U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

MSc

2.º CICLO

FCUP
BIAL
2013

U.PORTO

Opicapone Sulfation: Sulfotransferase Isoforms Characterization

Carlos Manuel Fernandes Lopes

FC

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Bial

Opicapone Sulfation: Sulfotransferase Isoforms Characterization

Carlos Manuel Fernandes Lopes

Dissertação de Mestrado apresentada à
Faculdade de Ciências da Universidade do Porto, Bial, Portela & Companhia – S.A.
Bioquímica

2013

Opicapone Sulfation: Sulfotransferase Isoforms Characterization

Carlos Manuel Fernandes Lopes
Mestrado em Bioquímica
Departamento de Química e Bioquímica
2013

Orientador
Prof. Doutor Patrício Soares da Silva, Professor Catedrático, FMUP

Coorientador
Prof. Doutor Nuno Mateus, Professor Associado, FCUP

Bial

U.PORTO
FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

To Susete, Tiago

and Matilde.

“Until we are better informed
respecting the nature of the disease,
the employment of internal medicine is
scarcely warrantable.”

J. Parkinson, 1817

ACKNOWLEDGEMENTS

It is a pleasure to thank to many people who made this dissertation possible.

First of all I would like to thank Doctor Luís Portela, Chairman and Dr. António Portela, CEO of BIAL – Portela & Companhia S.A., for made available all the conditions for the elaboration and completion of this dissertation.

To Professor Patrício Soares da Silva, my supervisor, I would like to thank for all the help, availability, support and encouragement over the course of this project.

I also would like to thank my FCUP supervisor, Professor Nuno Mateus, for all the availability, support and friendship demonstrated over these years.

To Ana I. Loureiro, I would like to thank for all scientific discussions that we had and help to understand the best way to perform the practical work of this dissertation.

I would like to thank also to all my colleagues of Bial's Laboratory of Pharmacological Research for the sharing of knowledge and experiences, as well as for the pleasant environment always present in the lab.

To all my dear friends, I would like to thank all their support.

My final words go to my dear wife Susete, my son Tiago and my little baby Matilde. Without their love, encouragement, support and infinite patience this work did not make sense. All adversities found over the course of this dissertation were overcome by their smile when I came home.

LIST OF ABBREVIATIONS

3-MT – 3-methoxytyramine
3-OMD – 3-*O*-methyldopa
AADC – aromatic amino acid descarboxylase
ACN – acetonitrile
ADP – adenosine diphosphate
APS – adenosine 5'-phosphosulfate
Arg - arginine
ATP – adenosine triphosphate
BBB – blood-brain barrier
Caco-2 – intestinal carcinoma cells
CNS – central nervous system
COMT – catechol-*O*-methyltransferase
CYP – cytochrome P450
CV – coefficient of variation
DCNP – 2,6-dichloro-4-nitrophenol
DHEA – dehydroepiandrosterone
DHEA ST – dehydroepiandrosterone sulfotransferase
DNA – deoxyribonucleic acid
DOPA – 3,4-dihydroxyphenylalanine
DOPAC – 3,4-dihydroxyphenylacetic acid
EST – estrogen sulfotransferase
FDA – food and drug administration
GST – glutathione S-transferase
HepG2 – hepatic carcinoma cells
His – histidine
HIS9 – human intestine S9
HLS9 – human liver S9
HPLC – high performance liquid chromatography
HVA – homovanilic acid
K_d – dissociation constant
K_i – inhibition constant
K_m – Michaelis-Menten constant
LC-MS/MS – liquid chromatography tandem mass spectrometry
L-DOPA – levodopa

LLOQ – lower limit of quantification

M-PST – monoamine phenol sulfotransferase

MAO – monoamine oxidase

MB-COMT – membrane catechol-*O*-methyltransferase

MRM – multiple reaction monitoring

mRNA – messenger ribonucleic acid

NAT – N-acetyltransferase

P-PST – phenol sulfotransferase

PAP – 3'-phosphoadenosine 5'-phosphate

PAPS – 3'-phosphoadenosine 5'-phosphosulfate

PD – parkinson disease

PPi – inorganic pyrophosphate

QC – quality control

S-COMT- soluble catechol-*O*-methyltransferase

SAM – S-adenosyl-L-methionine

SEM – standard error of mean

SULT - sulfotransferase

TH – tyrosine hydroxylase

TPMT – thiopurine S-methyltransferase

TL PST – thermolabile phenol sulfotransferase

TS PST – thermostable phenol sulfotransferase

ULOQ – upper limit of quantification

UGT – UDP-glucuronosyltransferase

V_{max} – maximum enzyme velocity

ABSTRACT

Parkinson's Disease (PD) is the second most prevalent neurodegenerative disease in the world, affecting primarily aged populations. The gold standard treatment for PD is still the administration of the combination levodopa/aromatic amino acid decarboxylase (AADC) inhibitor and, for the past decade, together with the administration of catechol-*O*-methyl transferase (COMT) inhibitors.

Opicapone is a novel COMT inhibitor, under phase III clinical trials for the treatment of PD. When metabolised, opicapone is directly 3-*O*-sulfated by sulfotransferases (SULT). Additionally, both 3-*O*-methylation and 3-*O*-glucuronidation compete with 3-*O*-sulfation for conjugation of the adjacent phenolic hydroxyls.

The purpose of this work was characterizing the kinetics for the conversion of opicapone into sulfated metabolite by intestinal, kidney and liver S9 fractions and by human recombinant SULTs, using sensitive and specific Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) equipment. The method to quantify the opicapone 3-*O*-sulfate was developed and validated according with FDA regulations for the validation of bioanalytical methods and the validation parameters tested were: selectivity/specificity, carryover/contamination, dilution integrity, linearity, intra- and inter-batch accuracy and precision. From the tested S9 fractions, significantly higher levels of opicapone 3-*O*-sulfate were observed in the human intestinal S9 fraction in comparison with the other S9 fractions studied. Human, rat, monkey and dog liver S9 fractions, produced small amounts of opicapone 3-*O*-sulfate and human kidney S9 fraction conjugate opicapone at extremely low levels over an incubation period of 60 min. The apparent kinetic parameters derived from human intestinal and liver S9 fraction curve fitted to the allosteric sigmoidal and Michaelis-Menten equations, respectively. Both apparent K_m and V_{max} values were higher for liver than for intestine S9 fraction (12 and 7-fold higher, respectively).

Furthermore, seven commercial available recombinant SULT enzymes (SULT1A1*1, 1A1*2, 1A2, 1A3, 1B1, 1E1 and 2A1) were also tested *in vitro* to evaluate opicapone sulfation capacity. From the tested SULTs only SULT1A1*1 and 1A1*2 produced significant amounts of opicapone 3-*O*-sulfate (1165.9 ± 127.4 and 1913.2 ± 126.1 fmol.mg $prot^{-1}$ min^{-1}, respectively). SULT1A2, 1A3, 1E1 and 2A1 produced small amounts of opicapone 3-*O*-sulfate (between 28.3 – 221.1 fmol.mg $prot^{-1}$ min^{-1}). No metabolite formation was detected with SULT1B1 over an incubation period of 60 min. The opicapone sulfation kinetics was performed for the alleles of SULT1A1, SULT1A1*1 and 1A1*2, which fitted with a substrate inhibition profile. The apparent affinity for the opicapone conjugation was similar for the two alleles, as shown by the apparent K_m close to 20 µM.

The interaction with the SULT substrates, acetaminophen, quercetin and 2,6-dichloro-4-nitrophenol (DCNP), was also tested. No inhibition was observed on opicapone (100 µM) sulfation in the presence of acetaminophen in human liver pooled S9 fraction. Quercetin and DCNP completely inhibited opicapone sulfation by human liver S9 fraction with an IC_{50} of 1795 nM and 383.3 nM, and with an IC_{50} of 1483 nM and 181 nM for human intestinal S9 fraction, respectively. An IC_{50} of 529 nM and 245 nM was obtained for SULT1A1*1 and an IC_{50} of 232 nM and 700 nM was obtained for SULT1A1*2 for quercetin and DCNP, respectively.

In conclusion, sulfation is a relevant pathway on the metabolic handling of opicapone and multiple SULTs are able of catalyzing the sulfation of opicapone. The results obtained in the present study also support the role of intestine in opicapone sulfation and strongly suggest that in humans, opicapone 3-*O*-sulfation is mainly catalyzed by SULT1A1 isoforms.

Keywords: opicapone; opicapone 3-*O*-sulfate; SULT; sulfotransferases; PAPS; validation; LC-MS/MS; S9 fraction; acetaminophen; quercetin; 2,6-dichloro-4-nitrophenol.

RESUMO

A Doença de Parkinson (DP) é a segunda doença neurodegenerativa mais frequente em todo o Mundo, afetando principalmente pessoas mais idosas. O principal tratamento aplicado atualmente nesta doença continua a ser a administração em combinação de levodopa/inibidor da aromatic amino acid descarboxílase (AADC), e mais recentemente, em conjunto com um inibidor da catechol-*O*-methyltransferase (COMT).

O opicapone é o mais recente inibidor de COMT, atualmente está a ser testado em ensaios clínicos de fase III para o tratamento da DP. O metabolito mais abundante do opicapone é o 3-*O*-sulfato conjugado pelas sulfotransferases (SULT). A formação deste metabolito compete em grande parte com a 3-*O*-metilação e a 3-*O*-glucuronização pela conjugação dos grupos hidróxilos fenólicos do anel nitrocatecol.

O objetivo deste trabalho prático foi caraterizar as cinéticas de conversão do opicapone no seu metabolito sulfatado através de frações S9 de fígado, rim e intestino, e por sulfotransferases recombinantes humanas, recorrendo a um equipamento de Cromatografia Liquida acoplada a um Espectrómetro de Massa (LC-MS/MS) altamente sensível e específico. O método para a quantificação do sulfato do opicapone foi desenvolvido e validado de acordo com as diretivas da FDA para a validação de métodos bio-analíticos e os parâmetros de validação testados foram os seguintes: seletividade/especificidade, carryover/contaminação, integridade da diluição, linearidade, exatidão e precisão intra- e inter-ensaio. Das frações S9 testadas, grandes quantidades de opicapone 3-*O*-sulfato foram observadas em fração S9 de intestino humano em comparação com outras frações S9 testadas. Frações S9 provenientes de fígado humano, rato, macaco e cão produziram pequenas quantidades de opicapone 3-*O*-sulfato e a fração S9 de rim humano conjuga o opicapone a níveis extremamente baixos para um tempo de incubação de 60 min. Os parâmetros cinéticos aparentes derivados da fração S9 do intestino e fígado humanos correlacionam segundo as equações de Michaelis-Menten e da alostérica sigmoidal, respetivamente. Os valores de K_m e V_{max} obtidos foram maiores para a fração S9 do fígado do que para a fração S9 do intestino (12 e 7 vezes maior, respetivamente).

Adicionalmente, sete sulfotransferases recombinantes humanas disponíveis comercialmente (SULT1A1*1, 1A1*2, 1A2, 1A3, 1B1, 1E1 e 2A1) foram testadas *in vitro* para avaliar a capacidade de sulfatação do opicapone. Das SULTs testadas só as SULT1A1*1 e 1A1*2 produziram quantidades significantes de opicapone 3-*O*-sulfato (1165.9 ± 127.4 e 1913.2 ± 126.1 fmol.mg $prot^{-1}$ min^{-1}, respetivamente). As SULT1A2, 1A3, 1E1 e 2A1 produziram pequenas quantidades do metabolito sulfatado (entre 28.3 – 221.1 pmol mg $prot^{-1}$ min^{-1}). Com a SULT1B1 não se observou a formação de opicapone 3-*O*-

sulfato durante um período de incubação de 60 min. A cinética da sulfatação do opicapone foi realizada para os alelos da SULT1A1, SULT1A1*1 e SULT1A1*2, que corresponderam a um perfil de inibição de um substrato. A aparente afinidade para a conjugação do opicapone mostrou-se similar para os dois alelos, como demonstrado para o K_m aparente perto dos 20 µM.

A interação com substratos conhecidos das SULT, como o acetaminofeno, a quercetina e o 2,6-dicloro-4-nitrofenol (DCNP), também foi testada. Não foi observada nenhuma inibição na sulfatação do opicapone (100 µM) na presença do acetaminofeno em fração S9 de figado humano. A quercetina e o DCNP inibiram completamente a sulfatação do opicapone com um IC_{50} de 1795 nM e 383.3 nM em fração S9 de fígado humano, e um IC_{50} de 1483 nM e 181 nM para a fração S9 de intestino humano, respetivamente. Um IC_{50} de 529 nM e 245 nM foi obtido para a SULT1A1*1 e um IC_{50} de 232 nM e 700 nM foi obtido para a SULT1A1*2 para a quercetina e para o DCNP, respetivamente.

Em conclusão, a sulfatação é uma via relevante no metabolismo do opicapone e muitas SULTs são capazes de conjugar o opicapone. Os resultados obtidos neste trabalho prático demonstram adicionalmente o papel do intestino na sulfatação do opicapone e sugerem que a 3-O-sulfatação do opicapone em humanos é principalmente catalizada por isoformas SULT1A1.

Palavras-chave: opicapone; opicapone 3-*O*-sulfato; SULT; sulfotransferases; PAPS; validação; LC-MS/MS; fração S9; acetaminofeno; quercetina; 2,6-dicloro-4-nitrofenol.

Table of Contents

LIST OF FIGURES

LIST OF TABLES

CHAPTER I

INTRODUCTION AND OBJECTIVE

1. PARKINSON DISEASE

Parkinson's disease (PD) is a chronic degenerative disease with a prevalence of just over 1 per 1000 patients and increasing incidence at older ages. It is a movement disorder with cardinal features of tremor, rigidity, bradykinesia (slowness of movement), and postural instability. Other primary symptoms are slowness, stiffness, and the inability to initiate movements. The symptoms of PD become apparent after about 240,000 nigral cells die (60% of the total). The peak onset of PD is at 60 years of age and it progresses slowly over the next 10 to 20 years. In normal, unaffected people approximately 2400 nigral cells die yearly. Thus if someone lives 100 years they are at risk for PD. In PD the nigral cell loss accelerates more than 2400 cells die each year. It is unknown why the loss accelerates but important clues point at the involvement of some combination of genetic and environmental factors. Paralleling the nigral cell loss, there is a loss of the neurotransmitter dopamine in another region of the brain: the striatum (Bonifacio et al. 2007).

The discoveries by Carlsson et al. (1957) (Carlsson et al. 1957) showing that 3,4-dihydroxyphenylalanine (DOPA) can reverse the inability to initiate movement (akinesia) induced by catecholamine depletion in mice. In 1960, Ehringer and Hornykiewicz (Hornykiewicz 2006) demonstrating for the first time striatal dopamine deficiency in PD, provided the background for the first clinical trials with DOPA. Initial trials led to inconsistent results, but some years later Cotzias et al. (Cotzias et al. 1967) reported impressive antiparkinsonian effects of long-term oral DL-DOPA, establishing the basis for the modern pharmacotherapy of PD. The improved efficacy of the combination of levodopa (L-DOPA) with a peripheral decarboxylase inhibitor and the major adverse effects of the treatment, including involuntary movements, motor fluctuations, and mental symptoms, were described shortly thereafter (Cotzias GC 1969). Levodopa therapy radically changed the life history of patients afflicted with PD to such an extent that a large scale, controlled, prospective study comparing levodopa versus placebo has never been conducted.

Levodopa is the most effective symptomatic agent in the treatment of PD and the ''gold standard'' against which new agents must be compared. However, there remain two areas of controversy: (1) whether levodopa is toxic, and (2) whether levodopa directly causes motor fluctuations and dyskinesias. Levodopa is toxic to cultured dopamine neurons, and this may be a problem in PD where there is evidence of oxidative stress in the substantia nigra. However, there is little firm evidence to suggest that levodopa is toxic in vivo or in PD. Levodopa is also associated with motor complications and increasing evidence suggests that they are related, at least in part, to the short half-life of the drug (and its potential to

induce pulsatile stimulation of dopamine receptors) rather than to specific properties of the molecule. Treatment strategies that provide more continuous stimulation of dopamine receptors provide reduced motor complications in PD patients (Olanow et al. 2004). These studies raise the possibility that more continuous and physiological delivery of levodopa might reduce the risk of motor complications.

The absorption of levodopa occurs primarily in the duodenum and jejunum by means of an amino acid carrier-mediated transport system. Factors that decrease gastric emptying, such as food intake, gastric acidity, and anticholinergic medication, can delay the delivery of levodopa to the small intestine, allowing more time for peripheral decarboxylation. Protein intake may interfere with levodopa treatment because neutral amino acids will compete with levodopa for transport across the gut and the blood-brain barrier (BBB) (Pinho et al. 2007a; Pinho et al. 2007b). Levodopa is also metabolized in periphery by catechol-*O*-methyltransferase (COMT), into 3-O-methyl-levodopa (3-OMD) which may also compete with levodopa for transport across these membranes.

Since its introduction in 1960s, levodopa has been the mainstay of treatment for PD. Unlike dopamine, levodopa crosses the BBB and it is then decarboxylated to dopamine by aromatic amino acid decarboxylase (AADC) and is released by presynaptic terminals in the striatum, where it replenishes the dopaminergic deficiency that is characteristic of PD (Figure 1). Given orally, levodopa is approximately 95% decarboxylated by the intestinal and liver AADC to dopamine, which, as mentioned above, cannot cross the BBB and by these conditions the amount of levodopa that reaches the brain is, nevertheless, very low, about 1% of the administered dose. The combination of levodopa with a decarboxylase inhibitor (carbidopa or benserazide), which is unable to penetrate the Central Nervous System (CNS), diminishes the decarboxylation of levodopa to dopamine in peripheral tissues and enable a greater proportion of levodopa to reach the target sites in the striatum. As a result the levodopa dose can be reduced until 70%-80% maintaining the clinical efficacy. Therefore, although the same dosage is used, the peripheral side effects of levodopa and dopamine (nausea, hypotension, etc.) were minimizes, since circulating dopamine returns to physiological levels (Bonifati et al. 1999; Deleu et al. 2002).

COMT is an enzyme that efficiently metabolize levodopa this being particularly evident when given with decarboxylase inhibitors. COMT (EC 2.1.1.6), initially described by Axelrod and Tomchick in 1958, catalyzes the transference of a methyl group from S-adenosyl-L-methionine (SAM) to a hydroxyl group of a catechol substrate in the presence of magnesium, the reaction products being the O-methylated catechol and S-adenosyl-L-homocysteine (Guldberg et al. 1975).

COMT inhibition prevents the normal *O*-methylation of levodopa to its metabolite 3-OMD, a reaction that diverts a portion of the levodopa from conversion to dopamine. By limiting this ‘‘metabolic loss’’ of levodopa to 3-OMD, COMT inhibitors increase the availability of levodopa for dopamine production. COMT inhibitors, taken orally in combination with levodopa and carbidopa or benserazide, reduce peripheral 3-OMD formation from levodopa, enabling more levodopa to enter the brain. Carbidopa and benserazide are also substrates for COMT, and COMT inhibitors may prolong the action of these decarboxylase inhibitors.

