 1.5–4.7 h for nebicapone. The pharmacokinetic properties of tolcapone, entacapone, and nebicapone remain similar after repeated administration, and no accumulation was observed when given at the therapeutic doses (100–200 mg) [126].

Taking into consideration the kinetics of absorption of tolcapone, entacapone, nitecapone and nebicapone a high bioavailability would be predictable for these compounds; however, owing to their extensive and variable first-pass metabolism, the oral bioavailability is only about 60–68% for tolcapone [197,198], approximately 35% for entacapone [194], 56% for nitecapone [148], and > 55% for nebicapone (in rat) [195]. The tolcapone, entacapone, and nitecapone bind to human plasma proteins in a high extension (> 99.9%, 98%, and 97%, respectively), leading to small volumes of distribution [148]. In line with those COMT inhibitors, nebicapone also demonstrated a very high binding to plasma proteins (> 99%) [199].

Metabolism is the main elimination route of tolcapone, entacapone, nitecapone and nebicapone. Indeed, all of them are extensively metabolized, mainly in liver, being the conjugation reactions the major metabolic pathways [148,194,197,200].

Particularizing, tolcapone is extensively metabolized and the metabolic pathways currently known to occur in human and rat species are summarized in Figure I.2.3 [201,202]. The glucuronidation of tolcapone is the most prominent metabolic route in human, with the 3-*O*-glucuronide appearing as the major metabolite in plasma, urine and faeces. In addition, methylation, oxidation and reduction, as well as, other chemical reactions, such as sulfation and *N*-acetylation, may also occur, but these are considered to be minor metabolic pathways [201]. As depicted in Figure I.2.3, there is a considerable homology between rat and human species regarding the metabolism of tolcapone,

suggesting that the rat is a good laboratory animal model to investigate the pharmacology and toxicity ascribed to tolcapone or its metabolites. Nevertheless, some new metabolites of tolcapone not reported in human were identified by Sun *et al.* [202] in rat urine, namely diglucuronide and sulfate conjugates.

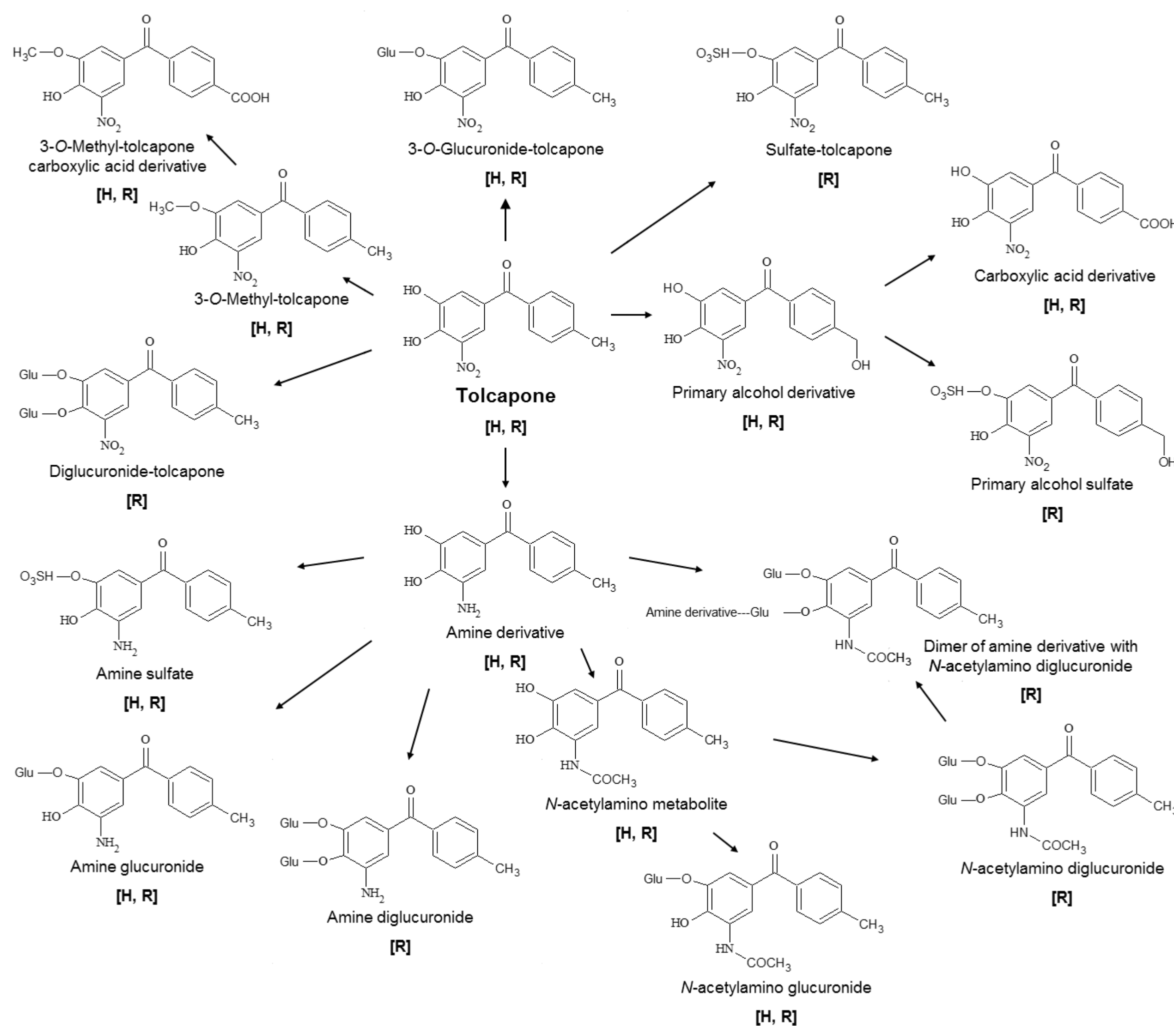


Figure I.2.3 Potential metabolic pathways for tolcapone in human (H) and in rat (R) (adapted from [146]).

Entacapone [(*E)*-entacapone] is also extensively metabolized in human and rat (Figure I.2.4), originating several metabolites which can be excreted in faeces (major route) and urine [194,203]. However, the metabolic pathways for entacapone appear to be simpler than those previously discussed for tolcapone. The major metabolite of entacapone identified in human and rat urine was the 3-*O*-glucuronide-entacapone, while its (*Z*)-isomer was the only found in human plasma. Other less abundant urinary

metabolites were also identified in both species, but some differences in their metabolic profiles were evidenced (Figure I.2.4), for example the 3-*O*-methyl-entacapone was not detected in humans. It is noteworthy that entacapone and its phase I metabolites are mostly excreted in their conjugated forms (only the glucuronide of entacapone is represented in Figure I.2.4) in rat and human urine. Specifically, they were found almost exclusively as glucuronides in human urine with a very small percentage of sulfates (0.5% of the urinary metabolites), and mainly as glucuronides and sulfates in rat urine [203].

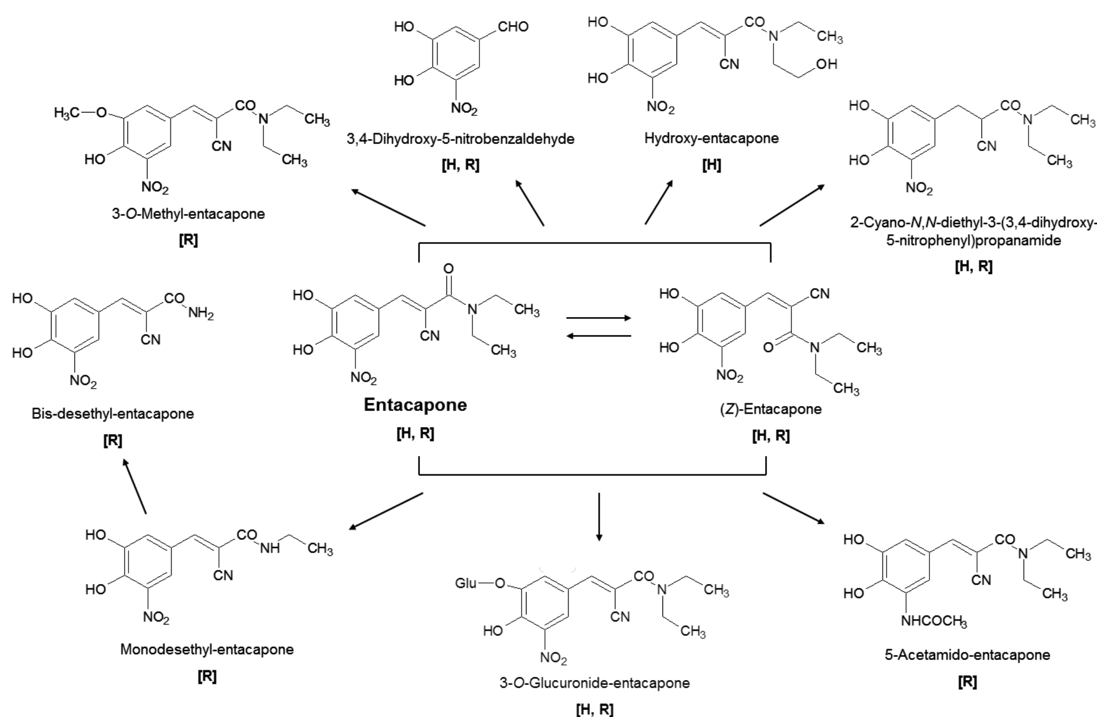


Figure I.2.4 Potential metabolic pathways for entacapone in human (H) and in rat (R) (adapted from [146]).

The metabolism of nitecapone was investigated in humans [204] and rats [205], and the proposed metabolic pathways are depicted in Figure I.2.5. Both in humans and rats, glucuronide conjugates were the most abundant metabolites in urine, being the glucuronide of nitecapone the major metabolite. However, while in humans the glucuronides of nitecapone and its phase I metabolites were the only detected conjugated metabolites, in rats were also found sulfate conjugates. Taskinen *et al.* [204] also analysed the glucuronidation process in humans and found it to be regioselective, because only one glucuronide was formed from nitecapone (parent drug) and each one of phase I metabolites. In contrast, the glucuronidation and sulfation processes appeared not be regioselective in the rat [205]. In addition, several minor metabolites were identified in

both species, but the metabolism of nitecapone in rats seems to be more complex than in humans, as shown in Figure I.2.5.

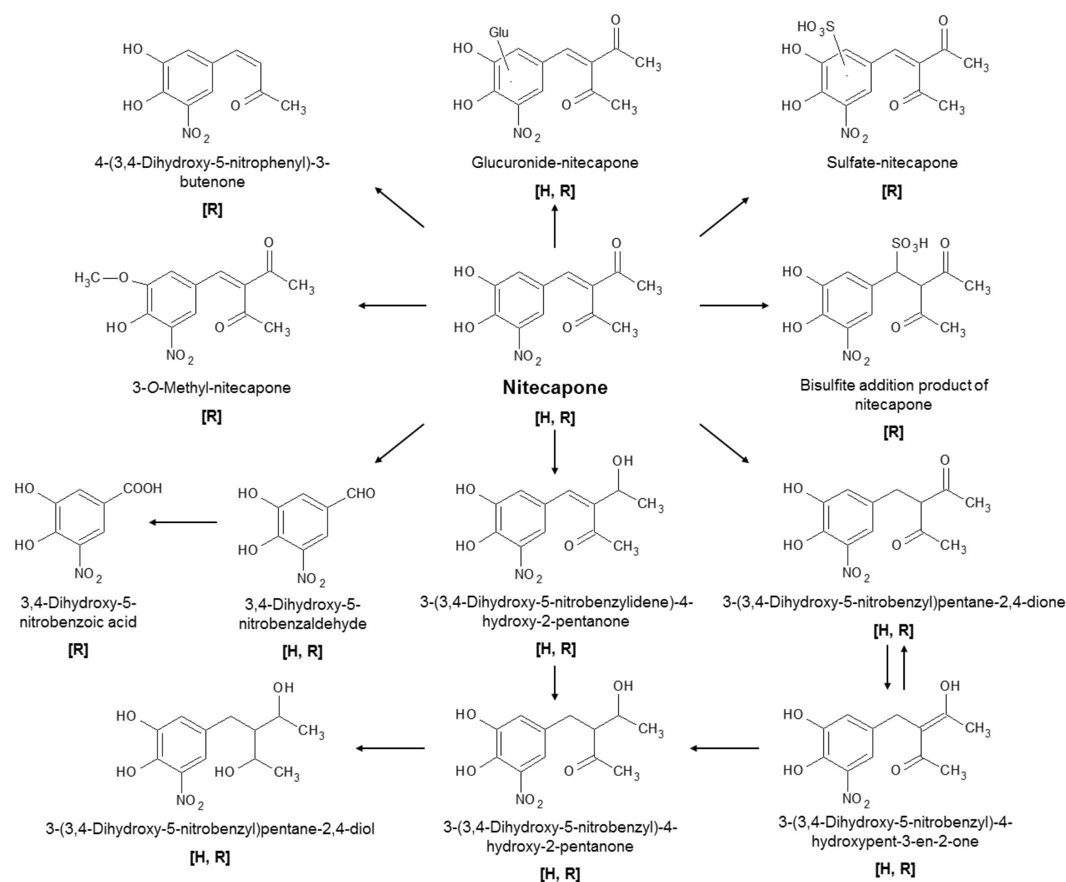


Figure I.2.5 Potential metabolic pathways for nitecapone in human (H) and in rat (R) (adapted from [146]).

The metabolism of nebicapone in humans was firstly investigated by Loureiro *et al.* [206] and later by Wright *et al.* [200]. As with tolcapone, entacapone and nitecapone, the major metabolic pathway involved in the metabolism of nebicapone is the glucuronidation of the hydroxyl group at position 3, originating the 3-*O*-glucuronide-nebicapone. The 3-*O*-methylated derivative is also formed, as result of the COMT activity, and is responsible for low but sustained presence of nebicapone-related material in plasma [200,206]. Other two very minor metabolites, which result from sulfation of the hydroxyl group at position 3 or *N*-acetylation of the 5-nitro group of nebicapone, were also detected in human plasma by Loureiro *et al.* [206]. Nevertheless, the study performed by Wright *et al.* [200] also provided information on other minor metabolites. Overall, in humans, after the administration of [^{14}C]-labelled nebicapone, eight metabolites were identified in plasma, twenty-two in urine

and four in faeces. The occurrence of species differences on the pharmacokinetics of nebicapone in rodents (rat and mouse) was also examined by Bonifácio *et al.* [207]; in consequence, some differences on its metabolic profile were identified between rat and mouse species, in particular an extra major metabolite was found in the rat resulting from sulfation. In fact, the major metabolites found in mouse were the glucuronide (the most abundant) and methylated derivatives, whereas in rat they were the glucuronide, methylated (the most abundant) and sulfate derivatives. The main metabolites of nebicapone identified in human, rat and mouse species are summarizes in Figure I.2.6.

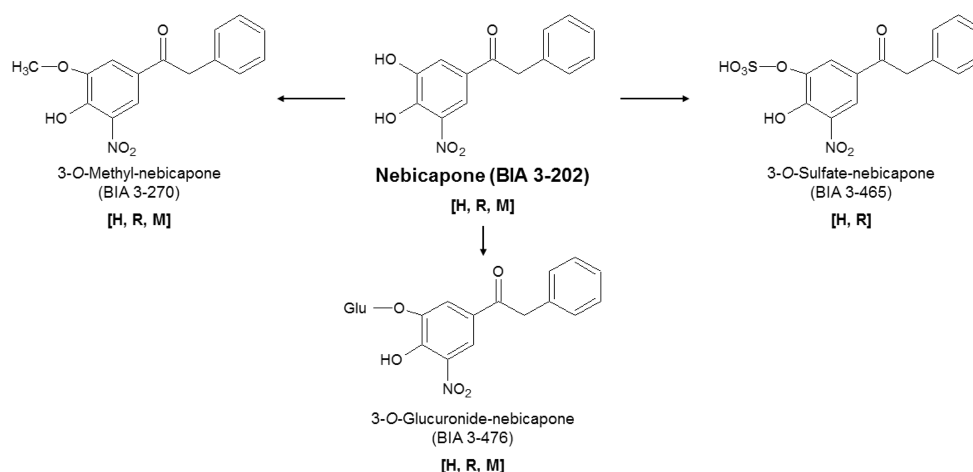


Figure I.2.6 Main metabolic pathways for nebicapone in rodents [rat (R) and mouse (M)] and in human (H) (adapted from [146]).

I.2.2.2. Effect of the Second-generation COMT Inhibitors in COMT Activity

The inhibition of a drug target is sometimes not only due to the parent compound itself but also to some active metabolites. In this context, it is noteworthy that the metabolites resulting from conjugation reactions are usually not pharmacologically active [208], and those resulting from isomerization, reduction or oxidation reactions may be bioactive [201], and thereby, they may contribute to drug efficacy or toxicity.

Regarding tolcapone, it is described that the carboxylic acid metabolite and the alcohol metabolite exert COMT inhibitory effects similar to those of the parent compound [209]. In contrast, the hepatotoxicity of the tolcapone appears to be mediated through the oxidation of amine and acetylamino metabolites of tolcapone to reactive species (*o*-quinone or quinone-imine) [210]. On the other hand, entacapone is the only COMT inhibitor that undergoes an isomerization process to its (*Z*)-isomer. Representing approximately 5% of the total of the two isomers in human plasma, (*Z*)-entacapone is an

active metabolite and acts as inhibitor of the COMT activity [122,194,203]. Some metabolites of nitecapone also appear to have COMT inhibitory activity comparable to nitecapone, such as the 3,4-dihydroxy-5-nitro-benzaldehyde and 3-(3,4-dihydroxy-5-nitro-benzylidene)-4-hydroxy-2-pentanone; in addition, other aglycone metabolites of nitecapone should also have some activity, but in a lower magnitude [204]. At last, for nebicapone there is no published data up to now on the presence or absence of active metabolites. Hence, taking into account these remarks, it is evident that the metabolites produced at low amounts should also be considered during the process of drug development.

According to studies performed in rats, the mentioned inhibitors present different selectivity profiles for peripheral and central COMT. Nitecapone does not inhibit the striatal COMT, and thus it is a strictly peripheral COMT inhibitor acting mainly in the gastrointestinal tract [211]. Entacapone is fundamentally a peripheral COMT inhibitor, which induces a relevant COMT inhibition in duodenum, erythrocytes and liver, with only slight and transient inhibition of central COMT [135,212]. In contrast, tolcapone demonstrated not only to be a more potent inhibitor of peripheral COMT than nitecapone and entacapone, but also to easily cross the BBB inhibiting the central COMT considerably [135,149]. Nebicapone showed to be relatively selective for peripheral COMT at low doses, but this selectivity was completely lost at higher doses [213].

In clinical trials, all the inhibitors reduced erythrocyte COMT activity in a dose-dependent and reversible manner. Moreover, the inhibition of erythrocyte COMT activity by tolcapone [125], entacapone [194], nitecapone [214], and nebicapone [215] was closely related to plasma concentrations of the drug. They act rapidly with the maximum inhibition of COMT activity (E_{max}) occurring within 2 h after their oral administration. However, the level of inhibition and the time for enzyme activity recovery may differ with inhibitor. Considering both E_{max} and the duration of inhibition, tolcapone seems to be stronger than entacapone and nitecapone, while nebicapone showed to have an inhibition profile comparable to that of tolcapone. In fact, after a single oral dose of 100 mg and 200 mg, the E_{max} was 55% and 65% for entacapone [194], 72% and 80% for tolcapone [193], and 69% and 80% for nebicapone [216], respectively. Moreover, COMT activity returned to the baseline within 8 h for entacapone, 15 h for tolcapone and 18 h for nebicapone. Nitecapone inhibited more duodenal COMT (58%) than erythrocyte enzyme (33%) at 1 h after oral administration of 100 mg, which suggested a limited penetration of it through the erythrocyte membrane. The erythrocyte COMT activity was fully recovered within 4 h after all tested doses [214]. During multiple-dose administration of tolcapone, entacapone or nebicapone, no indication of pharmacodynamic tolerance arose [122,215].

Since tolcapone and entacapone are the only available in clinical practice, their effect on levodopa pharmacokinetics was briefly discussed in *section I.1.3.1.*, as well as their clinical effects.

I.2.2.3. Safety and Toxicity of the Second-generation COMT Inhibitors

In non-clinical regulatory safety studies, tolcapone and entacapone demonstrated to be safe after being subjected to extensive toxicity testing in various animal species [188]. Regarding non-clinical studies of nebicapone, the compound also revealed to be well tolerated in several animal species. Moreover, in animal safety pharmacology studies, no significant adverse effects were observed concerning to central nervous system, hepatic, renal, cardiovascular, respiratory or gastrointestinal systems [195].

The adverse effects that result from COMT inhibitors therapy in humans can be classified as dopaminergic, which are related to the increased bioavailability of levodopa, and non-dopaminergic [188]. The dopaminergic adverse effects are normally moderate or mild including dyskinesia, nausea, dizziness, orthostatic hypotension, vomiting, anorexia, sleep disorders, somnolence, and hallucinations. Dyskinesia is the most relevant of these adverse effects and can be minimized by reducing the dose of levodopa. Among the non-dopaminergic adverse effects, the diarrhoea is the most common one and it is frequently more severe with tolcapone than with other COMT inhibitors. In addition to diarrhoea, other non-dopaminergic events appear, such as abdominal pain, headache, constipation, urine discoloration (due to the presence of COMT inhibitor and/or its metabolites in urine), and transaminases increase [126,188,217].

Elevated liver transaminases levels are observed very rarely in entacapone-treated patients and at a low occurrence (1-3%) in patients treated with tolcapone [148]. Unexpectedly, after the introduction of tolcapone into the market, three cases of fatal fulminant hepatitis were reported [139]. In this context, tolcapone was withdrawn from the European Union and Canada markets one year after its introduction and received a black box warning in the United States of America. Nevertheless, some years later, tolcapone was reintroduced into the market of the European Union since, during the period of suspension, its safety profile was reviewed based on new clinical safety data. Nowadays, its use requires failure or intolerance to other COMT inhibitors (in practice, entacapone but for a short time period since opicapone received very recently its marking authorisation) and strict liver function monitoring on a regular basis [138,141,155]. On the other hand, no cases of hepatitis or other serious liver failures have been reported for entacapone, which has been used as a negative reference substance in the studies on idiosyncratic liver toxicity *in vitro* and *in vivo* [126,188]. With respect to nebicapone, in phase II clinical trials, it

induced a clinically relevant increase in the liver transaminases in 4 of 46 patients receiving nebicapone at dose of 150 mg [217]. Hence, its toxicological profile reflects quite well the profile of tolcapone and thereby its clinical development was discontinued due to safety concerns [145]. As both tolcapone and nebicapone have induced elevations in liver transaminases, concern has arisen regarding hepatotoxicity of COMT inhibitors.

I.2.3. Why Is There a Current Need for New COMT Inhibitors?

As aforementioned, up to June of 2016, only two COMT inhibitors (tolcapone and entacapone) were approved for clinical use in PD patients. However, each of them presents problems related to their pharmacokinetics, pharmacodynamics, clinical efficacy and/or safety that restrict their success [138].

As denoted earlier, tolcapone is stronger and longer-acting inhibitor of COMT than entacapone (*section I.2.2.2.*). Moreover, tolcapone increases more levodopa AUC than entacapone being clinically more effective (s*ection I.1.3.1.*). Nevertheless, the greatest drawback of tolcapone is its hepatotoxicity which, albeit rare, limits its use (*section I.2.2.3.*). On the other hand, although entacapone is considered safer regarding hepatic effects (s*ection I.2.2.3.*), its low oral bioavailability, short duration of action, and low potency to inhibit peripheral COMT (*sections I.2.2.1.* and *I.2.2.2.*) restrict its clinical efficacy as an adjunct to levodopa/AADC inhibitor therapy in PD. For these reasons, there remains a need for the development of new COMT inhibitors with a better therapeutic profile. One possible "optimal" COMT inhibitor should be potent, safe and long-acting in order to be administered once-daily (more convenient dosage regimen) and lead to a more sustained levodopa levels in PD patients [138,155]. In this context, the object of study of the current thesis, opicapone (formerly known as BIA 9-1067) emerged as an attempt to fulfil that unmet therapeutic need.

I.2.4. The New Third-generation COMT Inhibitor: Opicapone

In resemblance to the second-generation COMT inhibitors, opicapone was rapidly absorbed, attaining its C_{max} within 1.5–3.5 h (median values) post-dosing, and presented a relatively short $t_{1/2el}$ (0.8–3.2 h, mean values) following the administration of single oral doses of 10 to 1200 mg to healthy humans. Furthermore, it exhibited a systemic exposure approximately dose-proportional at that wide dose range (10–1200 mg) [218]. After

multiple-dose administration, similar pharmacokinetic properties were observed for opicapone (5–50 mg once-daily) [219,220]. Moreover, when opicapone was administered once-daily at the therapeutic dose (50 mg), it did not undergo accumulation in plasma [220]. In line with the second-generation COMT inhibitors, a high percentage of plasma protein binding (> 99%) was reported for opicapone [221].

The metabolic profile of opicapone in humans (Figure I.2.7) is slightly different from the profiles commonly found for the second-generation COMT inhibitors. In contrast to the second-generation inhibitors, which undergo mainly *O*-glucuronidation, the main metabolic pathway of opicapone is the *O*-sulfation reaction, with formation of an inactive metabolite, the 3-*O*-sulfate-opicapone (BIA 9-1103) [218]. Opicapone also suffers a side-chain metabolic reaction in which the pyridine-*N*-oxide ring is reduced to a pyridine derivative, known as BIA 9-1079 [186]. In non-clinical studies, BIA 9-1079 demonstrated to be active as COMT inhibitor [half maximal inhibitory concentration (IC_{50}) of 429 nM against rat liver COMT] [218,220] and, in clinical trials, its concentrations were found to be lower than those of BIA 9-1103 [218,219]. Other minor metabolites were also identified, including *O*-methylated (BIA 9-1100 and BIA 9-1101) and *O*-glucuronide (BIA 9-1106) derivatives [218]. The published information about the metabolic profile of opicapone in rats is very limited. Nevertheless, it is known that all the metabolic pathways identified in humans (*O*-sulfation, *O*-glucuronidation, *O*-methylation and *N*-oxide reduction) are also present in rats (Figure I.2.7) [222,223]. BIA 9-1101 (3-*O*-methyl-BIA 9-1079) was identified in non-clinical studies, but no specific information in which species it was detected is currently available. Furthermore, in clinical trials performed at doses of opicapone up to 1200 mg, the concentrations of BIA 9-1101 were below the lower limit of quantification (LLOQ) at any time point post-dose [218]. In addition to BIA 9-1100 and BIA 9-1101, the occurrence of another methylated metabolite, BIA 9-1104, in humans [220] and rats [223] was recently reported. Moreover, two other metabolites were also recently mentioned: BIA 9-3752, which is the most abundant metabolite in human faeces, but its occurrence in other species has not been described; and M10, which is present in humans and rats. Although the M10 is probably the hydroxylated sulphate metabolite of opicapone (BIA 9-4588), its absolute identification was not yet possible. On the other hand, regarding BIA 9-3752, there is no indication neither of the metabolic pathway involved in its formation nor of its chemical structure [223]. Therefore, these two metabolites were not included in Figure I.2.7.

In relation to opicapone excretion, the hepatobiliary excretion appears to be the major elimination pathway in humans and rats [221,222].

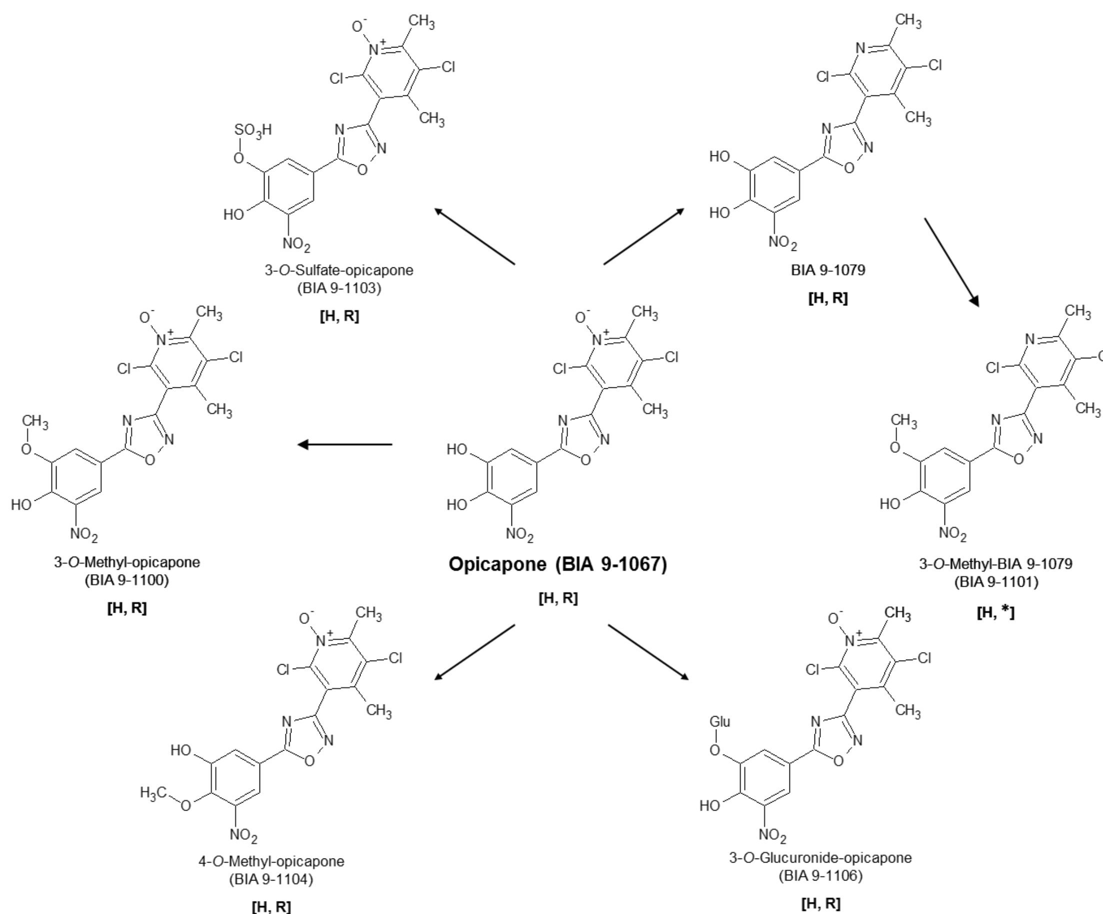


Figure I.2.7 Metabolic pathways for opicapone in human (H) and in rat (R). *Also identified during non-clinical studies but with no specific information regarding the species where it was detected.

