Glucuronidation of Inhibitors and Methylation of Substrates

by

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Academic Dissertation
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Contents

List of original publications

This dissertation is based on the following publications that are referred to in the text by their Roman numerals.

I Lautala, P., Salomies, H., Elovaara, E., and Taskinen, J. An HPTLC method for the assay of UDP-glucuronosyltransferase using p-nitrophenol as substrate. J. Planar Chromatogr. 9 (1996) 413-417.

II Lautala, P., Kivimaa, M., Salomies, H., Elovaara, E., and Taskinen, J. Glucuronidation of entacapone, nitecapone, tolcapone, and some other nitrocatechols by rat liver microsomes. Pharm. Res. 14 (1997) 1444-1448.

III Lautala, P., Ethell, B., Taskinen, J., and Burchell, B. The specificity of glucuronidation of entacapone and tolcapone by recombinant human UDP-glucuronosyltransferases. Accepted for publication in Drug Metab. Dispos.

IV Lautala, P., Ulmanen, I., and Taskinen, J. Radiochemical high-performance liquid chromatographic assay for the determination of catechol *O*-methyltransferase activity towards various substrates. J. Chromatogr. B. 736 (1999) 143-151.

V Lautala, P., Ulmanen, I., and Taskinen, J. Molecular mechanisms controlling the rate and specificity of catechol *O*-methylation by human soluble catechol *O*-methyltransferase. Submitted.

Also some unpublished data are included.

List of abbreviations

AdoMet	S-Adenosyl-L-methionine
^{14}C-AdoMet	S-Adenosyl-L-[*methyl*-^{14}C]methionine
AdoHcy	S-Adenosyl-L-homocysteine
cDNA	Complementary deoxyribonucleic acid
CYP	Cytochrome P450
COMT	Catechol *O*-methyltransferase
DHBA	3,4-Dihydroxybenzoic acid
DDC	Dopa decarboxylase
GST	Glutathione S-transferase
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin-layer chromatography
MB-COMT	Membrane-bound catechol *O*-methyltransferase
MEP	Molecular electrostatic potential
NAT	N-acetyltransferase
4NPG	4-Nitrophenyl-β-D-glucuronide
NSAID	Non-steroidal anti-inflammatoric drug
QSAR	Quantitative structure-activity relationships
RSD	Relative standard deviation
S-COMT	Soluble catechol *O*-methyltransferase
S^N2	Bimolecular nucleophilic substitution
SULT	Sulphotransferase
TLC	Thin-layer chromatography
UDPGA	Uridine diphosphoglucuronic acid
^{14}C-UDPGA	Uridine diphospho[U-^{14}C]glucuronic acid
UGT	Uridine diphosphoglucuronosyltransferase
UV	Ultraviolet

Abstract

Catechol structures can be found in many endogenous compounds including catecholamines and catechol estrogens and in various drugs and drug candidates. Catecholic hydroxyls provide reactive groups for phase II metabolic enzymes of which different forms of UGTs, SULTs and COMTs compete for their conjugation. Little is known, however, about the factors determining the substrate acceptance of these enzymes and their relative contribution to the metabolism of catechols with diverse structures.

In this study, two novel analytical methods were developed: an HPTLC method combining radioactivity measurement and densitometry for the assay of UGT, and a radiochemical HPLC method for the assay of COMT. The respective methods were utilised in studying the glucuronidation properties of a set of nitrocatechols in rat liver microsomes and in determining the apparent enzyme kinetic parameters of methylation for 41 structurally diverse catechols catalysed by human recombinant S-COMT. In addition, the *in vitro* glucuronidation of the COMT inhibitors entacapone and tolcapone was compared by determining the kinetic parameters using human liver microsomes and the relevant human recombinant UGT isoforms.