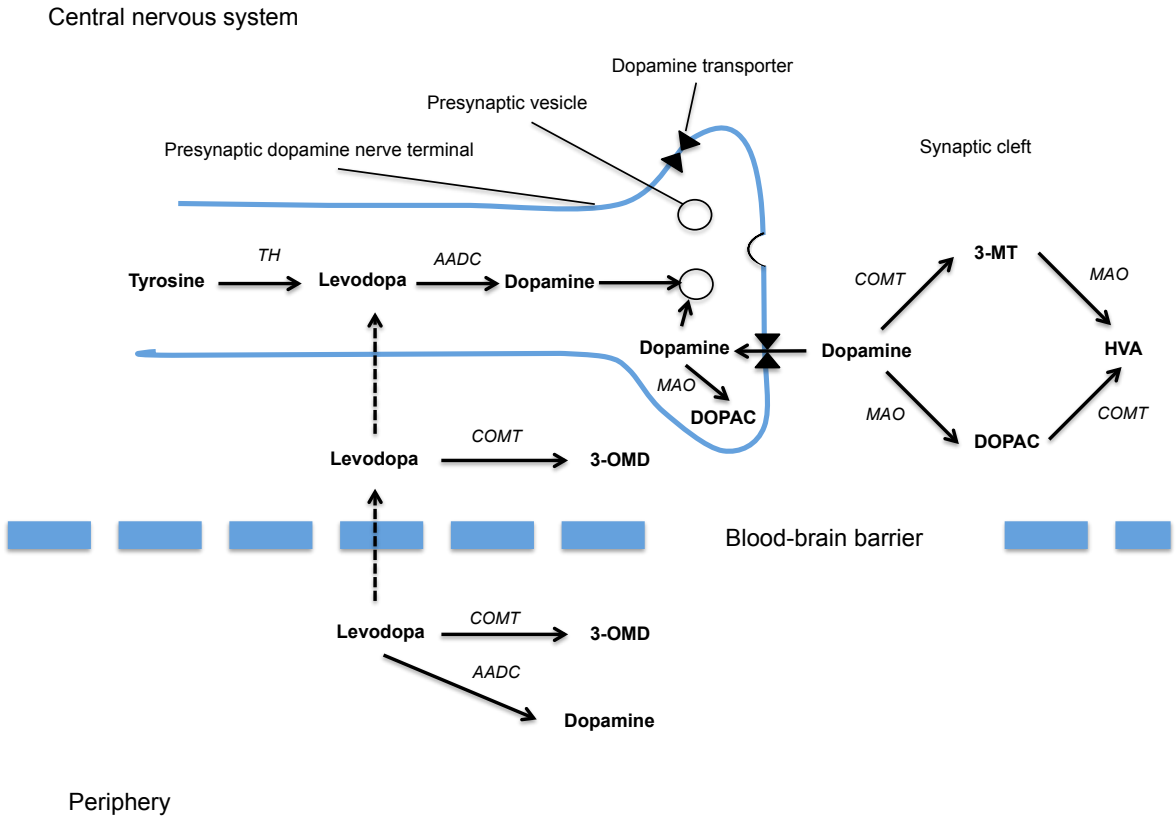


Figure 1 - Dopamine synthesis and metabolism: AADC, aromatic amino acid decarboxylase; COMT, catechol-*O*-methyltransferase; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanilic acid; MAO, monoamine oxidase; TH, tyrosine hydroxylase; 3-MT, 3-methoxytyramine; 3-OMD, 3-*O*-methyldopa.

2. COMT INHIBITORS

COMT substrates comprise a wide variety of endogenous and exogenous catechol derivatives such as catecholamines, their hydroxylated metabolites, catecholestrogens, ascorbic acid, dietary phytochemicals, and therapeutic compounds (Guldberg et al. 1975; Mannisto et al. 1992; Zhu et al. 2000). The major physiological role of the O-methylation reaction is the inactivation of biologically active or toxic catechols. Of particular relevance is the methylation of levodopa to 3-OMD in patients with PD (Sharpless et al. 1971; Mannisto et al. 1990). Recent studies also suggest a relevant role of COMT in modulating prefrontal dopamine neurotransmission (Egan et al. 2002; Huotari et al. 2002; Akil et al. 2003; Bilder et al. 2004). Two molecular forms of the enzyme have been described in mammals, a soluble form (S-COMT) and another form associated with membranes (MB-COMT) (Borchardt 1974; Jeffery et al. 1984; Grossman et al. 1985) both encoded by a single COMT gene (Salminen et al. 1990; Lundström et al. 1991; Winqvist et al. 1992).

MB-COMT and S-COMT have in common similar affinities for SAM (Jeffery et al. 1987; Lotta et al. 1995), the requirement for magnesium, the inhibition by calcium, and the similar optimal pH for activity (Mannisto et al. 1999). The affinity for substrates can, however, be significantly different. MB-COMT has typically higher affinity for catecholamines (10- to 100-fold higher) than S-COMT, and this characteristic appears to be horizontal to different species. The reason for this difference is not yet clarified; however, it is probably due to interactions between the protein and the membrane through lipids and other membrane proteins (Lotta et al. 1992; Bonifácio et al. 2000).

Following to the first purification and characterization of COMT, in the late 1950s (Axelrod et al. 1958), several classes of COMT inhibitors were identified. A comprehensive review of the pharmacological properties of these first-generation COMT inhibitors was published by Guldberg and Marsden (1975). Those compounds are typically competitive substrates of COMT and contain a catechol structure, or some related bioisosteric moiety. They include derivatives of pyrogallol and catechols, such as gallic acid, caffeic acid, 2-hydroxyoestrogens, or flavonoids like quercetin or rutin. Moreover, several other noncatecholic compounds like ascorbic acid, tropolones and derivatives of 8-hydroxyquinolines, and 3-hydroxylated pyrones and pyridones were identified as COMT inhibitors.

The potencies of these compounds were only moderate, with large dissociation constants typically within the micromolar range, but on the other hand, they proved to be useful for the study of adrenergic mechanisms. Some of these COMT inhibitors showed little value as

pharmacological agents, due to unfavorable pharmacokinetics, poor selectivity, or toxicology (Ericsson 1971; Guldberg et al. 1975; Reches et al. 1984).

The interest in COMT, especially as a therapeutic target, was strongly revived with the discovery of second-generation COMT inhibitors, in the late 1980s. Two independent groups developed simultaneously a new class of disubstituted catechols as a new generation of potent and selective COMT inhibitors (Bäckström et al. 1989; Borgulya et al. 1989; Männistö et al. 1989). Structure–activity relationship analysis established that enhanced potency was obtained by substitution with electron-withdrawing groups at a position *ortho* to a hydroxyl group of the catechol moiety. The best results were obtained with the nitro group, hence giving rise to a new class of nitrocatecholic COMT inhibitors. The concentration of the most potent nitrocatechols necessary to inhibit 50% of COMT activity *in vitro* was typically in the low nanomolar range, which indicates a potency three orders of magnitude higher than that of typical first-generation COMT inhibitors.

Nitrocatechols are kinetically characterized as reversible tight-binding inhibitors of COMT (Schultz et al. 1989; Lotta et al. 1995; Borges et al. 1997). Although poor substrates for the enzyme, they behave competitively with respect to the catechol substrate and are uncompetitive with respect to the co-substrate SAM (Schultz et al. 1989). Moreover, the new inhibitors showed increased selectivity for COMT over other enzymes involved in the metabolism of catecholamines, such as tyrosine hydroxylase, dopamine-*β*-hydroxylase, AADC, or monoamine oxidase (A and B forms) (Bäckström et al. 1989).

Of the initial series of nitrocatechol COMT inhibitors, nitecapone, entacapone, and tolcapone were characterized in detail. A direct comparison of the three inhibitors indicated values of Ki of 1.02, 0.30, and 0.27 nM for nitecapone, entacapone, and tolcapone, respectively (Lotta et al. 1995).

Ex vivo, tight-binding inhibitors are orally active and reversibly inhibit COMT activity, to a significant level, in a variety of tissues in the rat. While nitecapone has its main site of action in the duodenum (Nissinen et al. 1988), the structurally related entacapone shows significant COMT inhibition in other peripheral tissues, such as the liver and erythrocytes. However, it is essentially a peripheral COMT inhibitor, with only limited and transient inhibition of brain COMT at the higher doses tested (Nissinen et al. 1988; Learmonth et al. 2002). Tolcapone, on the other hand, by oral administration to rats has a higher potency and a longer duration of action than entacapone (Zurcher et al. 1990; Learmonth et al. 2002). Moreover, tolcapone can easily cross the BBB, showing almost indiscriminate inhibition of both peripheral and brain COMT (Zurcher et al. 1990; Zurcher et al. 1990; Borgulya et al. 1991; Learmonth et al. 2002).

More recently, several structure–activity relationship studies undertaken by another research group led to the development of two new nitrocatecholic COMT inhibitors, with superior pharmacological characteristics. Nebicapone (Learmonth et al. 2002) and BIA 3-335 (Learmonth et al. 2004) incorporate the nitrocatechol pharmacophore and show the typical tight-binding characteristics of other nitrocatechols, being potent and reversible competitive inhibitors of COMT (Bonifacio et al. 2003).

It has been demonstrated that these COMT inhibitors together with an AADC inhibitor such as carbidopa or benserazide, alter the metabolism of levodopa and effectively potentiate the action of orally administered levodopa. Indeed, the levels of 3-OMD are markedly reduced and the bioavailability of levodopa in the plasma is increased, when levodopa plus AADC inhibitor are orally administered to rats, together with nitecapone (Nissinen et al. 1988), entacapone (Nissinen et al. 1992), tolcapone (Borgulya et al. 1989; Zurcher et al. 1990; Zurcher et al. 1990) and nebicapone (Loureiro et al. 2006).

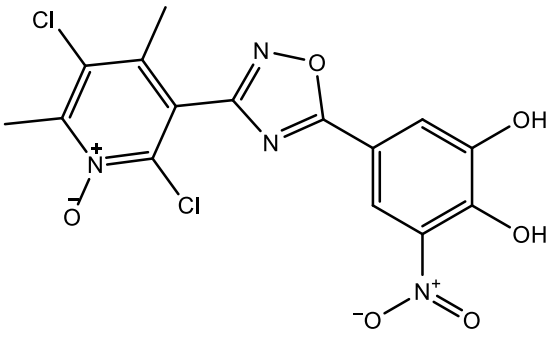
Tolcapone, entacapone, and nebicapone are extensively metabolized, mostly in liver. The major metabolic pathways of these compounds involve conjugative reactions. The 3 *O*-β,D-glucuronide conjugates of tolcapone and nebicapone are the major plasma metabolites. Tolcapone and nebicapone are also methylated (Jorga et al. 1999; Loureiro et al. 2006) and the respective metabolites, although present in minor concentrations in human plasma, have very long half-lives, which may suggest that accumulation could occur. However, during long-term administration only a relatively small accumulation of the methylated metabolite occurs, due to the suppression of its formation by the COMT inhibitor itself. Entacapone is not methylated in humans. Minor metabolic pathways in humans include oxidative or reductive reactions and further conjugation of the derived products for tolcapone (Jorga et al. 1999), and sulfate conjugation and nitro reduction followed by acetylation for nebicapone (Loureiro et al. 2006).

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 (Deleu et al. 2002; Keating et al. 2005; Bonifacio et al. 2007; Lees 2008).

Opicapone (2,5-dichloro-3-[5-(3,4-dihydroxy-5-nitrophenyl]-1,2,4-oxadiazol-3-yl)-4,6-dimethyl pyridine 1-oxide, also known as BIA 9-1067) (Figure 2) is a novel third generation COMT inhibitor currently under phase III clinical trials by BIAL – Portela & Cª, S.A. (S. Mamede do Coronado, Portugal) for use as adjunctive therapy in levodopa-treated PD patients. Opicapone was designed as a hydrophilic 1,2,4-oxadiazole analogue with a pyridine N-oxide

residue at position 3 to provide high COMT inhibitory potency and avoid cell toxicity (Kiss et al. 2010).

Actually, it is crucial the need for development of more potent, safer and longer acting COMT inhibitors, at this point, opicapone is emerging as a candidate for this unmet need. Opicapone is endowed with an exceptionally high binding affinity (sub-picomolar Kd) that translates into a slow complex dissociation rate constant and a long duration of action *in vivo* (Bonifácio et al. 2012a; Bonifácio et al. 2012b). In liver and brain homogenates from rats administered with opicapone, tolcapone and entacapone by gastric tube, opicapone showed to have a stronger and more sustained COMT inhibitory effect than the comparing COMT inhibitors tolcapone and entacapone. One hour after administration, COMT inhibition was 99% with opicapone versus 82% with tolcapone and 68% with entacapone. Nine hours after administration, entacapone showed no COMT inhibition and tolcapone produced minimal inhibitory effect (16%), whereas opicapone continued to inhibit COMT activity by 91% (Bonifácio et al. 2012b). Opicapone was well tolerated in studies in several animal species. On basis of these promising results, it was decided to proceed to further clinical trials with opicapone. In PD patients, opicapone as adjunctive therapy to a combination of levodopa/AADC inhibitor showed to increase levodopa systemic exposure, decrease 3-OMD exposure, decrease S-COMT activity and improves patients' motor performance in clinical studies (Kiss et al. 2010; Rocha et al. 2013).



Opicapone

Figure 2 - Chemical struture of opicapone (formerly known as BIA 9-1067).

3. OPICAPONE METABOLISM

The biotransformation of substances foreign to the body (xenobiotics) including drugs is divided into phase I and phase II. Phase I reactions are performed by the main enzymes cytochromes P450 (CYPs) and include the transformation of a parent compound to more polar metabolites, mainly hydroxylations and hence acting as monooxygenases, dioxygenases and hydrolases. The cytochromes P450 constitute a superfamily of heme enzymes responsible for the metabolism of xenobiotics and endobiotics. They are also involved in a variety of specific endogenous functions including the biosynthesis of steroid hormones, prostaglandins, bile acids, and others (Anzenbacher et al. 2001; Nebert et al. 2002). Only about a dozen enzymes belonging to the 1, 2, and 3 CYP-families are responsible for the metabolism of the majority of drugs and other xenobiotics. Despite the broad and overlapping substrate specificities of these enzymes, many drugs are metabolized at clinically relevant concentrations by one or few enzymes only, which limits the important redundancy of the phase I drug oxidation system. Knowledge of the intrinsic and extrinsic factors that influence expression and function of the responsible enzymes is thus a prerequisite for predicting variable pharmacokinetics and drug response. While monogenic polymorphisms explain a major part of the variability for only few enzymes (in particular CYP2D6), most enzymes are multifactorially controlled including additional polymorphisms in regulatory trans-genes and nongenetic host factors including sex, age, disease, hormonal and diurnal influences and other factors. Summarizing, the highest expressed forms in liver are CYPs 3A4, 2C9, 2C8, 2E1, and 1A2, while 2A6, 2D6, 2B6, 2C19 and 3A5 are less abundant and CYPs 2J2, 1A1, and 1B1 are mainly expressed extrahepatically.

Phase II enzymes play also an important role in the biotransformation of endogenous and xenobiotics compounds to more easily excretable forms as well as in the metabolic inactivation of pharmacologically active substances. The phase II reactions are currently designed by conjugating reactions. These include glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugation. In general, the respective conjugates are more hydrophilic than the parent compounds.

Phase II drug metabolizing enzymes are mostly transferases and include: UDP-glucuronosyltransferases (UGT), sulfotransferases (SULT), N-acetyltransferases (NAT),

glutathione S-transferases (GST) and various methyltransferases (mainly thiopurine S-methyltransferase, TPMT and catechol-*O*-methyl transferase, COMT) (Jancova et al. 2010). When metabolised, these nitrocatechol derivatives are extensively conjugated, usually undergo direct glucuronidation catalysed by UGTs, sulfation catalysed by SULTs and are then rapidly excreted in the urine (Lautala et al. 1997; Loureiro et al. 2006). Additionally, methylation may compete with other metabolic pathways for conjugation of the adjacent phenolic hydroxyls (Jorga et al. 1999; Loureiro et al. 2006). Minor metabolic pathways of COMT inhibitors in humans include oxidative or reductive reactions due to the presence of adjacent phenolic hydroxyls on nitrocatechol that is more favourable in metabolism involving conjugation reactions.

As have been shown for other COMT inhibitors, opicapone is metabolized into opicapone 3-*O*-sulfate, which is the major inactive metabolite in plasma, suggesting that the sulfation is the main metabolic pathway for opicapone (Figure 3). Additionally, both 3-*O*-methylation and 3-*O*-glucuronidation were observed as minor metabolites that compete with 3-*O*-sulfation for conjugation of the adjacent phenolic hydroxyls. The opicapone amine N-oxide reduced form, showed also to be a minor metabolite (Rocha et al. 2013).

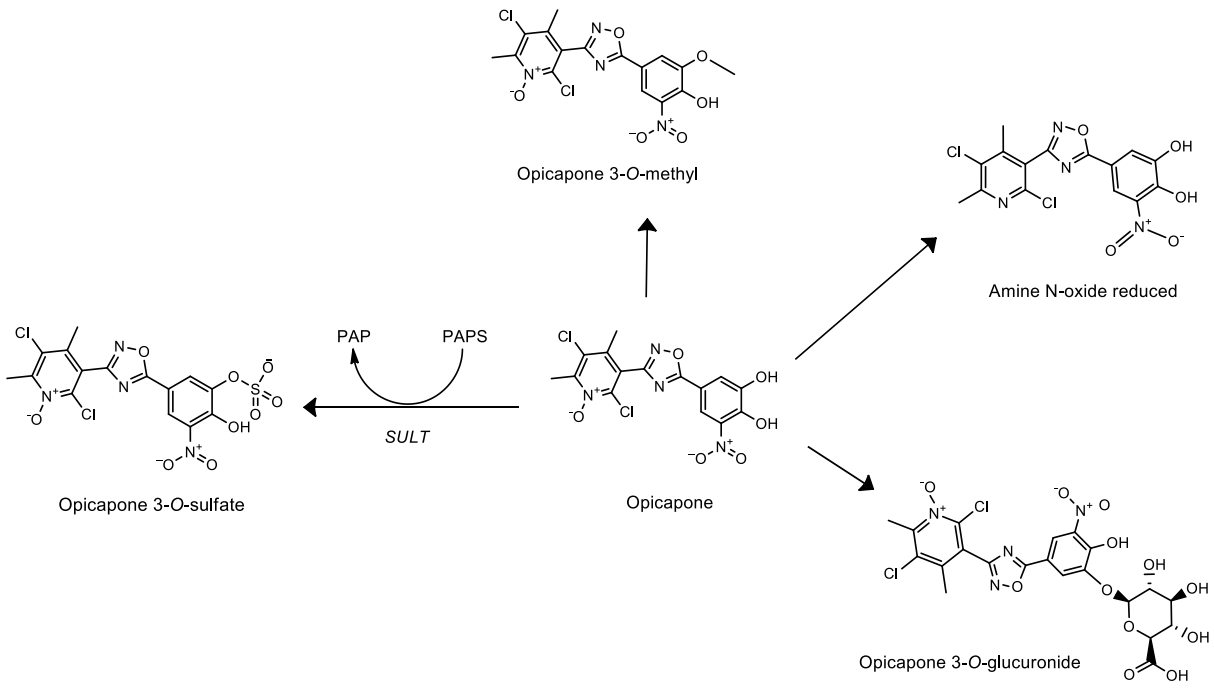


Figure 3 – Metabolic pathways for opicapone. Chemical structures of opicapone and its metabolites.. SULT, sulfotransferase; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PAP, 3'-phosphoadenosine 5'-phosphate.