In *in vitro* studies, opicapone, BIA 9-1103 and BIA 9-1079 demonstrated to be significant inhibitors of two cytochrome P450 (CYP) isoenzymes, the CYP2C8 and the CYP2C9. Hence, clinical interaction studies were carried out with repaglinide (CYP2C8 substrate) and warfarin (CYP2C9 substrate), evidencing that the inhibition of CYP2C8 is likely to occur *in vivo* while the inhibition of CYP2C9 could be excluded [223,224].

In addition, *in vitro* assays demonstrated that opicapone is transported by organic anion transporting polypeptide (OATP) 1B3 and effluxed by P-glycoprotein and breast cancer resistance protein, whereas BIA 9-1103 is transported by OATP1B1 and OATP1B3 and effluxed by breast cancer resistance protein. Furthermore, even though opicapone and BIA 9-1103 inhibited some transporters *in vitro*, only the inhibition of OATP1B1 may lead to interactions *in vivo* considering the plasma free fractions of both compounds detected in clinical trials. Nevertheless, no clinical studies have yet been performed to assess this aspect [223,224].

In non-clinical studies performed in rats and monkeys, opicapone showed to be unable to cross the BBB being a purely peripheral COMT inhibitor [189,225]. Moreover, in both species, it was demonstrated that opicapone is a potent peripheral COMT inhibitor, inducing a strong and sustained inhibition of COMT activity [189,225,226]. In fact, after 14 days of once-daily oral administration of opicapone (100 mg/kg) to monkeys, the erythrocyte COMT activity 23 h after the last dose remained between 16 and 24% of the baseline activity values [225]. In rats, around 50% of the liver COMT enzyme activity remained inhibited at 24 h following the administration of a single oral dose of opicapone (3 mg/kg) [189,226]. In opposition employing the same posologic regimen, entacapone did not produce any COMT inhibition and tolcapone induced only a minimal inhibitory effect (16%) at 9 h post-dose [189,218,227]. Moreover, with opicapone, rat liver COMT inhibition attained 99% at 1 h post-dose, while after tolcapone and entacapone administration it was 82 and 68%, respectively [218]. Therefore, opicapone presented a stronger and more sustained COMT inhibition than tolcapone and entacapone in rats. In contrast to the second-generation COMT inhibitors, the duration of opicapone-induced COMT inhibition is independent of its presence in the systemic circulation. Indeed, although opicapone is rapidly eliminated from bloodstream in rats (at 8 h post-dosing no opicapone was detected in plasma), its inhibitory effect is prolonged far beyond the observable point of plasma drug clearance [226]. In relation to the effect on the levodopa pharmacokinetics, opicapone induced a prolonged increment in systemic and central exposure to levodopa with a simultaneous reduction in 3-*O*-MD exposure in rats and monkeys [189,225,226]. Taking into account these promising results, opicapone advanced to clinical development.

Regarding clinical studies, it was observed that, after the administration of single oral doses of opicapone (10–1200 mg) to young healthy male volunteers [218], opicapone strongly inhibited the erythrocyte S-COMT activity in a dose-dependent manner, with E_{max} values ranging from 36.1 (10 mg) to 100% (200 mg and higher doses). Moreover, in spite of its rapid elimination from plasma (within 8–12 h), the opicapone-induced COMT inhibition was maintained for a long period of time independently of the administered dose, having an observed half-life of the enzyme activity recovery of 61.6 h. These exceptional pharmacodynamic properties were confirmed in a subsequent multiple-dose study in which once-daily doses (5–30 mg) of opicapone were administered for eight consecutive days to healthy male volunteers [219]. In that study, following the last dose of opicapone, E_{max} varied between 69.9 (5 mg) and 98.0% (30 mg) in a dose-dependent manner, and the observed half-life of erythrocyte S-COMT inhibition induced by opicapone was dose-independent, remaining around 130.4 h, which is much longer than the time of plasma opicapone elimination (6–10 h). Moreover, 24 h after the last dose of opicapone

the opicapone-induced S-COMT inhibition was still around 42.8% (5 mg), 52.4% (10 mg), 56.8% (20 mg) and 64.9% (30 mg). In this context, it is noteworthy that opicapone induces a much stronger and more prolonged COMT inhibitory effect than that described for tolcapone and entacapone (*section I.2.2.2.*), and has a unique pharmacodynamic profile adequate for a once-daily regimen. The long observed half-lives of opicapone-induced COMT inhibition in the above-mentioned single-dose and multiple-dose studies reflect the very slow dissociation rate of the COMT-opicapone complex and its long residence time [186,218,219].

Posteriorly, it was evaluated the effect of opicapone on levodopa pharmacokinetics in clinical trials conducted in healthy adult subjects [228] and in PD patients with motor fluctuations [229,230]. Remarkably, opicapone increased the levodopa bioavailability in all the mentioned studies. In the study performed in healthy subjects, repeated doses of 25, 50 or 75 mg opicapone or placebo were administered once-daily for 11 days, and, on the 12th day, the subjects of the opicapone groups received placebo concomitantly with each levodopa/carbidopa dose (three times separated by a 5 h interval), while those of the placebo group received 200 mg entacapone concomitantly with each levodopa/carbidopa dose. Four subjects in each group received placebo during the whole study. In all the opicapone treatments, the extent of levodopa exposure (assessed by AUC) was significantly increased in relation to that achieved after placebo treatment. Moreover, a significant increase in levodopa AUC was found in the 50 and 75 mg opicapone treatment groups when compared to entacapone treatment group. No significant differences were observed in the levodopa C_{max} between all active treatments (opicapone and entacapone) and placebo. In contrast, when compared to placebo, all active treatments substantially increased the levodopa C_{min}, with increment values ranging from 1.7-fold (200 mg entacapone) to 3.3-fold (75 mg opicapone). In relation to placebo, all opicapone treatments increased the levodopa $t_{1/2el}$ in approximately 1 h [228]. In PD patients, single-doses of 25, 50 or 100 mg opicapone co-administered with the morning dose of levodopa/carbidopa or levodopa/benserazide (levodopa/AADC inhibitor) increased the levodopa exposure (assessed by AUC) in relation to placebo (by 3.7, 16.4, or 34.8%, respectively, based on point estimates), as well as the levodopa C_{max} in a dose-dependent manner, but significant differences were only found for 100 mg opicapone [230]. In the other multiple-dosing clinical trial conducted in PD patients, opicapone increased the levodopa exposure (assessed by $AUC_{0\text{-}6}$) in relation to baseline by 24.7, 53.9 and 65.6% (based on point estimates) following 5, 15 and 30 mg opicapone, respectively, achieving a significant increase with both 15 and 30 mg opicapone. Additionally, an increase in levodopa C_{max} was also observed with all opicapone doses in relation to placebo and to baseline, but a

statistical difference was solely registered for the 30 mg opicapone in comparison with baseline. In this study, different doses of opicapone were administered once-daily for up to 28 days and approximately 1 h before the morning dose of levodopa/AADC inhibitor [229]. In both studies performed in PD patients, the mean levodopa $t_{1/2el}$ increased slightly with opicapone (between 10 to 50 min depending on the administration regimen and dose) when compared to placebo [229,230].

In those clinical trials, opicapone also markedly decreased the extent of exposure to 3-*O*-MD in relation to placebo [228,230] or to baseline [229] and the extent of S-COMT activity in relation to entacapone and placebo [228] or to placebo [229,230]. Furthermore, although the studies carried out in PD patients were not specifically designed to detect any significant differences in motor performance, the performed exploratory analysis revealed an improvement in various motor outcomes [229,230]. Considering all these favourable results, it was decided to proceed to further clinical trials.

Even though, in theory, the exposure to levodopa can be considered a surrogate efficacy marker, evaluation of drug effect on motor fluctuations is required to prove the clinical efficacy of a new COMT inhibitor. As such, two phase III clinical trials, BIPARK I [231] and BIPARK II [232], were conducted in PD patients with motor fluctuations in order to evaluate the efficacy and safety of opicapone as adjunct to levodopa/AADC inhibitor therapy. In both clinical trials, the primary efficacy endpoint was the change from baseline to study end in absolute time in the off state, based on daily patient diaries. The BIPARK II study was a randomised, double-blind, multinational, multicentre, placebo-controlled and parallel-group trial in which the efficacy of two different doses of opicapone (25 and 50 mg) administered once-daily for 14–15 weeks was compared with placebo. Interestingly, the mean reduction in absolute off-time in patient groups administered with opicapone at 25 and 50 mg was greater than in those administered with placebo (1.7, 2.0 and 1.1 h, respectively); nevertheless, only 50 mg opicapone was significantly better than placebo. In consonance with the observed reduction in absolute off-time, the mean increase in absolute on-time without or with non-troublesome dyskinesias (secondary efficacy endpoint) was higher in 25 mg and 50 mg opicapone groups than in placebo (1.4, 1.43 and 0.8 h, respectively) [232]. In BIPARK I, a randomised, double-blind, multinational, multicentre, parallel-group, placebo-controlled and active-controlled trial, opicapone (5, 25 or 50 mg once-daily at bedtime and at least 1 h after the last daily dose of levodopa), placebo or entacapone (200 mg with every levodopa dose) were orally administered for 14–15 weeks to assess the superiority of opicapone to placebo and its non-inferiority to entacapone. In these conditions, the mean change from baseline to the end of study treatment in absolute time in the off state was -56.0, -96.3, -91.3, -85.9 and -116.8 min for

placebo, entacapone, 5 mg opicapone, 25 mg opicapone and 50 mg opicapone, respectively. The analysis of these data followed a hierarchical procedure for each opicapone dose, requiring a prior establishment of superiority over placebo to evaluate non-inferiority to entacapone. In the superiority test, 50 mg opicapone and entacapone were superior to placebo, with a placebo-corrected mean difference in reduction of off-time from baseline of 60.8 min and 40.3 min, respectively, while treatment with 5 mg or 25 mg opicapone was not significantly different from treatment with placebo. Therefore, in the subsequent test of non-inferiority, only 50 mg opicapone was tested demonstrating to be non-inferior to entacapone. Relevantly, the reduction in off-time induced by 50 mg opicapone was complemented by a significant increase in on-time without troublesome dyskinesias (placebo-adjusted least-squares mean difference 62.6 min), while the duration of on-time with troublesome dyskinesia did not significantly alter. Other secondary efficacy endpoints were also assessed, including the proportion of patients with a reduction of at least 1 h in absolute off-time and the proportion of patients with an increase of at least 1 h in absolute on-time (key secondary endpoints). Overall, they also favoured 50 mg opicapone over placebo as adjunct to levodopa/AADC inhibitor therapy [231].

After the double-blind phase of both BIPARK clinical trials have been completed, the majority of the involved patients continued to a one-year, open-label extension phase of the respective BIPARK I [233,234] or BIPARK II [235] clinical trial. All patients received opicapone once-daily but the dose of opicapone and dosage of levodopa therapy were freely adjusted according to the clinical response and/or associated adverse effects. During those one-year extension studies, the efficacy of opicapone observed in the double-blind phases was maintained in patients who continued with adjunctive opicapone [234,235]. Moreover, patients who switched from placebo or entacapone to opicapone when finished the double-blind phase experienced significant improvements on motor fluctuations during the one-year BIPARK I extension phase, since it was observed a significant reduction of off-time and a significant increase of on-time without dyskinesia [234]. Hence, in addition to the proven clinical efficacy and non-inferiority against entacapone in the short-term studies, opicapone also maintained its efficacy in long-term use and improved significantly motor fluctuations in patients that switched from entacapone to opicapone.

Since the Val108/158Met single nucleotide polymorphism is a common *COMT* gene polymorphism and it has ethnic differences (the low COMT activity allele is more common in Caucasians than Asians), the pharmacokinetics and pharmacodynamics of opicapone were compared between healthy Japanese and matched Caucasian subjects in a clinical trial [220]. The results of that study showed no clinically relevant effects of *COMT* polymorphism or race on the pharmacokinetics and pharmacodynamics of opicapone.

Opicapone revealed to be well tolerated in several animal species [218] and devoid of toxicity in human primary hepatocytes and HepaRG cell line [226,236]. Furthermore, taking into account the central nervous system, renal, cardiovascular, respiratory and gastrointestinal systems, no relevant adverse effects were detected in the conventional animal safety pharmacological studies [218]. In phase III clinical trials (pooled safety data of double-blind phase of BIPARK I and BIPARK II studies), opicapone was generally well tolerated, being dyskinesia, constipation, insomnia and dry mouth the most common adverse events that emerged from opicapone administration. In general, the adverse events were mild to moderate without a dose relationship [237,238]. Importantly, also from the pooled safety data of both phase III clinical trials, no clinically relevant effects on hepatobiliary function arose after opicapone administration, with 2.0% and 3.5% of patients in opicapone groups and placebo group, respectively, experiencing hepatic-related adverse events and without cases of serious hepatotoxicity in opicapone groups [239]. In the one-year extension phase of both clinical trials, no new or unexpected safety concerns were identified with opicapone treatment. Moreover, it is noteworthy that there were no occurrences of urine discoloration, severe diarrhoea or severe hepatic events [240,241].

Considering all the information herein exposed about opicapone, it is expected that this new compound will add value to the treatment of motor complications, improving the PD patient's quality of life and expanding the available therapeutic armamentarium, which is far from being perfect. Moreover, in a near future, opicapone will probably become the first-choice COMT inhibitor due to its proven clinical efficacy and non-inferiority against entacapone, its once-daily administration regimen that can provide higher flexibility to the adjustment of daily levodopa dosage, and its favourable safety and tolerability profile.

CHAPTER I

I.3. Aims of this Thesis

I.3. Aims of this Thesis

At the beginning of the work underlying the present PhD thesis, opicapone was in clinical development and very little information was available in literature about this new and promising COMT inhibitor. Hence, in an attempt of complementing the information from clinical trials, the main goal of this thesis was to characterize the systemic and tissue (liver and kidneys) pharmacokinetics of opicapone and its active metabolite (BIA 9-1079), as well as the effect on erythrocyte, liver and kidney COMT activity after oral administration of opicapone to rats.

Sensitive, precise and accurate bioanalytical methods that allow the quantification of the compounds of interest in the desired matrices are essential to evaluate their pharmacokinetics. However, when this work started there was no published method for the quantification of opicapone and/or BIA 9-1079 neither in human samples nor in non-human species matrices. Thus, the development and validation of appropriate bioanalytical methods to extract these compounds from the biological samples and quantify them was indispensable to obtain reliable quantitative data.

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

- Development and full validation of an analytical high performance liquid chromatography (HPLC) method with diode array detection (DAD) to simultaneously separate and quantify opicapone and BIA 9-1079 in human plasma.

- Development and full validation of an analytical HPLC-DAD method to simultaneously quantify opicapone and BIA 9-1079 in rat matrices (plasma and liver and kidney tissue homogenates).

- Pharmacokinetic characterization of opicapone and BIA 9-1079 in rat plasma after the administration of three single oral rising doses of opicapone to rats.

- Comparison of plasma pharmacokinetics of opicapone and BIA 9-1079 following the oral administration of a selected dose of opicapone in single- and multiple-dose regimens to rats.

- Assessment of plasma, liver and kidney pharmacokinetics of opicapone and BIA 9-1079 and, in parallel, evaluation of COMT activity in erythrocyte, liver and kidney after single and repeated oral administrations of a selected dose of opicapone to rats.

CHAPTER II

BIOANALYSIS OF OPICAPONE AND BIA 9-1079

CHAPTER II

II.1. General Introduction

II.1. General Introduction

Bioanalysis is defined as the quantitative determination of drugs, their metabolites and/or endogenous substances in biological matrices, such as blood, plasma, serum, urine, other fluids (e.g. saliva, sputum, cerebrospinal fluid), faeces or tissues, by employing a bioanalytical method, which is a set of procedures implicated in the collection, processing, storage, and analysis of a biological matrix for a compound [242–244].

Currently, the bioanalysis is globally recognised as a fundamental component of the pharmacokinetic and pharmacodynamic characterization of a drug throughout all the stages of the drug discovery and development process, as well as of the development of new drug formulations and therapeutic drug monitoring during the post approval period [245,246]. The applied bioanalytical method determines the quality of the generated data from non-clinical, clinical and biopharmaceutical studies, playing a crucial role in the evaluation and interpretation of the obtained results [247,248]. Therefore, in order to guarantee the generation of high quality data and give confidence in the obtained results, the developed bioanalytical methods must be adequately characterised and validated prior to their implementation for routine use [244,247].

The bioanalytical method validation is an essential process to demonstrate and document, through the employment of specific laboratory investigations, that the performance characteristics of a particular bioanalytical method developed and used for the quantification of the analytes in a given biological matrix are suitable, reliable and reproducible for the intended analytical applications and thereby the method can provide accurate, precise and reproducible results [249,250].

As the method validation is a crucial step in bioanalysis, since 1990, when the first conference about this topic took place, many efforts have been developed to harmonize that process and define the parameters and acceptance criteria to be considered [250]. One of the first official documents with general recommendations on bioanalytical method validation was issued in 2001 by FDA [249,251]. This guidance was delineated based on the deliberations of two conferences: "Analytical methods validation: Bioavailability, bioequivalence, and pharmacokinetic studies" [252] and "Bioanalytical methods validation – A revisit with a decade of progress" [247]; and, it is considered the pillar of bioanalytical validation. Then, other regulatory agencies, such as EMA, have developed and issued their own guidelines to facilitate the regulatory submissions in their countries [253]. The EMA guideline on this topic was published in July of 2011 [254]. According to both FDA and EMA guidelines, at least the following parameters should be evaluated during a validation process: selectivity, LLOQ, calibration curve and linearity, precision, accuracy, recovery and stability. Since bioanalysis is a field in permanent evolution, the worldwide

recognised FDA guidance has been revised to reflect advances in science and technology and in September of 2013, an updated draft [255] of this document was released to enable public review and comment before it is finalized.

When this project was initiated, no analytical assays for the quantitative determination of opicapone and/or its active metabolite (BIA 9-1079) were published. Therefore, the experimental work began with the development, optimization and full validation of two bioanalytical methodologies, which will be described in this chapter. The validation of these assays was carried out according to the general recommendations described in both FDA and EMA guidelines. The first developed method (*section II.2.*) allows the quantification of opicapone and BIA 9-1079 in human plasma and can be presently applied in clinical studies. Moreover, it was the starting point for the development of the second assay (*section II.3.*) which enables the quantification of the compounds of interest in rat plasma, and liver and kidney tissues homogenates. This second method was developed and fully validated to support the analysis of the samples obtained from the pharmacokinetic studies that will be presented in the following chapters of this dissertation.

This chapter is based on two articles already published, thus the information herein presented is in accordance with the state of the art at the time of their submission.

CHAPTER II

II.2. An HPLC-DAD Method for the Simultaneous Quantification of Opicapone (BIA 9-1067) and its Active Metabolite in Human Plasma

II.2.1. Introduction

Opicapone [2,5-dichloro-3-(5-(3,4-dihydroxy-5-nitrophenyl)-1,2,4-oxadiazol-3-yl)-4,6-dimethylpyridine 1-oxide], formerly known as BIA 9-1067 (Figure II.2.1), is a novel peripheral selective third-generation COMT inhibitor presently under phase III clinical trials as adjunctive therapy in PD [189,256].

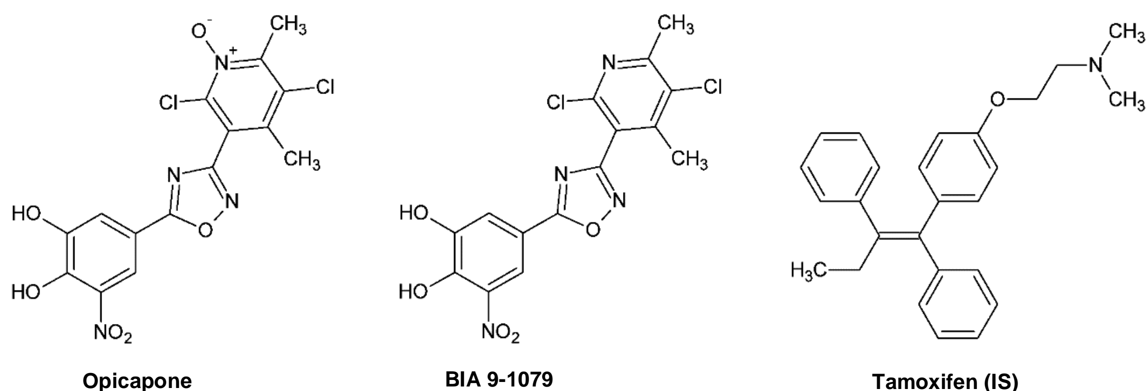


Figure II.2.1 Chemical structures of opicapone and its active metabolite (BIA 9-1079), and tamoxifen used as internal standard (IS).

Entacapone and tolcapone are the COMT inhibitors currently available in the market. Nevertheless, whilst they have improved the treatment of PD, both compounds exhibit important drawbacks that limit their clinical success; entacapone exhibits a limited clinical efficacy, while tolcapone must be used under strict hepatic monitoring due to its severe liver toxicity [106,126,152,154]. Therefore, the need for development of more potent, safer and longer-acting COMT inhibitors is undeniable and, at this point, opicapone is emerging as a candidate for this unmet need [189,218]. It is endowed with higher capacity for inhibiting COMT and for a longer time than entacapone and tolcapone; indeed, 50% of the peripheral inhibition achieved with opicapone was preserved up to 24 h after single administration to rats [189]. Furthermore, when administered with levodopa and benserazide to rats, opicapone was demonstrated to provide a consistent increase in levodopa plasma levels over a long period of time [189]. Due to the great potential of opicapone to be successfully used as an adjunct to levodopa/AADC inhibitor therapy, it is expected that a deep characterization of its pharmacokinetics and pharmacodynamics in humans will be carried out in a near future.

As HPLC is an analytical methodology widely used for separation and quantification of COMT inhibitors in biological samples [146], the availability of a reliable quantitative

HPLC assay for the determination of opicapone and its active metabolite (BIA 9-1079; Figure II.2.1) in human plasma is essential to support clinical studies. To date, only a liquid chromatography (LC) method coupled to tandem mass spectrometry (MS/MS) has been made available for quantification of opicapone and/or BIA 9-1079 [218]. Hence, the development and full validation of the first HPLC-DAD method able to separate and quantify opicapone and its active metabolite in human plasma is herein reported.

II.2.2. Materials and Methods

II.2.2.1. Chemicals and Reagents

Standards of opicapone (BIA 9-1067; batch number 54516-2-4) and BIA 9-1079 (batch number PC101220) were kindly supplied by BIAL-Portela & Cª S.A. (S. Mamede do Coronado, Portugal), while tamoxifen citrate salt (batch number 035K1270), used as internal standard (IS), was acquired from Sigma-Aldrich (St. Louis, MO, USA; Figure II.2.1). Acetonitrile (HPLC gradient grade), dimethyl sulfoxide (DMSO; HPLC grade) and ethyl acetate were purchased from Fisher Scientific (Leicestershire, UK), and ultra-pure water (HPLC, > 15 MΩ) was obtained by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). The 85% *ortho*-phosphoric acid was purchased from Panreac (Barcelona, Spain), while sodium dihydrogen phosphate dehydrate and 37% fuming hydrochloric acid were obtained from Merck KGaA (Darmstadt, Germany). Blank human plasma samples from healthy donors were kindly provided by the Portuguese Blood Institute after written consent of each subject.

II.2.2.2. Stock Solutions, Calibration Standards, and Quality Control Samples

Stock solutions of opicapone and BIA 9-1079 were individually prepared in DMSO at 1 mg/mL and then appropriately diluted with acetonitrile in order to give working solutions with final concentrations of 50 µg/mL. Appropriate volumes of stock and working solutions of opicapone and BIA 9-1079 were combined and diluted in acetonitrile to obtain six combined spiking solutions at final concentrations of 0.5, 1, 2, 10, 30, and 60 µg/mL. Each of these combined solutions was daily used for spiking aliquots of blank human plasma in order to prepare six plasma calibration standards at 25, 50, 100, 500, 1500 and 3000 ng/mL. The stock solution of IS was prepared in acetonitrile at 1 mg/mL and it was daily diluted with water-acetonitrile (50:50, v/v) to prepare the working solution (20 µg/mL). All solutions were stored at 4 °C and protected from light. Under these conditions, the stock solution of opicapone was stable for 30 days, while the stock solutions of BIA 9-1079 and IS were stable at least for 60 days. In addition, the working solutions of the analytes (opicapone and BIA 9-1079) and IS were stable for 30 days.

The quality control (QC) samples at the LLOQ (QC_{LLOQ}) and at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration range were independently prepared in the same biological matrix. Another QC sample was also prepared in order to evaluate the dilution effect (1:5) in plasma (QC_{Dil}). The final concentrations of opicapone and BIA 9-1079

in QC human plasma samples were 25 ng/mL in QC_{LLOQ}, 75 ng/mL in QC_1, 1500 ng/mL in QC_2, 2700 ng/mL in QC_3 and 10 000 ng/mL in QC_{Dil}.

II.2.2.3. Sample Preparation

Each aliquot (200 µL) of human plasma was added of 10 µL of IS working solution (20 µg/mL) and 600 µL of acetonitrile. After a thorough vortex mixing (30 s), the mixture was centrifuged at 13 400 rpm for 10 min in order to precipitate plasma proteins. The resulting supernatant was collected and acidified with 200 µL of 2 M hydrochloric acid. After vortexing for 30 s, 500 µL of the extraction solvent (ethyl acetate) were added and the mixture was vortex-mixed for 2 min and then centrifuged at 13 400 rpm (5 min). The organic layer was transferred to a glass tube and the aqueous layer was re-extracted two more times using the liquid-liquid extraction (LLE) procedure previously described. Organic phases were combined, evaporated to dryness under a gentle nitrogen stream at 45 °C and then reconstituted with 100 µL of solvent A–solvent B (50:50, v/v) of mobile phase. Afterwards, the reconstituted extract was transferred to an Eppendorf tube of 1.5 mL, centrifuged at 13 400 rpm for 2 min and 20 µL of the supernatant was injected into the chromatographic system.

II.2.2.4. HPLC-DAD Instrumentation and Chromatographic Conditions

The chromatographic analysis was carried out on a LC-AD Liquid Chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a DGU-$20A_5$ degasser, a SIL-$20A_{HT}$ autosampler, a CTO-$10AS_{VP}$ column oven and a SPD-M20A diode array detector. Data acquisition and instrumentation control were achieved by means of the LCsolution software (Shimadzu Corporation, Kyoto, Japan).

Chromatographic separation of opicapone, BIA 9-1079 and IS was accomplished in less than 10 min by gradient elution on a reversed-phase LiChroCART® Purospher Star® C_{18} column (55 x 4 mm; 3 µm particle size) purchased from Merck KGaA (Darmstadt, Germany) and thermostatted at 25 °C. A gradient elution was employed at a flow rate of 0.8 mL/min with a mobile phase composed of 0.05 M monosodium phosphate solution adjusted to pH 2.45 ± 0.05 with *ortho*-phosphoric acid (solvent A) and acetonitrile (solvent B). During the first 3 min of the run, a linear gradient was applied from 33% to 60% (solvent B) and it was kept until 7 min. Subsequently, the percentage of solvent B was restored to 33% within 1 min and maintained until the end of the run (13 min). The solvent A was filtered through a 0.45 µm filter before used. The detection wavelengths were set at 257 nm for BIA 9-1079 and at 271 nm for opicapone and IS.

II.2.2.5. Method Validation

The presented method was validated according to the guidelines for bioanalytical method validation from FDA and EMA [249,254] as well as other international recommendations for bioanalytical method validation [247,257]. The method was validated considering the following parameters: selectivity, linearity, precision and accuracy, limits of quantification and detection, sample dilution, recovery and stability.

Selectivity was studied by analysing the presence of potential chromatographic interferences of endogenous compounds (matrix effects) at the retention times of opicapone, BIA 9-1079 and IS; for that, blank plasma samples obtained from six different subjects were processed and their chromatograms were compared with those from spiked plasma samples. Furthermore, the interference from drugs commonly co-administered to parkinsonian patients was also investigated (levodopa, carbidopa, benserazide, biperiden, bromocriptine, ropinirole, selegiline and amantadine), as well as other potentially co-prescribed drugs such as acetylsalicylic acid, alprazolam, amitriptyline, azithromycin, carbamazepine, chlorothiazide, clozapine, diazepam, digoxin, donepezil, dopamine, enoxaparin, erythromycin, furosemide, hydrochlorothiazide, ibuprofen, ketoprofen, naproxen, omeprazole, paracetamol, penicillin-G, propranolol, ranitidine, venlafaxine and verapamil.

The linearity of the method for both analytes (opicapone and BIA 9-1079) was assessed within the range of 25–3000 ng/mL, using calibration curves prepared on five separate days (n = 5) with six spiked plasma calibration standards. These curves were constructed plotting analyte–IS peak height ratios *versus* the corresponding nominal concentrations in plasma. The data were subjected to a weighted linear regression analysis using $1/y^2$ and $1/y$ as weighting factors for, respectively, opicapone and BIA 9-1079. The weighting factors were selected taking the plots and the sums of absolute percentage relative error into account [258]. The LLOQ was defined as the lowest concentration of the calibration curve that is measured with precision not exceeding 20% (expressed as percentage of coefficient of variation, % CV), and accuracy within ±20% (expressed as the percentage of deviation from nominal concentration, % Bias). It was established using five replicates of plasma samples (n = 5) both intra- and inter-day. The limit of detection (LOD), defined as the lowest concentration that can be distinguished from the noise level, was determined by analysing plasma samples with known concentrations of opicapone and BIA 9-1079, after successive dilutions.