The results on the glucuronidation of nitrocatechols indicated that although they may be excellent UGT substrates, this property is greatly affected by the nature and position of substituents. Tolcapone was a slightly better substrate than entacapone in rat liver microsomes, whereas entacapone showed a 14-fold V_{max}/K_m value in human liver microsomes. Consequently, rat might be a poor animal model in predicting the glucuronidation of this type of compound in humans. The higher glucuronidation rate of entacapone compared with tolcapone in human microsomes may explain part of its approximately seven times faster elimination half-life *in vivo*. Both compounds, especially entacapone, were excellent substrates of UGT1A9, which knowledge may be useful in evaluating risks for metabolic interactions.

A great variation was detected in the methylation ability of structurally diverse catechols. For instance, among drugs used in the treatment of Parkinson's disease no methylation of entacapone or tolcapone was observed, L-dopa and carbidopa appeared to be poor COMT substrates, whereas benserazide exhibited a relatively high affinity and reactivity. The best endogenous substrate was 2-hydroxyestradiol. For QSAR analysis, the experimental data were combined with the calculation of substituent physicochemical properties and modelling of the compounds to the active site of rat S-COMT. The most decisive factor increasing affinity and simultaneously decreasing reactivity was the electron-withdrawing effect of substituents. In general, hydrophobic substituents increased and hydrophilic groups reduced the affinity, but the orientation of the side chains greatly affected the extent of interactions formed with the hydrophobic surroundings of the binding site. Most important of the several *ortho*-effects discovered, that bulky *ortho*-substituents worsened affinity and reactivity, was demonstrated by apomorphine that was not methylated under the conditions applied. Predictive models for affinity and reactivity were constructed, and they may be utilised, in conjunction with modelling of the active site, in assessing interactions between endogenous catechols and catecholic drugs and in designing catecholic drugs with controlled metabolic methylation.

1. Introduction

Once an orally administered drug has entered the body, it has to be absorbed, distributed, metabolised and finally excreted. All these stages can have a significant effect on the bioavailability of the drug and must be taken into account in drug development (Lin and Lu, 1997). Although drugs may be extensively excreted as such by the kidney, most drugs undergo some kind of metabolic transformation before excretion. Metabolic reactions can be divided into phase I and phase II reactions (Gibson and Skett, 1994). Phase I reactions, such as oxidation, reduction or hydrolysis, produce functional groups that are subsequently capable of conjugation in phase II reactions. Conjugation with endogenous compounds usually results in more water-soluble molecules, which facilitates excretion in the bile and urine. The most important enzyme family catalysing phase I reactions is cytochrome P-450 (CYP). UDP-glucuronosyltransferases (UGTs), sulphotransferases (SULTs), glutathione-S-transferases (GSTs) and different acetyltransferases and methyltransferases are mainly responsible for the phase II reactions. Different enzymes and enzyme families may contribute to the metabolism of a given drug and the rate and route can greatly affect the duration of action and safety of the drug. Besides poor absorption, inappropriate metabolism is one of the main reasons preventing the clinical use of many promising drug candidates (Prentis *et al.*, 1988).

Due to the complexity of metabolic enzymes and the many internal and external factors influencing them, the rate and route of metabolism is difficult to predict (Lin and Lu, 1997). Metabolic studies are traditionally initiated by measuring the elimination half-life of a drug candidate in laboratory animals and identifying the metabolites from plasma and urine samples. However, numerous examples show that remarkable differences exist between species and problems in metabolism may appear only in administration to humans. The increasing availability of human tissues and the advances of gene technology in producing individual enzymes have brought *in vitro* methods to routine use in drug metabolism studies. *In vitro* studies are suited for early assessment of metabolism and selection of the animal model for toxicity studies and for identification of the individual enzyme forms contributing to the metabolism of a drug candidate. *In vitro* data may be further utilised in drug interaction studies and sometimes even in predicting *in vivo* clearance.