3.1. SULFOTRANSFERASES

Sulfotransferases are a supergene family of enzymes that catalyse the conjugation of 3'-phosphoadenosine 5'- phosphosulphate (PAPS) with an O-, N- or S- acceptor group of an appropriate molecule. In general, *O*-sulfation represents the dominant cellular sulfation reaction. Nevertheless, *N*-sulfation is a crucial reaction in the modification of carbohydrate chains in macromolecules such as heparin and heparan sulfate, common components of proteoglycan. *N*-Sulfoconjugation is also involved in the metabolism of xenobiotics such as quinolones and amino drugs (Senggunprai et al. 2009). The PAPS is a universal sulfate (or, correctly sulfonate) donor molecule required for all sulfonation reactions and shown that it can be synthesized by all tissues in mammals (Strott 2002). All the human cells are capable to synthesize PAPS in cytoplasm by a two-step reaction utilizing ATP and inorganic sulfate (Figure 4). The inorganic sulfate used in the synthesis of PAPS may come from the diet, renal reabsorption or from the catabolism of the amino acids cysteine and methionine (Klaassen et al. 1997). The sulfation of drugs in humans may be limited by the availability of PAPS, because the synthesis of PAPS depends on the availability of inorganic sulfate in plasma. High doses of substrates for glutathione conjugation, sulfation, and amino acid synthesis as well as by genetic defects in PAPS synthesis may deplete the levels of inorganic sulfate in plasma. A sulfation decrease due to the depletion of PAPS may increase the toxicity of some drugs such as acetaminophen (Falany 1997; Klaassen et al. 1997).

Sulfation has a significant role in the biotransformation of a number of endogenous low-molecular compounds (e.g. steroids, catecholamines, serotonin, iodothyronines, eicosanoids, some tyrosine-containing peptides, retinol, 6-hydroxymelatonin, ascorbate and vitamin D) (Glatt et al. 2004). Furthermore, it is an important pathway in the biotransformation of numerous xenobiotics such as drugs and chemicals (Gamage et al. 2006). On the other hand, a number of compounds (procarcinogens) are converted by sulfation into highly reactive intermediates which can act as chemical carcinogens and mutagens by covalently binding to DNA (Surh 1998; Glatt et al. 2004; Jancova et al. 2010).

Human SULTs are divided into four families: SULT1 (phenol SULTs), SULT2 (hydroxylsteroid SULTs), SULT4 and SULT6. Sulfation of phenolic xenobiotics is performed by SULT1 family, and sometimes by SULT2 (Allali-Hassani et al. 2007). The SULT1 family involves 8 members divided into 4 subfamilies (1A1, 1A2, 1A3, 1B1; 1C1, 1C2 and 1C3; 1E1). The SULT2 family can be divided into two subfamilies, SULT2A (SULT2A1 and SULT2A3) and SULT2B that comprises two isoforms SULT2B1a and SULT2B1b. The SULT4A1 and SULT6B1 are the only members of the SULT4 and SULT6 family

respectively. The members of the same SULT gene family share at least 45 % amino acid sequence identity while members of subfamilies share at least >60 % identity in amino acid sequence. Two broad classes of sulfotransferases have been identified: namely the cytosolic and membrane-bound ones. Membrane-bound SULTs are localized in the Golgi apparatus and are responsible for the sulfation of peptides, proteins, lipids and glycosaminoglycans. Cytosolic SULTs catalyze sulfation of xenobiotics and small endobiotic molecules such as steroids, bile acids and neurotransmitters. SULTs exhibit wide tissue distribution. The members of SULT1A subfamily have been identified in liver, brain, breast, intestine, jejunum, lung, adrenal gland, endometrium, placenta, kidney and blood platelets and is subject to a common genetic polymorphism (Price et al. 1989). Common single nucleotide polymorphisms have been reported in the human *SULT1A1* gene (Jones et al. 1995; Raftogianis et al. 1996). The common SULT1A1 allozymes include *1 which as an Arg in position 213, the *2 variant defined by an $Arg^{213}His$ amino acid substitution due to a conversion from Guanine (G) to Adenine (A) at nucleotide 638. These single nucleotide polymorphisms lead to changes in thermal stability, enzymatic activity, and altered degradation of the allozymes (Raftogianis et al. 1997; Raftogianis et al. 1999; Nagar et al. 2006). SULT1A1 exhibits the highest level of expression of all SULT1 enzymes in the liver. In contrast, SULT1A3 is expressed in most tissues with the exception of adult liver and SULT1B1 in liver, small intestine, colon and leukocytes. Expression of the SULT1C subfamily is found predominantly in the human fetus, SULT1E1 is expressed in the human liver and jejunum. SULT2A1 shows the highest level of expression in liver, adrenal, duodenum and fetal adrenal gland. Subfamily SULT2B is localized in human prostate, placenta, adrenal gland, ovary, lung, kidney and colon (Glatt et al. 2004; Gamage et al. 2006; Lindsay et al. 2008). Human SULT4A1 has been identified in brain and SULT6B1 in testis and kidney (Jancova et al. 2010).

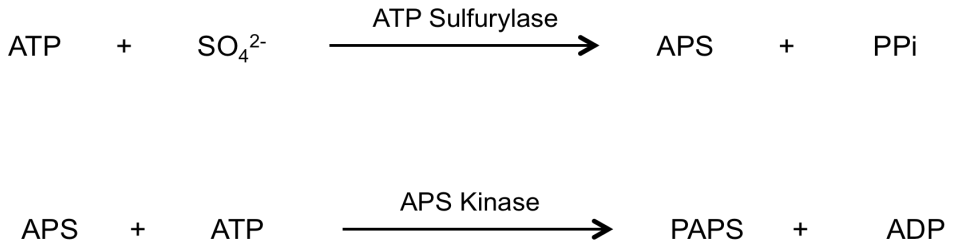


Figure 4 – The two-step synthesis of PAPS. ATP, adenosine triphosphate; APS, adenosine 5'-phosphosulfate; PPi, inorganic pyrophosphate; ADP, adenosine diphosphate.

SULT enzymes have different substrate preferences although there is evidence of substrate overlap at the levels of subfamilies and families.

SULT1A1 is a xenobiotic-conjugating enzyme with a broad substrate range with specificity for small phenolic compounds. It has also been termed phenol sulfotransferase (P-PST) and thermostable phenol sulfotransferase (TS PST1). This form is responsible for the sulfoconjugation of phenolic compounds such as monocyclic phenols, naphtols, benzylic alcohols, aromatic amines, hydroxylamines, dopamine and iodothyronines (Glatt et al. 2004). The SULT1A1 has high affinity for 4-nitrophenol, sensitivity to inhibition by 2,6-dichloro-4-nitrophenol (DCNP) and quercetin and its thermostability at 45 ºC (Dajani et al. 1998; De Santi et al. 2002; Hempel et al. 2007).

SULT1A2 (TS PST2) appears to be an efficient enzyme in sulfating some aromatic hydroxylamines (Meinl et al. 2002; Nowell et al. 2005), and this reaction forms charged species (the sulfoconjugates of hydroxylamines) which may suggest a potential role in mutagenicity and carcinogenicity. This is an example of a toxification reaction, contrary to detoxication reactions occurring in the majority of cases. The physiological role of SULT1A2 has not been identified yet (Nowell et al. 2005). SULT1A2 can sulfoconjugate substrates such as 2-naphtol or 4-nitrophenol (Glatt et al. 2004). Although, SULT1A2 shares >93 % amino acid identity with SULT1A1 and SULT1A3, this enzyme exhibits no activity toward dopamine as a substrate (Gamage et al. 2006).

SULT1A3 was previously known as thermolabile phenol SULT (TL PST) and monoamine phenol sulfotransferase (M-PST). It displays high affinity for monocyclic phenols. SULT1A3 has a strong substrate preference for endogenous catecholamines and as such is responsible for the regulation of the rapidly fluctuating levels of neurotransmitters. Dopamine

is often used as a selective substrate for the detection of SULT1A3 activity (Dajani et al. 1998). Norepinephrine, catechols, monocyclic phenols and aromatic molecules are other substrates of SULT1A3 (Gamage et al. 2006).

The substrate specifity of SULT1B1 is restricted to thyroid hormones (Fujita et al. 1997) and small phenolic compounds such as 1-naphtol and 4-nitrophenol (Pai et al. 2002).

SULT1C1 conjugates some iodothyronines, 4-nitrophenol and *N*-hydroxy-2-acetylaminofluorene (Li et al. 2000) but a good endogenous substrate for this enzyme has not been identified.

SULT1C2 showed activity for substrates as 4-nitrophenol and N-hydroxy-2-acetylaminofluorene (Yoshinari et al. 1998).

SULT1E1 is widely known as estrogen sulfotransferase (EST). This enzyme has a greater affinity for estrogen sulfation than any other SULTs that conjugate estrogen (Falany et al. 1995; Schrag et al. 2004). SULT1E1 may be important in both the metabolism of estrogens and in the regulation of their activities. This enzyme also shows activity towards iodothyronines, pregnenolon, 1-naphtol, naringenin, genistein and 4-hydroxytamoxifen (Gamage et al. 2006).

SULT2A1 was termed dehydroepiandrosterone-sulfotransferase (DHEA ST). This form is known to sulfonate steroids, drugs, and other xenobiotics and is highly selective for DHEA (dehydroepiandrosterone) (Comer et al. 1993; Cook et al. 2012).

SULT2A and SULT2B subfamilies conjugate similar substrates but members of the SULT2B subfamily are predominantly cholesterol sulfotransferases (Javitt et al. 2001).

To date no substrates have been identified for SULT4A1 or SULT6B1 (Lindsay et al. 2008; Minchin et al. 2008).

SULT activity may be inhibited in humans exposed to certain therapeutic drugs, dietary or environmental chemicals (Wang et al. 2006). The inhibitory effects of various compounds have been examined mainly for the SULT1A subfamily. Vietri et al. (Vietri et al. 2003) described curcumin as a potent inhibitor of SULT1A1 in human liver. De Santi et al. (De Santi et al. 2002) showed inhibition of SULT1A1 by quercetin in human adult and fetal liver. The inhibitory effects of various beverages and catechins in tea were investigated by Nishimuta et al. (Nishimuta et al. 2007). Their results showed inhibition of recombinant SULT1A1 and 1A3 by grapefruit juice, orange juice, green tea, black tea and oolong tea. An inhibitory effect of some non-steroidal anti-inflammatory agents on SULT1A1 and SULT1E1 activity was demonstrated by King et al. (King et al. 2006). Nimesulide, meclofenamate, piroxicam were selective inhibitors of SULT1A1 while sulindac and ibuprofen were more selective for SULT1E1 inhibition.

Maiti et al. (Maiti et al. 2005) found that retinoic acid can increase sulfotransferase expression and activity in cultured human cells. They reported retinoic acid induction of human SULT1A1, 2A1 and 1E1 in hepatic carcinoma cells (HepG2) and in intestinal carcinoma cells (Caco-2). Methotrexate induced human SULTs in HepG2 and Caco-2 cells (Chen et al. 2005). Chen el al. showed that protein and mRNA expression of human SULT1A1, 1A3, 2A1, 1E1 were induced in HepG2 cells; SULT1A3 and 2A1 were induced in Caco-2 cells. Sulfotransferase expression in HepG2 and Caco-2 cell lines was also investigated by Chen et al. (Chen et al. 2008). Their data suggested that genistein, a natural isoflavone found in soybean products induced SULT1A1 and SULT2A1 gene and protein expression in these cells (Jancova et al. 2010).

4. OBJECTIVES

4.1. GENERAL OBJECTIVE

Since identification of SULTs isoforms helps to predict potential drug-drug interaction mediated by particular enzyme isoform and polymorphism-related interindividual variability, we thought it should be useful to identify the SULTs involved in opicapone sulfation, using human liver and intestinal S9 fraction and recombinant SULTs.
The overall purpose of this study was to identify the SULTs involved in opicapone sulfation and to characterize their kinetics parameters using human intestinal and liver S9 fractions and human recombinant sulfotransferases.

4.2. SPECIFIC OBJECTIVES

- To develop and validate a LC-MS/MS method for the quantification of opicapone 3-*O*-sulfate;

- To characterize the kinetics parameters of opicapone conjugation into opicapone 3-*O*-sulfate by S9 fractions from different species;

- To characterize the kinetics parameters of opicapone conjugation into opicapone 3-*O*-sulfate by human recombinant sulfotransferases;

- To characterize the interaction between SULT substrates, acetaminophen, quercetin and DCNP in opicapone sulfoconjugation.

CHAPTER II

MATERIAL AND METHODS

1. REAGENTS

Opicapone (code name BIA 9-1067); 2,5-dichloro-3-(5-(3,4-dihydroxy-5-nitrophenyl)-1,2,4-oxadiazol-3-yl)-4,6-dimethylpyridine 1-oxide; opicapone 3-*O*-sulfate (code name BIA 9-1103); pyridinium 5-(3-(2,5-dichloro-4,6-dimethyl-1-oxidopyridin-3-yl)-1,2,4-oxadiazol-5-yl)-2-hydroxy-3 nitrophenyl sulfate; were synthesized in the Laboratory of Chemistry, BIAL (S. Mamede Coronado, Portugal), with purities >99.5%.

Acetaminophen, quercetin and 2,6-dichloro-4-nitrophenol were purchased from Sigma-Aldrich (St. Louis, MO). The 3'-phosphoadenosine 5'-phosphosulfate (PAPS), magnesium chloride ($MgCl_2$), potassium phosphate monobasic (KH_2PO_4) and potassium phosphate dibasic (K_2HPO_4) were purchased from Sigma-Aldrich (St. Louis, MO). All the compounds mentioned above were stored in accordance to the certificates of analysis retained in Bial's Laboratory of Pharmacological Research.

Pooled liver S9 from human, monkey, rat and dog were purchased from BD Gentest (Woburn, MA). Pooled human intestinal S9 fraction and kidney S9 fraction were purchased from XenoTech (Lenexa, KS). Recombinant human SULT expressed in Escherichia coli were purchased from Cypex (Scotland, UK, SULT: 1A1*1, 1A1*2, 1A2, 1A3, 1B1, 1E1 and 2A1). The protein contents were used as described in the data sheets provided by the manufacturers.

All the biological material was appropriately handled with gloves and all disposable materials were incinerated.

2. LABORATORY EQUIPMENT

The laboratory equipment used is present next:

- Automatic Pipettes Gilson PIPETMAN®;
- Multipette Plus Eppendorf®**;**
- Balance Kern® ABJ;
- Balance Mettler Toledo® XP26;
- Digital Timer Smiths;
- Magnetic Stirrer IKA®;
- MilliQ Water System (Millipore®);
- Shaking Water Bath GFL®;
- Digital Thermometer Checktemp 4 (HANNA® Instruments);
- Fume Hood mc6® Waldner;
- Centrifuge Eppendorf® 5417R;
- Vortex Mixer SA8 Stuart®;
- Agilent® LC TQ/MS (Agilent Technologies, CA, United States).

3. SOFTWARES

The softwares used for data collection and data analysis are the ones described next:

- GraphPad Prism® 5.02 (GraphPad Software, Inc.);
- Microsoft Office® Excel 2007;
- Agilent MassHunter® Workstation Software Version B.04.01.

4. OPICAPONE SULFATION

4.1. OPICAPONE SULFATION BY S9 FRACTIONS

The sulfation of opicapone by human, monkey, rat and dog S9 fractions were performed in the incubation mixture contained the following: 50 mM phosphate buffer (pH 7.4); 5 mM $MgCl_2$; 0.1 mM PAPS and S9 (0.4 mg/ml and 1.0 mg/ml) suspension in 50 mM phosphate buffer (pH 7.4). The mixture was pre-incubated for 5 min at 37ºC, and then the compound (10 µM) was added to initiate the assay. The samples were incubated at 37 ºC for 30 to 120 min. After incubation the reaction was stopped with acetonitrile 0.1% formic acid and samples, centrifuged (approx. 14000 g for 10 min), filtered (6000 g for 5 min) and injected into LC-MS/MS. The incubation without PAPS was performed for 60 min.

4.2. OPICAPONE SULFATION BY RECOMBINANT SULFOTRANSFERASES

Opicapone sulfation by SULT1A1*1, 1A1*2, 1A2, 1A3, 1B1, 1E1 and 2A1 was measured using the following assay conditions: the incubation mixture (100 µl total volume) contained 0.4 mg/ml total protein, 5 mM $MgCl_2$, 0.1 mM PAPS, in 50 mM phosphate buffer (pH 7.4) and 10 µM opicapone. Drug was dissolved in DMSO and the final concentration of DMSO in the reaction was below 0.5% (v/v). The mixture was pre-incubated for 5 min at 37ºC, and then the compound was added to initiate the assay. The samples were incubated for up to 60 min and stopped with 100 µl acetonitrile 0.1% formic acid. All incubations were performed in a shaking water bath at 37ºC. After removal of the protein by centrifugation for 10 min at 14000g, supernatant was filtered (6000 g for 5 min) and injected into LC-MS/MS.

4.3. Kinetic of Opicapone Sulfation by Human Recombinant Sulfotransferases and Pooled Human Liver and Intestinal S9 Fraction

Rates of sulfation were determined, as described above for SULT screening, with opicapone concentrations ranging 5-100 μM and with 60 min incubation time for all recombinant SULTs, SULT1A1*1, 1A1*2, 1A2, 1A3, 1B1, 1E1 and 2A1, and for human liver and intestinal S9 fractions. All preparations were evaluated for linearity of product formation with respect to incubation time (0-240 min) and protein concentration. The total protein concentration was 30 μg/ml for SULTs, 0.4 mg/ml for HIS9 and 1.0 mg/ml for HLS9. All experiments were performed with samples in duplicate.

4.4. Inhibition of Opicapone Sulfation by Typical Substrates for SULT Isoforms

In experiments designed to evaluate the inhibition of opicapone sulfation, human pooled S9 fractions and SULT1A1*1 and 1A1*2 were incubated for 60 min with 100 μM opicapone in the presence and in the absence of the following substrates: acetaminophen, (substrate for SULT) at the concentrations of 1, 5, 10, 50, 100, 200, 400 and 500 μM; quercetin (substrate for SULT1A1) and 2,6-dichloro 4-nitrophenol (substrate for SULT1A1) at 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 μM. The opicapone sulfation was determined as described above for SULT screening.

5. BIO-ANALYTICAL METHOD VALIDATION

Bio-analytical method validation included all the procedures that demonstrate that the method used for a quantitative measurement of opicapone 3-*O*-sulfate in a given matrix, is reliable and reproducible for the intended use.
Validation involved documenting, through the use of specific laboratory investigations, that the performance characteristics of the method were suitable and reliable for analytical applications.
The validation criteria were established in such a way to define the necessary parameters for validation of the analytical method (Blume et al. 2010; DeSilva et al. 2012).