Intra- and inter-day precision and accuracy were assessed in replicated (*n* = 5) for QC_1, QC_2 and QC_3. The intra- and inter-day precision and accuracy upon sample dilution (1:5) were also investigated using QC_{Dil} samples (*n* = 5) in order to ensure that concentrations exceeding the upper limit of quantification of the calibration range could be accurately determined after proper sample dilution with blank human plasma. The acceptance criterion for precision was a CV value ≤ 15% and for accuracy was a Bias value within ±15%.

The absolute recovery of the analytes (opicapone and BIA 9-1079) from human plasma samples was determined by comparing the analyte peak heights from extracted QC samples (QC_1, QC_2 and QC_3) with the corresponding heights obtained from non-extracted solutions at the same nominal concentrations (*n* = 5). Similarly, the recovery of the IS was also determined at the concentration used in sample analysis.

Human plasma stability of opicapone and BIA 9-1079 was evaluated using QC_1 and QC_3 samples exposed at room temperature for 4 h, at 4 °C for 24 h and at -30 °C for 30 days in order to simulate sample handling and storage time in the freezer before analysis. In plasma, the effect of three freeze-thaw cycles on the stability of the analytes was also investigated; briefly, aliquots of spiked plasma samples were stored at -30 °C for 24 h, thawed unassisted at room temperature and then refrozen for 24h under the same conditions until completing the three cycles. In order to assess the post-preparative stability of processed samples under usual autosampler conditions, the reconstituted extracts were analysed after 24 h at room temperature and at 4 °C. 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 analyte concentrations ratio of 85–115% was accepted as the stability criterion (*n* = 5).

II.2.3. Results and Discussion

II.2.3.1. Method Development

The reversed-phase LiChroCART® Purospher Star® C_{18} column was herein employed not only because two apolar compounds were intended to be separated (opicapone and BIA 9-1079), but also owing to the high efficiency, robustness and resistance previously reported for this column [259,260]. In order to establish the best chromatographic conditions to separate the analytes of interest (opicapone and BIA 9-1079), individual drug solutions were directly injected into the HPLC system and several mobile phases were tested. Firstly, different proportions of water–methanol–acetonitrile were investigated as mobile phases without any pH adjustment; however, these preliminary results were not promising. Indeed, observing the molecular structure of opicapone (Figure II.2.1), it is evident that it is a weak acidic compound and therefore the influence of mobile phase pH was investigated in the range between 2.0 and 4.0. The results clearly demonstrated that using 0.05 M monosodium phosphate aqueous solution at pH 2.45 ± 0.05, adjusted with *ortho*-phosphoric acid, and different proportions of acetonitrile, sharpened and symmetric peaks were obtained for both analytes (opicapone and BIA 9-1079). Interestingly, similar mobile phases have been frequently employed to separate other COMT inhibitors and their metabolites by HPLC [146]. Obviously, these preliminary studies were carried out under isocratic elution, but the tested conditions were not successful when combined solutions of opicapone and BIA 9-1079 were injected; particularly, because the analytes resolution and full separation of opicapone from initial impurities were not achieved within a practicable run time. Therefore, a gradient elution program was developed in order to achieve the best relationship between peak shape, resolution, selectivity and run time. Hence, the optimal chromatographic separation was accomplished applying the gradient elution described in *section II.2.2.4.* at a flow rate of 0.8 mL/min. Under these chromatographic conditions, the retention time of opicapone and BIA 9-1079 was approximately 4.4 min and 8.3 min, respectively. After that, among several compounds that were tested for potential use as IS, tamoxifen citrate appeared as the most appropriate one and presented a retention time of 7.6 min.

Regarding the sample preparation and extraction procedures, the simple protein precipitation with methanol, acetonitrile and 20% trichloroacetic acid, as well as different procedures of solid-phase extraction using Waters Oasis® HLB extraction cartridges (30 mg, 1 mL) were tested; however, a good relationship between analytes recovery and selectivity was not reached. Therefore, a single-step of plasma protein precipitation with acetonitrile followed by a LLE emerged as a new hypothesis; then, LLE procedure was evaluated using

different types and proportions of extraction solvents (hexane, ethyl acetate, dichloromethane and tetrahydrofuran). The best selectivity and recovery were found after acidification of the protein precipitation supernatant and using ethyl acetate as extraction solvent. This sample pre-treatment procedure is cheaper than any procedure employing solid-phase extraction.

At last, the selection of the wavelength values to monitor the chromatographic response of the compounds of interest (opicapone, BIA 9-1079 and IS) was accomplished aiming to obtain the minimal endogenous interferences at their retention times associated with the highest signal intensity. The best relationship was achieved at 271 nm for opicapone and IS, and at 257 nm for BIA 9-1079. Representative chromatograms of the extracts of blank and spiked plasma samples are shown in Figure II.2.2.

II.2.3.2. Method Validation

The method herein reported was demonstrated to be selective since the chromatograms of blank plasma samples from six healthy volunteers showed no interfering peaks at the retention times of the analytes (opicapone and BIA 9-1079) and IS (Figure II.2.2). Similarly, none of the drugs potentially co-administered with opicapone that were tested interfered with the peaks of the analytes or IS.

The linearity of the method within the concentration range of 25–3000 ng/mL was demonstrated for opicapone and BIA 9-1079 in human plasma ($r^2 > 0.997$). The weighted regression equations of calibration curves and the corresponding coefficients of determination were $y = 0.0018x + 0.0067$ ($r^2 = 0.9972$) for opicapone and $y = 0.0019x - 0.0143$ ($r^2 = 0.9986$) for BIA 9-1079, where y represents the analyte/IS peak height ratios and x represents the nominal concentrations of the analytes expressed in ng/mL. The LLOQ of the presented method was set at 25 ng/mL for both analytes (opicapone and BIA 9-1079) with good precision and accuracy, as summarized in Table II.2.1. The LOD was established at 15 ng/mL for both compounds. Hence, it should be highlighted that this assay is sufficiently sensitive and can be applied for the quantitative determination of opicapone and BIA 9-1079 in a wide concentration range (25–3000 ng/mL). Actually, taking into account as a reference method the LC-MS/MS assay reported by Almeida *et al.* [218], it deserves to be noted the similar sensitivity of both bioanalytical methods (HPLC-DAD *versus* LC-MS/MS) for quantification of opicapone and BIA 9-1079 in plasma samples.

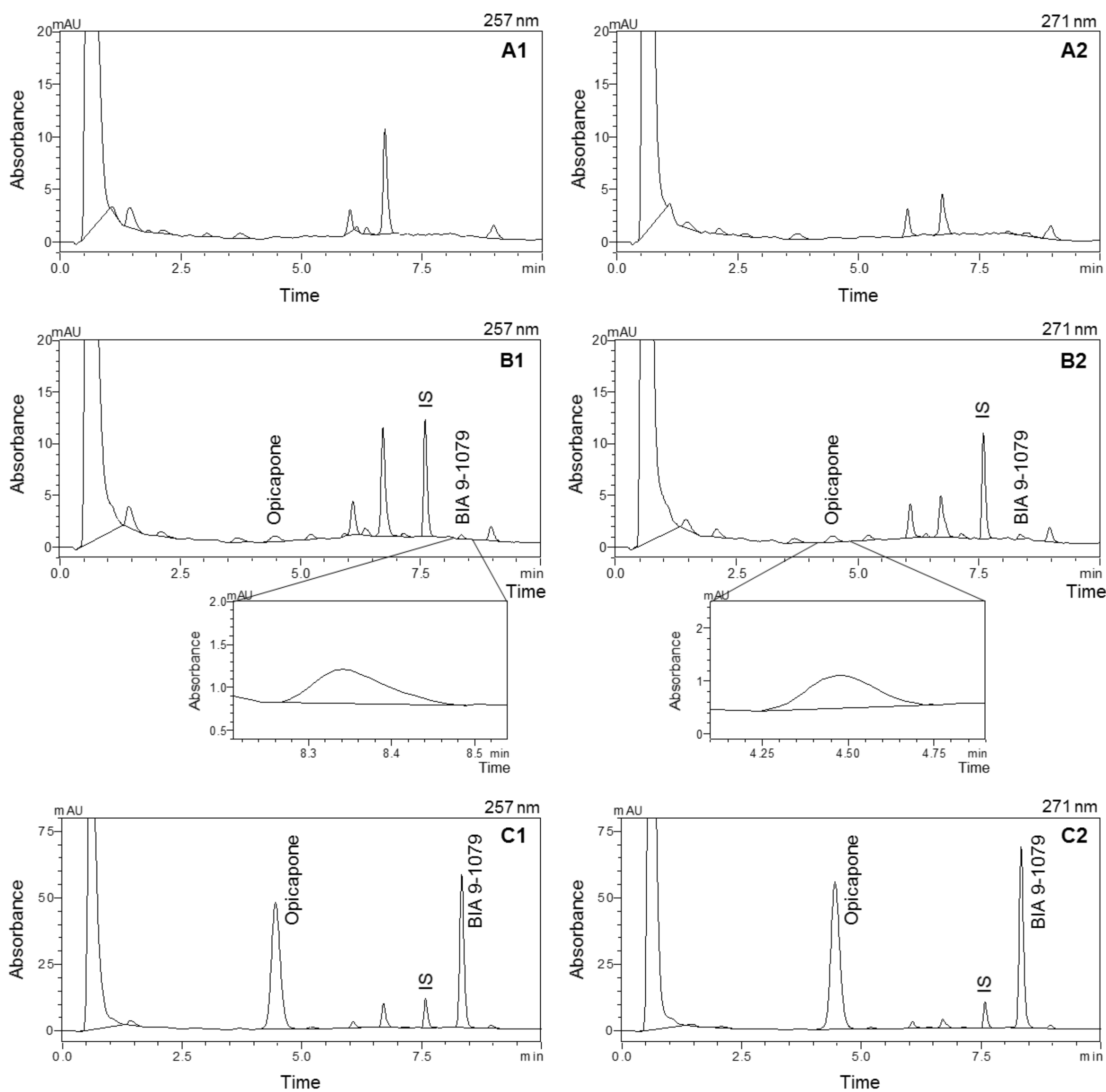


Figure II.2.2 Typical HPLC-DAD chromatograms of extracted human plasma samples: blank sample at 257 nm (A1) and at 271 nm (A2), sample spiked at level of the lower limit of quantification (25 ng/mL) at 257 nm (B1) and at 271 nm (B2), and sample spiked at level of the higher limit of the calibration range (3000 ng/mL) at 257 nm (C1) and at 271 nm (C2). IS, internal standard.

Precision and accuracy data for intra- and inter-day QC plasma samples are presented in Table II.2.1. All the data fulfilled the acceptance criteria, indicating that this HPLC-DAD method developed for the quantification of opicapone and BIA 9-1079 in human plasma is precise, accurate and reproducible; indeed, the CV value was at a maximum of 13.20% and the Bias values varied between -6.52% and 11.10%. For QC_{Dil} plasma samples the results shown in Table II.2.1 also support that a 5-fold sample dilution with blank human plasma can be appropriately applied when the concentrations of analytes in a tested sample exceed the upper limit of quantification of the calibration curve (3000 ng/mL).

Table II.2.1 Precision (% CV) and accuracy (% Bias) for the determination of opicapone and BIA 9-1079 in human plasma samples at concentrations of the lower limit of quantification (*), at low, middle and high concentrations of the calibration range and following a sample dilution ([#]) by a 5-fold factor (*n* = 5).

Nominal concentration	Intra-day			Inter-day		
	Experimental concentration[a]	% CV	% Bias	Experimental concentration[a]	% CV	% Bias
Opicapone						
25*	24.89 ± 3.25	10.81	-0.46	23.05 ± 2.41	9.02	-7.79
75	78.82 ± 4.96	5.91	11.10	76.59 ± 7.42	9.23	2.11
1500	1575.06 ± 67.22	4.25	5.00	1576.68 ± 114.23	7.23	5.11
2700	2779.63 ± 87.36	3.14	2.95	2719.17 ± 138.20	5.08	0.71
10 000[#]	10 391.35 ± 325.87	3.13	3.91	10 028.14 ± 698.69	6.96	0.28
BIA 9-1079						
25*	26.42 ± 1.04	5.88	5.68	24.53 ± 2.10	12.36	-1.88
75	73.72 ± 4.13	6.35	3.46	78.08 ± 5.99	8.48	4.11
1500	1456.51 ± 82.25	5.68	-2.90	1514.81 ± 198.95	13.20	0.99
2700	2565.95 ± 120.9	4.73	-4.96	2635.92 ± 169.40	6.44	-2.37
10 000[#]	9347.75 ± 487.10	5.22	-6.52	9537.49 ± 974.15	10.22	-4.63

[a] Results expressed as mean ± standard deviation. Nominal and experimental concentrations are expressed in ng/mL. Bias, deviation from nominal values; CV, coefficient of variation.

Although LLE is usually associated with low recovery values, the mean absolute recovery of opicapone and BIA 9-1079 achieved with the present methodology was higher than 70% for both compounds and showed very low CV values (≤ 4.63%; Table II.2.2). Furthermore, considering all the recovery values obtained at the three assessed concentration levels for each analyte, the CV values (*n* = 15) for opicapone and BIA 9-1079 were 4.08% and 8.94%, respectively, suggesting a consistent recovery over the evaluated concentration range. The absolute recovery of the IS was also evaluated and a mean value (*n* = 15) of 94.7% was obtained, with a CV of 2.67%.

Table II.2.2 Absolute recovery (%) of opicapone and BIA 9-1079 from human plasma (*n* = 5).

Analyte	Nominal concentration (ng/mL)	Recovery (%)	
		Mean ± SD	CV (%)
Opicapone	75	110.78 ± 2.07	1.87
	1500	102.42 ± 2.93	2.86
	2700	105.39 ± 2.80	2.65
BIA 9-1079	75	70.43 ± 2.93	4.15
	1500	81.57 ± 3.78	4.63
	2700	85.02 ± 2.51	2.95

CV, coefficient of variation; SD, standard deviation.

The stability of the opicapone and BIA 9-1079 in plasma was investigated under the conditions previously stated in *section II.2.2.5*. The obtained results are shown in Table II.2.3 and they evidence no significant degradation of opicapone and BIA 9-1079 under those conditions.

Table II.2.3 Stability (values in percentage) of opicapone and BIA 9-1079 in human plasma (*n* = 5).

Analytes	**Opicapone**		**BIA 9-1079**	
Nominal concentration (ng/mL)	**75**	**2700**	**75**	**2700**
Stability conditions	**Stability/reference analyte concentrations (%)**			
Plasma				
Room temperature (4 h)	95.81	97.96	102.67	93.84
4 °C (24 h)	98.10	97.42	92.60	93.28
Three freeze/thaw cycles (-30 °C)	88.86	86.05	90.63	85.67
-30 °C (30 days)	94.61	91.58	99.73	93.98
Processed plasma samples				
Room temperature (24 h)	98.46	106.83	101.39	105.92
4 °C (24 h)	92.72	104.72	102.55	103.45

II.2.3.3. Method Application

The application of the proposed HPLC-DAD method to real samples was also demonstrated through the quantification of opicapone (parent) and BIA 9-1079 in plasma samples obtained from a healthy subject treated with opicapone within a clinical trial. These samples were kindly supplied by BIAL-Portela & Cª S.A. (S. Mamede do Coronado, Portugal) and the plasma concentrations of opicapone and BIA 9-1079 measured by HPLC-DAD were closely similar to those determined by LC-MS/MS [218] in the

corresponding samples. Representative chromatograms obtained from one of those real plasma samples collected at 3 h after opicapone administration are shown in Figure II.2.3, and it is seen that peak shape and chromatographic resolution of the analytes are very similar to those obtained from spiked plasma samples.

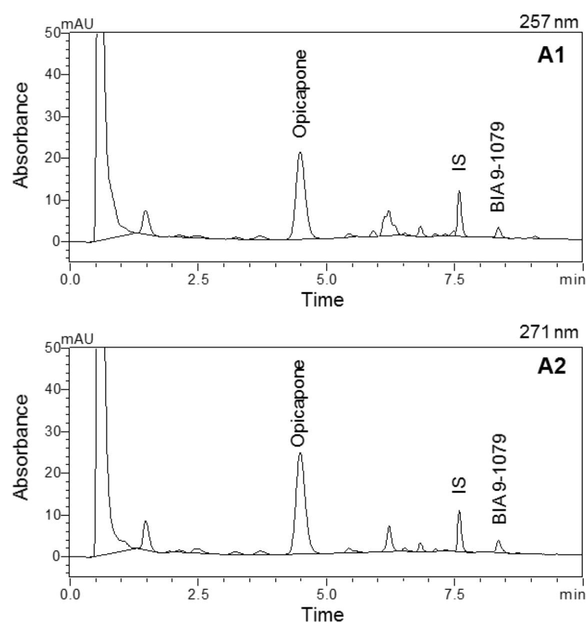


Figure II.2.3 Representative HPLC-DAD chromatograms of a real plasma sample from a healthy subject administered with opicapone at 257 nm (A1) and at 271 nm (A2). In this plasma sample, collected at 3.0 h post-dose, the plasma concentrations of opicapone and BIA 9-1079 measured by HPLC-DAD were respectively 1389.4 ng/mL (1361.9 ng/mL by LC-MS/MS – reference method) and 134.1 ng/mL (131.4 ng/mL by LC-MS/MS – reference method). IS, internal standard.

II.2.4. Conclusion

To date, to the best of our knowledge, this is the first report that describes the development and full validation of the first reversed-phase HPLC-DAD method able to quantify opicapone and BIA 9-1079 in human plasma and within a wide concentration range (25–3000 ng/mL). All compounds of interest (opicapone, BIA 9-1079 and IS) were also successfully extracted using a sample pre-treatment procedure involving protein precipitation followed by LLE, as demonstrated by their high absolute recoveries. The LLOQs of the method achieved for both analytes (opicapone and BIA 9-1079) were considerably low despite the nature of the procedures applied in sample preparation and taking into account the use of a HPLC-DAD system, which is simpler and cheaper than other more sensitive chromatographic systems such as LC coupled to mass spectrometry (MS) or MS/MS. Furthermore, the dilution integrity of 5-fold for human plasma samples at concentrations above the upper limit of the calibration range was successfully assessed. The application of this method was also successfully demonstrated using plasma samples from a healthy subject enrolled in a clinical trial of opicapone.

It is concluded that the developed bioanalytical assay using an HPLC-DAD system for the quantification of opicapone and BIA 9-1079 in human plasma is selective, sensitive, accurate, reliable, reproducible and suitable to support clinical pharmacokinetic studies with opicapone.

CHAPTER II

II.3. Development of a Liquid Chromatography Assay for the Determination of Opicapone and BIA 9-1079 in Rat Matrices

II.3.1. Introduction

Opicapone, also known as BIA 9-1067 (Figure II.2.1, *section II.2.*), is a novel potent, reversible and purely peripheral third-generation COMT inhibitor, which is currently under phase III clinical trials as an adjunct to levodopa therapy for PD [189,256,261].

More than 40 years after its clinical introduction, levodopa is still regarded as the most effective drug for the symptomatic treatment of PD [16,109]. Nevertheless, owing to its fast and extensive metabolism, only approximately 1% of levodopa reaches the brain after oral administration. Although the major metabolic pathway of levodopa is catalysed by the AADC, COMT enzyme expressed in extracerebral tissues is also significantly involved in the peripheral metabolism of levodopa [13,109,262]. Consequently, oral levodopa therapy has been improved using a combination of levodopa/AADC inhibitor plus a COMT inhibitor; this triple combination prolongs the half-life time of levodopa, increases its cerebral bioavailability and reduces its peripheral side effects [13,75,121,122]. However, the COMT inhibitors currently marketed (tolcapone and entacapone) exhibit important drawbacks that limit their clinical success [126,152,154] and, therefore, opicapone is emerging as a safer, more potent and longer-acting COMT inhibitor [189,218].

In fact, non-clinical studies performed in Wistar rats revealed that opicapone has a higher activity as a peripheral COMT inhibitor than tolcapone, with median effective doses at 3 h post-dose of 1.05 ± 0.04 and 1.77 ± 0.10 mg/kg, respectively [189]. In another study, opicapone also inhibited the rat peripheral COMT more efficiently than tolcapone [226]; however, as expected, contrary to tolcapone, opicapone does not inhibit the central COMT [189,226]. Anyway, opicapone is a longer-acting inhibitor than tolcapone and entacapone, with 50% of peripheral COMT inhibition maintained up to 24 h after a single administration to rats [189]. Furthermore, when administered with levodopa/benserazide (an AADC inhibitor), opicapone was demonstrated to increase two-fold the initial levodopa plasma levels in rats and sustain this ability for at least 24 h [189,226]. Recent clinical studies also revealed that opicapone increases more significantly the levodopa bioavailability in comparison to entacapone [228].

To date, the metabolic profile of opicapone has not been extensively documented in rats or other animal species. Even so, Almeida *et al.* [218] and Rocha *et al.* [219] reported five opicapone metabolites according to the results of non-clinical studies: two inactive methylated metabolites (BIA 9-1100 and BIA 9-1101), an inactive sulphated metabolite (BIA 9-1103), an inactive glucuronide metabolite (BIA 9-1106) and the BIA 9-1079 (Figure II.2.1, *section II.2.*), which was demonstrated to be active as a COMT inhibitor.

In humans, opicapone is rapidly absorbed and it is well tolerated after single- and multiple-dose regimens [218,219]. Additionally, in spite of its short apparent terminal

elimination half-life in healthy subjects, opicapone leads to a rapid, potent and sustained inhibition of erythrocyte S-COMT, making it suitable for a once-daily regimen. In these clinical studies, the opicapone metabolites listed above were also evaluated in plasma and urine; BIA 9-1103 was demonstrated to be the major metabolite in human plasma and BIA 9-1106 was the only metabolite found in urine; on the other hand, BIA 9-1100 and BIA 9-1101 were found to be minor metabolites in humans. Regarding BIA 9-1079 (the active metabolite), it was found to be the second most prevalent metabolite in plasma, representing between 6.3% to 16.0% of the systemic drug exposure following single or multiple oral doses of opicapone [218,219].

According to the aforementioned data, opicapone is a good drug candidate to be successfully administered in combination with levodopa/AADC inhibitor for the symptomatic treatment of PD. However, a deeper characterization of its pharmacokinetics and pharmacodynamics is essential. At present, pharmacokinetic and pharmacodynamic studies are being carried out in humans [218,219,228,256,261]; indeed, new questions emerge during the different phases of drug development program, which is a dynamic process that involves continuous feedback between non-clinical and clinical studies [263]. Moreover, additional non-clinical experiments are required along the clinical development and even during post-marketing period [264]. Particularly considering COMT inhibitors, practice has shown that toxicokinetic studies may be necessary in advanced stages of the drug development; for instance, tolcapone only revealed serious hepatotoxicity after its introduction into the market [126,152,154], and nebicapone, a COMT inhibitor developed in the last decade, only raised concerns about its liver safety in phase II clinical trials and further studies were required [217].

The rat is the most frequently employed species for pharmacokinetic and pharmacodynamic non-clinical studies with COMT inhibitors [202,203,205,207,265–268] and, therefore, we selected it as the most relevant animal model. To the best of our knowledge, there are only two liquid chromatographic techniques published to quantify opicapone and its active metabolite (BIA 9-1079) [218,269]. However, they were developed in human plasma and, consequently, a reliable and affordable HPLC assay that simultaneously quantifies opicapone and BIA 9-1079 in several rat matrices is still lacking, which will be important to support further non-clinical pharmacokinetic and toxicokinetic studies. Hence, the purpose of the present work was to develop and validate an HPLC-DAD method for the simultaneous quantification of opicapone and BIA 9-1079 in rat plasma, liver and kidney.

II.3.2. Materials and Methods

II.3.2.1. Chemicals and Reagents

Standards of opicapone (BIA 9-1067; batch number 81122-1-3) and BIA 9-1079 (batch number PC101220) were kindly supplied by BIAL-Portela & Cª S.A. (S. Mamede do Coronado, Portugal), while tamoxifen citrate salt (batch number 035K1270), used as IS, was acquired from Sigma-Aldrich (St. Louis, MO, USA; Figure II.2.1, *section II.2.*). Acetonitrile (HPLC gradient grade), DMSO (HPLC grade) and ethyl acetate were purchased from Fisher Scientific (Leicestershire, UK), and the ultra-pure water (HPLC, 18.2 MΩ.cm) was obtained by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). The 85% *ortho*-phosphoric acid was purchased from Panreac (Barcelona, Spain), while sodium dihydrogen phosphate dehydrate and di-sodium hydrogen phosphate anhydrous were obtained from Merck KGaA (Darmstadt, Germany) and 37% hydrochloric acid from Sigma-Aldrich (St. Louis, MO, USA).

II.3.2.2. Preparation of Stock Solutions, Calibration Standards, and Quality Control Samples

Stock solutions of opicapone and BIA 9-1079 (1 mg/mL) were individually prepared by dissolving the adequate amount of each compound in DMSO and then appropriately diluted with acetonitrile in order to achieve intermediate solutions (50 µg/mL). Afterwards, stock and intermediate solutions were adequately combined and diluted in acetonitrile to obtain six combined working solutions (final concentration of 0.4, 0.8, 3.0, 10, 35 and 60 µg/mL for opicapone and BIA 9-1079), which were used to spike blank rat plasma. Likewise, other six combined working solutions with the final concentration of 0.6, 1.2, 3.75, 12, 37.5 and 60 µg/mL for opicapone and 1.05, 2.1, 5.25, 12, 37.5 and 60 µg/mL for BIA 9-1079 were prepared and used to spike blank liver homogenate supernatant. Another set of six combined working solutions with the final concentrations of 0.6, 1.2, 3.75, 12, 37.5 and 60 µg/mL for opicapone and 0.9, 1.8, 4.5, 12, 37.5 and 60 µg/mL for BIA 9-1079 was also prepared and used to spike blank kidney homogenate supernatant. Specifically, 10 µL of each combined working solution were used for spiking aliquots of blank plasma (100 µL) and tissues homogenate supernatants (150 µL) in order to prepare six calibration standards in the concentration range of 0.04–6.0 µg/mL for both compounds in plasma, 0.04–4.0 µg/mL for opicapone in liver and kidney homogenate supernatants, and 0.07–4.0 µg/mL and 0.06–4.0 µg/mL for BIA 9-1079 in liver and kidney homogenate supernatants, respectively.

The QC samples at the LLOQ (QC_{LLOQ}) and at low (QC_1), middle (QC_2) and high (QC_3) concentrations of the calibration range were independently prepared in the three biological matrices. Another QC sample was prepared in order to evaluate the dilution effect (1:5) in plasma and tissues homogenate supernatants (QC_{Dil}).

The stock solution of the IS was prepared in acetonitrile at 1 mg/mL and it was daily diluted with water–acetonitrile (50:50, v/v) to prepare two working solutions: one at the final concentration of 90 µg/mL, which was used for the analysis of plasma samples, and a second one at 70 µg/mL to analyse tissues samples.

Stock and diluted solutions were stored at 4 °C and protected from light. Under these storage conditions, the stock solution of opicapone was stable for 30 days, while the stock solutions of BIA 9-1079 and IS were stable for at least 60 days. Moreover, the stability of the working solutions of opicapone, BIA 9-1079 and IS was demonstrated for at least 30 days.

II.3.2.3. Blank Rat Plasma and Tissues

Healthy adult male Wistar rats obtained from Harlan Laboratories (Barcelona, Spain) were used to collect the blank plasma and liver and kidney tissues required for validation studies. Rats were kept in local animal facilities under controlled environmental conditions (12 h light/dark cycle; temperature 22 ± 1 °C; relative humidity 50 ± 5%) and received a standard maintenance diet (4RF21, Mucedola, Italy) and tap water *ad libitum*. In order to obtain the blank matrices, rats were anaesthetized with an intraperitoneal (i.p.) injection of sodium pentobarbital (60 mg/kg) and decapitated. The blood samples were immediately collected into heparinised tubes while liver and kidneys were quickly removed. Blood samples were centrifuged at 2900 rpm (1514 *g*) for 10 min (4 °C) to obtain plasma samples, which were stored at -80 °C until used. The liver and kidney tissues were weighed and homogenized in 0.05 M sodium phosphate buffer pH 7.4 (4 mL/g tissue) using a Thomas® teflon pestle tissue homogenizer. The tissues homogenates were centrifuged at 4800 rpm (4147 *g*) for 15 min at 4 °C and the corresponding supernatants were also collected and stored at -80 °C until used.

All animal experimentation was conducted in accordance to the European Directive 2010/63/EU [270] regarding the protection of laboratory animals used for scientific purposes.

II.3.2.4. Chromatographic System and Conditions

The analysis was carried out on a LC-AD Liquid Chromatograph (Shimadzu Corporation, Kyoto, Japan) equipped with a DGU-20A$_5$ degasser, a SIL-20AHT autosampler, a CTO-10ASVP column oven and a SPD-M20A diode array detector. Data acquisition and instrumentation control were achieved by means of the LCsolution software (Shimadzu Corporation, Kyoto, Japan).

Chromatographic separation of opicapone, BIA 9-1079 and IS was accomplished in less than 10 min on a reversed-phase LiChroCART® Purospher Star® C_{18} column (55 x 4 mm; 3 µm particle size from Merck KGaA, Darmstadt, Germany), employing a gradient elution program for 14 min at 25 °C. The mobile phase was composed of 0.05 M monosodium phosphate solution adjusted to pH 2.45 ± 0.05 with *ortho*-phosphoric acid (solvent A) and acetonitrile (solvent B) and it was pumped at a flow rate of 0.8 mL/min. The solvent A was filtered through a 0.45 µm filter before use. During the first 4 min of the run, a linear gradient was applied varying the proportion of solvent B from 33% to 60%; this final proportion was maintained for an additional 4 min and, subsequently, the percentage of solvent B was restored to 33% within 1 min and maintained until the end of the run (14 min). The detection of analytes and IS was set at different wavelengths depending on the compound and the rat matrix in study. Thus, in plasma, opicapone and BIA 9-1079 were monitored at 271 nm and IS at 300 nm, while in tissues samples, opicapone was assessed at 257 nm and BIA 9-1079 and IS at 300 nm.

II.3.2.5. Sample Preparation

The final sample pre-treatment herein optimized was very similar for all the rat matrices and it combined two different methodologies: protein precipitation and LLE.

After rat plasma samples had been thawed at room temperature, they were centrifuged at 13 400 rpm (12 045 *g*) for 2 min and 100 µL were spiked with 10 µL of IS working solution. Afterwards, 400 µL of acetonitrile were added to these plasma samples and the mixture was then vortex-mixed for 30 s and centrifuged at 13 400 rpm (12 045 *g*) for 10 min in order to precipitate plasma proteins. The resulting supernatant was collected and acidified with 200 µL of 2 M hydrochloric acid, vortexed for 30 s, and then 500 µL of ethyl acetate was added. This mixture was vortex-mixed for 2 min and then centrifuged at 13 400 rpm (12 045 *g*) for 5 min in order to extract the analytes and the IS. The upper organic layer was transferred to a clean glass tube and the aqueous layer was re-extracted again using the above-mentioned LLE procedure. Organic phases were combined and evaporated to dryness under a gentle nitrogen stream at 45 °C, and the residue was

reconstituted with 100 µL of solvent A–solvent B (50:50, v/v) of mobile phase. The reconstituted extract was then centrifuged at 13 400 rpm (12 045 *g*) for 2 min and 20 µL of the supernatant was injected into the chromatographic system.

The supernatant samples of rat tissues (liver and kidney) homogenates were also thawed at room temperature and 150 µL were spiked with 10 µL of the IS working solution. These tissues supernatants were then prepared to be injected into the chromatographic system, employing the same procedures (protein precipitation and LLE) as described above for plasma samples.

II.3.2.6. Method Validation

The developed method was validated according to the guidelines for bioanalytical method validation from FDA and EMA [249,254] as well as other international recommendations for bioanalytical method validation [247,257]. The method was validated considering the acceptance criteria for the following parameters: selectivity, linearity, precision and accuracy, LLOQ, LOD, sample dilution, recovery and stability.

Selectivity was evaluated by analysing the presence of potential chromatographic interferences from endogenous compounds of rat matrices at the retention times of opicapone, BIA 9-1079 and IS. For that, blank samples (plasma and liver and kidney homogenate supernatants) obtained from six different rats were processed and their chromatograms were compared with those from samples spiked with the analytes and the IS. Furthermore, the interference from pentobarbital and heparin, herein used and frequently employed in experimental protocols of pharmacokinetic studies, was also investigated, as well as drugs potentially co-administered with opicapone (levodopa, carbidopa, benserazide, amantadine, ropinirole, selegiline, biperideno and bromocriptine).

The linearity of the method for opicapone and BIA 9-1079 was evaluated using calibration curves prepared on five separate days (n = 5) with six calibration standards. These curves were constructed plotting peak height ratios (analyte/IS) *versus* the corresponding nominal concentrations. The data were subjected to a weighted linear regression analysis using $1/x^2$ as weighting factor. This weighting factor was selected since it generated the best fit of height ratios *versus* concentration revealed by the lowest value of the sum of absolute percentage relative error [258]. The LLOQ was defined as the lowest concentration that could be measured with a precision not exceeding 20% (expressed as % CV), and an accuracy within ±20% (expressed as % Bias). It was stipulated using five replicates of plasma and tissues (liver and kidney) homogenate supernatant samples (n = 5) both intra- and inter-daily. The LOD, defined as the lowest concentration that can

be distinguished from the noise level, was determined by analysing successive dilutions of rat samples with known concentrations of opicapone and BIA 9-1079 and it was established as the concentration that yields a signal-to-noise ratio of 3:1.

Intra- and inter-day accuracy and precision were assessed in replicate (*n* = 5) using QC_1, QC_2 and QC_3. In order to ensure that concentrations exceeding the upper limit of quantification of the calibration range could be accurately determined after proper sample dilution with blank matrices, the intra- and inter-day accuracy and precision of a 5-fold dilution were also investigated in all matrices using QC_{Dil} samples (*n* = 5). The acceptance criteria for accuracy and precision were a Bias value within ±15% and a CV value ≤ 15%, respectively.

The absolute recovery of opicapone and BIA 9-1079 from plasma and tissues homogenate supernatant samples was determined by comparing the analyte peak heights from QC samples submitted to the previously described pre-treatment process with the corresponding heights obtained from non-extracted solutions at the same nominal concentrations. This procedure was repeated five times (*n* = 5) using the QC samples at three different concentrations (QC_1, QC_2 and QC_3). Similarly, the recovery of the IS was also determined at the concentration used in sample analysis.

The stability of opicapone and BIA 9-1079 was evaluated in all rat matrices and in the processed samples using QC_1 and QC_3. Spiked rat samples were exposed at room temperature for 4 h, at 4 °C for 24 h and at -80 °C for 30 days in order to simulate sample handling and storage time in the refrigerator and freezer before analysis. The effect of three freeze-thaw cycles on the stability of the analytes was also investigated. Briefly, aliquots of spiked plasma or tissue homogenate supernatant samples were stored at -80 °C for 24 h, thawed unassisted at room temperature and then refrozen for 24h under the same conditions until completing the three cycles. Additionally, in order to assess the post-preparative stability of processed samples under autosampler conditions, the reconstituted extracts were analysed after 12 h at room temperature and 24 h at 4 °C. 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 analyte concentrations ratio of 85–115% is accepted as the stability criterion (*n* = 5).

II.3.2.7. Method Application and Pharmacokinetic Analysis

To demonstrate the applicability of the proposed method to real samples, a single-dose (100 mg/kg) of opicapone, suspended in 0.5% carboxymethylcellulose, was administered by oral gavage to male Wistar rats (270–310 g). Then, blood samples (~ 0.3 mL) were collected into heparinised tubes, through a cannula introduced in the tail vein of rats, at several pre-defined post-dose time points (0.5, 1, 2, 4, 8 and 12 h). At the end of the study (12 h post-dosing) the rats were sacrificed and the liver and kidney tissues were also excised. These biological samples were immediately processed, as described in *section II.3.2.3*. All animal experimentation was conducted in accordance with the European Directive 2010/63/EU [270] for the accommodation and care of laboratory animals.

The maximum plasma concentration (C_{max}) of opicapone and BIA 9-1079 and the corresponding time to reach C_{max} (t_{max}) were obtained from the experimental data. The remaining pharmacokinetic parameters were derived by non-compartmental pharmacokinetic analysis from the mean plasma concentration values (n = 3) determined at each time point using the WinNonlin® version 5.2 (Pharsight Co, Mountain View, CA, USA). The estimated pharmacokinetic parameters included the area under the plasma concentration-time curve (AUC) from time zero to the last measurable drug concentration (AUC_{0-t}) calculated by the linear trapezoidal rule; the AUC from time zero to infinity (AUC_{0-inf}) calculated from AUC_{0-t} + (C_{last}/k_{el}), where C_{last} is the last measurable plasma concentration and k_{el} is the apparent plasma elimination rate constant calculated by log-linear regression of the terminal segment of the plasma concentration-time profile; and the plasma elimination half-life ($t_{1/2el}$).

II.3.3. Results and Discussion

II.3.3.1. Method Development

To the best of our knowledge, no liquid chromatography methods have yet been described in the literature to simultaneously quantify opicapone and its active metabolite (BIA 9-1079) in rat matrices. Hence, the HPLC-DAD method herein reported is the first bioanalytical assay published for the quantification of opicapone and BIA 9-1079 in rat.

Employing the HPLC-DAD method that quantifies both analytes in human plasma [269] (described in *section II.2.* of this chapter), the impurity profile obtained for rat plasma revealed interferences at the retention time of BIA 9-1079, preventing its straightforward application. Figure II.3.1 compares blank plasma samples from human and rat applying the conditions reported by Gonçalves *et al.* [269] and described in *sections II.2.2.3.* and *II.2.2.4.* of this chapter. At the retention time of BIA 9-1079, it is evident the presence of an impurity which height is twice the height of BIA 9-1079 at the LLOQ level.

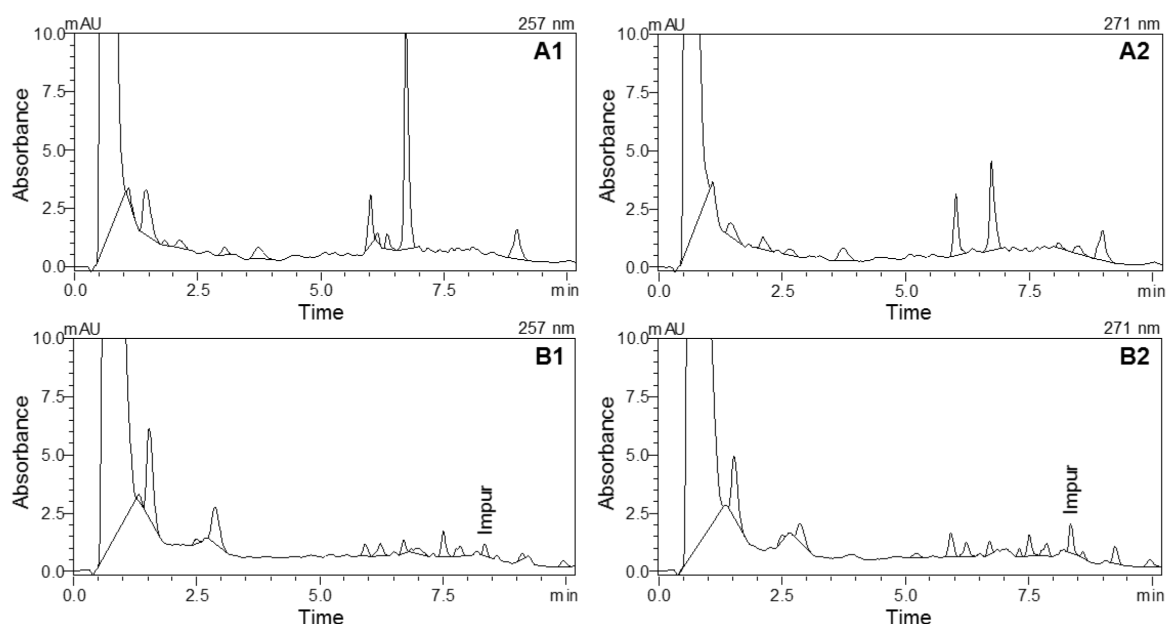


Figure II.3.1 Chromatograms of extracted blank plasma samples applying the conditions reported by Gonçalves *et al.* [269]: human plasma at 257 nm (A1) and at 271 nm (A2), and rat plasma at 257 nm (B1) and at 271 nm (B2). In blank rat plasma samples, a relevant impurity (Impur) appears at the retention time of BIA 9-1079 (8.3 min using the conditions reported by Gonçalves *et al.* [269]).

With the purpose of reducing endogenous impurities of rat matrices and simultaneously extracting opicapone and its active metabolite, simple protein precipitation with methanol, acetonitrile or 20% trichloroacetic acid and distinct LLE procedures using

ethyl acetate, dichloromethane or *n*-hexane (alone or in combination) were investigated. In fact, using methanol, the impurity profile was not improved in relation to acetonitrile, and the use of dichloromethane instead of ethyl acetate was also not favourable. On the other hand, when 20% trichloroacetic acid was used for protein precipitation before LLE, impurities were not eluted at the retention times of the analytes; however, the IS and the metabolite were not detected. Similar results were found with *n*-hexane, which did not enable the extraction of opicapone and BIA 9-1079. Thus, acceptable selectivity and recovery values were only achieved when the samples were previously submitted to a protein precipitation step with acetonitrile and the remaining supernatant was acidified with hydrochloric acid and submitted to two subsequent LLE steps using small volumes of ethyl acetate. The acidification step is important as it decreases the pH value of the mixture, which is essential to increase the amount of analytes extracted by the organic solvent. This fact is probably due to the chemical nature of the nitrocatechol group (in some second-generation COMT inhibitors $pK_{a1} = 4.5$) present in analytes [146].

In parallel, chromatographic conditions, including the gradient elution and the detection wavelengths, were optimized to quantify opicapone and BIA 9-1079 in rat matrices. Using a short reversed-phase LiChroCART® Purospher Star® C_{18} column, the chromatographic conditions were optimized in order to enable the separation of both analytes (opicapone and BIA 9-1079) in a short period of time and to obtain resolved, sharpened and symmetric peaks. Several gradient elution programs were tested in order to improve the selectivity of the method, allowing the separation of the analytes from endogenous interferences of rat matrices. The best relationship between selectivity, resolution, peak shape and run time was achieved applying the gradient elution described in *section II.3.2.4*. Under these chromatographic conditions, the retention times of opicapone and BIA 9-1079 were approximately 4.6 min and 9.0 min, respectively. Tamoxifen was selected as IS owing to its similar behaviour to the analytes throughout sample pre-treatment and chromatographic procedures and also because it is commercially available. Applying the chromatographic conditions described in *section II.3.2.4.*, the IS presented a retention time of 8.2 min. On the other hand, the wavelength values were selected in order to minimize the endogenous interferences eluted at analytes retention times and, simultaneously, keep enough signal intensity. Since one maximum absorption peak of opicapone and BIA 9-1079 is achieved at approximately 271 nm, this wavelength was initially selected to quantify opicapone and BIA 9-1079 in plasma. However, at 271 nm significant endogenous interfering peaks appeared in tissues matrices at the retention times of both analytes. Therefore, in tissues rat matrices the detection wavelengths were changed in order to achieve the best compromise in terms of

selectivity and sensitivity. Hence, opicapone was quantified at 257 nm and BIA 9-1079 at 300 nm, because, as shown in Figure II.3.2C1, BIA 9-1079 has a relevant interfering peak at 257 nm (with a height similar to the LLOQ), which is not detected at 300 nm. Finally, the IS was monitored at 300 nm in all the studied rat matrices, due to the endogenous interferences observed at 271 (Figure II.3.2A1) and 257 nm (Figure II.3.2C1).

Thus, comparing the presented method with the method developed by Gonçalves *et al.* [269] (described in *section II.2.* of this chapter), the gradient elution and the detection wavelengths are different, allowing a suitable selectivity in rat matrices. The lower volume of acetonitrile (human plasma 600 µL *versus* rat matrices 400 µL) and the reduced number of LLE (human plasma three LLE *versus* rat matrices two LLE) employed in rat matrices in relation to human plasma permitted the simplification and the cost reduction of sample processing. Moreover, the volume of sample was reduced for all rat matrices (human plasma 200 µL *versus* rat plasma 100 µL and rat tissues 150 µL). This small plasma sample volume is particularly important to enable multiple blood samples collection from the same animal, as it is desired in pharmacokinetic/toxicokinetic non-clinical studies. Taking into account all these modifications, as well as the species differences, according to the international guidelines [249,254] a partial validation of the method herein described would not be enough to ensure a reliable assay to quantify opicapone and BIA 9-1079; therefore, a full validation was considered.

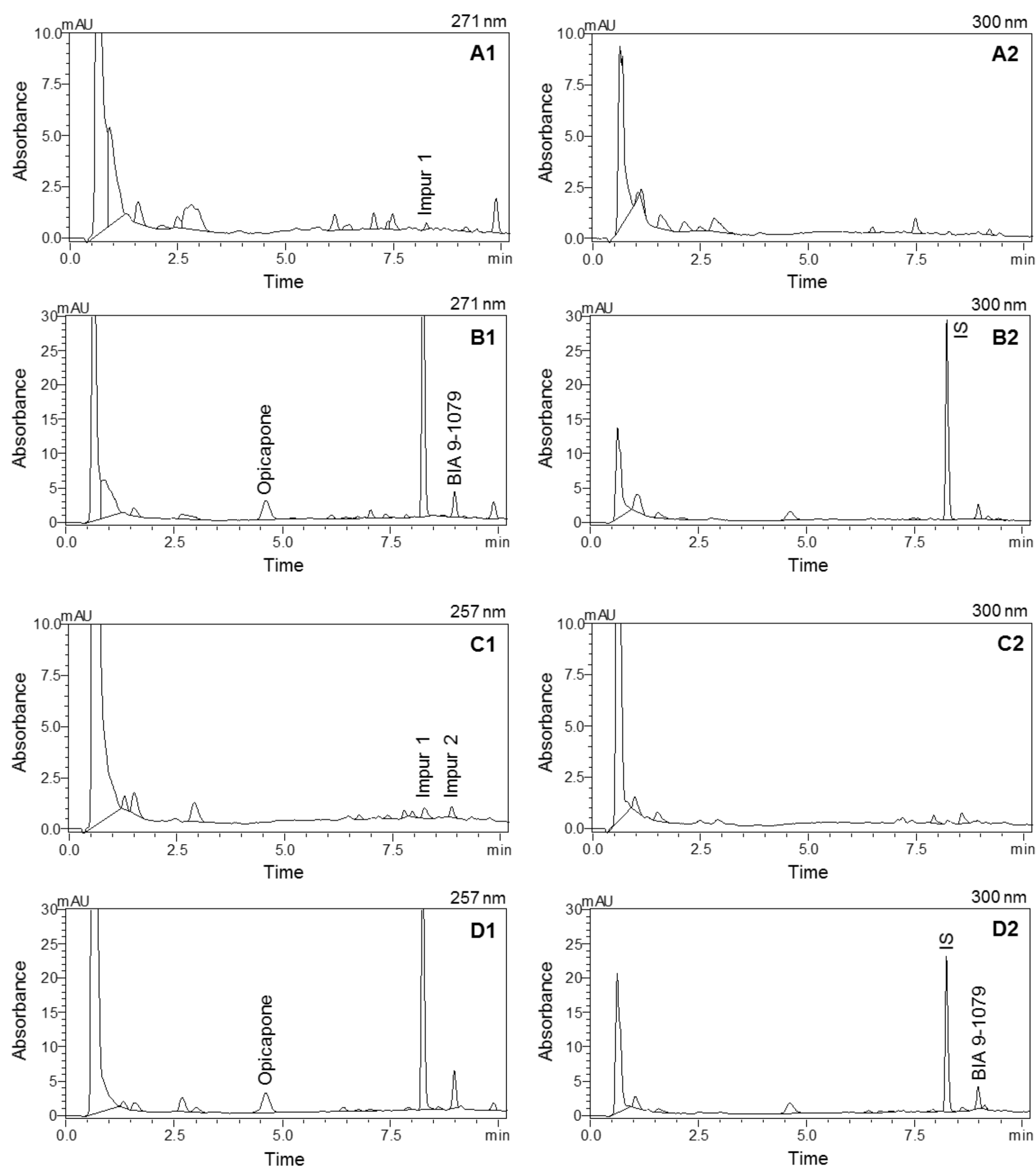


Figure II.3.2 Typical HPLC-DAD chromatograms of extracted rat samples: blank plasma at 271 nm (A1) and at 300 nm (A2); plasma spiked with internal standard (IS) and analytes (0.3 µg/mL) at 271 nm (B1) and at 300 nm (B2); blank liver homogenate supernatant at 257 nm (C1) and at 300 nm (C2); and liver homogenate supernatant spiked with IS and analytes (opicapone at 0.25 µg/mL and BIA 9-1079 at 0.35 µg/mL) at 257 nm (D1) and at 300 nm (D2). Impur 1, impurity at the retention time of IS; Impur 2, impurity at the retention time of BIA 9-1079. Peak shape and chromatographic behaviour of the analytes in kidney homogenate supernatant were very similar to those obtained in liver homogenate supernatant.

II.3.3.2. Method Validation

Again, bearing in mind the international recommendations for bioanalytical method validation, a partial validation could be acceptable for different matrices within the same animal species [249,254]; however, independent full validations were herein carried out for all the rat matrices (plasma and liver and kidney homogenate supernatants) in order to increase the method confidence for its application in further pivotal pharmacokinetic studies.

The selectivity was evaluated and demonstrated in all the intended rat matrices applying the aforementioned sample pre-treatment process and chromatographic conditions. Representative chromatograms of blank and spiked plasma and tissues homogenate supernatants are shown in Figure II.3.2. Accordingly, no interferences were found at the retention times of the studied compounds. Similarly, pentobarbital and heparin, which are used in experimental protocols of pharmacokinetic studies, as well as drugs potentially co-administered with opicapone, did not interfere with the peaks of the analytes or IS.

The linearity of the method within the defined concentration ranges (Table II.3.1) was demonstrated for opicapone and BIA 9-1079 in all the rat matrices in study ($r^2 \geq 0.994$), ensuring a consistent correlation between peak height ratios (analyte/IS) and the corresponding nominal concentrations in plasma or tissues homogenate supernatants. The regression equations of calibration curves and the corresponding coefficients of determination are indicated in Table II.3.1. The LLOQ of the presented method was set at 0.04 µg/mL for opicapone and BIA 9-1079 in plasma, 0.04 µg/mL for opicapone and 0.07 µg/mL for BIA 9-1079 in liver homogenate supernatant, and 0.04 µg/mL for opicapone and 0.06 µg/mL for BIA 9-1079 in kidney homogenate supernatant (Table II.3.2). The LOD was established at 0.030 µg/mL for opicapone and 0.015 µg/mL for BIA 9-1079 in plasma, and 0.025 µg/mL for opicapone and BIA 9-1079 in homogenate supernatants of both tissues.

Table II.3.1 Calibration curve parameters (mean values) of opicapone and its metabolite (BIA 9-1079) in rat matrices (n = 5).

Matrix **Analyte**	**Calibration standards (µg/mL)**	**Regression equation**[a]	r^2
Plasma			
Opicapone	0.04, 0.08, 0.3, 1, 3.5, 6	$y = 0.3319x - 0.0040$	0.998
BIA 9-1079	0.04, 0.08, 0.3, 1, 3.5, 6	$y = 0.4322x - 0.0020$	0.994
Liver homogenate supernatant			
Opicapone	0.04, 0.08, 0.25, 0.8, 2.5, 4	$y = 0.5453x - 0.0051$	0.997
BIA 9-1079	0.07, 0.14, 0.35, 0.8, 2.5, 4	$y = 0.4772x - 0.0074$	0.996
Kidney homogenate supernatant			
Opicapone	0.04, 0.08, 0.25, 0.8, 2.5, 4	$y = 0.5250\text{x} - 0.0056$	0.998
BIA 9-1079	0.06, 0.12, 0.30, 0.8, 2.5, 4	$y = 0.4949x - 0.0030$	0.999

[a] Equation of the calibration curve is given by $y=mx+b$, where x is the analyte concentration, expressed in µg/mL, and y is the analyte to internal standard peak height ratio, expressed in arbitrary height units. r^2, coefficient of determination.

The results of intra- and inter-day accuracy and precision analysis are shown in Table II.3.2. The overall intra- and inter-day accuracy (Bias) ranged from -12.68% to 7.70% and the precision (CV) values did not exceed 11.95%. All the data fulfilled the acceptance criteria of the international guidelines, and hence, the sample pre-treatment procedure followed by the HPLC-DAD method herein described is accurate, precise and reproducible. The dilution integrity of samples was also investigated and the results are also presented in Table II.3.2, demonstrating that a 5-fold sample dilution with corresponding blank rat matrix can be appropriately applied if the concentration of a trial sample surpasses the upper limit of quantification of the calibration curve.

Table II.3.2 Intra- and inter-day precision (% CV) and accuracy (% Bias) achieved for the determination of opicapone and BIA 9-1079 in rat matrices at the concentration of the lower limit of quantification (*), at low, middle and high concentrations of the calibration range and following a sample dilution ([#]) by a 5-fold factor (*n* = 5).

Nominal concentration	Intra-day			Inter-day		
	Experimental concentration[a]	% CV	% Bias	Experimental concentration[a]	% CV	% Bias
Plasma						
Opicapone						
0.04*	0.040 ± 0.002	6.39	-0.43	0.042 ± 0.001	2.56	4.76
0.12	0.129 ± 0.005	4.40	7.70	0.127 ± 0.002	1.35	6.03
3.00	3.026 ± 0.079	2.62	0.88	3.051 ± 0.047	1.53	1.70
5.40	5.338 ± 0.137	2.57	-1.14	5.400 ± 0.093	1.73	-0.003
20[#]	17.858 ± 1.452	8.16	-10.71	17.939 ± 0.508	2.84	-10.31
BIA 9-1079						
0.04*	0.040 ± 0.0003	1.07	0.43	0.039 ± 0.002	6.78	-2.77
0.12	0.118 ± 0.005	4.10	-1.50	0.119 ± 0.005	4.55	-1.16
3.00	3.109 ± 0.119	3.84	3.63	3.116 ± 0.075	2.41	3.88
5.40	5.473 ± 0.263	4.80	1.35	5.634 ± 0.170	3.02	4.34
20[#]	17.716 ± 0.609	3.44	-11.42	18.064 ± 0.177	0.98	-9.68
Liver homogenate supernatant						
Opicapone						
0.04*	0.041 ± 0.002	6.64	2.47	0.039 ± 0.003	8.60	-2.10
0.12	0.111 ± 0.006	5.70	-7.39	0.118 ± 0.005	4.61	-1.94
2.00	1.876 ± 0.076	4.10	-6.22	2.003 ± 0.110	5.54	0.17
3.60	3.265 ± 0.211	6.49	-9.31	3.605 ± 0.126	3.50	0.15
10[#]	8.973 ± 0.093	1.04	-10.27	9.440 ± 0.705	7.50	-5.60
BIA 9-1079						
0.07*	0.064 ± 0.004	7.12	-8.66	0.068 ± 0.005	9.85	-2.28
0.21	0.183 ± 0.010	5.71	-12.68	0.201 ± 0.006	3.41	-4.25
2.00	1.884 ± 0.103	5.50	-5.78	2.011± 0.108	5.42	0.56
3.60	3.313 ± 0.097	2.93	-7.96	3.609 ± 0.103	2.87	0.26
10[#]	9.572 ± 0.188	1.97	-4.28	9.806± 0.383	3.94	-1.94
Kidney homogenate supernatant						
Opicapone						
0.04*	0.042 ± 0.003	8.37	4.62	0.040 ± 0.002	5.63	0.71
0.12	0.115 ± 0.002	1.88	-3.99	0.112 ± 0.007	6.79	-6.45
2.00	2.092 ± 0.104	5.02	4.58	2.115 ± 0.079	3.73	5.73
3.60	3.569 ± 0.202	5.67	-0.87	3.658 ± 0.134	3.68	1.60
10[#]	9.864 ± 0.612	6.24	-1.36	9.527 ± 0.348	3.67	-4.73

Table II.3.2 *(Continued)*

Nominal concentration	Intra-day			Inter-day		
	Experimental concentration[a]	% CV	% Bias	Experimental concentration[a]	% CV	% Bias
Kidney homogenate supernatant						
BIA 9-1079						
0.06*	0.056 ± 0.005	8.99	-6.22	0.062 ± 0.007	11.95	2.79
0.18	0.164 ± 0.008	4.73	-8.80	0.182 ± 0.005	2.69	0.94
2.00	2.037 ± 0.117	5.74	1.85	2.084 ± 0.115	5.52	4.18
3.60	3.458 ± 0.123	3.55	-3.94	3.597 ± 0.116	3.24	-0.09
10[#]	9.799 ± 0.296	3.00	-2.01	9.402 ± 0.191	2.03	-5.98

[a] Results expressed as mean ± standard deviation. Nominal and experimental concentrations are expressed in µg/mL. Bias, deviation from nominal values; CV, coefficient of variation.

The absolute recoveries of opicapone and BIA 9-1079 from rat matrices were estimated and are shown in Table II.3.3. LLE is frequently associated with low recovery values; however, the mean absolute recovery achieved was higher than 69% for both compounds in all the rat matrices, and low CV values were also found (≤ 7.91%). The absolute recovery of the IS in all the rat matrices was also assessed and the mean values (n = 15) obtained in plasma and in liver and kidney homogenate supernatants were 88.44% (CV = 3.87%), 84.67% (CV = 4.11%) and 81.86% (CV = 6.14%), respectively. The low CV values coupled to the high values of recovery yields clearly corroborate that the sample preparation and extraction procedures employed are reproducible. Furthermore, considering all the recovery values obtained at the three assessed concentration levels for each analyte, the CV values (n = 15) in plasma and in liver and kidney homogenate supernatants were respectively 4.25, 5.68 and 4.05% for opicapone and 6.71, 7.66 and 5.26% for BIA 9-1079, suggesting a consistent recovery over the evaluated concentration ranges.

Table II.3.3. Absolute recovery from rat matrices (%) of opicapone and BIA 9-1079 obtained employing the optimized sample treatment and extraction procedures. Low, medium and high quality control samples were used (*n* = 5).

Matrix Analyte	Nominal concentration (µg/mL)	Recovery (%)	
		Mean ± SD	CV (%)
Plasma			
Opicapone	0.12	101.55 ± 5.38	5.30
	3.00	100.73 ± 2.27	2.25
	5.40	98.31 ± 4.74	4.82
BIA 9-1079	0.12	78.92 ± 5.66	7.17
	3.00	81.69 ± 4.78	5.85
	5.40	84.13 ± 5.74	6.82
Liver homogenate supernatant			
Opicapone	0.12	83.86 ± 6.63	7.91
	2.00	87.72 ± 1.99	2.27
	3.60	87.39 ± 4.98	5.70
BIA 9-1079	0.21	69.88 ± 2.73	3.90
	2.00	76.37 ± 1.81	2.37
	3.60	82.99 ± 1.34	1.61
Kidney homogenate supernatant			
Opicapone	0.12	88.32 ± 3.48	3.94
	2.00	92.16 ± 2.04	2.22
	3.60	91.16 ± 4.56	5.01
BIA 9-1079	0.18	76.93 ± 2.16	2.81
	2.00	76.63 ± 2.74	3.58
	3.60	81.73 ± 5.19	6.35

CV, coefficient of variation; SD, standard deviation.