5.1. REFERENCE STOCK SOLUTIONS

Two separate stock solutions (one for calibration standards and other for quality controls specimens) of opicapone 3-*O*-sulfate were prepared by accurately weighing an appropriate amount of compound and dissolving it in DMSO to obtain 10 mM solutions. Opicapone 3-*O*-sulfate stock solutions were prepared freshly every day.

5.2. CALIBRATION STANDARDS

The calibration standards were prepared with phosphate buffer 50 mM, pH 7.4: Acetonitrile (ACN) 0.1 % formic acid (50:50, v:v) by dilution of the calibration reference stock solution to have eight calibration points: 1000, 400, 200, 100, 50, 10, 5 and 1 nM.

5.3. QUALITY CONTROLS

The Quality Control (QC) solutions were prepared with phosphate buffer 50 mM, pH 7.4: ACN 0.1% formic acid (50:50, v:v) by dilution of the QC stock solution to have 1000, 400, 100, 50, 5 and 1 nM solutions.

5.4. VALIDATION PARAMETERS

5.4.1. SELECTIVITY/SPECIFICITY

This is the ability of the analytical procedure to assess unequivocally the analyte in the presence of components that may be expected to be present. This parameter was assessed by injecting a blank sample prepared in the same conditions of the experiment but without opicapone 3-*O*-sulfate.
The specificity of the assay against opicapone, quercetin, acetaminophen and DCNP was evaluated. To blank matrix was added a certain amount of these compounds (100 µM) and the samples were treated and analysed to check if there is any interference of these compounds at the same retention time of opicapone 3-*O*-sulfate.

5.4.2. CARRYOVER/CONTAMINATION

When validating a chromatographic analysis method, tests should be performed to look for characteristic peaks occurring at the same retention times as the analytes, or that may interfere with subsequent analysis. Contamination or blank response from matrix or reagents can affect, also, the accuracy and precision at all concentrations. However low concentrations samples are most affected as a percentage of concentrations.
Carryover was assessed on the chromatographic system by making a single injection of an extracted blank matrix after of the last highest concentration of the calibration standard (1000 nM).

5.4.3. WORKING AND LINEAR RANGE

The range of an analytical procedure is the interval between the upper and the lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision and accuracy. At the lower end of the concentration range the limiting factors are the values of the limits of quantification (LLOQ). At the upper end of the concentration range (ULOQ) limitations will be imposed by various effects, depending on the instrument response system.

The working and linear range was evaluated using three calibration curves obtained from three separated runs. The calibration curve consists of eight calibrations standards (1000, 400, 200, 100, 50, 10, 5 and 1 nM) covering the range including the LLOQ and ULOQ.

5.4.3.1. LLOQ and ULOQ

The LLOQ and ULOQ of an individual analytical procedure is strictly the lowest or highest amounts, respectively, of analyte in a sample that can be quantitatively determined with suitable precision and accuracy. The quantification limit is a parameter of quantitative assays for low and high levels of compounds in sample matrices, and is used particularly for determination of, for example, degradation products. It may also be referred to as the sensitivity of a quantitative assay.

5.4.3.2. Calibration Curve

Within the working range there may exist a linear response range. Within the linear range, signal response will have a linear relationship to analyte concentration or property value. The extent of this range may be established during the evaluation of the working range.

5.4.4. ACCURACY

The accuracy of an analytical procedure expresses the closeness of agreement between the value, which is accepted either as a conventional true value or an accepted reference value, and the determined value.
Accuracy was measured using five determinations for each of the following concentrations: 1000, 400, 100, 50, 5 and 1 nM.

5.4.5. PRECISION

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. The precision was divided into repeatability and intermediate precision assessment. Repeatability expresses the precision under the same operating conditions over a short interval of time, during a single analytical run (Intra-batch).

The intermediate precision expresses within laboratory variations (Inter-batch) that was measured in different days.

5.4.5.1. Repeatability (Intra-batch)

Repeatability was measured using five determinations for each of the following concentrations 1000, 400, 100, 50, 5 and 1 nM, which include LLOQ and ULOQ.

5.4.5.2. Intermediate Precision (Inter-batch)

Intermediate precision was measured using 90 determinations, from 3 different days, for each of the six concentrations, 1000, 400, 100, 50, 5 and 1 nM, that fall within the range including LLOQ and ULOQ.

5.4.6. DILUTION INTEGRITY

Tests whether, in the event that a test sample contains concentrations of analyte falling above the validated range of the assay, the sample can be diluted in the appropriate matrix into the validated range of the assay, with acceptable accuracy and precision. To assess this parameter, a validation specimen was prepared in triplicate with a concentration above the ULOQ (10000 nM) and diluted 20-fold in the appropriate matrix into the validated range (1 nM to 1000 nM).

6. LC-MS/MS METHOD

The analysis of samples extracts for opicapone 3-*O*-sulfate was performed using a LC-MS/MS equipment. An Agilent G6460 triple quadrupole mass spectrometer (Agilent Technologies, CA, United States) coupled to an Agilent 1200 Infinity HPLC system consisting of infinity binary pump G4220A, infinity sampler G4226A, FC/ALS thermostated G1330B and infinity column thermostat G1316C was used. The ionization mode was electrospray, polarity negative. Electrospray jetstream conditions were as follows: capillary voltage, 3500 V; drying gas flow, 10 l/min nitrogen; drying gas temperature, 300 ºC; nebulizer pressure, 45 psi; sheath gas temperature, 400 ºC; and sheath gas flow, 11 l/min. The mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. The MRM transition was *m/z* 491→394; dwell time, 200 ms; fragmentor voltage, 100 V; and collision energy, 25 V. HPLC separation was achieved on a Zorbax Eclipse XDB-C8 column (4.6 x 150 mm; 5 µm; Agilent, USA) using (A) ammonium acetate 25 mM and (B) acetonitrile as mobile phases at a flow rate of 0.5 ml/min. Gradient conditions were programmed as follows: 20 % B for the first minute, a linear increase to 100 % B to 4 min, remaining at 100 % B to 8 min, and then reequilibration to 20 % B. A volume of 5 µL was injected and the column temperature kept at 40 ºC for the run time of 10 min.

7. CALCULATIONS AND STATISTICS

7.1. CALCULATIONS

Bias

For analytical samples, accuracy is determined by replicate analysis of samples containing a known amount of the analyte.
Accuracy may be reported using the % of deviation from the expected value Bias (% inaccuracy):

$$\% \text{ Bias} = \frac{\text{Mean Concentration Determined} - \text{Expected Concentration}}{\text{Expected Concentration}} \times 100$$

The mean value should be within 15% of the actual value except at the LLOQ, where it should not deviate by more than 20%.

Standard Deviation

The standard deviation, relative standard deviation (% CV) and/or confidence interval should be reported for each type of precision investigated.

$$\% \text{ CV} = \frac{\text{Standard Deviation}}{\text{Mean Conc. Determined}} \times 100$$

The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV.

7.2. STATISTICAL ANALYSIS

MS data were acquired and processed (integrated) using the Agilent MassHunter Workstation software version B.04.01. Calibration plots of analyte peak area versus opicapone 3-*O*-sulfate nominal concentrations were constructed and a weighted $1/x^2$ linear regression was used. Concentrations of opicapone 3-*O*-sulfate in validation samples were determined from the appropriate calibration curve and used to calculate the bias and precision of the method with a Microsoft Excel® worksheet previously validated.

Analysis of the data and curve-fitting (to obtain K_m and V_{max} values) was performed using nonlinear regression and the algorithms contained in the program GraphPad Prism® 5.02 (GraphPad Software, Inc.).

The formation rates of opicapone 3-*O*-sulfate (nanomole per minute per milligram of protein of human intestine S9 fraction) from incubations with a broad substrate concentration range were evaluated by fitting the data to the allosteric sigmoidal equation, V = V_{max} . S^h/(K_{prime} + S^h), where S is the inhibitor concentration, V_{max} is the maximum enzyme velocity, K_{prime} is related to the K_m, but is not equal the substrate concentration needed to achieve a half-maximum enzyme velocity (unless h=1), and h is the Hill slope.

The formation rates of opicapone 3-*O*-sulfate (nanomole per minute per milligram of protein of human liver S9 fraction) from incubations with a broad substrate concentration range were evaluated by fitting the data to the Michaelis-Menten equation, V = V_{max} . S/(K_m + S), where S is the inhibitor concentration, V_{max} is the maximum enzyme velocity and K_m is the Michaelis-Menten constant.

The formation rates of opicapone 3-*O*-sulfate (nanomole per minute per milligram of protein of SULT1A1*1 or SULT1A1*2) from incubations with a broad substrate concentration range were evaluated by fitting the data to the substrate inhibition equation, V = V_{max} . S/(K_m + S*(1+S/Ki)), where S is the inhibitor concentration, V_{max} is the maximum enzyme velocity, K_m is the Michaelis-Menten constant, and K_i is the dissociation constant for substrate binding in such a way that two substrates can bind to an enzyme.

The IC_{50} was calculated by fitting the percentage of activity versus control: IC_{50} = 100/(1+10^((LogIC_{50}-X)*HillSlope))), where X is the inhibitor concentration and the HillSlope describes the steepness of the family of curves.

The one-way ANOVA analysis of variance and Tukey's multiple comparison tests were used to compare the results obtained for liver S9 fraction of the species tested. A value of $P<0.05$ was considered to represent a significant difference.

CHAPTER III

RESULTS

1. BIO-ANALYTICAL METHOD VALIDATION

1.1. SELECTIVITY/SPECIFICITY

No co-eluted peaks were observed that could compromise the selectivity of the assay for opicapone 3-*O*-sulfate quantification. A typical chromatogram of extracted blank matrix is shown in Figure 5.

The specificity of the assay against opicapone, quercetin, acetaminophen and DCNP was evaluated. To blank matrix was added 100 µM of these compounds and the samples were treated and analysed. There is no any interference at the same retention time of opicapone 3-*O*-sulfate.

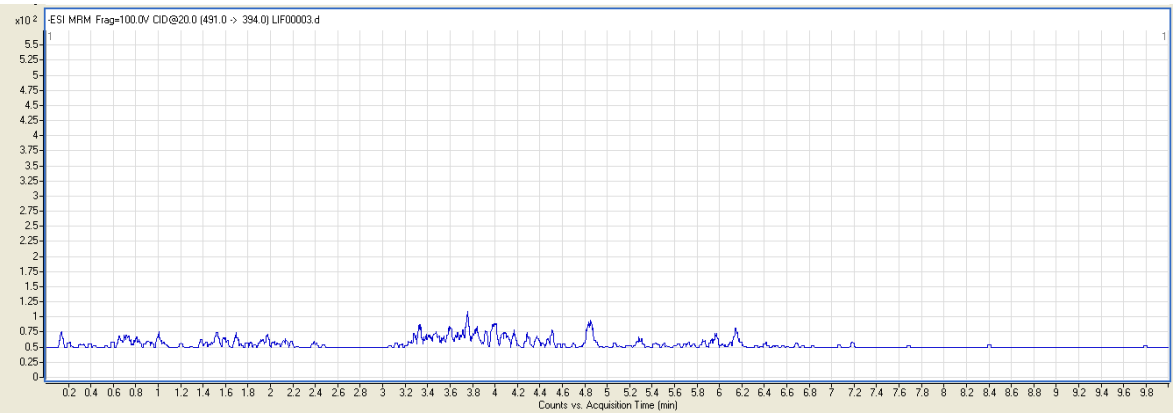


Figure 5 - Representative chromatogram of extracted blank matrix

1.2. CARRYOVER/CONTAMINATION

A control sample was injected after the injection of the highest calibration sample for carryover assessment. This procedure was repeated three times. No peaks were observed on control samples, the analytical method is considered to be without contaminations between subsequent samples analysed.

1.3. WORKING AND LINEAR RANGE

1.3.1. CALIBRATION CURVE

The linearity of the assay was assessed using three calibration curves prepared on three different days. Calibration curves were obtained by weighted ($1/x^2$) linear regression for the peak area of the analyte against the amount of the analyte. The correlation coefficient (r) was greater than 0.9965 (Table 2). The % bias for the back-calculated concentrations of the calibration specimens which was within 15 % for the entire range, were varying from -8.7 % to 11.0 % (Table 1). There was no calibration specimens rejected in the three calibration curves.

Table 1 - Inter-assay variation for calibration standards of opicapone 3-*O*-sulfate

	Concentration (nM)							
	1	5	10	50	100	200	400	1000
Run 1	1.02	4.56	9.60	46.00	97.01	196.78	449.52	1105.82
Run 2	1.01	4.92	9.46	45.45	96.83	197.25	439.03	1099.20
Run 3	1.01	4.74	9.85	45.46	93.10	197.03	441.77	1124.07
Mean	1.02	4.74	9.64	45.64	95.65	197.02	443.44	1109.70
Standard deviation	0.01	0.18	0.20	0.32	2.21	0.24	5.44	12.88
Precision (%CV)	0.6	3.7	2.0	0.7	2.3	0.1	1.2	1.2
Bias (%)	1.6	-5.2	-3.6	-8.7	-4.4	-1.5	10.9	11.0

Table 2 - Calibration curve parameters for opicapone 3-*O*-sulfate

	linear equation: ax + b		Correlation coefficient
	a	b	
Run 1	530.67	-24.58	0.9965
Run 2	519.54	-40.36	0.9975
Run 3	287.61	-84.32	0.9974

Typical chromatograms of extracts of low, medium and high calibration standards are shown in Figures 6 to 8.

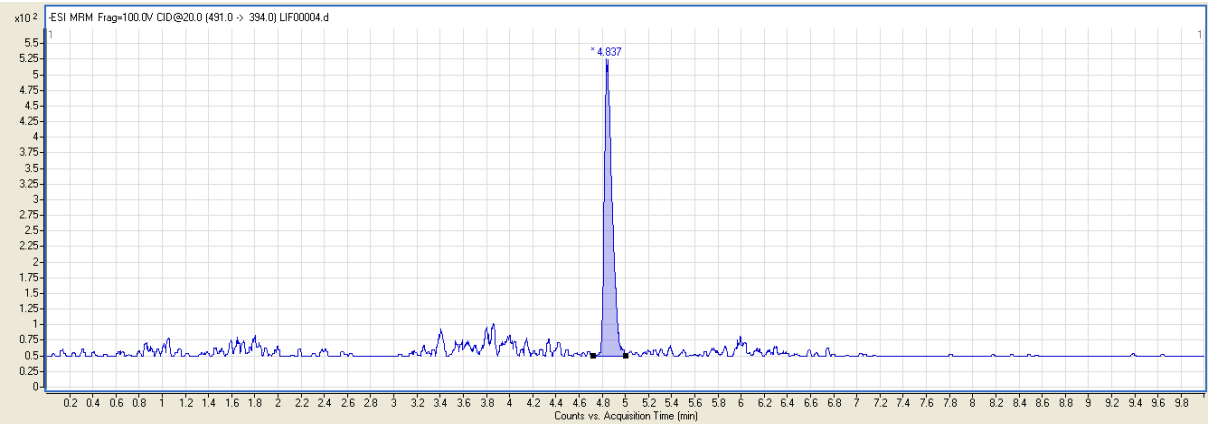


Figure 6 - Representative chromatogram of 1 nM opicapone 3-*O*-sulfate in matrix.

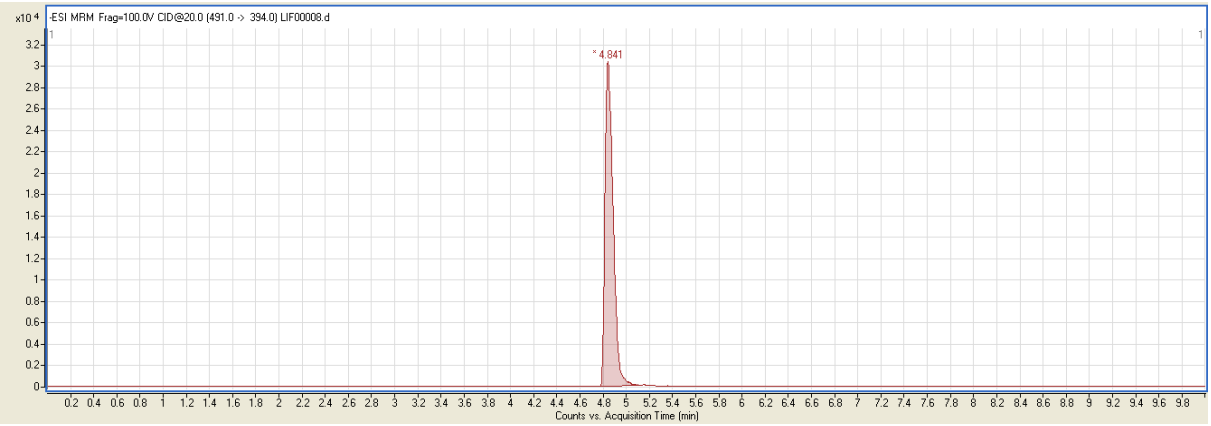


Figure 7 - Representative chromatogram of 100 nM opicapone 3-*O*-sulfate in matrix.

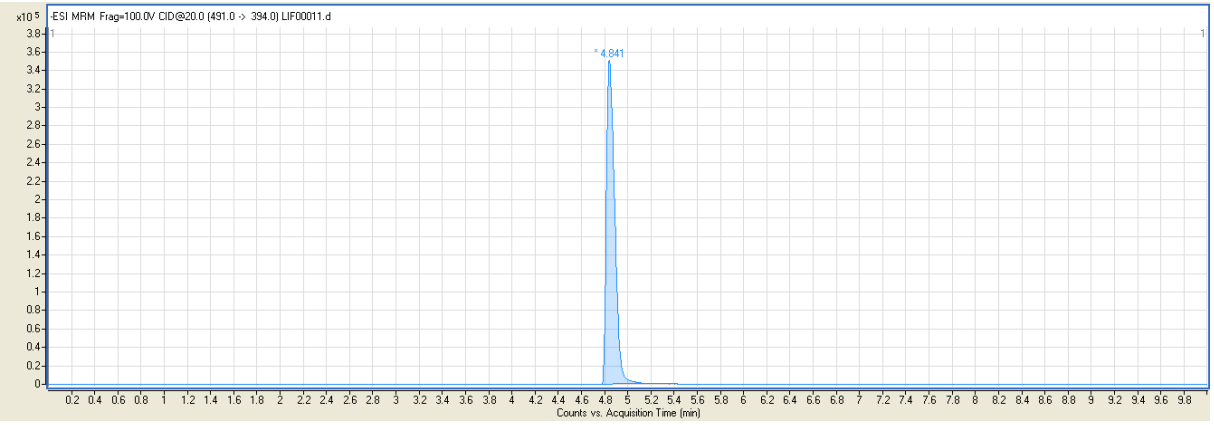


Figure 8 - Representative chromatogram of 1000 nM opicapone 3-*O*-sulfate in matrix.