The stability of opicapone and BIA 9-1079 in rat matrices was investigated under the conditions previously stated in *section II.3.2.6.* and the obtained results are reported in Table II.3.4. From the obtained stability data, it is evident that no significant loss of opicapone and BIA 9-1079 was observed in rat plasma, as well as in processed liver and kidney homogenate supernatants. In unprocessed tissue homogenate supernatants, opicapone and BIA 9-1079 were demonstrated to be stable in all the conditions indicated in Table II.3.4, with the exception of opicapone in the kidney homogenate supernatant at room temperature, which presented considerable degradation at all times considered. Nevertheless, these results do not comprise the application of the methodology described here, as long as kidney homogenate supernatant samples are always prepared on ice.

Table II.3.4 Stability (values in percentage) of opicapone and BIA 9-1079 in rat matrices (n = 5) under the conditions that mimic sample handling and storage.

Stability conditions	Stability/reference analyte concentrations (%)			
	Opicapone		BIA 9-1079	
	$C_{Nominal}$[a] (μg/mL)		$C_{Nominal}$[a] (μg/mL)	
Plasma	**0.12**	**5.40**	**0.12**	**5.40**
Unprocessed plasma				
Room temperature (4 h)	105.58	97.94	113.32	98.28
4 °C (24 h)	110.43	101.36	109.67	101.59
Three freeze/thaw cycles (-80 °C)	105.51	103.73	102.94	100.15
-80 °C (30 days)	111.23	106.46	113.05	99.55
Processed plasma				
Room temperature (12 h)	110.48	105.96	105.04	102.07
4 °C (24 h)	110.05	103.77	93.14	103.23
Liver	**0.12**	**3.6**	**0.21**	**3.6**
Unprocessed liver homogenate supernatant				
Room temperature (2 h)	95.14	98.62	107.83	98.95
4 °C (12 h)	91.81	95.19	102.00	98.36
Two freeze/thaw cycles (-80 °C)	87.07	96.93	99.42	106.34
-80 °C (15 days)	93.30	93.97	92.79	100.73
Processed liver homogenate supernatant				
Room temperature (12 h)	111.60	111.53	103.68	99.54
4 °C (24 h)	105.80	104.80	100.92	102.92
Kidney	**0.12**	**3.6**	**0.18**	**3.6**
Unprocessed kidney homogenate supernatant				
Room temperature (2 h)	72.39	86.39	86.51	96.09
4 °C (5 h)	105.25	94.31	106.47	99.13
Three freeze/thaw cycles (-80 °C)	95.18	97.27	101.87	101.32
-80 °C (30 days)	98.90	91.94	102.46	100.38
Processed kidney homogenate supernatant				
Room temperature (12 h)	108.56	95.76	102.75	94.67
4 °C (24 h)	112.36	95.93	110.54	93.65

[a] $C_{Nominal}$, Nominal concentration

II.3.3.3. Method Application and Pharmacokinetic Study

The reported HPLC-DAD method was mainly developed to support non-clinical pharmacokinetic/toxicokinetic studies in rats. Hence, a preliminary study was performed with a small number of Wistar rats (n = 3), and it was carried out with the purpose of demonstrating the applicability of the method for analysis of opicapone and BIA 9-1079 in plasma and tissues (liver and kidney). After opicapone (100 mg/kg) administration to the rats, multiple blood samples were collected at different time points during a period of 12 h in order to obtain the plasma pharmacokinetic profiles for opicapone and its active metabolite. The mean plasma concentration-time profiles (n = 3) of opicapone and BIA 9-1079 are depicted in Figure II.3.3. In addition, the corresponding pharmacokinetic parameters are summarized in Table II.3.5. The t_{max} of opicapone and BIA 9-1079 occurred at 2 and 8 h post-dose, respectively. Indeed, similar t_{max} values were observed for opicapone in humans (t_{max} ranged between 1.5 and 3.5 h) following single oral doses of opicapone [218]. Taking into account the AUC_{0-t} values achieved for both analytes (opicapone and BIA 9-1079) in rat, it is evident that BIA 9-1079 represents 32.9% of the systemic exposure to opicapone. Once again, comparing these results with those obtained in humans [218], it is also clear that the systemic exposure to BIA 9-1079 is higher in rat than in man after opicapone administration. Therefore, it is relevant to quantify and evaluate simultaneously the biodisposition of BIA 9-1079 and opicapone in rat. The concentrations of opicapone and BIA 9-1079 found in liver and kidney homogenate supernatant samples at 12 h post-dosing were also determined, showing concentration levels equal to or below the LLOQ of the method. The low concentrations of opicapone and BIA 9-1079 measured in liver and kidney tissues at 12 h post-dosing do not compromise the application of the method. However, it is suggested that shorter post-dosing times should be considered in subsequent pharmacokinetic studies designed to assess the tissue disposition of opicapone and its active metabolite.

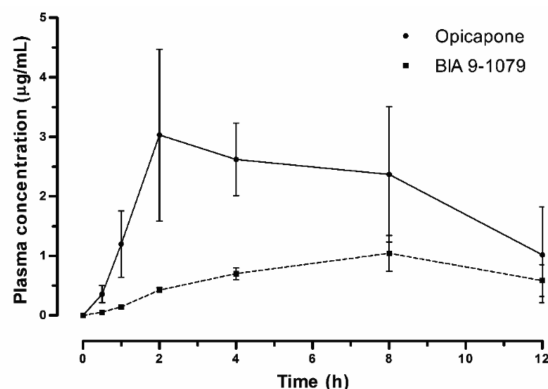


Figure II.3.3 Mean plasma concentration-time profiles of opicapone and BIA 9-1079 following a single oral dose of opicapone (100 mg/kg) to rats. Symbols represent the mean values ± standard error of the mean (SEM) of three determinations per time point obtained from different rats (n = 3).

Table II.3.5 Plasma pharmacokinetic parameters of opicapone and BIA 9-1079 following a single oral dose of opicapone (100 mg/kg) to rats.

Pharmacokinetic parameters[a]	**Opicapone**	**BIA 9-1079**
t_{max} **(h)**	2.00	8.00
C_{max} **(µg/mL)**	3.031	1.042
AUC_{0-t} **(µg.h/mL)**	24.997	8.211
AUC_{0-inf} **(µg.h/mL)**	35.014	NC
k_{el} **(h^{-1})**	0.102	NC
$t_{1/2el}$ **(h)**	6.81	NC

[a] t_{max} and C_{max} are experimental values; AUC_{0-t}, AUC_{0-inf}, k_{el} and $t_{1/2el}$ values were calculated by non-compartmental analysis from mean concentrations at each time point (n = 3). AUC_{0-inf}, area under the drug concentration-time curve from time zero to infinity; AUC_{0-t}, area under the drug concentration-time curve from time zero to the time of the last measurable concentration; C_{max}, maximum plasma concentration; k_{el}, apparent plasma elimination rate constant; NC, not calculated; $t_{1/2el}$, plasma elimination half-life; t_{max}, time to reach the maximum plasma concentration.

II.3.4. Conclusion

A novel selective, sensitive, precise, accurate and reproducible HPLC-DAD method was herein developed and fully validated for the simultaneous quantification of opicapone and BIA 9-1079 in rat plasma and liver and kidney homogenate supernatants. The applied sample pre-treatment procedure is inexpensive, since it combines a protein precipitation step followed by a LLE, which require small volumes of solvents. Moreover, it showed high and precise absolute recovery values. Another important aspect of this method is the small plasma 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 used animals. This is particularly relevant because of the particular attention given to the implementation of principles of the 4R's (replacement, reduction, refinement and responsibility) [271]. The reported bioanalytical method was also successfully applied to quantify opicapone and BIA 9-1079 in real biological samples.

Therefore, it can be concluded that this HPLC-DAD method is a useful tool to support *in vivo* non-clinical studies designed to investigate the pharmacokinetics and biodisposition of opicapone in rats.

CHAPTER III

PHARMACOKINETICS OF OPICAPONE, A THIRD-GENERATION COMT INHIBITOR, AFTER SINGLE AND MULTIPLE ORAL ADMINISTRATION: A COMPARATIVE STUDY IN THE RAT

III.1. Introduction

PD is a chronic and progressive degenerative brain condition, recognized as the second most common neurodegenerative disorder worldwide, only surpassed by Alzheimer's disease [19]. PD is more predominant in the elderly with a prevalence of about 1–2% [19,35]. As the elderly population is increasing, if no cure arises in a near future, the number of individuals with PD is expected to rise very sharply [25]. In fact, several new drugs have emerged over the last decades for the PD treatment (e.g., rasagiline, ropinirole, pramipexole). However, the dopamine replacement therapy with levodopa is still considered the most effective pharmacological treatment for the symptomatic management of the disease [16]. Nevertheless, as levodopa is extensively metabolized in peripheral tissues, mainly by AADC and by COMT, the inhibition of this peripheral metabolism is essential to increase the access of levodopa to the brain. Thus, in order to optimize the therapeutic effect of oral levodopa therapy, levodopa is prescribed in combination with an AADC inhibitor and, for patients suffering from motor fluctuations, a COMT inhibitor is also recommended to be associated with the previous combination [16,272].

Up to June of 2016, when opicapone was approved to be use in the European Union by the EMA, there were only two COMT inhibitors clinically used (entacapone and tolcapone). However, both exhibit drawbacks related to their pharmacokinetics, pharmacodynamics, clinical efficacy and/or safety which hamper their success [138]. After the introduction of tolcapone into the market, severe hepatotoxicity adverse reactions were notified and, consequently, its prescription has been restricted to patients whose therapy with entacapone has failed. In addition, strict monitoring of the liver function is required during the intake of tolcapone. With regard to entacapone, although it is not associated with liver toxicity, its low oral bioavailability (~ 35%), short duration of action, and low potency (maximum erythrocyte COMT inhibition is around 60% and the enzyme recovers full activity within 8 h post-dosing) at the recommended therapeutic dose of 200 mg limit its clinical efficacy [138]. In this context, the reversible and purely peripheral third-generation COMT inhibitor, opicapone arose as an attempt to fulfil the need of a safer, more potent and long-acting COMT inhibitor [138,189].

Non-clinical studies performed in rats demonstrated that opicapone is a longer-acting and more potent peripheral COMT inhibitor than tolcapone and, thereby, also than entacapone [189]. Indeed, after the administration of a single oral dose of opicapone (3 mg/kg) to rats, around 50% of the peripheral (liver and kidney) COMT enzyme activity was still inhibited at 24 h post-dose, in opposition to tolcapone, which activity against liver COMT remained only up to 9 h post-dose [189,226]. The interaction of

opicapone with levodopa has also been evaluated and compared to tolcapone in rats. Two hours after the COMT inhibitor had been administered, levodopa and benserazide, an AADC inhibitor, were administered together and the bioavailability of levodopa increased similarly in the presence of opicapone and tolcapone. However, when the combination levodopa/benserazide was given 24 h after the COMT inhibitor, only opicapone improved the bioavailability of levodopa [226]. These findings are favourable to support a once-daily administration regimen of opicapone. Thus, in phase III clinical trials, opicapone was administered once-daily to PD patients with motor fluctuations as an adjunct to levodopa/AADC inhibitor therapy, revealing to be not only safe and well tolerated in short- and long-term use (BIPARK I and II studies) [233,238] but also effective in reducing the off-time (BIPARK I study) [231] and increasing the on-time without troublesome dyskinesia (BIPARK I and II studies) [273].

As tolcapone was associated with hepatotoxicity in humans, the potential hepatic toxicity induced by opicapone was assessed *in vitro* using primary human hepatocytes and HepaRG cells and revealed a wider safety margin for opicapone than for tolcapone or entacapone [226,236].

In non-clinical studies various metabolites of opicapone were identified, among which BIA 9-1079 stood out as an inhibitor of the COMT enzyme [218]; nevertheless, the metabolic profile of opicapone is not extensively documented in rats or other non-human species. Hence, in order to deepen the non-clinical pharmacological knowledge about opicapone and complement the data generated during its clinical development program, the present work was planned to evaluate the systemic pharmacokinetics of opicapone and its active metabolite after oral administration of the parent drug to rats. Thus, firstly, the plasma pharmacokinetics of opicapone and BIA 9-1079 was evaluated following three single oral rising doses and, then, a specific dose was selected to compare the pharmacokinetics of opicapone and BIA 9-1079 after single- and multiple-dose regimens.

III.2. Materials and Methods

III.2.1. Drugs and Reagents

Opicapone (BIA 9-1067) and BIA 9-1079 were kindly supplied by BIAL – Portela & Cª., S.A. (S. Mamede do Coronado, Portugal). Hydroxypropyl methylcellulose (HPMC) for preparation of opicapone oral suspensions was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). Pentobarbital sodium salt, tamoxifen citrate salt, sodium dihydrogen phosphate dehydrate and 37% hydrochloric acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile (HPLC gradient grade) and ethyl acetate were acquired from Fisher Scientific (Leicestershire, UK), and the ultra-pure water (HPLC, 18.2 MΩ.cm) was obtained by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). The 85% *ortho*-phosphoric acid was purchased from Panreac (Barcelona, Spain). Other used compounds were sodium chloride 0.9% solution for injection and heparin sodium 5000 I.U./mL for injection (B. Braun Medical, Portugal).

III.2.2. Animals

Healthy adult male Wistar rats (RccHan:WIST), weighting between 300 and 370 g, were supplied by Harlan Laboratories (Barcelona, Spain) and housed under controlled environmental conditions (12 h light/dark cycle; temperature 22 ± 1 °C; relative humidity 50 ± 5%) with free access to a standard maintenance diet (4RF21, Mucedola, Italy) and tap water *ad libitum*. Animals were acclimated for at least one week before use.

Opicapone was daily suspended in a HPMC (0.2%, w/v) solution and it was orally given (4 mL/kg of body weight) by gavage using a stainless steel curved feeding needle. In single-dose studies and on the last dosing day of the multiple-dose study, rats were fasted for 12–15 h before the oral administration, and the food was only provided at 4 h post-dose in order to avoid its effect on the oral bioavailability of opicapone. On the remaining days of the multiple-dose study, rats were maintained fasted for at least 12 h before administration and until 2 h post-dosing. Rats had free access to water throughout the experimental period in all the studies.

All the animal procedures were conducted in conformity with the international regulations of the European Directive (2010/63/EU) [270] regarding the protection of laboratory animals used for scientific purposes, the Portuguese law on animal welfare (Decreto-Lei 113/2013) [274] and the employed experimental procedures were reviewed and approved by the Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção-Geral de Alimentação e Veterinária).

III.2.3. Experimental Design

The present work comprised two pharmacokinetic studies. The first one consisted in evaluating the plasma pharmacokinetics and dose-proportionality of opicapone after single oral rising doses, and the second one was performed to investigate plasma pharmacokinetics and accumulation of opicapone and BIA 9-1079 after multiple-dose administration of opicapone for seven consecutive days.

III.2.3.1. Single-dose Pharmacokinetic Study

In order to study the plasma pharmacokinetics and dose-proportionality of opicapone, twenty-four rats were randomly divided into three groups (n = 8 per group). Each group was treated with a single oral dose of opicapone (30, 60 or 90 mg/kg) and blood samples (~ 0.3 mL) were collected into heparinized tubes at 0.5, 1, 2, 3, 4, 5, 6, 8, 12 and 24 h post-administration.

At the night of the day before opicapone administration, rats were anaesthetized with sodium pentobarbital (60 mg/kg, i.p. administration), and their lateral tail vein was cannulated by inserting the Introcan® Certo IV indwelling cannula (22G; 0.9 x 2.5 mm; from B. Braun, Melsungen, Germany). This procedure allowed to obtain a complete plasma concentration-time profile from each rat. After full overnight recovery from anaesthesia, rats were dosed and blood was collected through the cannula in conscious rats appropriately restrained only at the moment of the oral administration and blood sampling. The collected blood volume was replaced by the injection of sterile heparinized saline (5 I.U./mL). The blood samples were processed for drug analysis.

III.2.3.2. Multiple-dose Pharmacokinetic Study

To assess the systemic accumulation of opicapone and BIA 9-1079, rats (n = 8) were orally treated with opicapone (30 mg/kg) once-daily for seven consecutive days. Between the 2nd and 6th days, a blood sample from the lateral vein of each rat was collected at 1 h pre-dose. At the night before the 7th day, rats were anaesthetized for cannulation of the lateral tail vein as described in the *section III.2.3.1*. On the 7th day, multiple serial blood samples (~ 0.3 mL) were collected through the cannula into heparinized tubes 1 h before opicapone administration (pre-dose collection) and at 0.5, 1, 2, 3, 4, 5, 6, 8, 12 and 24 h post-dose; the collected blood volume was replaced by the injection of sterile heparinized saline (5 I.U./mL). The blood samples were processed for drug analysis.

III.2.4. Drug Analysis

After being collected, blood samples were immediately centrifuged at 2900 rpm (1514 *g*), for 10 min, at 4 °C to obtain plasma, which was stored at -80 °C until analysis.

The concentrations of opicapone and BIA 9-1079 in plasma were determined according to the method previously developed and fully validated by Gonçalves *et al.* [275] (*Chapter II*, *section II.3.*). In brief, aliquots of plasma (100 µL) were spiked with 10 µL of the IS working solution (tamoxifen citrate, 90 µg/mL). Afterwards, samples were submitted to a deproteinization with acetonitrile (400 µL) and the resulting supernatant was acidified with 2 M hydrochloric acid (200 µL) and then submitted to a double LLE with ethyl acetate (2 x 500 µL). The whole organic extract was evaporated to dryness under a nitrogen stream at 45 °C and the obtained residue was reconstituted and injected into a HPLC-DAD system (LC-AD Liquid Chromatograph, Shimadzu Corporation, Kyoto, Japan). The separation of opicapone and BIA 9-1079 was performed on a reversed-phase LiChroCART® Purospher Star® C_{18} column (3 µm, 55 mm x 4 mm; Merck KGaA, Darmstadt, Germany) at 25 °C employing a gradient elution program with a mobile phase composed of 0.05 M monosodium phosphate solution (pH 2.45 ± 0.05 adjusted with *ortho*-phosphoric acid) and acetonitrile pumped at 0.8 mL/min. The calibration curves were established within the range of 0.04–6.0 µg/mL for both analytes. When necessary, a 5-fold dilution of plasma samples with blank matrix was applied.

III.2.5. Pharmacokinetic Analysis

The maximum plasma concentration (C_{max}) of opicapone and BIA 9-1079 and the corresponding time to reach C_{max} (t_{max}) were directly obtained from the experimental data. The remaining pharmacokinetic parameters were estimated from the individual plasma concentration-time profiles by non-compartmental pharmacokinetic analysis using the WinNonlin® (Pharsight Co, Mountain View, CA, USA). The estimated pharmacokinetic parameters included the area under the plasma drug concentration-time curve (AUC) from time zero to the time of the last measurable drug concentration (AUC_{0-t}) which was calculated by the linear trapezoidal rule; the AUC from time zero to infinity (AUC_{0-inf}) that was calculated based on the equation 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 plasma elimination rate constant calculated by the log-linear regression of the terminal segment of the concentration-time profile; and the percentage of AUC extrapolated from t_{last} to infinity [AUC_{extrap} (%)]. It was also determined the plasma elimination half-life ($t_{1/2el}$) as well as the mean residence time (MRT). Plasma

concentrations below the LLOQ of the analytical method were considered as zero for the pharmacokinetic data analysis.

The theoretical accumulation ratio (R_0) was calculated according to $R_0 = 1/(1–e^{–ke l\tau})$, where τ is the dosing interval (herein, 24 h). The observed accumulation ratio (R_{ac}) was estimated by dividing the mean AUC within the dosing interval (AUC_τ) of the 7th day of the multiple-dose study by the mean AUC from time zero to 24 h ($AUC_{0\text{-}24h}$) of the single-dose study ($R_{ac} = AUC_\tau /AUC_{0\text{-}24h}$).

III.2.6. Statistical Analysis

As the t_{max} in the present pharmacokinetic studies is a non-continuous variable and determined by the sampling schedule, it was expressed as median (range) and the non-parametric tests Kruskal-Wallis and Mann-Whitney were used to analyse the t_{max} data between three or two groups, respectively. The log-transformed $t_{1/2el}$ and the dose-normalized $AUC_{0\text{-}t}$, $AUC_{0\text{-}inf}$ and C_{max} were compared between three groups with the test one-way ANOVA followed by Tukey post-test. Comparisons of the pharmacokinetic parameters between two groups were made using unpaired two-tailed Student's t-test and when appropriated the parameters were previously log-transformed. A difference was considered statistically significant for a p-value lower than 0.05 ($p < 0.05$).

III.3. Results

III.3.1. Single-dose Pharmacokinetic Study

The mean plasma concentration-time profiles (n = 8) of opicapone and its active metabolite (BIA 9-1079) following the administration of single oral doses (30, 60 or 90 mg/kg) of the parent drug (opicapone) to rats are depicted in Figure III.1. The corresponding mean plasma pharmacokinetic parameters estimated by non-compartmental analysis of each individual concentration-time profile are summarized in Table III.1. The median values for t_{max} of opicapone ranged from 1.5 h (60 and 90 mg/kg) to 2 h (30 mg/kg), but these differences were not statistically different ($p > 0.05$). On the other hand, as expected, the C_{max} of BIA 9-1079 was attained later at 4.5 or 5.0 h post-dose (no statistical significance among the tested doses was observed, $p > 0.05$). Analysing the AUC_{0-inf}, AUC_{0-t} and C_{max} of opicapone, its extent of systemic exposure augmented as the dose increased. For a successive dose level increment in the ratio of 2.0:1.5 (30 to 60 mg/kg and 60 to 90 mg/kg), the AUC_{0-inf}, AUC_{0-t} and C_{max} values increased in the proportion of 1.9:1.5, 1.9:1.5 and 1.4:1.8, respectively. These results suggest an approximately dose-proportional increase in AUC_{0-inf} and AUC_{0-t} values. Additionally, no significant changes in dose-normalized AUC_{0-inf}, AUC_{0-t} and C_{max} values were observed over the three tested doses ($p > 0.05$). Regarding BIA 9-1079, for a dose level increase of opicapone in the ratio of 2.0:1.5, AUC_{0-inf}, AUC_{0-t} and C_{max} values ranged in the proportion of 1.9:0.9, 2.0:0.9 and 1.6:1.0, respectively. Thus, apparently, the extent of systemic exposure to BIA 9-1079 proportionally increased from 30 to 60 mg/kg considering the AUC_{0-inf} and AUC_{0-t}, but it remained unchanged when the dose increased from 60 to 90 mg/kg (Table III.1; Figure III.1B). The $t_{1/2el}$ of opicapone slightly increased as the dose of the drug was heightened, but a statistically significant increase was only detected between the doses of 30 and 90 mg/kg ($p < 0.05$), while the $t_{1/2el}$ of BIA 9-1079 remained unchanged. Considering the achieved AUC_{0-t} values, BIA 9-1079 represented 53.7% and 56.5% of the systemic exposure to opicapone after doses of 30 and 60 mg/kg had been administered, but it only contributed to 33.6% after administering the dose of 90 mg/kg.

As opicapone apparently has a dose-independent pharmacokinetics in rat plasma after a single oral dose in the studied range (30–90 mg/kg), the dose of 30 mg/kg was chosen for the following multiple-dose pharmacokinetic study.

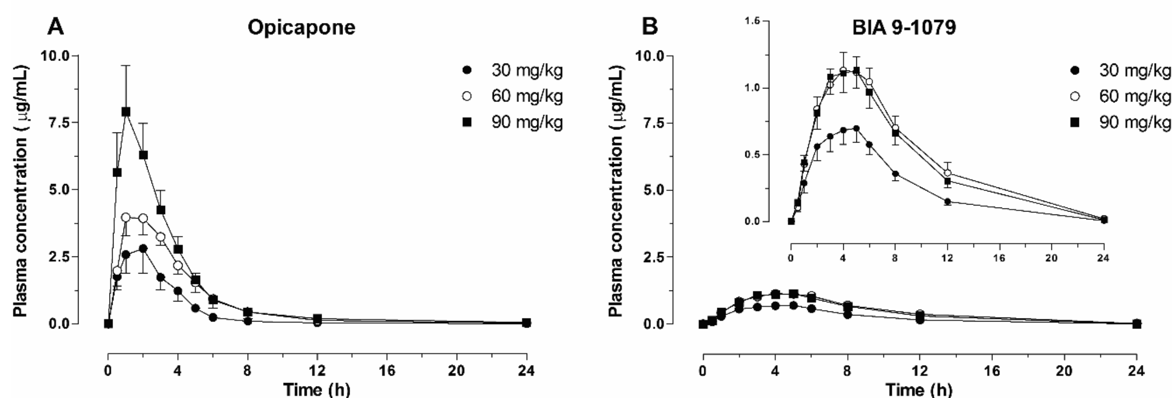


Figure III.1 Mean plasma concentration-time profiles of opicapone (A) and its active metabolite (BIA 9-1079; B) following single oral administration of opicapone (30, 60 and 90 mg/kg) to Wistar rats. The inset in B shows an expanded representation of the concentration-time profiles of BIA 9-1079 in a shorter y-axis scale. Symbols represent the mean values ± standard error of the mean (SEM) of eight determinations per time point (n = 8).

Table III.1 Plasma pharmacokinetic parameters of opicapone and its active metabolite (BIA 9-1079) following single oral doses of opicapone (30, 60 and 90 mg/kg) to rats (n = 8 per group, unless otherwise noted).

Compound	Pharmacokinetic parameters[a]	Dose		
		30 mg/kg	60 mg/kg	90 mg/kg
Opicapone	t_{max} (h)	2.0 (1.0–4.0)	1.5 (1.0–3.0)	1.5 (0.5–3.0)
	C_{max} (μg/mL)	3.46 ± 2.58	4.85 ± 1.65	8.67 ± 4.54
	$AUC_{0\text{-}t}$ (μg.h/mL)	9.76 ± 7.21	18.16 ± 7.01	28.00 ± 10.65
	$AUC_{0\text{-}inf}$ (μg.h/mL)	9.97 ± 7.39	18.61 ± 7.34	28.58 ± 10.88
	AUC_{extrap} (%)	3.02 ± 2.86	2.08 ± 1.69	2.08 ± 1.12
	k_{el} (h^{-1})	0.59 ± 0.33	0.50 ± 0.39	0.21 ± 0.14
	$t_{1/2el}$ (h)	1.58 ± 0.93	2.89 ± 2.94	4.50 ± 2.28
	MRT (h)	3.12 ± 1.34	3.78 ± 1.47	3.83 ± 0.96
BIA 9-1079	t_{max} (h)	4.5 (3.0–6.0)	5.0 (4.0–6.0)	5.0 (3.0–5.0)
	C_{max} (μg/mL)	0.74 ± 0.30	1.21 ± 0.36	1.19 ± 0.39
	$AUC_{0\text{-}t}$ (μg.h/mL)	5.24 ± 2.27	10.27 ± 3.95	9.41 ± 4.21
	$AUC_{0\text{-}inf}$ (μg.h/mL)	6.20 ± 2.35[b]	11.47 ± 3.94[b]	10.36 ± 4.12
	AUC_{extrap} (%)	11.37 ± 5.99[b]	8.04 ± 5.94[b]	10.26 ± 6.22
	k_{el} (h^{-1})	0.22 ± 0.05[b]	0.20 ± 0.04[b]	0.21 ± 0.03
	$t_{1/2el}$ (h)	3.28 ± 1.00[b]	3.50 ± 0.62[b]	3.38 ± 0.40
	MRT (h)	6.58 ± 1.08[b]	7.37 ± 0.97[b]	7.07 ± 0.80

[a] Parameters values are expressed as mean ± standard deviation, except t_{max} values expressed as median (range); [b] n = 7. AUC_{extrap}, area under the drug concentration-time curve extrapolated from the time of the last measurable concentration to infinity; $AUC_{0\text{-}inf}$, area under the drug concentration-time curve from time zero to infinity; $AUC_{0\text{-}t}$, area under the drug concentration-time curve from time zero to the time of the last measurable concentration; C_{max}, maximum plasma concentration; k_{el}, apparent plasma elimination rate

constant; MRT, mean residence time; $t_{1/2el}$, plasma elimination half-life; t_{max}, time to reach the maximum plasma concentration.

III.3.2. Multiple-dose Pharmacokinetic Study

The mean plasma concentration-time profiles (n = 8) of opicapone and its active metabolite following the 7th and last daily oral dose of 30 mg/kg opicapone to rats are shown in Figure III.2, and the respective mean plasma pharmacokinetic parameters are summarized in Table III.2. For an easier comparison, the concentration-time profiles and the pharmacokinetic parameters of the single oral dose of 30 mg/kg were also presented in Figure III.2 and Table III.2, respectively. The concentrations of both compounds (opicapone and BIA 9-1079) in plasma samples collected at 1 h pre-dose between day two and day seven were equal or below the LLOQ of the method, and hence, the statistical assessment of steady-state could not be carried out. Nevertheless, these results suggested that opicapone and BIA 9-1079 were almost completely eliminated from the systemic circulation before the following administration of opicapone, showing a low probability of systemic accumulation in the tested dosage regimen (30 mg/kg/day).

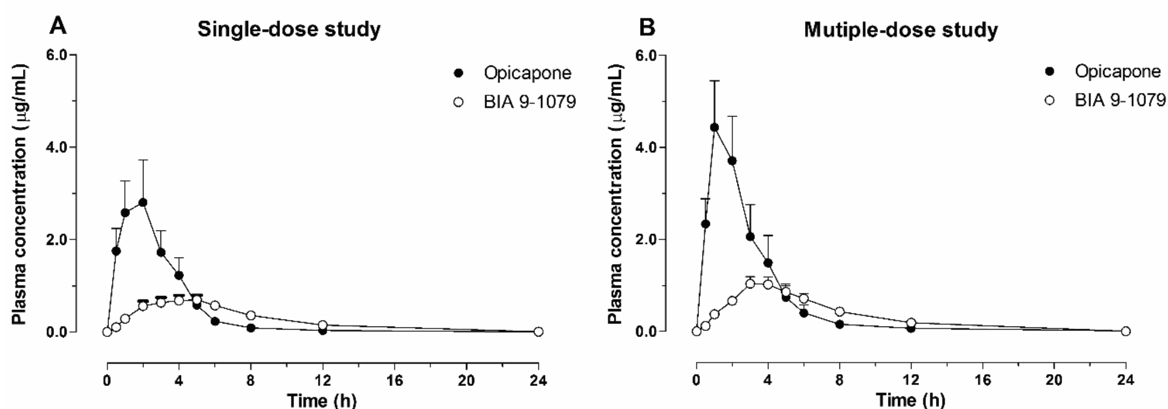


Figure III.2 Mean plasma concentration-time profiles of opicapone and its active metabolite (BIA 9-1079) following a single oral administration of opicapone 30 mg/kg (A; these profiles were also shown in Figure III.1, but they were here replicated to facilitate the comparison of both dosage regimens) and the last dose of a seven day once-daily regimen with opicapone 30 mg/kg (B) to Wistar rats. Symbols represent the mean values ± standard error of the mean (SEM) of eight determinations per time point (n = 8).

Table III.2 Plasma pharmacokinetic parameters of opicapone and its active metabolite (BIA 9-1079) following single and multiple oral doses of opicapone (30 mg/kg) to rats (n = 8 per group, unless otherwise noted).

Pharmacokinetic parameters[a]	Opicapone		BIA 9-1079	
	Single-dose[b]	Multiple-dose[c]	Single-dose[b]	Multiple-dose[c]
t_{max} (h)	2.0 (1.0–4.0)	1.0 (1.0–2.0)	4.5 (3.0–6.0)	3.0 (3.0–4.0)*
C_{max} (µg/mL)	3.46 ± 2.58	5.12 ± 3.18	0.74 ± 0.30	1.08 ± 0.49
AUC_{0-t} (µg.h/mL)	9.76 ± 7.21	14.00 ± 10.10	5.24 ± 2.27	6.64 ± 2.69
AUC_{0-inf} (µg.h/mL)	9.97 ± 7.39	14.18 ± 10.14	6.20 ± 2.35[d]	7.57 ± 3.18
AUC_{extrap} (%)	3.02 ± 2.86	1.78 ± 1.02	11.37 ± 5.99[d]	11.73 ± 4.61
k_{el} (h^{-1})	0.59 ± 0.33	0.43 ± 0.20	0.22 ± 0.05[d]	0.22 ± 0.04
$t_{1/2el}$ (h)	1.58 ± 0.93	1.96 ± 0.95	3.28 ± 1.00[d]	3.26 ± 0.61
MRT (h)	3.12 ± 1.34	2.67 ± 0.82	6.58 ± 1.08[d]	5.45 ± 0.88*

[a] Parameters values expressed as mean ± standard deviation, except t_{max} values expressed as median (range). [b] These parameters were also presented in Table III.1, but they were here replicated to facilitate the comparison of both dosage regimens. [c] Parameters were obtained after last dosing on day seven. [d] n = 7. * $p < 0.05$, significantly different from the single-dose study. AUC_{extrap}, area under the drug concentration-time curve extrapolated from the time of the last measurable concentration to infinity; AUC_{0-inf}, area under the drug concentration-time curve from time zero to infinity; AUC_{0-t}, area under the drug concentration-time curve from time zero to the time of the last measurable concentration; C_{max}, maximum plasma concentration; k_{el}, apparent plasma elimination rate constant; MRT, mean residence time; $t_{1/2el}$, plasma elimination half-life; t_{max}, time to reach the maximum plasma concentration.

After administering multiple oral doses, the C_{max} of opicapone and BIA 9-1079 were reached earlier than after the administration of single oral doses (Table III.2), even though a statistically significant difference was only obtained for BIA 9-1079 (p = 0.01). The same tendency was observed for the MRT, which showed a decrease for both compounds in the multiple-dose study. Again, a statistical significance was only found for BIA 9-1079 (p = 0.03). The $t_{1/2el}$ of opicapone and BIA 9-1079 were relatively short and remained similar to those obtained after single-dose administration ($p > 0.05$) as well as their k_{el} ($p > 0.05$). Additionally, considering the dosing interval (24 h) and the obtained k_{el} values, the estimated R_0 values of opicapone and BIA 9-1079 were 1.00 ± 0.004 and 1.01 ± 0.01, respectively. Even so, the systemic exposure parameters (C_{max} and AUC; Table III.2) observed after repeated oral doses of opicapone (30 mg/kg/day) increased in comparison to the single-dose administration (30 mg/kg). Nevertheless, no statistically significant differences ($p > 0.05$) were detected between both groups. As expected, taking into account the increase of these parameters, the calculated R_{ac} values of opicapone and BIA 9-1079 were slightly higher than the unity (1.41 and 1.29 for opicapone and BIA 9-1079, respectively), suggesting thus a small systemic accumulation of both compounds after seven consecutive daily doses of 30 mg/kg opicapone.

III.4. Discussion

The present work was developed to study the plasma pharmacokinetics of opicapone and its active metabolite after the administration of single and multiple oral doses of opicapone to Wistar rats. To the best of our knowledge, no data in rats have yet been published about the opicapone pharmacokinetics after multiple-dose administration or drug dose-proportionality after single oral rising doses. Likewise, the available information about the pharmacokinetics of opicapone and BIA 9-1079 in rats is scarce [222,226,275].

The rat was herein chosen as the animal model to study the pharmacokinetics of opicapone not only because of the reduced costs and uncomplicated handling but also because the rat is the most frequently employed species for pharmacokinetic and pharmacodynamic non-clinical studies to evaluate COMT inhibitors [202,203,205,207,226,265–268]. Importantly, although only limited information has been reported about the metabolic profile of opicapone in rats, it seems to be similar to that in humans with various metabolites in common, such as a methylated metabolite (BIA 9-1100), a sulfated metabolite (BIA 9-1103), a glucuronide metabolite (BIA 9-1106) and the active BIA 9-1079 (an amine *N*-oxide reduced form of opicapone) [218,222].

The dose range used in these experiments (30–90 mg/kg) is broader than the therapeutically recommended dose in humans (50 mg of opicapone once-daily [224]). However, it was planned not only to evaluate the relationship between dose and systemic drug exposure in rat, but also to provide evidence on the potential pharmacokinetic impact in eventual situations of overdose in man.

The single-dose pharmacokinetic study showed that opicapone was quickly absorbed ($t_{max} \leq 2$ h, considering median values) after oral administration of the three dose levels to rats. Moreover, the delay found between the t_{max} of BIA 9-1079 and that of the parent drug suggests that opicapone is not immediately converted into its active metabolite. The same trend was also observed in humans after single oral doses of opicapone with similar median t_{max} values. Specifically, at the recommended therapeutic dose (50 mg), the t_{max} ranged from 1.5 to 4.0 h for opicapone and varied between 2.0 and 5.0 h for BIA 9-1079 [218]. Both compounds showed a relatively short $t_{1/2el}$ as it was also observed in humans [218]. Regarding the MRT, the BIA 9-1079 remained in rat plasma in its unchanged form for, approximately, a twice-longer period than opicapone. In addition, BIA 9-1079 exhibited a high relative percentage (33.6–56.5%) of the systemic exposure of opicapone, suggesting that this active metabolite may contribute to the pharmacological activity of opicapone in rats. In contrast, the systemic exposure to BIA 9-1079 is considerably lower in humans after opicapone administration, representing only 11.6% of

the systemic exposure to opicapone at a dose of 50 mg [218]. Hence, although this metabolic pathway is present in both species, the obtained data indicate the existence of some quantitative differences between rat and man.

Apparently, the wide dose range (30–90 mg/kg) herein investigated led to a dose-proportional increase of the extent of systemic exposure to opicapone, allowing the prediction of its pharmacokinetic profile. On the other hand, it is noteworthy that, after the administration of 60 and 90 mg/kg of opicapone, the systemic exposure to BIA 9-1079 was equivalent, suggesting a saturation of this metabolic pathway at the higher tested doses. However, within the studied dose range, the pharmacokinetics of opicapone was dose-independent, proposing that other metabolic pathways, such as glucuronidation and sulfation, probably compensate the saturation of opicapone reduction pathway.

After the multiple-dose regimen, no statistically significant changes of opicapone plasma pharmacokinetic parameters in relation to single-dose administration were observed, suggesting that each daily administration behaves like a single-dose regimen, at least from the pharmacokinetic point of view. Indeed, considering solely the MRT, $t_{1/2\mathrm{el}}$, k_{el} and R_0 data of opicapone and BIA 9-1079, none of the compounds seemed to suffer systemic accumulation after the administration of opicapone once-daily for seven consecutive days. However, their systemic exposure parameters ($C_{\max}$ and AUC) were slightly greater following the multiple-dose administration, evidencing that a small systemic accumulation may occur. This finding was corroborated by the R_{ac} values a little higher than the unity observed for opicapone (1.41) and BIA 9-1079 (1.29). This small systemic accumulation could be related to the inter-individual variability among distinct experimental animals and/or to the mechanism of COMT inhibition by opicapone. Regarding this second hypothesis, it is known that the prolonged COMT inhibitory effect of opicapone is independent of its presence in the bloodstream not only in humans [218,219] but also in rats [226], and thus, the long-lasting pharmacodynamic effect of opicapone seems to be due to the long half-life of the reversible COMT-opicapone complex [186,276]. Although in rats the tissues half-lives of COMT inhibition reported by Bonifácio *et al.* [226] were significantly shorter than those found in human erythrocytes [218,219], the same hypothesis can be applied. Therefore, in the multiple-dose regimen, when the next dose of opicapone is given the active site of COMT enzymes may be still occupied by opicapone molecules from the previous dose. This might lead to an initial augment of opicapone levels ($C_{\max}$ and AUC) in systemic circulation, although, then, it is rapidly eliminated ($t_{1/2\mathrm{el}} < 2$ h) before the following administration. Hence, even administering higher doses than those used in other studies performed in rats (3 mg/kg) [189,226], opicapone, as well as its active metabolite, exhibit only a minor systemic accumulation.

The potential hepatotoxicity is a safety concern related to COMT inhibitors [138,139] and, very recently, Bonifácio *et al.* [226,236] evaluated in *in vitro* conditions the toxicity of opicapone. They showed that concentrations around 40.5 and 74.8 µg/mL of opicapone were required to decrease 50% (IC_{50}) of ATP content and mitochondrial membrane potential (cytotoxicity indicators) in primary human hepatocytes, respectively, while, in HepaRG cells, opicapone reduced mitochondrial membrane potential with a IC_{50} of 206 µg/mL [226,236]. These concentrations are considerably higher than those herein achieved for the plasma C_{max} values of opicapone after single- or multiple-dosing; thus, taking all these data into account, no liver toxicity concerns are expected to be associated with the dosing regimens tested in these pharmacokinetic studies. Nevertheless, it is interesting to deepen the pharmacokinetics and disposition of opicapone in liver and other tissues in future studies. Despite the scarcity of available information concerning tissue distribution, according to a whole body autoradiography study in which [^{14}C]-opicapone was administered to Wistar rats (10 mg/kg *per os*), the radioactivity was rapidly absorbed and distributed throughout the body, but predominantly to liver and kidney [222,223]. It is not surprising since these tissues play a relevant role in metabolism and excretion of drugs. Moreover, knowing that opicapone binds to the rat plasma proteins in a very high percentage (> 99.7%), as well as in humans (99.9%), and in a concentration-independent manner [223,224], a limited tissue exposure to opicapone is expected to occur.

In conclusion, after oral administration to rats, opicapone was rapidly absorbed and eliminated, presenting an extent of systemic exposure which was approximately dose-proportional within the studied dose range (30–90 mg/kg). Moreover, the systemic accumulation of opicapone and BIA 9-1079 seemed to be negligible.

CHAPTER IV

A SINGLE- AND MULTIPLE-DOSE STUDY TO INVESTIGATE THE PHARMACOKINETICS AND PHARMACODYNAMICS OF OPICAPONE, A NOVEL COMT INHIBITOR, IN THE RAT

IV.1. Introduction

COMT (EC 2.1.1.6) is a widespread intracellular enzyme in mammalian tissues that catalyses the *O*-methylation of catechol-containing compounds. In the presence of magnesium ion, COMT enzyme catalyses the transfer of a methyl group from SAM to one of the hydroxyl groups of a catechol substrate, originating a mono-*O*-methylated catechol and *S*-adenosyl-*L*-homocysteine [147]. The main physiological role of COMT is the inactivation and/or elimination of a wide variety of biologically active or toxic endogenous and exogenous catechols [145]. COMT is expressed in two different isoforms, the soluble (S-COMT) and the membrane-bound (MB-COMT) one, with the highest activity found in the liver followed by the kidneys and gastrointestinal tract. COMT is also present in a variety of other tissues and cells, including erythrocytes that are the most convenient source of the enzyme in humans [163,167,168].

COMT inhibitors are commonly used as an adjunct to levodopa/AADC inhibitor therapy to treat motor fluctuations of patients with PD [16,272]. The co-administration of a COMT inhibitor with levodopa/AADC inhibitor therapy enables a more continuous delivery of levodopa into the brain due to the more sustained and less fluctuating plasma levels, which lead to a more constant central dopaminergic stimulation, improving motor complications [126,155]. The well-known COMT inhibitors, tolcapone and entacapone, have been assigned to some limitations regarding their pharmacokinetics, pharmacodynamics, clinical efficacy and/or safety, limiting the therapy benefits that PD patients may have [138]. Even though tolcapone is more potent than entacapone, it was associated with severe hepatotoxicity. Consequently, its prescription requires strict hepatic monitoring and it has been restricted to patients whose therapy with other COMT inhibitors has previously failed. On the other hand, entacapone does not require special restrictions, but its low bioavailability, short duration of action, and low potency limit its clinical efficacy [138]. Hence, the new COMT inhibitor, opicapone (formerly known as BIA 9-1067), emerges as an attempt to fulfil the unmet need of a more potent, safer and long-acting COMT inhibitor [155]. Overall, opicapone has shown a favourable non-clinical and clinical pharmacological profile [189,218,219,225,226,230,231] and has been recently approved for use in the European Union by the EMA.

During the non-clinical development studies, different metabolites of opicapone were identified, including a sulphated metabolite (BIA 9-1103), a glucuronide metabolite (BIA 9-1106), two methylated metabolites (BIA 9-1100 and BIA 9-1101), and BIA 9-1079 (amine *N*-oxide reduced form), which was demonstrated to be active as a COMT inhibitor (IC_{50} = 429 nM against rat liver COMT) [218–220]. Curiously, although opicapone (3 mg/kg, *per os*) was rapidly metabolized and/or eliminated from bloodstream in rats (at 8 h

post-dosing no opicapone was detected in plasma), it induced a prolonged COMT inhibition [226]. In addition, employing the same dosage regimen, opicapone presented a stronger and more sustained COMT inhibition than tolcapone in rats and, therefore, also than entacapone [189]. Furthermore, opicapone revealed to be devoid of toxicity in human primary hepatocytes and HepaRG cells [226,236]. Nevertheless, the metabolic profile of opicapone is not extensively documented in rats or other non-human species neither its pharmacokinetics in tissues. Additionally, up to today, the data available in literature about opicapone pharmacodynamics in different tissues concerns mainly single-dosing in the rat [189,226]. Solely, the erythrocyte COMT activity has been evaluated after multiple-dosing in monkeys [225] and after single- and multiple-dosing in humans [218,219]. This work was designed to characterize the systemic and tissue pharmacokinetics of opicapone and its active metabolite (BIA 9-1079) and, in parallel, evaluate the COMT activity not only in erythrocytes but also in liver and kidneys after single and multiple oral administrations of opicapone to rats.

IV.2. Materials and Methods

IV.2.1. Drugs and Reagents

Opicapone (BIA 9-1067) and BIA 9-1079 were kindly supplied by BIAL – Portela & Cª S.A. (S. Mamede do Coronado, Portugal). HPMC for preparation of opicapone oral suspensions was obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). SAM, *L*-epinephrine bitartrate salt, *DL*-metanephrine hydrochloride, pargyline hydrochloride, magnesium chloride ($MgCl_2$), ethylene glycol-bis(2-aminoethylether)-*N,N,N′,N′*-tetraacetic acid (EGTA), pentobarbital sodium salt, tamoxifen citrate salt and 37% hydrochloric acid were purchased from Sigma-Aldrich (St Louis, MO, USA). Sodium dihydrogen phosphate dehydrate and di-sodium hydrogen phosphate dehydrate used to prepare the different sodium phosphate buffers were obtained from Sigma-Aldrich (St Louis, MO, USA) and Chem-Lab NV (Zedelgem, Belgium), respectively. Acetonitrile (HPLC gradient grade) and ethyl acetate were acquired from Fisher Scientific (Leicestershire, UK), and the ultra-pure water (HPLC, 18.2 MΩ.cm) was obtained by means of a Milli-Q water apparatus from Millipore (Milford, MA, USA). The 85% *ortho*-phosphoric acid and the 60% perchloric acid were purchased from Panreac (Barcelona, Spain). Other used compounds were sodium chloride 0.9% solution for injection and heparin sodium 5000 I.U./mL for injection (B. Braun Medical, Portugal).

IV.2.2. Animals

Healthy adult male Wistar rats (RccHan:WIST), weighting 300–355 g, were supplied by Harlan Laboratories (Barcelona, Spain) and housed under controlled environmental conditions (12 h light/dark cycle; temperature 22 ± 1 °C; relative humidity 50 ± 5%) with free access to a standard maintenance diet (4RF21, Mucedola, Italy) and tap water *ad libitum*. All animals were acclimated for at least one week before use.

Opicapone was daily suspended in a HPMC (0.2%, w/v) solution and it was orally given (4 mL/kg of body weight) by gavage using a stainless steel curved feeding needle. To avoid the interference of food on the oral bioavailability of opicapone, rats were fasted for 12–15 h before drug administration and they were only fed at 4 h or 2 h post-dose in single-dose study and on the 7th day of the multiple-dose study or on the remaining days of the multiple-dose study, respectively. Animals had free access to water throughout the experimental period in both studies.

All the animal procedures were conducted in conformity with the international regulations of the European Directive (2010/63/EU) [270] regarding the protection of laboratory animals used for scientific purposes, the Portuguese law on animal welfare

(Decreto-Lei 113/2013) [274] and the employed experimental procedures were reviewed and approved by the Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção-Geral de Alimentação e Veterinária).

IV.2.3. Experimental Design

In order to evaluate the pharmacokinetics of opicapone and BIA 9-1079 in plasma, liver and kidney as well as the erythrocyte, liver and kidney COMT time-activity profiles in rats, two studies were performed: in the first study, a single oral dose of opicapone was administered, while in the second, opicapone was orally administered once-daily for seven consecutive days.

IV.2.3.1. Single-dose Study

Rats were orally treated with 30 mg/kg of opicapone suspended in 0.2% HPMC (n = 24) and, 20 min before sample collection, they were anaesthetized with sodium pentobarbital (60 mg/kg, i.p. administration). Blood, liver and kidneys were collected at 1, 2, 4, 8, 12, 24, 48 and 72 h post-dosing (n = 3 per time point). On the other hand, rats of the control group were administered with the drug vehicle 0.2% HPMC (n = 3), anaesthetized and then the matrices of interest were collected.

Blood and tissues collection and processing were based on the procedures previously described by Soares-da-Silva *et al.* [268]. Briefly, at the predefined time points, blood (~ 10 mL) was collected from anaesthetized rats by cardiac puncture with a heparinized needle and syringe, which was processed to obtain plasma for pharmacokinetic analysis and an erythrocyte pellet for the erythrocyte COMT activity assay. More specifically, collected blood samples were centrifuged at 1514 *g* for 10 min (4 °C) and the resulting plasma supernatant was stored at -80 °C until HPLC analysis (*section IV.2.4.*); then, the buffy coat was removed and the erythrocyte pellet was washed with cold 0.9% sodium chloride. The washing process of the erythrocytes consisted in the addition of two volumes of cold 0.9% sodium chloride to the erythrocyte pellet, and then samples were gently mixed and centrifuged at 1514 *g* for 10 min (4 °C); the supernatant was rejected and the washing process was repeated twice. Aliquots of washed erythrocytes (250 µL) were stored in Eppendorfs at -80 °C until the COMT enzyme assay (*section IV.2.5.*). Immediately after blood collection and before harvesting tissues, the rats were perfused by cardiac route with 0.9% sodium chloride (40 mL) in order to reduce the amount of blood found in liver and kidneys and determine more reliable concentration values for opicapone and BIA 9-1079 in those tissues; this procedure also reduces the contamination of hepatic and renal COMT

with the erythrocyte COMT. After cardiac perfusion, liver and kidneys were immediately removed and washed with 0.9% sodium chloride. Pieces of liver and the left kidney were used to evaluate COMT activity and stored in plastic tubes with 5 mM sodium phosphate buffer pH 7.8 at -80 °C until the *in vitro–ex vivo* COMT activity assay (*section IV.2.5.*). The remaining hepatic tissue and the right kidney were weighed and homogenized in 50 mM sodium phosphate buffer pH 7.4 (4 mL/g tissue), using a Thomas® Teflon pestle tissue homogenizer, in order to study the tissue pharmacokinetics. The obtained tissues homogenates were centrifuged at 4147 *g* for 15 min (4 °C) and the corresponding supernatants were collected and stored at -80 °C until HPLC analysis *(section IV.2.4.)*.

IV.2.3.2. Multiple-dose Study

For seven consecutive days, rats received once-daily oral opicapone suspension (30 mg/kg, *n* = 24) or 0.2% HPMC (control group, *n* = 3). After the last dose on the 7th day, rats were anaesthetized and blood, liver and kidneys were collected at the same time points post-dose (*n* = 3 per time point) defined for the single-dose study. Similarly, after the last administration of drug vehicle 0.2% HPMC on the 7th day, rats of the control group were also anaesthetized and then blood and tissues were collected. Blood and tissues collection and processing were performed as described in the *section IV.2.3.1*.

IV.2.4. Opicapone and BIA 9-1079 Bioanalysis

The concentrations of opicapone and BIA 9-1079 in plasma, liver and kidney tissues were determined according to the method previously developed and fully validated by Gonçalves *et al.* [275] (*Chapter II*, *section II.3.*). In brief, after spiked with the IS (tamoxifen citrate), aliquots of plasma (100 µL) and tissues homogenate supernatants (150 µL) were submitted to a deproteinization process with acetonitrile and the resulting supernatant was submitted to a double LLE with ethyl acetate after acidification with 2 M hydrochloric acid. The whole organic extract was evaporated to dryness under a nitrogen stream at 45 °C and the obtained residue was reconstituted and injected into a HPLC-DAD system (LC-AD Liquid Chromatograph, Shimadzu Corporation, Kyoto, Japan). The separation of opicapone and BIA 9-1079 was performed on a reversed-phase LiChroCART® Purospher Star® C_{18} column (3 µm, 55 mm x 4 mm; Merck KGaA, Darmstadt, Germany) at 25 °C employing a gradient elution program with a mobile phase composed of 0.05 M monosodium phosphate solution (pH 2.45 ± 0.05 adjusted with *ortho*-phosphoric acid) and acetonitrile pumped at 0.8 mL/min. The calibration curves were established within the ranges of 0.04–6.0 µg/mL for both analytes in plasma, 0.04–4.0 µg/mL for opicapone in the

supernatant of liver and kidney homogenates, and 0.07–4.0 µg/mL and 0.06–4.0 µg/mL for BIA 9-1079 in the supernatant of liver and kidney homogenates, respectively. When necessary, a 5-fold dilution of plasma samples with blank matrix was applied.

IV.2.5. COMT Activity Determination

Erythrocyte, liver and kidney COMT preparations were obtained according to the method described by Soares-da-Silva *et al.* [268] with slight changes. In brief, on the day of the assay, the frozen erythrocytes and tissues were slowly thawed on ice. Erythrocytes were haemolysed by adding ice-cold ultrapure water (4:1, water:erythrocytes, *v/v*) and mixing vigorously. After remaining for 10 min on ice, the samples were centrifuged at 15 000 *g* for 20 min (4 °C) and the resulting supernatants used for measuring erythrocyte COMT activity. Liver and kidney were homogenized using a Thomas® Teflon pestle tissue homogenizer and maintaining the grinding vessel on ice. The homogenates were centrifuged at 15 000 *g* for 20 min (4 °C) and the collected supernatants were used to evaluate the liver and kidney COMT activity. Before the enzymatic activity assay, the amount of protein in the supernatants was determined using the Bio-Rad Protein Assay (BioRad, Hercules, California, USA) with bovine serum albumin as standard (0.1–0.4 mg/mL).

COMT activity was evaluated as previously described by Bonifácio *et al.* [226,277] and Soares-da-Silva *et al.* [268] taking into account the ability of COMT enzyme to methylate epinephrine to metanephrine (*Chapter I*, Figure I.2.2). Concisely, triplicate samples (500 µL of enzyme preparations with a protein concentration of 4 mg/mL) were pre-incubated with 400 µL of incubation medium in a water bath at 37 °C for 20 min. The incubation medium contained 100 µM $MgCl_2$, 1 mM EGTA, 100 µM pargyline and 500 µM SAM (liver and erythrocytes) or 250 µM SAM (kidney) in 5 mM sodium phosphate buffer pH 7.8 (final concentrations in the reaction mixture). Enzymatic reactions were initiated by adding the enzyme substrate, epinephrine, at the final concentration of 1 mM in the reaction mixture. Liver samples were incubated for 5 min, while erythrocytes and kidney samples were incubated for 10 min. The pre-incubation and incubation were performed at 37 °C with continuous shaking and light protection. After the incubation period, reaction was stopped on ice by adding 200 µL of ice-cold 2 M perchloric acid. After protein precipitation, samples were centrifuged at 16 000 *g* for 3 min at 4 °C and the supernatants filtered through 0.22 µm Costar® Spin-X® filter tubes (Corning Inc., Corning, NY) and stored at -80 °C until metanephrine quantification. Metanephrine was quantified by HPLC with electrochemical detection in Laboratory of Pharmacological Research (BIAL – Portela & Cª

S.A., S. Mamede do Coronado, Portugal) as previously described by Bonifácio *et al.* [226,277].

IV.2.6. Data Analysis

IV.2.6.1. Pharmacokinetic Analysis

The maximum concentration (C_{max}) in plasma and tissues of opicapone and BIA 9-1079 and the corresponding time to reach C_{max} (t_{max}) were directly obtained from the experimental data. The remaining pharmacokinetic parameters were estimated from the mean concentration values (n = 3) determined at each time point by non-compartmental pharmacokinetic analysis using the WinNonlin® (Pharsight Co, Mountain View, CA, USA). The estimated pharmacokinetic parameters included the area under the drug concentration-time curve (AUC) from time zero to the time of the last measurable drug concentration ($AUC_{0\text{-}t}$) which was calculated by the linear trapezoidal rule; the AUC from time zero to infinity ($AUC_{0\text{-}inf}$) that was calculated from $AUC_{0\text{-}t}$ + (C_{last}/k_{el} or k_{tis}), where C_{last} is the quantifiable concentration at the time of the last measurable drug concentration (t_{last}) and k_{el} (or k_{tis}) is the apparent plasma (or tissue) elimination rate constant calculated by the log-linear regression of the terminal segment of the concentration-time profile; and the percentage of AUC extrapolated from t_{last} to infinity [AUC_{extrap} (%)]. It was also determined the plasma (or tissue) elimination half-life ($t_{1/2el}$, or $t_{1/2tis}$) from ln(2)/k_{el} (or k_{tis}) and the mean residence time (MRT). Plasma and tissue concentrations below the LLOQ of the analytical method were considered as zero for the pharmacokinetic data analysis.

The theoretical accumulation ratio (R_0) was calculated according to the following equation (Eq. 1):

$$R_0 = \frac{1}{1-e^{-k_{el}\tau}} \qquad (1)$$

where τ is the dosing interval (herein, 24 h), while the observed accumulation ratio (R_{ac}) was estimated employing the equation (Eq. 2):

$$R_{ac} = \frac{AUC_{\tau}}{AUC_{0-24h}} \qquad (2)$$

where AUC_{τ} refers to the AUC within the dosing interval (24 h) after the last dose administered on the 7th day of multiple-dose study and $AUC_{0\text{-}24h}$ corresponds to the AUC from time zero to 24 h of the single-dose study.

IV.2.6.2. Pharmacodynamic Analysis

The mean values of the observed COMT activities in control groups (n = 3) were taken as the baseline value in the corresponding single- or multiple-dose study and specific COMT activities were transformed as percentages of the control. The maximum inhibition of COMT activity (E_{max}) and the corresponding time to achieve E_{max} (t_{Emax}) were directly obtained from the experimental data. The remaining pharmacodynamic parameters included the area under the effect-time curve (AUEC) from time zero to 24 h ($AUEC_{0-24h}$) and from time zero to 72 h ($AUEC_{0-72h}$) and they were estimated from the mean inhibition of COMT activity values (n = 3) determined at each time point using the WinNonlin® (Pharsight Co, Mountain View, CA, USA). For determination of the AUEC and presentation of E_{max}, the percentages of COMT inhibition were calculated as follows (Eq. 3):

$$\frac{(Control\ activity - Sample\ activity)}{Control\ activity} \times 100 \qquad (3)$$

The obtained values of COMT activity at 24 h post-dosing on the 7th day of the multiple-dose study were also considered for time zero of the same day since it was previously demonstrated that COMT inhibition reaches the equilibrium state after three to four days of once-daily administrations of opicapone in a multiple-dose regimen [219,228].