1.3.2. LLOQ AND ULOQ

The ULOQ and LLOQ is the highest and lowest limits of quantification of the range examined, which has acceptable precision and accuracy according to the criteria. Accordingly to the results presented in section 1.3.1, the LLOQ is 1 nM and the ULOQ is 1000 nM.

1.4. ACCURACY

The accuracy of the method was analysed over the analytical range of 1 to 1000 nM by measuring the % bias, which was within 15 % for the entire range, varying from -8.0 % to 10.2 % (Table 3).

Table 3 - Intra-batch precision and accuracy for opicapone 3-*O*-sulfate

	Concentration (nM)					
	1	5	50	100	400	1000
Run 1, n=5	1.07	5.10	47.59	99.23	420.50	1123.96
	0.87	4.97	47.36	99.17	407.89	1121.19
	0.83	4.50	42.75	93.99	405.18	1085.13
	1.15	4.71	50.14	92.07	398.34	1095.17
	0.99	4.26	42.15	95.86	417.64	1083.07
Mean	0.98	4.71	46.00	96.06	409.91	1101.70
Precision (%CV)	13.8	7.3	7.4	3.3	2.2	1.8
Bias (%)	-1.7	-5.9	-8.0	-3.9	2.5	10.2

1.5. PRECISION

1.5.1. REPEATABILITY (INTRA-BATCH)

The repeatability was evaluated using the coefficient of variation and it was analysed over the analytical range of 1 to 1000 nM. The coefficient of variation determined in the first intra-batch at each concentration level (1, 5, 50, 100, 400 and 1000 nM) covering the entire range was within 15%, varying from 1.8 % to 13.8 % (Table 3).

1.5.2. *INTERMEDIATE PRECISION (INTER-BATCH)*

The Intermediate precision was evaluated using the coefficient of variation determined in the inter-batch. For each concentration level (1, 5, 50, 100, 400 and 1000 nM) covering the entire range the coefficient of variation was less than 15 %, varying from 4.1 % to 11.3 % (Table 4).

Table 4 - Inter-batch precision and accuracy for opicapone 3-*O*-sulfate

	Concentration (nM)					
	1	5	50	100	400	1000
Run 1, n=5	1.07	4.65	48.87	105.89	466.86	1145.04
	1.16	4.99	48.76	107.58	453.48	1141.58
	1.01	4.14	48.26	102.50	467.10	1086.70
	1.00	4.57	47.21	102.76	405.17	1139.70
	1.09	4.68	52.51	105.31	513.11	1200.94
Mean	1.07	4.61	49.12	104.81	461.14	1142.79
Precision (%CV)	6.1	6.6	4.1	2.1	8.4	3.5
Bias (%)	6.6	-7.9	-1.8	4.8	15.3	14.3
Run 2, n=5	1.02	4.79	48.13	90.70	386.86	1050.14
	0.95	4.21	40.89	88.55	422.39	1139.73
	1.29	4.30	43.02	88.36	371.25	1047.64
	0.96	4.04	42.72	83.72	361.41	1044.58
	1.11	4.40	38.39	90.41	395.76	1057.49
Mean	1.07	4.35	42,63	88.35	387.53	1067.92
Precision (%CV)	13.2	6.4	8,4	3.2	6.1	3.8
Bias (%)	6.6	-13.0	-14,7	-11.7	-3.1	6.8
Run 3, n=5	1.07	5.10	47.59	99.23	420.50	1123.96
	0.87	4.97	47.36	99.17	407.89	1121.19
	0.83	4.50	42.75	93.99	405.18	1085.13
	1.15	4.71	50.14	92.07	398.34	1095.17
	0.99	4.26	42.15	95.86	417.64	1083.07
Mean	0.98	4.71	46.00	96.06	409.91	1101.70
Precision (%CV)	13.8	7.3	7.4	3.3	2.2	1.8
Bias (%)	-1.7	-5.9	-8.0	-3.9	2.5	10.2
Overall totals, n=15						
Mean	1.04	4.55	45.92	96.41	419.53	1104.14
Precision (%CV)	11.3	7.2	8.6	7.7	9.6	4.1
Bias (%)	3.8	-8.9	-8.2	-3.6	4.9	10.4

1.6. DILUTION INTEGRITY

Five validation specimens were prepared with a concentration above the ULOQ (10000 nM) and diluted 20-fold in the appropriate matrix, that brought the analyte concentration within the assay range. The determined value for the analyte after dilution was 2.4 %, less than 15 % established by criteria.

Table 5 - Opicapone 3-*O*-sulfate dilution integrity

Dilution factor	Nominal Concentration	Found Concentration	Found Conc x Dilution factor	Mean	SD	CV	Bias
	nM	nM	nM	nM		%	%
		517.38	10347.59				
		511.63	10232.57				
20	10000	489.07	9781.45	10235.11	270.99	2.6	2.4
		524.85	10497.01				
		515.85	10316.94				

2. OPICAPONE SULFATION BY S9 FRACTIONS OF DIFFERENT SPECIES

Liver, intestine and kidney S9 fractions commercially available from different species were used to evaluate their ability to conjugate opicapone to opicapone 3-*O*-sulfate (Table 6, Figure 9).

Table 6 - Apparent opicapone 3-*O*-sulfate rates catalysed by human, rat, dog and monkey S9 fractions following 60 min incubation. Rates were determined at 10 µM of opicapone. Values represent mean ± SEM of 2 determinations.

	PAPS	No PAPS
S9 fraction	Mean ± SEM (fmol.mg $prot^{-1}$ min^{-1})	
Human liver*	45.2 ± 0.6	6.5 ± 0.0
Human intestine	516.0 ± 97.6	8.2 ± 0.7
Human kidney	21.0 ± 0.5	7.8 ± 0.1
Monkey liver*	74.5 ± 0.6	5.8 ± 0.6
Rat liver*	86.2 ± 1.2	5.4 ± 0.1
Dog liver*	91.8 ± 0.4	7.2 ± 0.9

* significantly different according with one-way ANOVA analysis of variance and Tukey's multiple comparison test

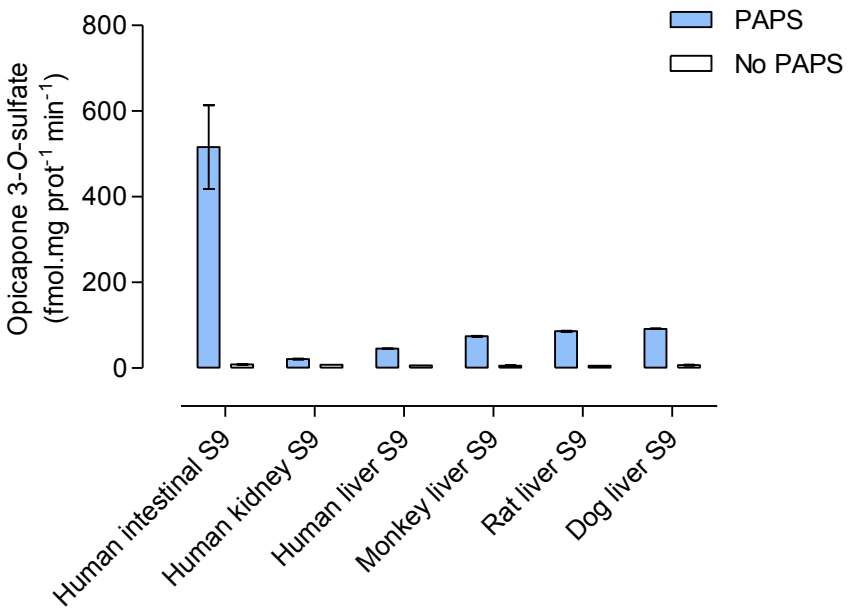


Figure 9 - Apparent sulfation rates catalysed by human, rat, dog and monkey S9 fraction (0.4 and 1.0 mg/ml) following 60 min incubation. Rates were determined at 10 μM opicapone. Values represent mean ± SEM of duplicates.

From the tested S9 fractions, significant higher amounts of opicapone 3-*O*-sulfate were detected in human intestinal S9 fraction in comparison with the other S9 fractions studied. Human, rat, monkey and dog liver S9 fractions, produced small amounts of opicapone sulfated and human kidney S9 fraction conjugate opicapone at extremely low levels over an incubation period of 60 min. Differences were observed in the results obtained for opicapone 3-*O*-sulfation by liver S9 fraction across the species tested.

The linearity of opicapone 3-*O*-sulfation was obtained over 120 min, in all species studied, as presented in Table 7 and Figure 10.

Table 7 - Apparent sulfation catalysed by human, rat, dog and monkey S9 fractions over 120 min. Rates were determined at 10 µM of opicapone. Values represent mean ± SEM of duplicates.

	S9 fraction				
	Human liver	Human intestine	Monkey liver	Rat liver	Dog liver
Time (min)	Mean ± SEM (nmol/mg)				
30	1.3 ± 0.0	5.7 ± 0.2	2.1 ± 0.0	2.2 ± 0.2	2.9 ± 0.0
60	3.0 ± 0.1	31.3 ± 2.0	3.6 ± 0.1	6.2 ± 1.6	5.9 ± 0.5
120	12.2 ± 1.7	103.2 ± 3.1	6.1 ± 0.1	26.5 ± 0.2	12.8 ± 0.8

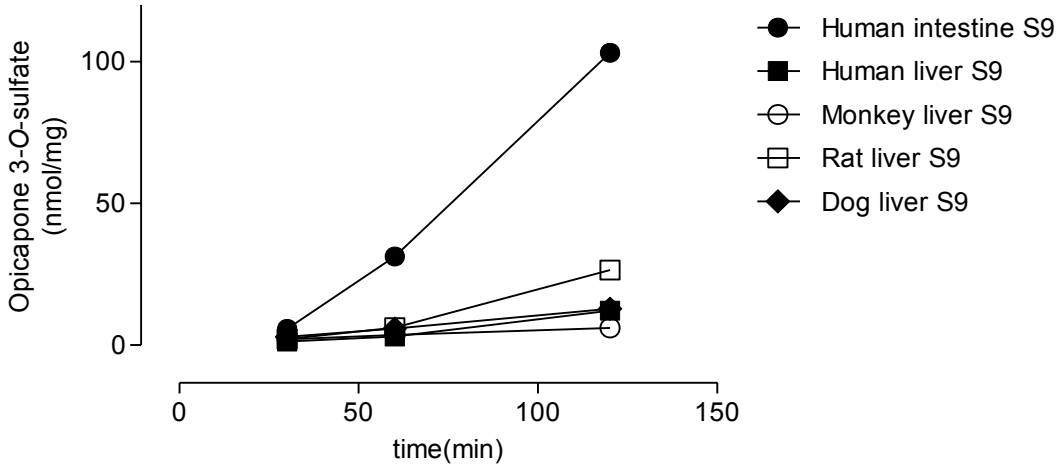


Figure 10 - Apparent opicapone sulfation catalysed by human, rat, dog and monkey S9 fractions over 120 min. Rates were determined at 10 µM opicapone. Values represent mean ± SEM of duplicates.

2.1. OPICAPONE SULFATION BY HUMAN S9 FRACTIONS

Kinetic analysis of opicapone sulfation was performed in human pooled liver and intestine S9 fractions. The linearity for the product formation with respect to the protein concentration was obtained over the range of 0.1 to 0.4 mg/ml for human intestinal S9 fraction and over the range of 0.5 to 2.0 mg/ml for human liver S9 fraction, as presented in Figure 11. As it can be observed in Figure 12**,** the linearity for opicapone 3-*O*-sulfate formation was obtained over 120 min for human intestinal S9 fraction and human liver S9 fraction.

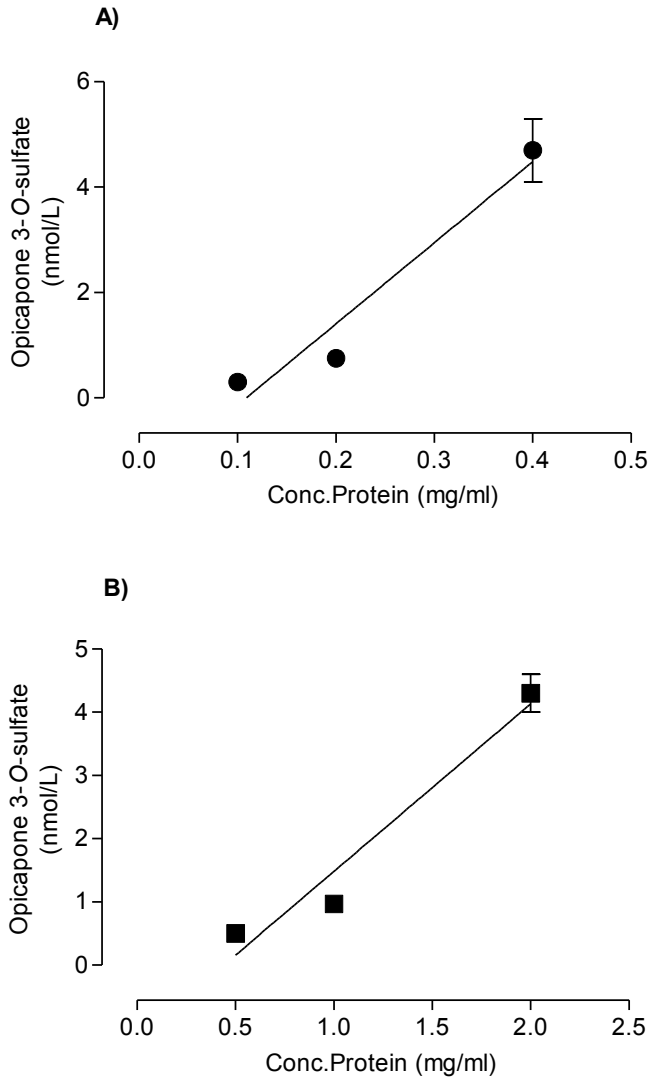


Figure 11 - Establishment of linear protein concentration for the *in vitro* sulfation of opicapone by A) human intestinal S9 (0.1, 0.2 and 0.4 mg/ml) and B) human liver S9 (0.5, 1.0 and 2.0 mg/ml) incubated for 60 min with opicapone (10 µM). Values represent mean ± SEM of duplicates.

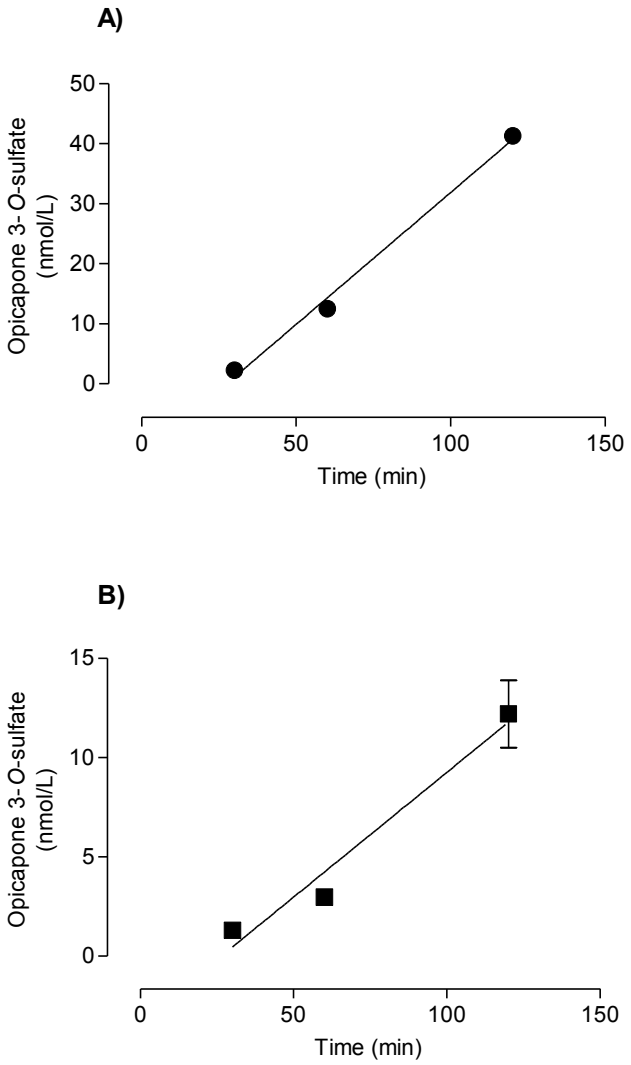


Figure 12 - Establishment of linear time conditions for the *in vitro* sulfation of opicapone by A) human intestinal S9 (0.4 mg/ml) and B) human liver S9 (1.0 mg/ml) fractions incubated at different time points with opicapone (10 µM). Values represent mean ± SEM of duplicates.

Each human pooled S9 fraction was incubated with different concentrations of opicapone (1-100 µM) and the initial rates of sulfation determined. As shown in Figure 13, both preparations displayed typical hyperbolic kinetics. The Eadie-Hofstee plots for opicapone sulfation in the tested S9 fractions were biphasic, indicating the involvement of more than one SULT isoform in opicapone sulfation. The apparent kinetic parameters derived from intestinal and liver S9 fraction curve fitted to the allosteric sigmoidal and Michaelis-Menten equations, respectively, and kinetic parameters are listed in Table 8. Both apparent K_m and

V_{max} values were higher for liver than for intestine S9 (12 and 7 fold higher, respectively). The intrinsic clearance ($Cl_{int} = V_{max}/K_m$) calculated for intestine was 82.1 µl.mg prot^{-1} min^{-1} and the one calculated for liver was 44.7 µl.mg prot^{-1} min^{-1}, about 2-fold lower than the one for intestine.