IV.2.6.3. Pharmacodynamic Modelling

With the purpose of estimating the half-life of the enzyme activity recovery, the time-course of COMT activity in erythrocytes, liver and kidneys after the administration of a single-dose and multiple-doses of opicapone were modelled. The simple empirical model, $E_a = f(t)$, expressed in Eq. 4 and previously used by Bonifácio *et al.* [226] was employed with a slight adaption for including also a pre-dose effect (Eq. 5).

$$E_a = 100 - \frac{D \times t}{L + t} \times e^{-kt} \qquad (4)$$

$$E_a = 100 - \left[(100 - P) + \frac{D \times t}{L + t}\right] \times e^{-kt} \qquad (5)$$

The Eq. 5 expresses the percentage enzyme activity (E_a) as a function of time (t) and is composed of two distinct parts. The first part describes the onset of enzyme inhibition, where parameters D and L relate to the extent of COMT inhibition (E_{max}) and to the time to achieved 50% of the E_{max}, respectively, and the parameter P corresponds to the

value of enzyme activity at t = 0. The parameter P was included in Eq. 5 since, in a multiple-dose study, the initial value of the enzyme activity may be lower than baseline, as a result of the effect of a previous dose administration. In this context, the parameter P is set to the value of enzyme activity at t = 0 in the matrices from the multiple-dose study, while for those obtained from the single-dose study (absence of a previous dose) P is set to 100 and Eq. 5 simplifies to Eq. 4. The second term of the Eq. 5 (e^{-kt}) describes the recovery of enzyme activity as an exponential function, with the kinetic recovery constant (k). The parameter L constrained to values greater than zero and D and k as free parameters were fitted to the experimental data points by non-linear least-squares. Having the best-fit value for parameter k in the different matrices, the half-live of the enzyme activity recovery was calculated from ln(2)/k.

IV.3. Results

IV.3.1. Pharmacokinetics of Opicapone and BIA 9-1079

The mean plasma and liver concentration-time profiles (*n* = 3) of opicapone and its active metabolite following single and multiple oral administrations of opicapone to rats are depicted in Figure IV.1, while the corresponding pharmacokinetic parameters estimated by non-compartmental analysis are compiled in Table IV.1. In kidney tissue, opicapone and BIA 9-1079 were either not detected or, when detected, they appeared below the LLOQ of the bioanalytical method.

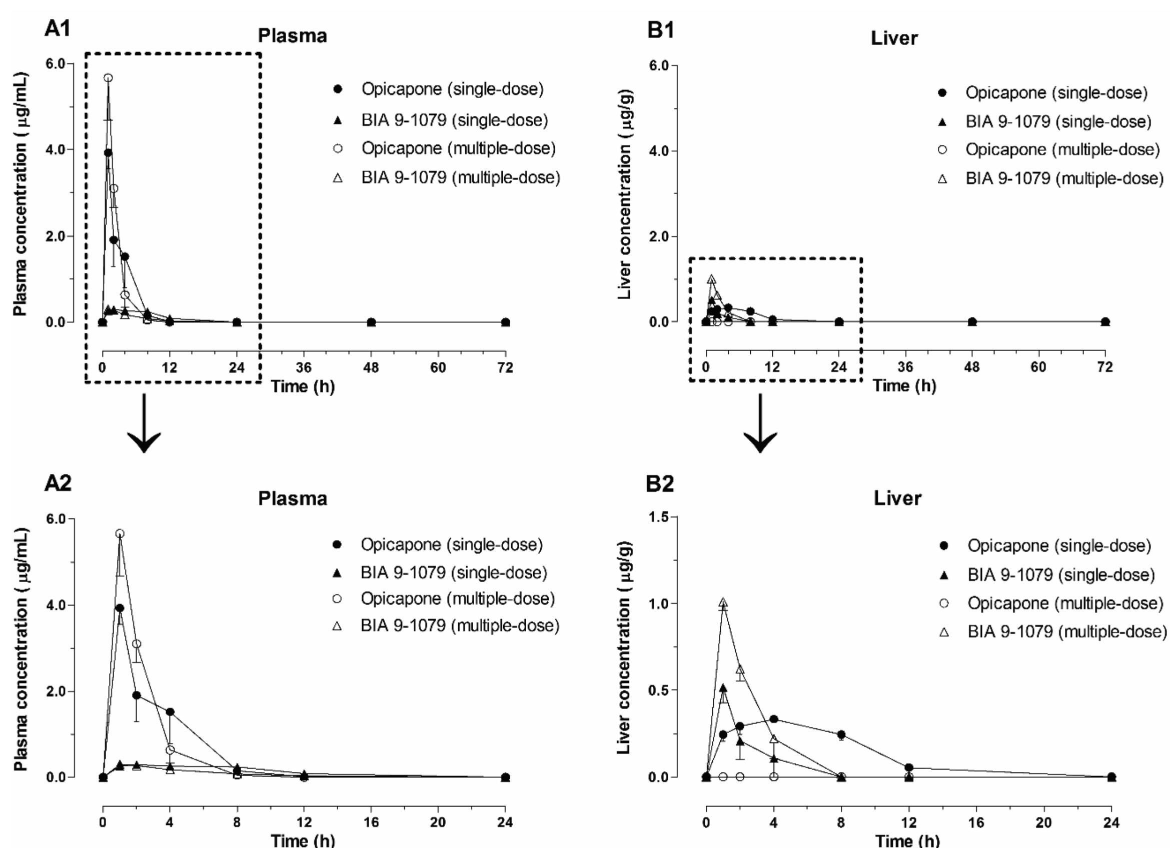


Figure IV.1 Mean concentration-time profiles of opicapone and its active metabolite (BIA 9-1079) in plasma (A1) and liver (B1) following a single oral administration and the last oral dose of a seven day once-daily regimen of opicapone (30 mg/kg) to Wistar rats. The expanded partial representation of the corresponding concentration-time profiles of opicapone and BIA 9-1079 up to 24 h post-dosing are also plotted for plasma (A2) and liver (B2), respectively. Symbols represent the mean values ± standard error of the mean (SEM) of three determinations per time point (*n* = 3).

In the single-dose study, the t_{max} of opicapone in plasma was achieved earlier than that of BIA 9-1079 (1.0 h *versus* 2.0 h). After administration of multiple oral doses, the t_{max} of opicapone was the same as that of the single-dose study, but the C_{max} of BIA 9-1079 was reached earlier than after the single oral dose administration. Interestingly, the $t_{1/2el}$ of opicapone and BIA 9-1079 in plasma were relatively short and slightly decreased after multiple-dose administration. As expected, the same tendency was also observed for the MRT in plasma. In addition, considering the dosing interval (24 h) and the obtained k_{el} values, the estimated R_0 values of opicapone and BIA 9-1079 were 1.00 and 1.01, respectively. Moreover, although the peak of systemic exposure (assessed by C_{max}) of opicapone was 1.4-fold higher after repeated oral dosing, the extent of systemic exposure (considering the AUC values) remained similar after both dosage regimens (Table IV.1). On the other hand, analysing the pharmacokinetic parameters of BIA 9-1079, its C_{max} remained unchanged after multiple-dosing, while AUC_{0-t} and AUC_{0-inf} values slightly decreased being 1.6-fold and 1.8-fold lower after multiple oral administration, respectively. Therefore, in line with the majority of systemic exposure parameters, the secondary parameters $t_{1/2el}$, k_{el} and MRT, and the estimated R_0, the calculated R_{ac} values of opicapone and BIA 9-1079 were approximately equal or below the unity (1.02 and 0.59 for opicapone and BIA 9-1079, respectively), suggesting no systemic accumulation of both compounds.

As shown in Figure IV.1, at all sampling times, liver concentrations of opicapone after multiple-dosing were below the LLOQ of the analytical method (0.04 µg/mL ≈ 0.16 µg/g). In contrast, after multiple oral doses of opicapone, the C_{max} of BIA 9-1079 in liver was 1.9-fold greater than after single-dosing and its extent of hepatic exposure assessed by AUC_{0-t} and AUC_{0-inf} was respectively 2.3-fold and 2.2-fold higher in the multiple-dosing study. Furthermore, although it presented a short $t_{1/2tis}$ and MRT and a R_0 value equal to the unity, BIA 9-1079 exhibited a R_{ac} value of 2.23, suggesting that the metabolite partially accumulates in the liver following the multiple-dose administration of the parent compound.

Comparing the plasma and liver pharmacokinetic parameters presented in Table IV.1 and the concentration-time profiles exhibited in Figure IV.1, AUC values of opicapone and BIA 9-1079 found in liver following both administration regimens were lower than they were in plasma, except for BIA 9-1079 after repeated administration. In order to evaluate the extent of hepatic exposure to opicapone and its active metabolite in comparison with the systemic exposure, the AUC_{0-t} and AUC_{0-inf} liver/plasma ratios were calculated when possible. After single-dose administration, the values of AUC_{0-t} and AUC_{0-inf} liver/plasma ratios of opicapone were approximately 0.23 and 0.24, respectively. On the other hand, the AUC_{0-t} and AUC_{0-inf} liver/plasma ratios of BIA 9-1079 were

respectively 0.36 and 0.35 after single-dose, but they substantially increased after multiple-dosing (1.33 and 1.40, respectively). These results suggest that particularly the opicapone presents a poor exposure in liver. In kidney, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}inf}$ kidney/plasma ratios of both compounds could not be calculated since opicapone and BIA 9-1079 kidney concentrations were lower than the LLOQ of the bioanalytical method, also suggesting that the opicapone has a poor exposure in kidney.

Table IV.1 Pharmacokinetic parameters of opicapone and its active metabolite (BIA 9-1079) following single and multiple oral doses of opicapone (30 mg/kg) to rats (*n* = 3 per time point).

Matrix	Pharmacokinetic parameters[a]	Opicapone		BIA 9-1079	
		Single-dose	Multiple-dose[b]	Single-dose	Multiple-dose[b]
Plasma	t_{max} (h)	1.0	1.0	2.0	1.0
	C_{max} (µg/mL)	3.93	5.66	0.30	0.30
	$AUC_{0\text{-}t}$ (µg.h/mL)	12.00	12.30	2.63	1.63
	$AUC_{0\text{-}inf}$ (µg.h/mL)	12.03	12.37	3.31	1.85
	AUC_{extrap} (%)	0.31	0.59	20.51	11.95
	k_{el} (h^{-1})	0.54	0.68	0.13	0.18
	$t_{1/2el}$ (h)	1.28	1.02	5.23	3.83
	MRT (h)	2.80	1.89	8.28	4.88
Liver	t_{max} (h)	4.0	NA	1.0	1.0
	C_{max} (µg/g)	0.33	NA	0.52	1.01
	$AUC_{0\text{-}t}$ (µg.h/g)	2.73	NC	0.95	2.16
	$AUC_{0\text{-}inf}$ (µg.h/g)	2.94	NC	1.17	2.59
	AUC_{extrap} (%)	7.22	NC	19.20	16.66
	k_{tis} (h^{-1})	0.24	NC	0.49	0.51
	$t_{1/2tis}$ (h)	2.94	NC	1.41	1.36
	MRT (h)	5.92	NC	2.52	2.04

[a] Pharmacokinetic parameters were estimated by non-compartmental analysis from the mean concentration-time profiles of opicapone and BIA 9-1079. [b] Parameters were obtained after last dosing on day seven. AUC_{extrap}, area under the drug concentration-time curve extrapolated from the time of the last measurable concentration to infinity; $AUC_{0\text{-}inf}$, area under the drug concentration-time curve from time zero to infinity; $AUC_{0\text{-}t}$, area under the drug concentration-time curve from time zero to the time of the last measurable drug concentration; C_{max}, maximum concentration; k_{tis}, apparent tissue elimination rate constant k_{el}, apparent plasma elimination rate constant; MRT, mean residence time; NA, not available; NC, not calculated; $t_{1/2tis}$, tissue elimination half-life; $t_{1/2el}$, plasma elimination half-life; t_{max}, time to reach the maximum concentration.

IV.3.2. COMT Inhibition by Opicapone

The erythrocyte, liver and kidney COMT activities (assessed as % of control) over time after single and multiple oral administrations of opicapone (30 mg/kg) to rats are shown in Figure IV.2 and the pharmacodynamic parameters are presented in Table IV.2.

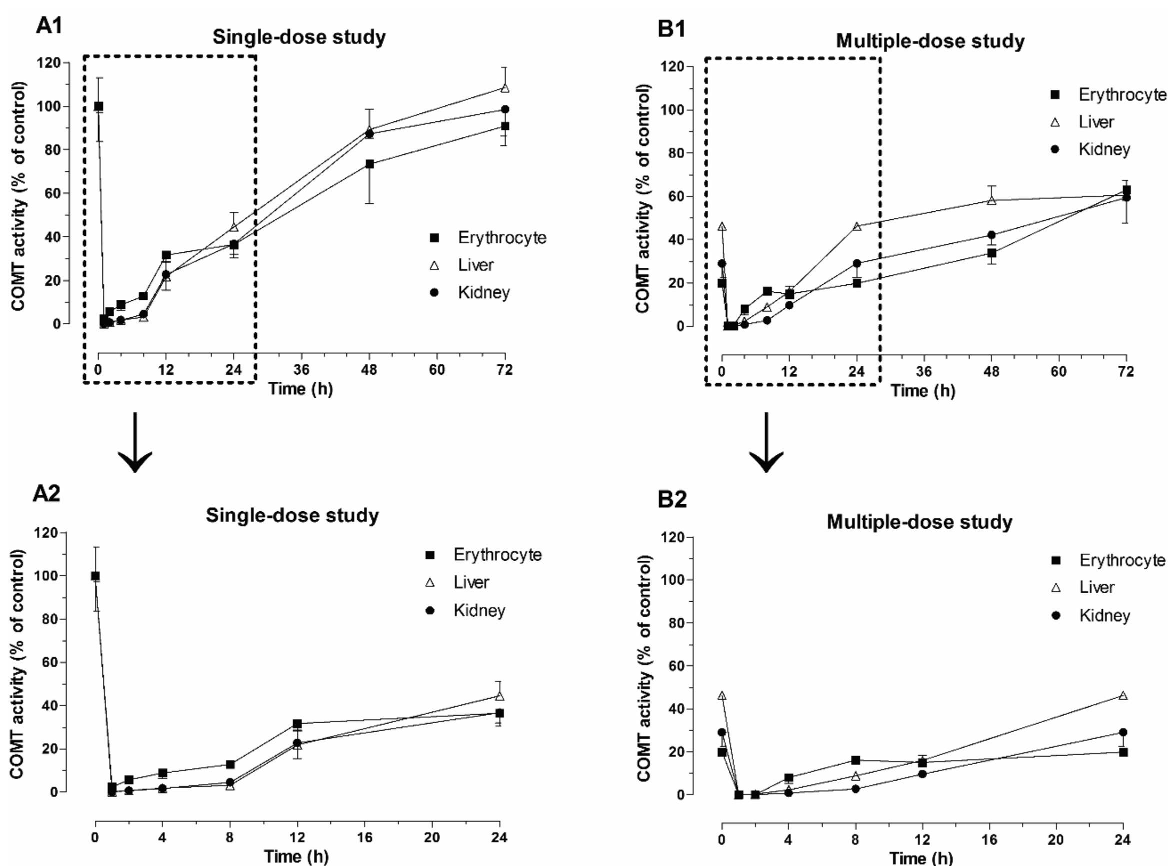


Figure IV.2 Mean catechol-*O*-methyltransferase (COMT) activity (% of control)-time profiles in erythrocyte, liver and kidney following a single oral administration (A1) and the last oral dose of a seven day once-daily regimen (B1) of opicapone (30 mg/kg) to Wistar rats. The expanded partial representation of the corresponding erythrocyte, liver and kidney COMT activity-time profiles up to 24 h post-dosing are also plotted for single-dose (A2) and multiple-dose (B2) studies, respectively. Symbols represent the mean values ± standard error of the mean (SEM) of three determinations per time point (n = 3), except for erythrocytes in the single-dose study at 2 h post-dose and liver in the multiple-dose study at 48 h post-dose which result from two determinations (n = 2) due to loss of experimental samples.

In the single-dose study, the basal COMT activities [mean ± standard error of the mean (SEM); n = 3] in erythrocytes, liver and kidneys were 2.45 ± 0.40, 152.81 ± 20.00 and 57.04 ± 1.66 nmol of metanephrine/mg of protein/h, respectively; while in the multiple-dose study, they were 3.12 ± 0.37, 197.47 ± 22.57 and 119.57 ± 20.47 nmol of metanephrine/mg of protein/h, respectively. It is noteworthy that the erythrocyte, liver and kidney inhibitory profiles obtained after single-dose administration of opicapone are reasonably comparable. However, the erythrocyte COMT activity recovery until 12 h post-dose was slightly faster than in liver and kidney, after that time period the reverse was observed. Additionally, a similar pattern was observed after multiple-dose administration of opicapone, although the COMT activity recovery was slower than after single-dosing.

The COMT activity in all the three biological matrices was nearly or fully abolished within 1 h after opicapone administration in both dosage regimens (Table IV.2). Moreover, a sustained inhibitory effect of opicapone upon erythrocyte, liver and kidney COMT activity was noticed after both dosage regimens since more than 50% of the COMT activity was still inhibited at 24 h post-dosing. The obtained mean ± SEM values of erythrocyte, liver and kidney COMT activity were 36.4 ± 4.5%, 44.5 ± 6.5% and 36.8 ± 6.3%, respectively, in the single-dose study, and 19.8 ± 1.3%, 46.2 ± 1.2% and 29.0 ± 6.6%, respectively, after the multiple-dose regimen. Indeed, in the single-dose study, only at 72 h post-dose the erythrocyte, liver and kidney COMT activity (mean ± SEM; 90.8 ± 9.1%, 108.5 ± 9.0% and 98.5 ± 12.3%, respectively) almost or completely recovered to control values (baseline activity), while in the multiple-dose study a more extended inhibitory activity was found because at 72 h post-dose it remained at 63.0 ± 4.9%, 60.6 ± 6.7% and 59.5 ± 11.9%, respectively. Although the $AUEC_{0\text{-}72h}$ values achieved after multiple-dosing were considerably higher than those observed after single-dosing, they were quite similar in all the studied matrices.

Table IV.2 Pharmacodynamic parameters of erythrocyte, liver and kidney catechol-*O*-methyltransferase (COMT) inhibition following single and multiple oral doses of opicapone (30 mg/kg) to rats (*n* = 3 per time point, unless otherwise noted).

Pharmacodynamic parameters[a]	E_{max} (%)	t_{Emax} (h)	$AUEC_{0\text{-}24h}$ (%.h)	$AUEC_{0\text{-}72h}$ (%.h)
Single-dose				
Erythrocyte[b]	97.6	1.0	1789.3	3399.9
Liver	100.0	1.0	1889.7	2873.3
Kidney	100.0	1.0	1923.8	3082.8
Multiple-dose[c]				
Erythrocyte	100.0	1.0	2063.1	5056.8
Liver[d]	100.0	1.0	1929.6	4052.9
Kidney	100.0	1.0	2120.7	4922.7

[a] Parameters were derived from the mean COMT activity inhibition-time profiles. [b] *n* = 2 at 2 h post-dosing. [c] Parameters were obtained after last dosing on day seven. [d] *n* = 2 at 48 h post-dosing. $AUEC_{0\text{-}24h}$, area under the effect-time curve from time zero to 24 h; $AUEC_{0\text{-}72h}$, area under the effect-time curve from time zero to 72 h; E_{max}, maximum inhibition of COMT activity; t_{Emax}, time to achieve the E_{max}.

Aiming to evaluate the half-life of the COMT activity recovery in the matrices of interest, the percentage of enzyme activity time-course was modelled applying the Eq. 5 (the fitting of experimental data-points is illustrated in Figure IV.3) and the *k* was estimated for each matrix in both studies. In the single-dose study, liver and kidneys presented

comparable k of 0.043 ± 0.008 h^{-1} and 0.037 ± 0.006 h^{-1} (parameter estimate ± SEM), respectively, which correspond to an inhibition half-life of 16.07 and 18.69 h, respectively. On the other hand, the erythrocytes presented a lower k value (0.027 ± 0.003 h^{-1}), which translates into a half-life time of 25.45 h. As expected, after repeated oral doses of opicapone, the values of estimated k were slightly lower than those obtained in single-dose study for the three studied matrices. Additionally, they were close to each other among the studied tissues, although the same decreasing tendency from liver to kidneys and to erythrocytes was maintained. In liver, kidneys and erythrocytes, the best-fit value for k was 0.017 ± 0.003 h^{-1}, 0.013 ± 0.001 h^{-1} and 0.011 ± 0.002 h^{-1}, respectively. These k values correspond to the inhibition half-lives of 40.07, 51.54 and 62.67 h, respectively.

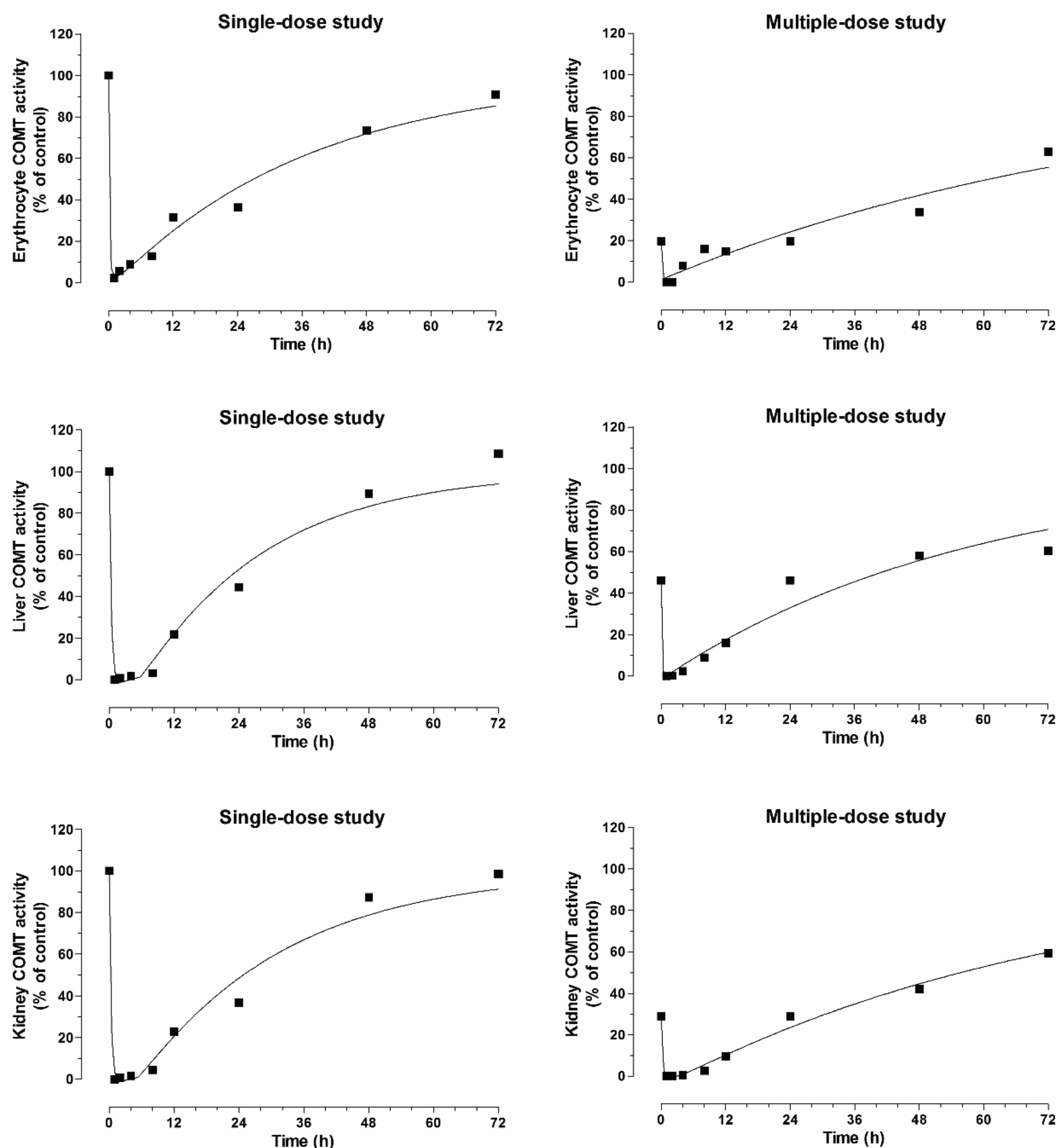


Figure IV.3 Non-linear regression fittings of erythrocyte, liver and kidney COMT activity (% of control) *versus* time following a single oral dose administration and the last oral dose of a seven day once-daily regimen of opicapone (30 mg/kg) to Wistar rats. Symbols represent means of three determinations per time point (n = 3). Curves represent enzyme inhibition modelled using Eq. 5, by adjusting the model parameters to the experimental data with non-linear least-squares fitting. The R^2 values of the fitting ranged from 0.93 (erythrocyte COMT activity in multiple-dose study) to 0.99 (kidney COMT activity in multiple-dose study).

IV.4. Discussion

As opicapone is a promising new COMT inhibitor, the present study was planned with the intent of investigating the systemic and tissue (liver and kidney) pharmacokinetics of opicapone and its active metabolite, in parallel with the evaluation of the erythrocyte, liver and kidney COMT activity after single and multiple oral administrations of opicapone to rats. The rat was herein chosen as the animal model to study the pharmacokinetics and pharmacodynamics of opicapone since it is one of the animal species most used in non-clinical studies and it exhibits a similar metabolic profile of opicapone and a comparable COMT enzyme distribution to those observed in humans [163,167,168,207,218,222].

Opicapone was rapidly absorbed (t_{max} = 1 h) and eliminated after both dosage regimens presenting a very short MRT in the systemic circulation. The active metabolite of opicapone also showed a short $t_{1/2el}$, but it remained in the plasma in its unchanged form for a period of time that was approximately three times longer than that observed for opicapone. Moreover, considering the obtained AUC_{0-t} values, the metabolite represented 13.3% (multiple-dose study) and 21.9% (single-dose study) of the systemic exposure to opicapone, suggesting that it may contribute to the pharmacological activity of opicapone in rats. In addition, the systemic pharmacokinetic results herein described revealed that none of the compounds undergoes systemic accumulation after the administration of opicapone 30 mg/kg once-daily for seven consecutive days.

At the studied dose level (30 mg/kg), opicapone led to a relatively fast (within 1 h post-dosing) and strong (> 97%) COMT inhibition in all the evaluated biological matrices independently of the dosage regimen. Despite its short $t_{1/2el}$ and $t_{1/2tis}$, opicapone showed a long-lasting COMT inhibition with a sustained COMT inhibition in erythrocytes, liver and kidneys. Indeed, this inhibition was registered far beyond the point in which the drug was quantifiable in plasma. These results are in contrast to those observed with entacapone for which the recovery of COMT activity is a mirror of its plasma concentration [267]. Furthermore, comparing the liver COMT inhibition profile herein obtained after a single-dose of opicapone with those reported for tolcapone and entacapone in the rat (30 mg/kg) [135], opicapone has undoubtedly a longer duration of action than tolcapone and entacapone.

The extended inhibitory profile observed after opicapone administration was also formerly noticed in rats [189,226], in monkeys [225] and in humans [218,219]. Accordingly, the extended inhibitory profile seems to be due to the long half-life of the reversible COMT-opicapone complex [186,276] because it is independent of opicapone presence in the bloodstream. To date, the documented data about COMT inhibition by opicapone in

rats were only obtained following the administration of a single oral dose of 3 mg/kg [189,226], where a lesser extent of inhibition was observed in the erythrocytes than in liver and kidneys and, consequently, the inhibition half-life found for erythrocytes (3.5 h) is lower than those found in liver (13.0 h) and kidney (15.3 h) [226]. Interestingly, in the present study where a 10-fold higher dose of opicapone (30 mg/kg) was used, a prolonged COMT inhibition and comparable extent of inhibition were found in all the matrices (erythrocytes, liver and kidneys). As a result, after single-dosing, the estimated values of the inhibitory half-lives in liver (16.07 h) and kidney (18.69 h) were similar to those obtained by Bonifácio *et al.* [226], but, in contrast, in erythrocytes a higher value (25.45 h) was obtained in our study. Despite the achieved differences in the extent of erythrocyte COMT inhibition, the results herein described appear to be in line with one of the hypothesis raised by Bonifácio *et al.* [226], who consider that the different ability of opicapone to penetrate membranes of different tissues may justify the shorter inhibition observed in erythrocytes in comparison with the other two tissues at 3 mg/kg. The higher dose herein administered allows a higher amount of opicapone to be available in plasma, mitigating those differences in membrane penetration.

The estimated AUEC values were slightly higher after the multiple-dose administration of opicapone than after single-dosing (Table IV.2) and the same trend was also observed for the estimated half-lives of the enzyme activity recovery in all the tissues, suggesting that the COMT enzyme is continuously inhibited in an once-daily dosage regimen. This is not surprising considering that 24 h after the single-dose administration of opicapone, the COMT activity was still less than 50% in all the studied matrices. The continuous inhibition of COMT activity found in multiple-dosing regimen is clinically important to reduce fluctuating levodopa levels and provide a more continuous and sustained dopamine levels in the brain, which has been regarded as the ideal therapy to reduce motor complications and improve patient's quality of life [134,225,226].

Regarding the tissue pharmacokinetics of opicapone, a low exposure to the drug in liver and kidneys was observed in relation to plasma. The high percentage of plasma protein binding of opicapone might be on the basis of this poor drug exposure in both tissues. Indeed, opicapone binds to human plasma proteins in a percentage higher than 99% [221], similarly to tolcapone and entacapone [278]. As it is well known, such high levels of plasma protein binding hamper drug distribution since only unbound fraction is able to cross membranes, limiting the access to the tissues [279]. Moreover, the extent of exposure to opicapone and its active metabolite was higher in liver than in kidneys, where no measurable concentrations of both compounds were found. These results are in agreement with the finding that hepatobiliary excretion is the major elimination pathway

of opicapone in rat, monkey and man [221,222]. Notwithstanding to the poor tissue-systemic drug exposure of liver and kidneys, a strong and prolonged COMT inhibition was achieved in these tissues, indicating that opicapone is a very potent COMT inhibitor.