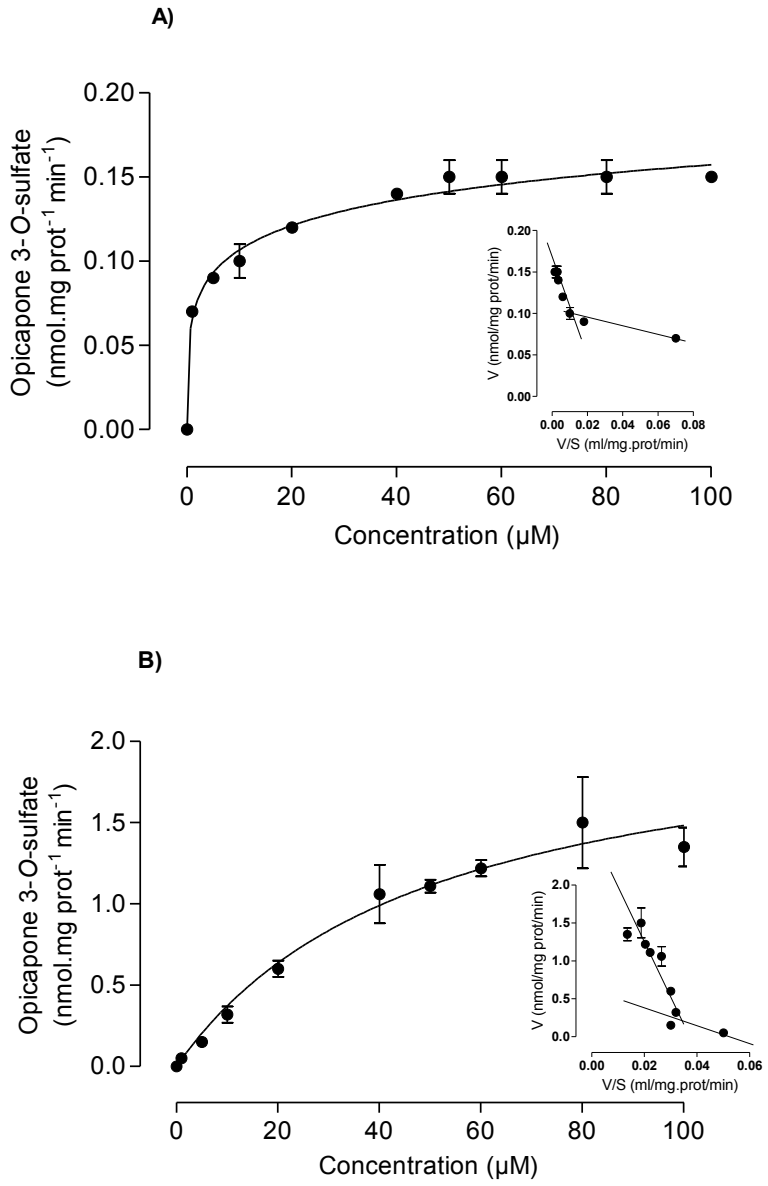


Figure 13 - Kinetics of opicapone sulfation in: A) human intestine S9 (0.4 mg/ml) and B) human liver S9 (1.0 mg/ml) fractions. Opicapone concentrations ranged 1-100 µM. Values represent mean ± SEM of duplicates. The insets show Eadie-Hofstee plots for 3-*O*-sulfation of opicapone.

Table 8 - Apparent kinetic parameters of opicapone sulfation in human liver and intestine S9 fractions.

	K_m	V_{max}		V_{max}/K_m
	*μ*M	nmol.mg prot^{-1} min^{-1}		μl.mg prot^{-1} min^{-1}
Human intestinal S9 [a]	3.90± 2.94	0.32± 0.21	*h*=0.284± 0.10 *μ*M	82.1
Human liver S9 [b]	49.69± 12.27	2.22± 0.25		44.7

a) rates were fitted to allosteric sigmoidal and b) Michaelis-Menten equation; *h*) is Hill slope. Values represent best fit values ± SEM.

2.2. OPICAPONE SULFATION BY HUMAN RECOMBINANT SULTS

Seven commercially available SULT enzymes were used to evaluate their ability to conjugate opicapone to opicapone 3-*O*-sulfate (Table 9, Figure 14). From the tested SULTs only SULT1A1*1 and 1A1*2 produced significant amounts of opicapone sulfated (1165.9 ± 127.4 and 1913.2 ± 126.1 fmol.mg prot^{-1} min^{-1}, respectively). SULT1A2, 1A3, 1E1 and 2A1 produced small amounts of opicapone 3-*O*-sulfate (between 28.3-221.1 fmol.mg prot^{-1} min^{-1}). No conjugated metabolite formation was detected with SULT1B1 over an incubation period of 60 min.

Table 9 - Apparent sulfation rates catalysed by human recombinant SULTs following 60 min incubation. Rates were determined at 10 μM of opicapone. Values represent mean ± SEM of duplicates.

Human Recombinant	Mean ± SEM (fmol.mg prot^{-1} min^{-1})
SULT1A1*1	1165.9 ± 127.4
SULT1A1*2	1913.2 ± 126.1
SULT1A2	43.8 ± 0.2
SULT1A3	221.1 ± 3.1
SULT1B1	4.0 ± 0.1
SULT1E1	28.3 ± 0.9
SULT2A1	210.2 ± 1.1

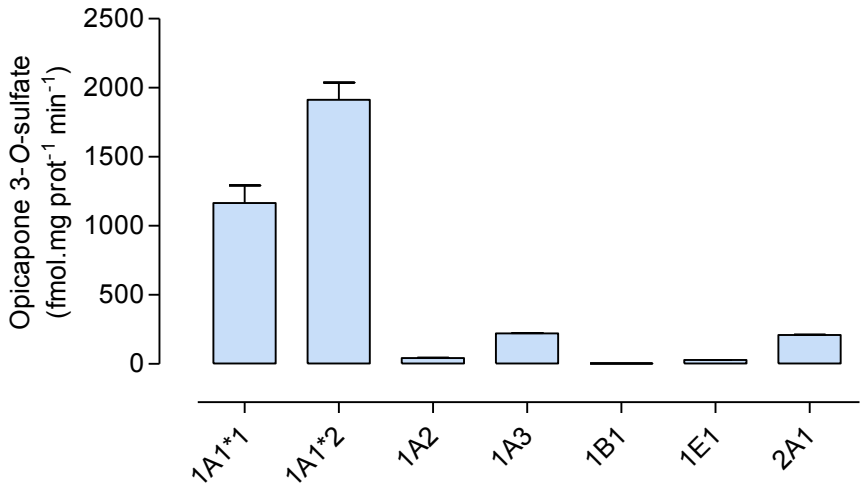


Figure 14 - Apparent sulfation rates catalysed by recombinant human SULT (0.4 mg/ml) isoforms. Rates were determined at 10 μM opicapone. Values represent mean ± SEM of duplicates.

2.3. KINETIC OF OPICAPONE SULFATION BY HUMAN RECOMBINANT SULT

The characterization of opicapone sulfation kinetics was performed for SULT1A1*1 and 1A1*2. The linearity for the protein concentration was obtained over the range of 10 to 40 µg/ml, as presented in Figure 15. As it can be observed in Figure 16, the linearity for opicapone 3-*O*-sulfate production was obtained over 240 min for SULT1A1*1 and 1A1*2 using a protein concentration of 30 µg/ml.

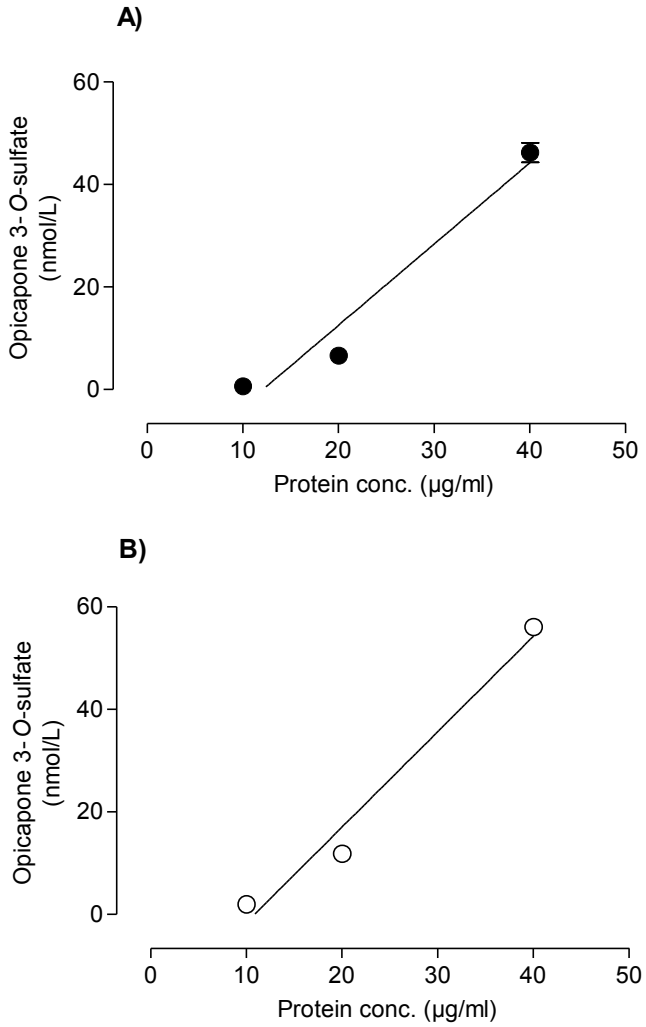


Figure 15 - Establishment of linear protein concentration for the *in vitro* sulfation of opicapone. A) Human recombinant SULT1A1*1 (10, 20 and 40 µg/ml) and B) human recombinant SULT1A1*2 (10, 20 and 40 µg/ml) were incubated for 60 min with opicapone (10 µM). Values represent mean ± SEM of duplicates.

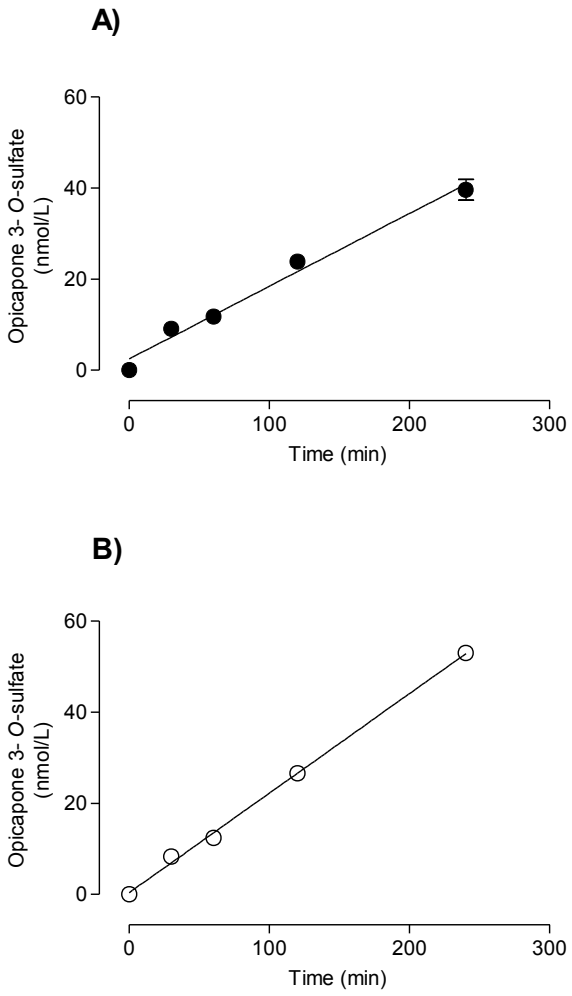


Figure 16 - Establishment of linear time conditions for the *in vitro* sulfation of opicapone. A) Human recombinant SULT1A1*1 (30 µg/ml) and B) human recombinant SULT1A1*2 (30 µg/ml) were incubated at different time points with opicapone (10 µM). Values represent mean ± SEM of duplicates.

Each recombinant enzyme was incubated with different concentrations of opicapone (1-100 µM) and the initial rates determined. Accordingly, the experimental data from SULT1A1*1 and 1A1*2 was fitted with a substrate inhibition profile. The resulting curves are represented in Figure 17 and the apparent kinetic parameters K_m and V_{max} derived from these curves, are shown in Table 10. SULT1A1*1 and 1A1*2 had similar affinities for the conjugation of opicapone as shown by the apparent K_m values determined. SULT1A1*1 and 1A1*2 had apparent affinities close to 20 µM.

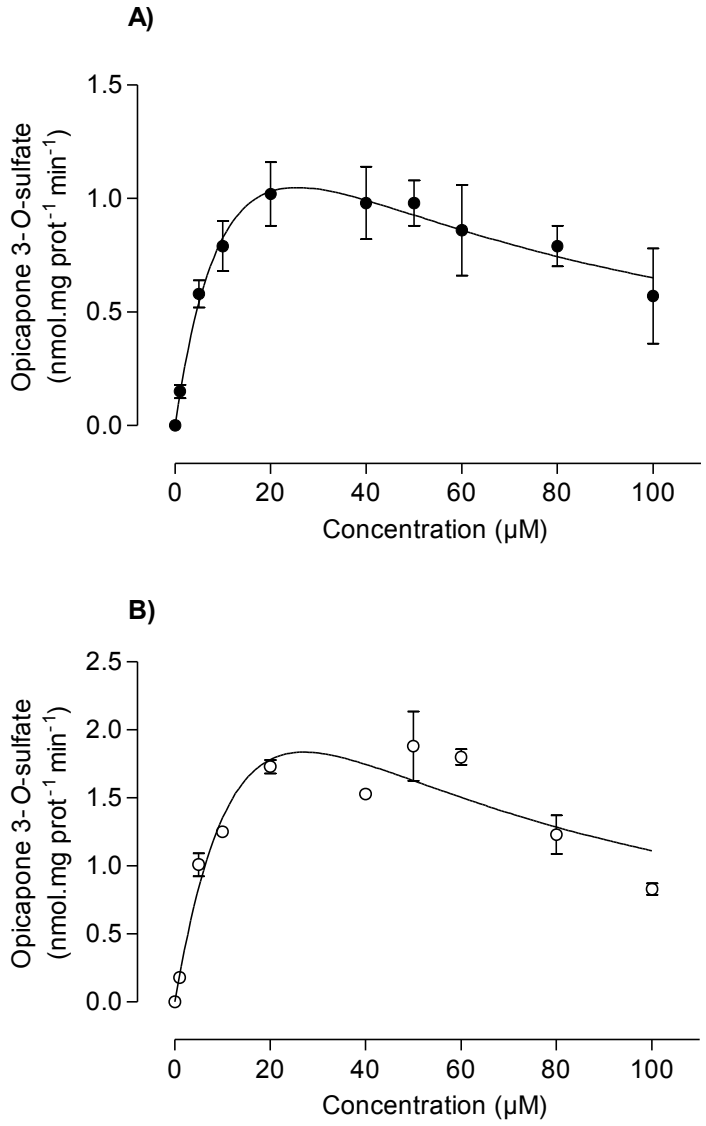


Figure 17 - Kinetics of opicapone sulfation by recombinant human SULT (30 µg/ml) isoforms: A) SULT1A1*1 and B) SULT1A1*2. Opicapone concentrations ranged 1-100 µM. Values represent mean ± SEM of duplicates. Lines represent the fitting curves to substrate inhibition equation.

Table 10 - Apparent kinetic parameters of opicapone sulfation in human recombinant SULT enzymes.

	K_m	V_{max}		V_{max} / K_m
	μM	*nmol mg prot^{-1} min^{-1}*		μl.mg prot^{-1} min^{-1}
SULT1A1*1[c]	16.62± 8.89	2.41± 0.82	*Ki*=39.37±21.33	145.0
SULT1A1*2[c]	26.71± 21.55	5.46± 3.19	*Ki* =27.50±22.48	204.4

c) Substrate inhibition; *Ki* is the substrate inhibition constant. Values represent best fit values ± SEM.

2.4. Inhibition of Opicapone Sulfation by Typical Substrates for SULT Isoforms

To further evaluate the potential interaction of typical substrates for SULT isoforms in opicapone sulfation, selective inhibitors as acetaminophen (substrate for SULT), quercetin (substrate for SULT1A1) and DCNP (substrate for SULT) were tested.

No inhibition was observed on opicapone sulfation in the presence of acetaminophen (1, 5, 10, 50, 100, 200, 400 and 500 µM), in liver pooled S9 fraction as presented in Figure 18.

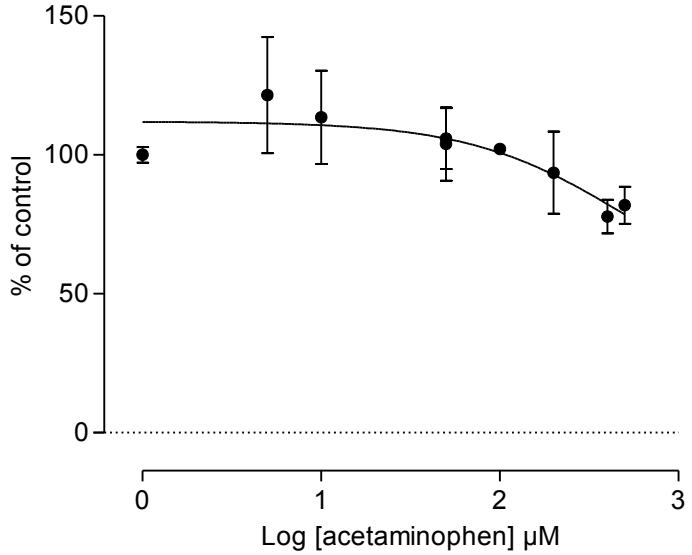


Figure 18 - Inhibitory effect of acetaminophen on opicapone sulfation. Opicapone sulfation was evaluated in pooled liver S9 fraction (1.0 mg/ml) at the concentration of 100 µM opicapone in the presence of inhibitor (1, 5, 10, 50, 100, 200, 400 and 500 µM). Values represent mean ± SEM of duplicates.

The effect of quercetin and DCNP (0.05, 0.1, 0.5, 1, 5, 10, 50, 100 µM) on opicapone (100 µM) sulfation activities in human pooled S9 fractions was also investigated.

As shown in Figure 19 and Table 11, quercetin and DCNP completely inhibited opicapone sulfation with an IC_{50} of 1795 nM and 383.3 nM in human liver S9 fraction, and an IC_{50} of 1483 nM and 181 nM for human intestinal S9 fraction, respectively.

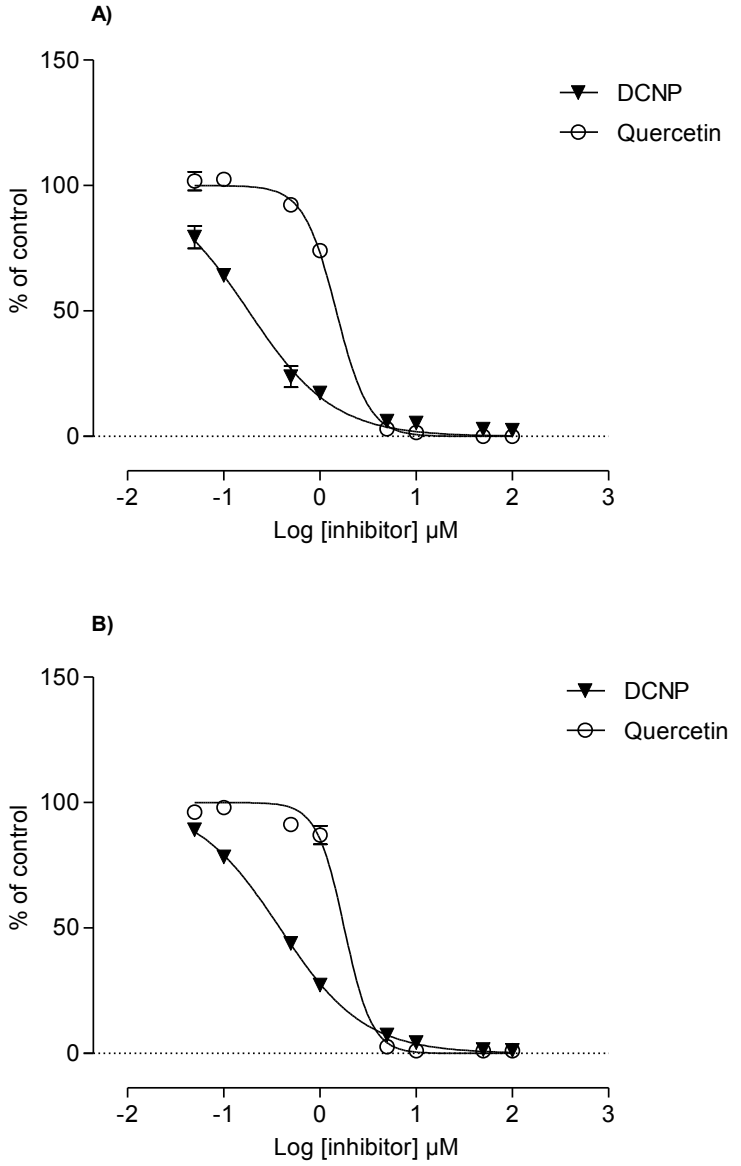


Figure 19 - Inhibitory effect of quercetin and DCNP on opicapone sulfation. Opicapone sulfation was evaluated in: A) pooled intestinal S9 fraction (0.4 mg/ml); B) pooled liver S9 fraction (1.0 mg/ml), at the concentration of 100 µM opicapone in the presence of inhibitors at the concentrations of 0.05, 0.1, 0.5, 1, 5, 10, 50, 100 µM. Values represent mean ± SEM of duplicates.