Another interesting and noteworthy point of discussion is the complete decline of the hepatic exposure to opicapone after multiple oral dosing. This change in the hepatic exposure probably resulted from the increased metabolism of opicapone into BIA 9-1079 and other possible metabolic pathways not evaluated in the current work but that may be also involved. This hypothesis is based on the hepatic accumulation found for BIA 9-1079 (R_{ac} = 2.2) after repeated oral doses of opicapone, even though BIA 9-1079 had a short $t_{1/2tis}$ in liver (Table IV.1) and the liver concentration-time profiles evidenced its elimination until 8 h post-dosing (Figure IV.1). Nevertheless, this seems to have no impact in the systemic pharmacokinetics of opicapone and BIA 9-1079, since no increase in the systemic exposure to both compounds (no systemic accumulation) was observed after multiple-dose administration. Taking into account the aforementioned findings and since in liver the C_{max} of BIA 9-1079 was reached earlier than that of opicapone and earlier than it was reached in plasma after single-dosing, the liver seems to be involved in the metabolism of opicapone into BIA 9-1079, which probably occurs straightaway after opicapone reach the hepatocytes. The liver involvement in the formation of BIA 9-1079 was also evidenced in a former clinical trial designed to evaluate the effect of moderate liver impairment on the pharmacokinetics of opicapone in humans [221]. According to that clinical trial, although plasma concentrations of BIA 9-1079 were below the LLOQ at any time point for patients with moderate liver impairment, it was quantifiable on six healthy subjects at some time points, suggesting the involvement of hepatic enzymes in the opicapone–BIA 9-1079 metabolic pathway. Hence, a possible explanation for the observed accumulation of BIA 9-1079 in liver but not in plasma may be related to the excretion of some molecules of this metabolite by hepatobiliary route (the main opicapone excretion pathway) immediately after its formation in liver, preventing all the compound to reach the systemic circulation.

The liver tissue was selected to quantify opicapone and its active metabolite because it represents an important target not only from a pharmacokinetic point of view but also from a toxicological perspective. Indeed, the potential hepatotoxicity is a safety concern related to COMT inhibitors since three cases of fatal fulminant hepatic failure were attributed to the use of tolcapone following its introduction into the market [138,139]. The results herein found revealed that opicapone had greater systemic exposure than hepatic, reaching higher concentration values in plasma; moreover, opicapone concentrations in liver were below the LLOQ after multiple-dose administration. Furthermore, the C_{max} values

of opicapone herein achieved in liver after single- and multiple-dosing are clearly within a good safety margin considering the *in vitro* cytotoxic investigations performed by Bonifácio *et al.* [226,236]; hence, taking all these data into account, no evidence of liver toxicity was found at the studied dose.

In summary, our results suggest a low exposure of the liver and kidneys to opicapone, and no relevant systemic, renal or hepatic accumulation after repeated opicapone administration, except for BIA 9-1079 in liver. In addition, despite the rapid drug elimination, a strong, sustained and long-lasting peripheral COMT inhibition was shown in all the assessed matrices. Accordingly, it can be concluded that opicapone presents some improved pharmacological properties over other COMT inhibitors, and the generated results argue in favour of the opicapone fulfil the need of a more potent, safer and long-lasting COMT inhibitor.

CHAPTER V

GENERAL DISCUSSION

V. GENERAL DISCUSSION

Currently, PD is the second most common neurodegenerative disease and the number of individuals with this condition is expected to increase substantially in the next years. With only symptomatic treatments presently available, PD is a chronic and progressive neurodegenerative disorder without cure. Curiously, almost 50 years after the clinical introduction of levodopa, it remains the mainstay treatment of the disease. In fact, levodopa is still considered the most effective therapy for the management of motor symptoms of PD and, for patients suffering from motor fluctuations, a COMT inhibitor is widely recommended to be associated with the levodopa/AADC inhibitor therapy. Nevertheless, as discussed in the *General Introduction* (*section I.2.3.*), the older COMT inhibitors, tolcapone and entacapone, have relevant drawbacks that have limited their clinical success, and thereby opicapone arose to fulfil the unmet clinical need of a more potent, safer and longer-acting COMT inhibitor. Importantly, in the field of chronic diseases without cure like PD, a new drug that simplifies the levodopa regimen and reduces the symptomatology of disease without safety concerns is indubitably a new hope for patients and physicians. In this context and given the scarcity of public information available about opicapone when this project was initiated (2011), the work presented in this dissertation aimed at providing non-clinical data on the pharmacokinetics of opicapone and its effect on COMT activity after single and multiple oral administrations to Wistar rats.

Notwithstanding the existence of a discussion section in each experimental chapter/subchapter of this dissertation, this section entitled “General Discussion” is intended to discuss in a more integrative manner all the studies carried out to achieve the initially proposed objectives.

To attain the major and above-mentioned aim of the present thesis, *in vivo* studies were performed using male Wistar rats as a whole-animal model. In fact, humans and animals are complex systems being difficult to replicate at the laboratory bench the complex interactions that occur within them at the same time. Hence, only a whole-animal model enables an assessment of the evolution of systemic and tissues concentrations over time, in parallel (or not) with the assessment of pharmacodynamic aspects of the administered drug. The rat was herein selected not only because it allows the collection of several blood samples from the same animal (ethically more appropriate when a systemic pharmacokinetic characterization of a drug at different doses is desired, as it was in the present work), but also because it is the most frequently employed species for pharmacokinetic and pharmacodynamic *in vivo* non-clinical studies to evaluate COMT inhibitors [202,203,205,207,226,265–268]. In addition to this, despite the existence of

some quantitative differences as referred in *sections II.3.3.3.* and *III.4.*, the rat exhibits a similar metabolic profile for opicapone and a comparable COMT enzyme distribution to those observed in humans. Indeed, although only scarce information has been published about the metabolic profile of opicapone in rats, as stated in *Chapter I section I.2.4.*, at least six of the eight opicapone metabolites up to now reported in humans are also present in rats, including BIA 9-1079. Furthermore, similarly to humans, the amount of S-COMT isoform in the rat liver (tissue with the highest COMT activity and enzyme quantity) dominates over the MB-COMT isoform [167,168,207]. In opposition, in mouse, both isoforms present a similar proportion [163,207]. Regarding the gender of animals, only male rats were used with the intention of avoiding potential pharmacokinetic interferences from the female hormonal cycle. Moreover, it was defined to administer opicapone by oral route because this is the intended human therapeutic route and, in the multiple-dose studies, a once-daily dosage regimen was used since this was the expected human therapeutic regimen and, nowadays, it is effectively the recommended one [142].

At the beginning of this project, we were faced with the lack of bioanalytical methods available in literature able to quantify opicapone and/or its metabolites in human matrices or in other non-human matrices. However, the availability of appropriately validated methods is essential to support non-clinical, clinical and biopharmaceutical studies that require pharmacokinetic evaluation [247]. Thus, it was vital to develop and validate a bioanalytical method that could be applied to quantify opicapone and its active metabolite (BIA 9-1079) in the different biological matrices obtained from the planned pharmacokinetic studies.

Before a bioanalytical method can be routinely applied for the intended use, it must be adequately developed, optimised and validated. Undoubtedly, the development phase of a bioanalytical method is a complex and laborious process of creating a procedure to enable the identification and quantification of the compound of interest in a specific biological matrix [280]. Notwithstanding, it can be conducted in multiple ways, ranging from simply adapting an existing method (by making minor changes so that it is suitable for a new application), to starting out with few ideas and applying expertise and experience to develop a suitable method for the intended use [281]. In the absence of a published method that could be adapted, the development of the proper and reliable analytical tool to support the succeeding pharmacokinetic studies of the present thesis revealed to be a challenging task. In this context, before beginning the method development, it was helpful to perform an extensive review [146] of the bioanalytical chromatographic methods available in the literature for the quantification of other COMT inhibitors, gathering an

appropriate background to start out the development of a novel method. Chromatographic methods are the preferred analytical methodology in bioanalytical laboratories. Moreover, among chromatographic methods, HPLC remains the one of choice for drugs since it is able to separate quite complex mixtures of compounds present in the same sample, and also enables the quantitative measurement of very small amounts of the analytes, affording a good selectivity and sensitivity needed in the bioanalysis of samples from pharmacokinetic studies [146,246]. Indeed, reverse-phase HPLC has been used as the major approach for the quantification of COMT inhibitors [146]. Hence and aiming at using a relatively simple and widespread technology, reverse-phase HPLC coupled with DAD was the analytical methodology selected to support the execution of the intended pharmacokinetic studies.

Firstly, a reverse-phase HPLC-DAD method was developed for the simultaneous quantification of opicapone and BIA 9-1079 in human plasma. The method proved to be selective, sensitive, accurate, precise, and linear in a wide concentration range. Among these characteristics, one that deserves to be highlighted is the great sensitivity achieved for the quantification of both analytes (LLOQ = 25 ng/mL), which is similar to that obtained by the LC-MS/MS method (LLOQ = 10 ng/mL) [218] published a few months earlier than ours (*Chapter II, section II.2.*). This way, the developed method revealed to be a reliable and suitable bioanalytical tool to support clinical pharmacokinetic studies involving opicapone. Although it was not the first method to be available in the literature for the quantification of opicapone and BIA 9-1079, it was provided as a novel bioanalytical technique based on a simple, relatively cheap and widespread technology (HPLC-DAD), and thereby it can be easily adopted by the majority of clinical pharmacokinetic departments. Moreover, it showed to be a viable alternative to the LC-MS/MS method since, in addition to the similar sensitivity of both methods, our method was successfully applied to real samples of healthy subjects enrolled in a clinical trial and the determined concentrations for opicapone and BIA 9-1079 were equivalents to those measured by the LC-MS/MS method (as shown in *Chapter II,* Figure II.2.3).

Another purpose of developing this method in human plasma, in reality the main purpose under the scope of the present doctoral project, was to acquire know-how in the bioanalytical field as well as test and optimise the best analytical conditions that could be later transposed into the analysis of animal matrices. In fact, the development of the method in animal matrices was expected to be a complex process in which a great amount of animal matrix would be necessary for testing and optimising all the analytical conditions. Therefore, in order to reduce the number of animals to the minimum indispensable respecting the 4R's principle [271], the optimization of the various analytical conditions begun with a surrogate matrix. As human plasma is a relatively complex and abundant

matrix, easily accessible and associated with few ethical restrictions, it seems to be the most appropriate one.

Hence, based on the conditions of the former bioanalytical method, the development and validation of the HPLC-DAD method able to quantify opicapone and BIA 9-1079 in rat plasma, liver and kidney homogenates (the matrices that were considered to be the most relevant from the pharmacokinetic point of view) was faster. However, owing to the lack of selectivity for BIA 9-1079, the straightforward application of the technique that quantifies both analytes in human plasma was prevented. As a result of that, some adjustments in chromatographic conditions as well as sample preparation were performed to ensure the generation of accurate, precise and reproducible data and, in this manner, guarantee a valid interpretation of the obtained analytical results. The developed and fully validated method (*Chapter II, section II.3.*) gathers some favourable aspects, including the inexpensive and highly reproducible sample preparation procedure employed (protein precipitation followed by LLE), which is relevant when a large amount of samples derived from pharmacokinetic studies need to be analysed, and the simple, relatively cheap and widespread technology used (HPLC-DAD). Most importantly, the small plasma sample volume required (100 µL) allows the collection of several blood samples from the same animal during non-terminal pharmacokinetic studies and, consequently, reduces the number of animals needed. In particular, this last aspect was fundamental to support the pharmacokinetic studies of the current work presented in *Chapter III*, which involved harvesting multiple blood samples from each animal. Another LC technique was reported for the quantification of opicapone in rat plasma [226]. However, besides not being fully described and not including validation parameters, the technique employs MS detection, a more expensive methodology which is not routinely used in all laboratories. On the other hand, our method can also be employed to analyse tissue samples and BIA 9-1079, in opposition to that stated by Bonifácio *et al.* [226].

After the development and validation of the bioanalytical tool to quantify opicapone and BIA 9-1079 in rat matrices, the project advanced in order to evaluate the systemic pharmacokinetics of opicapone and BIA 9-1079 in *in vivo* conditions after the oral administration of the parent drug (opicapone). Consequently, two studies were carried out: one tested the single oral administration of opicapone at three different doses and the second tested multiple oral administration of opicapone at one selected dose (*Chapter III*).

Nevertheless, before starting those *in vivo* studies, some preliminary experiments were required to define the sampling times and verify whether the sensitivity of the developed method was enough for the quantitative determination of both compounds in

study. These aims were achieved in *Chapter II* (Figure II.3.3), but the great inter-individual variability observed (as already expected taking into account the high variability also observed in humans [218,219]) implied to use eight animals; on the other hand, the number of collected samples had to be increased between 2 and 8 h post-dosing and also prolonged over time until 24 h post-dosing, in order to obtain a complete characterization of the systemic pharmacokinetic profiles of both opicapone and BIA 9-1079. Moreover, this preliminary investigation demonstrated that a dose higher than 10 mg/kg was required to employ our analytical technique. At that moment, there was a study published about opicapone, reporting its pharmacodynamics in rats after the administration of a dose of 3 mg/kg [189]. Hence, we intended to study doses at least 10-fold higher than that used, and thereby the doses of 30, 60 and 90 mg/kg were chosen for the single-dose studies reported in *Chapter III*. In the first preliminary experiments, opicapone was orally administered suspended in 0.5% carboxymethylcellulose, which is a frequently used vehicle for the oral administration of COMT inhibitors [135,189,207]. Nevertheless, aiming to reduce the observed inter-individual variability, it was also tested to administer opicapone suspended in 0.2% HPMC (vehicle used for oral administration of opicapone to monkeys [225]). Interestingly, it was observed that the use of 0.2% HPMC improved the opicapone suspension in relation to 0.5% carboxymethylcellulose, allowing to get results as described in *Chapter III* and *IV*.

Concerning the studies carried out for assessing the systemic pharmacokinetics of opicapone and BIA 9-1079 (*Chapter III*) after single oral administration, opicapone was quickly absorbed (t_{max} ≤ 2 h, considering the median values) at the three doses tested and both opicapone and its active metabolite presented a relatively short $t_{1/2el}$ (1.58– 4.50 h). Regarding the extent of systemic exposure, BIA 9-1079 represented a percentage between 33.6–56.5% in relation to opicapone, suggesting that this active metabolite may contribute to the pharmacological activity of the parent compound in rats. Furthermore, in the studied dose range, opicapone showed an approximately dose-proportional increase in its extent of systemic exposure. However, the systemic exposure to BIA 9-1079 was comparable after the administration of 60 and 90 mg/kg of opicapone. These results suggest that there is a saturation of opicapone reduction pathway at the higher tested doses, but it is probably compensated by other opicapone metabolic pathway, such as glucuronidation and sulfation.

The dose of 30 mg/kg was chosen for the multiple dose pharmacokinetic study also executed in the present PhD thesis work, mainly due to two reasons. The first one was the apparent dose-proportional increase in the opicapone extent of exposure observed in rat plasma after a single oral dose in the studied range (30–90 mg/kg). The second reason was

related to the doses under investigation which were higher than the range of probable therapeutic doses taking into consideration the FDA Guidance for Industry on conversion of animal doses to human equivalent doses based on body surface area [282] (at the time of these studies the range of 25–50 mg was indicated as having potential to provide clinical benefits in humans [283]). Thus, as described in *Chapter III*, after multiple-dose administrations, no statistically significant changes of opicapone plasma pharmacokinetic parameters in relation to single-dose administration were observed, suggesting that each daily administration behaves like a single-dose administration, at least from the pharmacokinetic perspective. Indeed, considering solely the MRT, $t_{1/2el}$, k_{el} and R_0 data of opicapone and BIA 9-1079, none of the compounds seemed to suffer systemic accumulation after the administration of opicapone once-daily for seven consecutive days. However, their systemic exposure parameters (C_{max} and AUC) were slightly greater following the multiple-dose administration, evidencing that a small systemic accumulation may occur. This finding was corroborated by the R_{ac} values observed for opicapone (1.41) and BIA 9-1079 (1.29), both a little higher than the unity. This small systemic accumulation could be related to the inter-individual variability among distinct experimental animals and/or to the mechanism of COMT inhibition by opicapone, as discussed in *Chapter III* (*section III.4.*).

After the complete characterization of the systemic pharmacokinetics of opicapone and BIA 9-1079, it sought to assess the liver and kidney disposition of both compounds, in parallel with the effect of opicapone on erythrocyte, liver and kidney COMT activity following its single and multiple oral administrations at 30 mg/kg. In point of fact, due to ethical and practical reasons, these types of studies cannot be performed in humans, making the use of adequate animal models a viable alternative. In the studies herein performed, the systemic pharmacokinetics of opicapone was again evaluated, mainly to understand the tissue-systemic exposure relationships and the relation between the erythrocyte COMT activity and plasma concentrations.

In previous studies (*Chapter III*) it was possible to obtain a complete pharmacokinetic profile from each animal, reducing the total number of animals needed. However, the evaluation of pharmacokinetic profiles and COMT activity in liver and kidney implied the sacrifice of the animals at each time point. Therefore, some modifications in the study design were outlined, including the reduction of the number of sampling time points, based on the previous knowledge of the systemic pharmacokinetics of opicapone (*Chapter III*) and its long duration of action [189], and the decrease in the number of samples per sampling time point. Furthermore, to ensure the availability of a sufficient quantity of erythrocytes for the COMT activity assay, the blood was collected by cardiac

puncture. Afterwards, the animals were submitted to intracardiac perfusion with saline solution to reduce residual blood found in liver and kidneys. This decreased the contamination of hepatic and renal COMT with erythrocyte COMT, and allowed a stricter quantification of opicapone and BIA 9-1079 in those tissues, by minimizing the interference of the quantity of those compounds present in blood.

With the intention of assessing the COMT activity after the administration of opicapone, an *in vitro–ex vivo* assay previously developed and used in BIAL Laboratories was adopted [226,277] with minor modifications. In brief, it is based on the ability of COMT enzyme to methylate epinephrine to metanephrine (*Chapter I*, Figure I.2.2). Nevertheless, due to the equipment available in our laboratory, the velocity of the performed centrifugation after the erythrocyte haemolysis was altered. Furthermore, in opposition to the developed method, the liver and kidney homogenates were centrifuged and the obtained supernatants were used to determine COMT activity, because of the difficulty of pipetting the crude homogenates in a reproducible way. Importantly, before starting the evaluation of samples from *in vivo* studies, it was ensured that the modifications did not interfere with the assay conditions. All the steps of the *in vitro–ex vivo* assay were performed in our laboratory, with the exception of the quantification of metanephrine (reaction product) which was determined by HPLC with electrochemical detection in Laboratory of Pharmacological Research (BIAL – Portela & Cª S.A., S. Mamede do Coronado, Portugal).

Overall, even though a direct comparison between the systemic pharmacokinetic parameters derived from the two different single- and multiple-dose studies (*Chapter III* and *Chapter IV*) carried out under the scope of the present dissertation may not be appropriate because of the difference in the study designs, the opicapone data between them are concordant. In the second single- and multiple-dose studies reported in *Chapter IV*, opicapone showed, again, a relatively rapid absorption from the gastrointestinal tract and elimination from the systemic circulation, and a similar peak and extent of systemic exposure to those of the first studies. Moreover, no systemic accumulation of opicapone and BIA 9-1079 was observed after the multiple-dose regimen (30 mg/kg/day for seven consecutive days), corroborating that the small accumulation observed in the study described in *Chapter III* might be negligible. Hence, the systemic accumulation of both compounds seems to be minor or, probably, non-existent at the dosage studied regimen. Once again, bearing in mind the systemic data of the first (*Chapter III*) and the second (*Chapter IV*) single- and multiple-dose studies, a feature in which they differ is the systemic exposure to BIA 9-1079. In fact, in the second single- and multiple-dose studies (*Chapter IV*, Table IV.1) the peak and extent of BIA 9-1079 systemic

exposure are 2- to 4-fold lower than those in the first studies (*Chapter III*, Table III.2). Possible explanations may be related to the difference in the sampling times that inevitably affect the concentration-time profile of a compound, as well as, to the inter-individual variability among animals.

In relation to the tissue pharmacokinetics, a low exposure to opicapone in liver and kidneys relative to plasma was observed, which might be related to its very high percentage of plasma protein binding (> 99%) [221]. Furthermore, the extent of exposure to opicapone and BIA 9-1079 was higher in liver than in kidneys, where no measurable concentrations of both compounds were found. These findings are in concordance with the fact that hepatobiliary excretion has been ascribed as the major elimination pathway of opicapone in rat, monkey and man [221,222]. Moreover, with the exception of BIA 9-1079 in liver, no renal or hepatic accumulation of both compounds was observed following the repeated administration of opicapone. Probably, the hepatic accumulation of BIA 9-1079 resulted from the increase in the metabolism of opicapone by this metabolic pathway, since a complete decline of the hepatic exposure to opicapone simultaneously with an augment in the exposure to BIA 9-1079 was observed after multiple-dose administration of the parent compound. Moreover, these data suggest the involvement of the liver in the formation of BIA 9-1079. Taking into consideration the C_{max} values attained in liver in these studies (*Chapter IV*, Table IV.1) and the IC_{50} values for some cytotoxicity indicators determined in *in vitro* conditions using human primary hepatocytes and HepaRG cells by Bonifácio *et al.* [226,236], no liver toxicity is evidenced at the dose under evaluation.

Independently of the adopted dosage regimen (single or multiple), the results regarding COMT inhibition demonstrated that opicapone led to a relatively fast (within 1 h post-dosing) and strong (> 97%) COMT inhibition in all the evaluated biological matrices. Despite its short $t_{1/2el}$ and $t_{1/2tis}$, opicapone showed a long-lasting pharmacodynamic effect with a sustained COMT inhibition in erythrocytes, liver and kidneys. Indeed, this inhibition was registered far beyond the point in which the drug was quantifiable in plasma, showing that the duration of the opicapone pharmacological effect is independent of its rate of clearance, in contrast to entacapone [267]. This prolonged COMT inhibition, far beyond the observable point of drug clearance, was also observed in humans after single [218] and multiple [219] oral administrations of opicapone. Hence, it was proposed that the long duration of action of opicapone is a result of the very long residence time of the reversible opicapone-COMT complex [186]. According to a study based on computer simulations [276], opicapone has an outstandingly high binding affinity to human COMT, with an estimated dissociation constant of 0.19 pM. In the same study, using the estimated dissociation constant value, it was calculated the dissociation rate constant (1.9×10^{-6} s^{-1}).

On the other hand, the long half-lives of opicapone-induced COMT inhibition in human erythrocytes observed in the above-mentioned single- (61.6 h) [218] and multiple-dose (130.4 h) [219] studies, translate into an underlying dissociative rate constants of approximately 3.1 x 10^{-6} s^{-1} and 1.5 x 10^{-6} s^{-1}, respectively. Remarkably, these dissociation rate constants are similar to the estimated dissociation rate constant, indicating that the sustained opicapone-induced COMT inhibition, far beyond the observable point of clearance of circulating drug, is a reflex of the long residence time of the reversible human opicapone-COMT complex [186].

In this context, the percentage of enzyme activity time-course in each studied matrix after both dosage regimens was modelled to estimate the half-life of the COMT activity recovery, which showed to be tissue-dependent despite relatively comparable among the three considered matrices. In the single-dose study, the inhibition half-lives were found to be 16.07, 18.69 and 25.45 h in liver, kidneys and erythrocytes, respectively. These liver and kidney half-lives of rat enzyme inhibition are similar to those determined in another study, where a 10-fold lower opicapone dose (3 mg/kg) was used [226]. This aspect is noteworthy since, even administering a higher dose than one close to that equivalent to the recommended therapeutic dose in humans, the recovery of the COMT physiological function did not change. Nevertheless, in contrast to our results, the recovery of COMT activity was considerably faster in erythrocytes (3.5 h) than in liver (13.0 h) and kidney (15.3 h), after a dose of 3 mg/kg. According to the authors of that work, the different ability of opicapone to penetrate membranes may be one hypothesis to explain the shorter inhibition in the erythrocytes compared with liver and kidneys. Interestingly, our results seem to be in line with that hypothesis, since the higher dose administered in our study allows a higher amount of opicapone to be available in plasma, attenuating those differences in membrane penetration. In order to confirm this explanation for the difference observed in the duration of erythrocyte COMT inhibition between the two studies, it could be interesting to evaluate the concentration-time profiles of opicapone in erythrocytes following the oral administration of 3 and 30 mg/kg opicapone doses to rats. After the administration of opicapone once-daily for seven consecutive days, as expected, the AUEC values slightly increased in relation to the single-dose administration, indicating that the COMT enzyme is continuously inhibited after once-daily doses. Moreover, the estimated half-lives of COMT activity recovery for the three matrices are longer, but the increasing tendency from liver to kidneys and to erythrocytes was maintained, with values of 40.07, 51.54 and 62.67 h, respectively. The erythrocyte, liver and kidney half-lives of rat COMT inhibition estimated in the present thesis are shorter than those found in human erythrocytes (61.6 h after opicapone single-dose [218] and 130.4 h following a

multiple-dose regimen [219]). As referred by Bonifácio *et al*. [226], the smaller duration of the pharmacodynamic effect in the rat in relation to man might be explained by a lower affinity of opicapone for rat COMT than for human enzyme.

In summary, with the work developed under the scope of this thesis, it was possible to generate information about the systemic pharmacokinetics of opicapone and BIA 9-1079 at different doses, their liver and kidney disposition and their effect on erythrocyte, liver and kidney COMT activity at one selected dose in rat.

CHAPTER VI

CONCLUSIONS AND FUTURE PERSPECTIVES

VI. CONCLUSIONS AND FUTURE PERSPECTIVES

Focusing on the study of the pharmacokinetic and pharmacodynamic (COMT inhibition) characteristics of opicapone, the work presented in this dissertation was dedicated to the investigation of this novel potent and purely peripheral COMT inhibitor that emerged to fulfil the need of a safer and more efficacious COMT inhibitor. After the development and full validation of bioanalytical assays for the quantification of this new COMT inhibitor and its active metabolite (BIA 9-1079), the systemic and tissue (liver and kidney) pharmacokinetics of opicapone and BIA 9-1079 were assessed, as well as, their impact on COMT activity, after single and multiple oral administrations of opicapone to Wistar rats.

In short, the most relevant achievements and findings obtained from all the experimental work underlying the present PhD thesis are the following:

- The first HPLC-DAD method for the simultaneous quantification of opicapone and BIA 9-1079 in human plasma was successfully developed and fully validated. Its main advantages comprise the employment of a simple, relatively cheap and widespread technology (HPLC-DAD), allowing to be easily adopted by the majority of clinical pharmacokinetic departments, and the good sensitivity. In addition to being selective, sensitive, accurate, precise and linear in a wide concentration range, this method was successfully applied to real samples of healthy subjects enrolled in a clinical trial. In this way, the developed method revealed to be a reliable and suitable bioanalytical tool to support future clinical pharmacokinetic studies involving opicapone.

- The first HPLC-DAD technique to simultaneously quantify opicapone and BIA 9-1079 in rat matrices (plasma and liver and kidney homogenates) was developed and fully validated. Its favourable characteristics include the inexpensive and highly reproducible sample preparation procedure employed (protein precipitation followed LLE); the simple, relatively cheap and widespread technology used (HPLC-DAD); and the small plasma sample volume (100 µL) required, which allows the collection of several blood samples from the same animal during non-terminal pharmacokinetic studies. This method was the bioanalytical tool that supported the pharmacokinetic studies presented in this dissertation.

- The gastrointestinal absorption of opicapone was relatively rapid as well as its elimination from systemic circulation. The active metabolite, BIA 9-1079, was

also quickly eliminated and represented a high relative percentage of the systemic exposure of opicapone, suggesting that this active metabolite may contribute to the pharmacological activity of the parent compound in rats. Furthermore, in the single-dose solely systemic pharmacokinetic study (*Chapter III*), it was noticed that, following the single oral administration of 60 and 90 mg/kg of opicapone, the systemic exposure to BIA 9-1079 was comparable which is suggestive of a saturation of this metabolic pathway at the higher tested doses. Nevertheless, this saturation of opicapone reduction pathway is probably compensated by other opicapone metabolic pathways, such as glucuronidation and sulfation, since the extent of systemic exposure to opicapone apparently increased in an approximately dose-proportional manner in the studied dose range (30–90 mg/kg).

- Taking into account the two different single- and multiple-dose studies carried out, the systemic accumulation of opicapone and BIA 9-1079 seems to be minor or, perhaps, non-existent at the studied dosage regimen (30 mg/kg once-daily for seven consecutive days). In addition, from the results of the single- and multiple-dose pharmacokinetic/pharmacodynamic studies (*Chapter IV*), no hepatic or renal accumulation of both compounds was detected after the multiple-dose administration of opicapone, except for BIA 9-1079 in liver. As a consequence, BIA 9-1079 accumulation in liver might result from the increased metabolism of opicapone by this metabolic pathway.

- The tissue-systemic exposure relationships to opicapone indicated a low drug exposure in the liver and kidneys, which is probably a consequence of its very high percentage of plasma protein binding. Among the two analysed tissues, the exposure to opicapone and BIA 9-1079 was higher in liver in consonance with the hepatobiliary excretion being the major elimination pathway of opicapone.

- Following the oral administration of 30 mg/kg opicapone, the COMT inhibition profiles were reasonably comparable in all the evaluated biological matrices (erythrocytes, liver and kidneys). Furthermore, in spite of its poor exposure in tissues (liver and kidney) and short $t_{1/2\mathrm{el}}$ and $t_{1/2\mathrm{tis}}$, opicapone showed a strong (> 97%) and long-lasting (> 50% at 24 h post-dosing) pharmacological effect, independently of the dosage regimen, although a slightly higher extent of COMT inhibition was observed after repeated administrations. In fact, the duration of the opicapone pharmacological effect showed to be independent of its rate of clearance.

- Considering all the results found during this work, it seems that no pharmacokinetic concerns or related to the physiological restoration of COMT activity in the studied matrices are expected when opicapone is administered once-daily. Moreover, the generated results support that opicapone fulfils the need of a more potent and long-lasting COMT inhibitor.

In conclusion, the execution of the experimental work described in this thesis allowed to enhance the present non-clinical pharmacological knowledge of a new drug that was recently approved by EMA to be used as adjunct to levodopa/AADC therapy in PD patients with motor fluctuations. In addition, this work also enabled to develop and provide two bioanalytical methods that will probably support future pharmacokinetic studies with opicapone.

Importantly, although a long way has already been travelled before the marketing approval of opicapone, further *in vivo* studies to evaluate the potential interaction of this novel COMT inhibitor with commonly prescribed drugs to PD patients, particularly other anti-parkinsonian drugs, as well as with OATP1B1 substrates are pertinent.

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