Table 11 - Inhibition of opicapone 3-*O*-sulfate by DCNP and quercetin in the presence of human S9 fractions. IC_{50} (nM) values for DCNP and quercetin at the concentration of 100 μM opicapone. Values presented as a parameter estimate with 95% Confidence Intervals.

	IC_{50} DCNP	IC_{50} Quercetin
	nM	nM
Human liver S9 fraction	383.3 (360.6 to 407.2)	1795.0 (1494.0 to 2156.0)
Human intestine S9 fraction	181.0 (157.4 to 208.2)	1483.0 (1350.0 to 1629.0)

An IC_{50} of 529 nM and 245 nM were obtained for SULT1A1*1 for quercetin and DCNP, respectively, and an IC_{50} of 232 nM and 700 nM for SULT1A1*2, for quercetin and DCNP, respectively (Figure 20, Table 12). The human S9 fraction IC_{50} values for DCNP and quercetin are closest to IC_{50} of SULT1A1*1 suggesting that SULT1A1*1 (the highest frequent allele) may plays a predominant role in opicapone 3-*O*-sulfation.

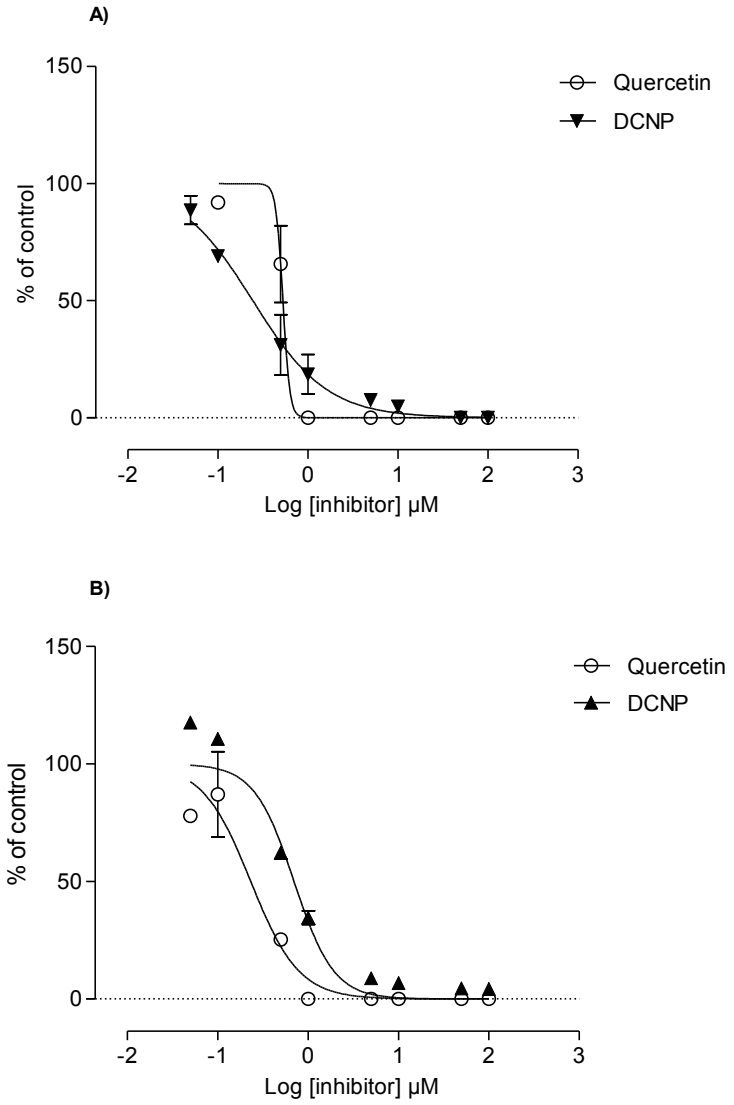


Figure 20 - Inhibitory effect of quercetin and DCNP on opicapone sulfation. Opicapone sulfation was evaluated in: A) SULT1A1*1 and B) SULT1A1*2 with 30.0 µg/ml of protein concentration, at the concentration of 100 µM opicapone in the presence of inhibitors (0.05, 0.1, 0.5, 1, 5, 10, 50, 100 µM). Values represent mean ± SEM of duplicates.

Table 12 - Inhibition of opicapone sulfate by DCNP and quercetin in the presence of human recombinant SULTs. IC_{50} (nM) values for DCNP and quercetin at the concentration of 100 µM opicapone. Values presented as a parameter estimate with 95% Confidence Intervals

	IC_{50} DCNP	IC_{50} Quercetin
	nM	nM
Human Rec SULT1A1*1	245.0 (186.4 to 321.8)	529.0 (162.7 to 1721.0)
Human Rec SULT1A1*2	700.0 (553.6 to 886.3)	232.0 (150.2 to 359.4)

CHAPTER IV

DISCUSSION AND CONCLUSION

Opicapone, is a novel third generation COMT inhibitor currently under phase III clinical trials for use as adjunctive therapy in levodopa-treated PD patients. In humans, opicapone is mainly conjugated by sulfotransferases giving opicapone 3-*O*-sulfate, a major opicapone inactive metabolite present in human plasma. Other minor metabolites that compete with the sulfated metabolite in the opicapone metabolism are: 3-*O*-methyl, 3-*O*-glucuronide and the amine N-oxide reduced metabolite (Rocha et al. 2013).

The purpose of this work was to characterize the kinetics for the conversion of opicapone into sulfated metabolite by intestinal, kidney and liver S9 fractions and by human recombinant SULTs.

In this study, a sensitive and selective method for the quantification of opicapone 3-*O*-sulfate based on LC–MS/MS technology was established. The assay demonstrated excellent accuracy, precision, linearity and specificity for the intended purpose in accordance with FDA regulations for the validation of bioanalytical methods. Linearity was proven over the range of 1 to 1000 nM. The intra- and inter-day coefficient of variation was less than 13.8 % and the accuracy was between -8.0 % and 10.2 %. The lower limit of quantification (LLOQ) was 1 nM and the method was precise and accurate for 20-fold dilution of samples. Validation data shows that method is selective, sensitive and robust.

To better characterize the extent of the formation of opicapone sulfated, S9 fractions of different species were initially used. S9 fraction was used because it is a routine model system used in phase II metabolic studies involving sulfation of drugs and other xenobiotics (Tang et al. 2012).

Results indicate that all tested S9 fractions sulfated opicapone. Human intestinal S9 fraction (516.0 ± 97.6 fmol.mg $prot^{-1}$ min^{-1}) has the highest sulfating capacity. Human, rat, monkey and dog liver S9 fractions and human kidney S9 fraction produced small amounts (ranged 21.0 - 91.8 fmol.mg $prot^{-1}$ min^{-}) of the sulfated metabolite. The values obtained for liver S9 fraction of the species tested were significantly different according applying one-way ANOVA analysis of variance and Tukey's multiple comparison test for 95% significance. However, no conclusion can be taking not only because of the differential expression of SULTs in the various tissues, but also because it is not possible to quantify the levels of the individual enzymes in the tissues preparations.

Human liver and intestinal S9 fractions displayed typical hyperbolic kinetics (Figure 13). The kinetic analysis indicates that more than one enzyme appeared to participate in the formation of opicapone metabolite in S9 fractions. Eadie-Hofstee plots for opicapone sulfation reactions were biphasic for the tested human S9 fractions, suggestive of

involvement of at least two components, one with a high affinity and low capacity, and another with a low affinity and high capacity.

The apparent kinetic parameters K_m and V_{max} values were higher for human liver than for human intestine S9 fraction. The intrinsic clearance (Cl_{int}) values of human liver and intestine S9 fractions for the formation of opicapone 3-*O*-sulfate were 44.7 and 82.1 µl.mg $prot^{-1}$ min^{-1}, respectively, suggesting that the formation of opicapone sulfated in human intestine was efficient. The Cl_{int} value of human intestine S9 fraction was 2-fold higher than of human liver S9 fraction, that suggest the presence of SULT isoforms in intestine that contribute for opicapone metabolism and elimination.

Among seven human recombinant SULTs tested, several enzymes (SULT1A1, 1A2, 1A3, 1E1 and 2A1) were capable of conjugating opicapone albeit at different levels. In this study, we identified SULT1A1 (SULT1A1*1 and SULT1A1*2) isoform as the major SULT involved in 3-*O*-sulfation of opicapone with a K_m values of 16.62 ± 8.89 and 26.71 ± 21.55 µM, for the alleles SULT1A1*1 and SULT1A1*2, respectively. SULT1A1 is generally recognized as the major xenobiotic-metabolizing SULT, as a result of its capacity to accept a very wide range of substrates, and this enzyme accounted for more than 50 % of total SULT protein in the liver. SULT2A1 a major isoform in the liver, second in abundance to SULT1A1, contribute to opicapone sulfation in lower extent as well as SULT1A3 an extrahepatic enzyme involved to selectively sulfate catecholamines (Riches et al. 2009).

Opicapone sulfation by SULT1A1 showed deviation from the typical Michaelis-Menten kinetics resulting in a concentration-dependent self-inhibition. The affinity of opicapone 3-*O*-sulfate by human recombinant SULT1A1 is 2 to 3-fold higher than the human liver S9 fraction, suggesting that SULT1A1 may be the primary isoform involved in opicapone sulfation in liver. However, the involvement of other isoforms at higher substrate concentrations should be considered. In the small intestine, the kinetic analysis of opicapone sulfation revealed an affinity for the conjugation 12-fold higher for intestinal S9 fraction than for liver S9 fraction, with a K_m of 3.90 ± 2.94 and 49.69 ± 12.27 µM, respectively. The observed highest affinity of opicapone sulfation may indicate that intestinal opicapone sulfation may be performed by other isoform. Additionally SULT1A1, was much less prevalent in small intestine and it was present at less than half the level of the liver (representing less than 20 % of total SULT protein) (Riches et al. 2009). The opicapone sulfation clearance (Cl_{int}) was significantly lower for the human intestine and liver S9 fraction than for SULT1A1 isoforms, suggesting that although the first-pass sulfation of opicapone in intestine may represent a significant contribution to the opicapone metabolism, liver sulfation may represent the major contributor due to the high prevalence of SULT1A1 in liver tissues.

In the present study, quercetin and DCNP selectively inhibit opicapone sulfation in liver and intestinal S9 fractions, with IC50 values of 383.3 nM and 1795 nM for liver S9 fraction and 181.0 nM and 1483.0 nM for intestine S9 fraction (Figure 19). In contrast, acetaminophen that is a known substrate for SULT1A1 and SULT1A3 (Reiter et al. 1982; Pacifici 2004) had no significant effect on the formation of opicapone 3-*O*-sulfate in human liver S9 fraction incubated with 100 μM of opicapone. The result obtained may indicate that the opicapone sulfation and acetaminophen sulfation may be performed by different isoforms, or the concentrations of acetaminophen used were not sufficient to inhibit the formation of opicapone 3-*O*-sulfate.

The comparison of the apparent K_m values obtained for the human recombinant enzymes evaluated with those for human S9 fractions, did not clarify which enzymes were involved in S9 fraction sulfation and clearly indicate that more than one SULT are involved in opicapone sulfation. Among the hepatic SULTs, SULT1A1 had the highest formation rate of opicapone 3-*O*-sulfate, however, the K_m was significantly lower than the K_m obtained for human liver S9 fraction, which may indicate that higher affinity SULTs may contribute to the opicapone sulfation. Furthermore, the high affinity of opicapone sulfation by the intestinal S9 fraction may suggest that other SULT isoform than SULT1A1 may be involved in opicapone sulfation. Besides the enzymes involved, sulfation will be dependent not only on the kinetics of the reaction but also on the amount of compound that reaches the respective tissue and most significantly on the enzyme levels present in the tissues. Clinical studies with opicapone showed that the maximal plasma concentration with the therapeutic dose of 50 mg is 522 ng/ml (1.29 μM), which is much less than the K_m obtained for S9 fraction. Given the high catalytic efficiency and expression levels of SULTs in intestinal tissue, it can be concluded that the first-pass metabolism within the intestinal cells contributes significantly to the formation of opicapone 3-*O*-sulfate, the major opicapone metabolite found *in vivo*, in human and rat plasma.

In conclusion, sulfation is an important metabolic pathway of opicapone and multiple SULTs are capable of catalyzing sulfation of opicapone. The results obtained in the present study further support the role of intestine in opicapone sulfation and strongly suggest that opicapone 3-*O*-sulfation is mainly catalyzed by SULT1A1 isoforms in humans.

CHAPTER V

REFERENCES

Akil, M., B. S. Kolachana, D. A. Rothmond, T. M. Hyde, D. R. Weinberger and J. E. Kleinman (2003). "Catechol-o-methyltransferase genotype and dopamine regulation in the human brain." J Neurosci **23**(6): 2008-13.

Allali-Hassani, A., P. W. Pan, L. Dombrovski, R. Najmanovich, W. Tempel, A. Dong, P. Loppnau, F. Martin, J. Thornton, A. M. Edwards, A. Bochkarev, A. N. Plotnikov, M. Vedadi and C. H. Arrowsmith (2007). "Structural and chemical profiling of the human cytosolic sulfotransferases." PLoS Biol **5**(5): e97.

Anzenbacher, P. and E. Anzenbacherova (2001). "Cytochromes P450 and metabolism of xenobiotics." Cell Mol Life Sci **58**(5-6): 737-47.

Axelrod, J. and R. Tomchick (1958). "Enzymatic O-methylation of epinephrine and other catechols." J. Biol. Chem. **233**: 702-705.

Bäckström, R., E. Honkanen, A. Pippuri, P. Kairisalo, J. Pystynen, K. Heinola, E. Nissinen, I. B. Linden, P. T. Männistö and S. Kaakkola (1989). "Synthesis of some novel potent and selective catechol-*O*-methyltransferase inhibitors." Journal of Medicinal Chemistry **32**(4): 841-6.

Bilder, R. M., J. Volavka, H. M. Lachman and A. A. Grace (2004). "The Catechol-O-Methyltransferase Polymorphism: Relations to the Tonic-Phasic Dopamine Hypothesis and Neuropsychiatric Phenotypes." Neuropsychopharmacology **29**: 1943-61.

Blume, H., E. Brendel, M. Brudny-Kloppel, S. Grebe, B. Lausecker, G. Rohde and C. Siethoff (2010). "Workshop/conference report on EMA draft guideline on validation of bioanalytical methods." Eur J Pharm Sci **42**(3): 300-5.

Bonifacio, M. J., P. N. Palma, L. Almeida and P. Soares-da-Silva (2007). "Catechol-O-methyltransferase and its inhibitors in Parkinson's disease." CNS Drug Rev **13**(3): 352-79.

Bonifácio, M. J., J. S. Sutcliffe, L. Torrao, L. Wright and P. Soares-da-Silva (2012a). "Brain and peripheral levodopa pharmacokinetics in the Cynomolgus monkey following administration of opicapone, a novel catechol-O-methyltransferase inhibitor." Parkinsonism Relat Disord **18**(S2): S125.

Bonifácio, M. J., L. Torrao, A. I. Loureiro, L. Wright and P. Soares-da-Silva (2012b). "Opicapone: Characterization of a novel peripheral long-acting catechol-O-methyltransferase inhibitor." Parkinsonism Relat Disord **18**(S2): S125.

Bonifácio, M. J., M. A. Vieira-Coelho, N. Borges and P. Soares-da-Silva (2000). "Kinetics of rat brain and liver solubilized membrane-bound-catechol-O-methyltransferase." Archives of Biochemistry and Biophysics **384**(2): 261-367.

Bonifacio, M. J., M. A. Vieira-Coelho and P. Soares-da-Silva (2003). "Kinetic inhibitory profile of BIA 3-202, a novel fast tight-binding, reversible and competitive catechol-O-methyltransferase inhibitor." Eur J Pharmacol **460**(2-3): 163-70.

Bonifati, V. and G. Meco (1999). "New, selective catechol-O-methyltransferase inhibitors as therapeutic agents in Parkinson's disease." Pharmacology & therapeutics **81**(1): 1-36.

Borchardt, R. T. (1974). "A rapid spectrophotometric assay for catechol-O-methyltransferase." Anal Biochem **58**(2): 382-9.

Borges, N., M. A. Vieira-Coelho, A. Parada and P. Soares-da-Silva (1997). "Studies on the tight-binding nature of tolcapone inhibition of soluble and membrane-bound rat brain catechol-O-methyltransferase." J Pharmacol Exp Ther **282**(2): 812-7.

Borgulya, J., H. Bruderer, K. Bernauer, G. Zurcher and M. Da Prada (1989). "Catechol-O-methyltransferase - Inhibiting Pyrocatechol Derivatives: Synthesis and Structure-Activity Studies." Helvetica Chimica Acta **72**: 952-968.

Borgulya, J., M. Da Prada, J. Dingemanse, R. Scherschlicht, B. Schläppi and G. Zürcher (1991). "RO 40-7592." Drugs Fut **16**(8): 719-721.

Carlsson, A., M. Lindqvist and T. Magnusson (1957). "3,4-Dihydroxyphenylalanine and 5-hydroxytryptophan as reserpine antagonists." Nature **180**(4596): 1200.

Chen, X., S. M. Baker and G. Chen (2005). "Methotrexate induction of human sulfotransferases in Hep G2 and Caco-2 cells." J Appl Toxicol **25**(5): 354-60.

Chen, Y., C. Huang, T. Zhou and G. Chen (2008). "Genistein induction of human sulfotransferases in HepG2 and Caco-2 cells." Basic Clin Pharmacol Toxicol **103**(6): 553-9.

Comer, K. A., J. L. Falany and C. N. Falany (1993). "Cloning and expression of human liver dehydroepiandrosterone sulphotransferase." Biochem J **289 (Pt 1)**: 233-40.

Cook, I., T. Wang, C. N. Falany and T. S. Leyh (2012). "A nucleotide-gated molecular pore selects sulfotransferase substrates." Biochemistry **51**(28): 5674-83.

Cotzias GC, P. P., Gellene R (1969). "Modification of Parkinsonism-Chronic treatment with L-dopa." N Engl J Med **280**: 337-345.

Cotzias, G. C., M. H. Van Woert and L. M. Schiffer (1967). "Aromatic amino acids and modification of parkinsonism." N Engl J Med **276**(7): 374-9.

Dajani, R., A. M. Hood and M. W. Coughtrie (1998). "A single amino acid, glu146, governs the substrate specificity of a human dopamine sulfotransferase, SULT1A3." Mol Pharmacol **54**(6): 942-8.

De Santi, C., A. Pietrabissa, F. Mosca, A. Rane and G. M. Pacifici (2002). "Inhibition of phenol sulfotransferase (SULT1A1) by quercetin in human adult and foetal livers." Xenobiotica **32**(5): 363-8.

Deleu, D., M. G. Northway and Y. Hanssens (2002). "Clinical pharmacokinetic and pharmacodynamic properties of drugs used in the treatment of Parkinson's disease." Clin Pharmacokinet **41**(4): 261-309.

DeSilva, B., F. Garofolo, M. Rocci, S. Martinez, I. Dumont, F. Landry, C. Dicaire, G. Szekely-Klepser, R. Weiner, M. Arnold, S. Bansal, K. Bateman, R. Bauer, B. Booth, S. Davis, S. Dudal, D. Gouty, J. Grundy, S. Haidar, R. Hayes, M. Jemal, S. Kaur, M. Kelley, M. Knutsson, O. Le Blaye, J. Lee, S. Lowes, M. Ma, T. Mitsuoka, J. T. Neto, R. Nicholson, E. Ormsby, J. Sailstad, L. Stevenson, D. Tang, J. Welink, C. T. Viswanathan, L. Wang, E. Woolf and E. Yang (2012). "2012 white paper on recent issues in bioanalysis and alignment of multiple guidelines." Bioanalysis **4**(18): 2213-26.

Egan, M., D. Goldman and D. Weinberger (2002). "The human genome: mutations." Am J Psychiatry **159**(1): 12.

Ericsson, A. D. (1971). "Potentiation of the L-Dopa effect in man by the use of catechol-O-methyltransferase inhibitors." J Neurol Sci **14**(2): 193-7.

Falany, C. N. (1997). "Enzymology of human cytosolic sulfotransferases." FASEB J **11**(4): 206-16.

Falany, C. N., V. Krasnykh and J. L. Falany (1995). "Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase." J Steroid Biochem Mol Biol **52**(6): 529-39.

Fujita, K., K. Nagata, S. Ozawa, H. Sasano and Y. Yamazoe (1997). "Molecular cloning and characterization of rat ST1B1 and human ST1B2 cDNAs, encoding thyroid hormone sulfotransferases." J Biochem **122**(5): 1052-61.

Gamage, N., A. Barnett, N. Hempel, R. G. Duggleby, K. F. Windmill, J. L. Martin and M. E. McManus (2006). "Human sulfotransferases and their role in chemical metabolism." Toxicol Sci **90**(1): 5-22.

Glatt, H. and W. Meinl (2004). "Pharmacogenetics of soluble sulfotransferases (SULTs)." Naunyn Schmiedebergs Arch Pharmacol **369**(1): 55-68.

Grossman, M. H., C. R. Creveling, R. Rybczynski, M. Braverman, C. Isersky and X. O. Breakefield (1985). "Soluble and particulate forms of rat catechol-O-

methyltransferase distinguished by gel electrophoresis and immune fixation." J Neurochem **44**(2): 421-32.

Guldberg, H. C. and C. A. Marsden (1975). "Catechol-O-methyltransferase: pharmacological aspects and physiological role." Pharmacol Rev **27**(2): 135-206.

Hempel, N., N. Gamage, J. L. Martin and M. E. McManus (2007). "Human cytosolic sulfotransferase SULT1A1." Int J Biochem Cell Biol **39**(4): 685-9.

Hornykiewicz, O. (2006). "The discovery of dopamine deficiency in the parkinsonian brain." J Neural Transm Suppl(70): 9-15.

Huotari, M., J. A. Gogos, M. Karayiorgou, O. Koponen, M. Forsberg, A. Raasmaja, J. Hyttinen and P. T. Mannisto (2002). "Brain catecholamine metabolism in catechol-O-methyltransferase (COMT)-deficient mice." Eur J Neurosci **15**(2): 246-56.

Jancova, P., P. Anzenbacher and E. Anzenbacherova (2010). "Phase II drug metabolizing enzymes." Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub **154**(2): 103-16.

Javitt, N. B., Y. C. Lee, C. Shimizu, H. Fuda and C. A. Strott (2001). "Cholesterol and hydroxycholesterol sulfotransferases: identification, distinction from dehydroepiandrosterone sulfotransferase, and differential tissue expression." Endocrinology **142**(7): 2978-84.

Jeffery, D. R. and J. A. Roth (1984). "Characterization of membrane-bound and soluble catechol-O- methyltransferase from human frontal cortex." J Neurochem **42**(3): 826-32.

Jeffery, D. R. and J. A. Roth (1987). "Kinetic reaction mechanism for magnesium binding to membrane-bound and soluble catechol O-methyltransferase." Biochemistry **26**(10): 2955-8.

Jones, A. L., M. Hagen, M. W. Coughtrie, R. C. Roberts and H. Glatt (1995). "Human platelet phenolsulfotransferases: cDNA cloning, stable expression in V79 cells and identification of a novel allelic variant of the phenol-sulfating form." Biochem Biophys Res Commun **208**(2): 855-62.

Jorga, K., B. Fotteler, P. Heizmann and R. Gasser (1999). "Metabolism and excretion of tolcapone, a novel inhibitor of catechol-O- methyltransferase." Br J Clin Pharmacol **48**(4): 513-20.

Keating, G. M. and K. A. Lyseng-Williamson (2005). "Tolcapone: a review of its use in the management of Parkinson's disease." CNS Drugs **19**(2): 165-84.

King, R. S., A. A. Ghosh and J. Wu (2006). "Inhibition of human phenol and estrogen sulfotransferase by certain non-steroidal anti-inflammatory agents." Curr Drug Metab **7**(7): 745-53.

Kiss, L. E., H. S. Ferreira, L. Torrao, M. J. Bonifacio, P. N. Palma, P. Soares-da-Silva and D. A. Learmonth (2010). "Discovery of a long-acting, peripherally selective inhibitor of catechol-O-methyltransferase." Journal of Medicinal Chemistry **53**(8): 3396-411.

Klaassen, C. D. and J. W. Boles (1997). "Sulfation and sulfotransferases 5: the importance of 3'-phosphoadenosine 5'-phosphosulfate (PAPS) in the regulation of sulfation." FASEB J **11**(6): 404-18.

Lautala, P., M. Kivimaa, H. Salomies, E. Elovaara and J. Taskinen (1997). "Glucuronidation of entacapone, nitecapone, tolcapone, and some other nitrocatechols by rat liver microsomes." Pharm Res **14**(10): 1444-8.

Learmonth, D. A., P. N. Palma, M. A. Vieira-Coelho and P. Soares-da-Silva (2004). "Synthesis, Biological Evaluation, and Molecular Modeling Studies of a Novel, Peripherally Selective Inhibitor of Catechol-O-methyltransferase." J Med Chem **47**(25): 6207-17.

Learmonth, D. A., M. A. Vieira-Coelho, J. Benes, P. C. Alves, N. Borges, A. P. Freitas and P. Soares-da-Silva (2002). "Synthesis of 1-(3,4-dihydroxy-5-nitrophenyl)-2-phenyl-

ethanone and derivatives as potent and long-acting peripheral inhibitors of catechol-O-methyltransferase." J Med Chem **45**(3): 685-95.

Lees, A. J. (2008). "Evidence-based efficacy comparison of tolcapone and entacapone as adjunctive therapy in Parkinson's disease." CNS Neurosci Ther **14**(1): 83-93.

Li, L., J. L. Popko, T. Umezawa and V. L. Chiang (2000). "5-hydroxyconiferyl aldehyde modulates enzymatic methylation for syringyl monolignol formation, a new view of monolignol biosynthesis in angiosperms." J Biol Chem **275**(9): 6537-45.

Lindsay, J., L. L. Wang, Y. Li and S. F. Zhou (2008). "Structure, function and polymorphism of human cytosolic sulfotransferases." Curr Drug Metab **9**(2): 99-105.

Lotta, T., J. Taskinen, R. Backstrom and E. Nissinen (1992). "PLS modelling of structure-activity relationships of catechol O- methyltransferase inhibitors." J Comput Aided Mol Des **6**(3): 253-72.

Lotta, T., J. Vidgren, C. Tilgmann, I. Ulmanen, K. Melen, I. Julkunen and J. Taskinen (1995). "Kinetics of human soluble and membrane-bound catechol O- methyltransferase: a revised mechanism and description of the thermolabile variant of the enzyme." Biochemistry **34**(13): 4202-10.

Loureiro, A. I., M. J. Bonifacio, C. Fernandes-Lopes, L. Almeida, L. C. Wright and P. Soares-Da-Silva (2006). "Human metabolism of nebicapone (BIA 3-202), a novel catechol-o-methyltransferase inhibitor: characterization of in vitro glucuronidation." Drug Metab Dispos **34**(11): 1856-62.

Lundström, K., M. Salminen, A. Jalanko, R. Savolainen and I. Ulmanen (1991). "Cloning and characterization of human placental catechol-O- methyltransferase cDNA." DNA and Cell Biology **10**(3): 181-9.

Maiti, S., X. Chen and G. Chen (2005). "All-trans retinoic acid induction of sulfotransferases." Basic Clin Pharmacol Toxicol **96**(1): 44-53.

Mannisto, P. T. and S. Kaakkola (1999). "Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors." Pharmacological Reviews **51**(4): 593-628.

Männistö, P. T. and S. Kaakkola (1989). "New selective COMT inhibitors: useful adjuncts for Parkinson's disease?" Trends in Pharmacological Sciences **10**(2): 54-6.

Mannisto, P. T., P. Tuomainen, M. Toivonen, M. Tornwall and S. Kaakkola (1990). "Effect of acute levodopa on brain catecholamines after selective MAO and COMT inhibition in male rats." J Neural Transm Park Dis Dement Sect **2**(1): 31-43.

Mannisto, P. T., P. Tuomainen and R. K. Tuominen (1992). "Different in vivo properties of three new inhibitors of catechol O- methyltransferase in the rat." Br J Pharmacol **105**(3): 569-74.

Meinl, W., J. H. Meerman and H. Glatt (2002). "Differential activation of promutagens by alloenzymes of human sulfotransferase 1A2 expressed in Salmonella typhimurium." Pharmacogenetics **12**(9): 677-89.

Minchin, R. F., A. Lewis, D. Mitchell, F. F. Kadlubar and M. E. McManus (2008). "Sulfotransferase 4A1." Int J Biochem Cell Biol **40**(12): 2686-91.

Nagar, S., S. Walther and R. L. Blanchard (2006). "Sulfotransferase (SULT) 1A1 polymorphic variants *1, *2, and *3 are associated with altered enzymatic activity, cellular phenotype, and protein degradation." Mol Pharmacol **69**(6): 2084-92.

Nebert, D. W. and D. W. Russell (2002). "Clinical importance of the cytochromes P450." Lancet **360**(9340): 1155-62.

Nishimuta, H., H. Ohtani, M. Tsujimoto, K. Ogura, A. Hiratsuka and Y. Sawada (2007). "Inhibitory effects of various beverages on human recombinant sulfotransferase isoforms SULT1A1 and SULT1A3." Biopharm Drug Dispos **28**(9): 491-500.

Nissinen, E., I. B. Linden, E. Schultz and P. Pohto (1992). "Biochemical and pharmacological properties of a peripherally acting catechol-O-methyltransferase inhibitor entacapone." Naunyn Schmiedebergs Arch Pharmacol **346**(3): 262-6.

Nissinen, E., R. Tuominen, V. Perhoniemi and S. Kaakkola (1988). "Catechol-O-methyltransferase activity in human and rat small intestine." Life Sci **42**(25): 2609-14.

Nowell, S., B. Green, Y. M. Tang, R. Wiese and F. F. Kadlubar (2005). "Examination of human tissue cytosols for expression of sulfotransferase isoform 1A2 (SULT1A2) using a SULT1A2-specific antibody." Mol Pharmacol **67**(2): 394-9.

Olanow, C. W. and F. Stocchi (2004). "COMT inhibitors in Parkinson's disease: can they prevent and/or reverse levodopa-induced motor complications?" Neurology **62**(1 Suppl 1): S72-81.

Pacifici, G. M. (2004). "Inhibition of human liver and duodenum sulfotransferases by drugs and dietary chemicals: a review of the literature." Int J Clin Pharmacol Ther **42**(9): 488-95.

Pai, T. G., T. Sugahara, M. Suiko, Y. Sakakibara, F. Xu and M. C. Liu (2002). "Differential xenoestrogen-sulfating activities of the human cytosolic sulfotransferases: molecular cloning, expression, and purification of human SULT2B1a and SULT2B1b sulfotransferases." Biochim Biophys Acta **1573**(2): 165-70.

Pinho, M. J., V. Pinto, M. P. Serrao, P. A. Jose and P. Soares-da-Silva (2007a). "Underexpression of the Na+-dependent neutral amino acid transporter ASCT2 in the spontaneously hypertensive rat kidney." American Journal of Physiology Regulatory **293**(1): R538-47.

Pinho, M. J., M. P. Serrao, P. A. Jose and P. Soares-da-Silva (2007b). "Overexpression of non-functional LAT1/4F2hc in renal proximal tubular epithelial cells from the spontaneous hypertensive rat." Cell Physiol Biochem **20**(5): 535-48.

Price, R. A., R. S. Spielman, A. L. Lucena, J. A. Van Loon, B. L. Maidak and R. M. Weinshilboum (1989). "Genetic polymorphism for human platelet thermostable phenol sulfotransferase (TS PST) activity." Genetics **122**(4): 905-14.

Raftogianis, R. B., C. Her and R. M. Weinshilboum (1996). "Human phenol sulfotransferase pharmacogenetics: STP1 gene cloning and structural characterization." Pharmacogenetics **6**(6): 473-87.

Raftogianis, R. B., T. C. Wood, D. M. Otterness, J. A. Van Loon and R. M. Weinshilboum (1997). "Phenol sulfotransferase pharmacogenetics in humans: association of common SULT1A1 alleles with TS PST phenotype." Biochem Biophys Res Commun **239**(1): 298-304.

Raftogianis, R. B., T. C. Wood and R. M. Weinshilboum (1999). "Human phenol sulfotransferases SULT1A2 and SULT1A1: genetic polymorphisms, allozyme properties, and human liver genotype-phenotype correlations." Biochem Pharmacol **58**(4): 605-16.

Reches, A. and S. Fahn (1984). "Catechol-O-methyltransferase and Parkinson's disease." Adv Neurol **40**: 171-9.

Reiter, C. and R. M. Weinshilboum (1982). "Acetaminophen and phenol: substrates for both a thermostable and a thermolabile form of human platelet phenol sulfotransferase." J Pharmacol Exp Ther **221**(1): 43-51.

Riches, Z., E. L. Stanley, J. C. Bloomer and M. W. Coughtrie (2009). "Quantitative evaluation of the expression and activity of five major sulfotransferases (SULTs) in human tissues: the SULT "pie"." Drug Metab Dispos **37**(11): 2255-61.

Rocha, J. F., L. Almeida, A. Falcão, P. N. Palma, A. I. Loureiro, R. Pinto, M. J. Bonifácio, L. C. Wright, T. Nunes and P. Soares-da-Silva (2013). "Opicapone: a short lived and very long acting novel catechol-O-methyltransferase inhibitor following multiple-dose administration in healthy subjects." British Journal of Clinical Pharmacology.

Salminen, M., K. Lundstrom, C. Tilgmann, R. Savolainen, N. Kalkkinen and I. Ulmanen (1990). "Molecular cloning and characterization of rat liver catechol-O-methyltransferase." Gene **93**(2): 241-7.

Schrag, M. L., D. Cui, T. H. Rushmore, M. Shou, B. Ma and A. D. Rodrigues (2004). "Sulfotransferase 1E1 is a low km isoform mediating the 3-O-sulfation of ethinyl estradiol." Drug Metab Dispos **32**(11): 1299-303.

Schultz, E. and E. Nissinen (1989). "Inhibition of rat liver and duodenum soluble catechol-O-methyltransferase by a tight-binding inhibitor OR-462." Biochem Pharmacol **38**(22): 3953-6.

Senggunprai, L., K. Yoshinari and Y. Yamazoe (2009). "Selective role of sulfotransferase 2A1 (SULT2A1) in the N-sulfoconjugation of quinolone drugs in humans." Drug Metab Dispos **37**(8): 1711-7.

Sharpless, N. S. and D. S. McCann (1971). "Dopa and 3-O-methyldopa in cerebrospinal fluid of Parkinsonism patients during treatment with oral L-dopa." Clin Chim Acta **31**(1): 155-69.

Strott, C. A. (2002). "Sulfonation and molecular action." Endocr Rev **23**(5): 703-32.

Surh, Y. J. (1998). "Bioactivation of benzylic and allylic alcohols via sulfo-conjugation." Chem Biol Interact **109**(1-3): 221-35.

Tang, L., Q. Feng, J. Zhao, L. Dong, W. Liu, C. Yang and Z. Liu (2012). "Involvement of UDP-glucuronosyltranferases and sulfotransferases in the liver and intestinal first-pass metabolism of seven flavones in C57 mice and humans in vitro." Food Chem Toxicol **50**(5): 1460-7.

Vietri, M., A. Pietrabissa, F. Mosca, R. Spisni and G. M. Pacifici (2003). "Curcumin is a potent inhibitor of phenol sulfotransferase (SULT1A1) in human liver and extrahepatic tissues." Xenobiotica **33**(4): 357-63.

Wang, L. Q. and M. O. James (2006). "Inhibition of sulfotransferases by xenobiotics." Curr Drug Metab **7**(1): 83-104.

Winqvist, R., K. Lundstrom, M. Salminen, M. Laatikainen and I. Ulmanen (1992). "The human catechol-O-methyltransferase (COMT) gene maps to band q11.2 of chromosome 22 and shows a frequent RFLP with BglI." Cytogenet Cell Genet **59**(4): 253-7.

Yoshinari, K., K. Nagata, M. Shimada and Y. Yamazoe (1998). "Molecular characterization of ST1C1-related human sulfotransferase." Carcinogenesis **19**(5): 951-3.

Zhu, B. T., U. K. Patel, M. X. X. Cai and A. H. Conney (2000). "O-methylation of tea polyphenols catalyzed by human placental cytosolic catechol-O-methyltransferase." Drug Metabolism & Disposition **28**(9): 1024-1030.

Zurcher, G., A. Colzi and M. Da Prada (1990). "Ro 40-7592: inhibition of COMT in rat brain and extracerebral tissues." J Neural Transm Suppl **32**: 375-80.

Zurcher, G., H. H. Keller, R. Kettler, J. Borgulya, E. P. Bonetti, R. Eigenmann and M. Da Prada (1990). "Ro 40-7592, a novel, very potent, and orally active inhibitor of catechol-O-methyltransferase: a pharmacological study in rats." Adv Neurol **53**: 497-503.