Cloning and expression of individual human enzymes that catalyse metabolic reactions has enabled investigations on their structures and mechanisms underlying their catalytical actions and substrate selectivities (Lin and Lu, 1997). At the moment, it is not possible to predict metabolism on the basis of molecular structure, yet the ability to control affinity to certain metabolic enzymes or alter the rate of metabolism by rational structure modification would be useful in the drug discovery and development process. In order to aspire after that goal, however, great efforts in investigating individual enzyme forms and other factors influencing drug metabolism *in vivo* are required.

In this study *in vitro* methods have been utilised in the study of the glucuronidation of nitrocatecholic catechol *O*-methyltransferase (COMT) inhibitors and in the development of predictive models for methylation catalysed by human soluble COMT.

2. Review of the literature

2.1. Role of conjugation reactions in drug and xenobiotic metabolism

Phase II enzymes play an important role in the biotransformation of endogenous and xenobiotic compounds to more easily excretable forms as well as in the metabolic inactivation of pharmacologically active compounds. An especially important function is to detoxify carcinogenic compounds formed in phase I reactions. For example, carcinogenic diol epoxides, formed in phase I reactions from polycyclic aromatic hydrocarbons, are normally conjugated with glutathione and thereby readily excreted from the body (Guengerich, 1992). However, reduced capacity of the phase II enzymes may lead to the appearance of toxic compounds. For instance, 2-hydroxybiphenyl, an antimicrobial agent used to protect edible crops, is metabolised to non-toxic glucuronide and sulphate conjugates at low doses, whereas at high doses these pathways become saturated and oxidative metabolism starts to produce toxic compounds capable of initiating bladder cancer (Reitz *et al.*, 1983). Although phase II reactions are basically detoxifying, the formed conjugates may also mediate adverse effects, for example acting as carriers for carcinogenic compounds. 2-Naphthylamine, found in cigarette smoke, is N-hydroxylated to a carcinogen in the liver, but subsequently glucuronidated to inactive N-hydroxy-N-glucuronide. The glucuronide is, however, hydrolysed in the slightly acidic environment of the bladder and decomposed to a nitrenium ion that can bind to DNA and initiate cancer (Kadlubar *et al*., 1981, Miller and Miller, 1981). The site of conjugation may have undesirable effects, for example biotransformation of a drug to a more hydrophilic conjugate in the gastrointestinal tract usually deteriorates its absorption. A special case of potentially harmful phase II metabolites is 1-*O*-acylglucuronides in which UDP-glucuronic acid is conjugated with a carboxyl acid group by ester linkage. These compounds are chemically labile and the glucuronic acid moiety may be displaced by nucleophiles. This leads to either hydrolysis of the glucuronide, intramolecular rearrangement by acyl migration or intermolecular transacylation. Covalent binding of 1-*O*-acylglucuronides and ester isomers to proteins by transacylation, or glycosylation, are suspected to cause cytotoxicity, carcinogenecity and allergic reactions (Fenselau, 1994).

The fact that interindividual differences in metabolic response occur is not related to phase I enzymes only, but external as well as internal factors including age, sex, diseases and genetics are known to influence also phase II enzymes. A well-known example of the effect of age on glucuronidation is the 'grey baby' syndrome that is caused by the decreased excretion of chloramphenicol glucuronide in new-borns (Weiss *et al*., 1960). In old age the metabolic clearance sometimes declines, but this is caused more likely by the lowered liver blood flow than by the decreased activity of metabolic enzymes (Miners and Mackenzie, 1991). However, decreased clearance of codeine to its 6-*O*-glucuronide has been observed in the elderly (Bochner *et al.*, 1990). Higher capacity in males than in females for glucuronidation of some drugs, including paracetamol (Abernethy *et al*., 1982, Miners *et al*., 1983) diflunisal (Macdonald *et al*., 1990) and propranolol (Walle *et al*., 1989), has been reported suggesting that the activity of some UGTs may be affected by sex hormones. Risks for adverse effects caused by polymorphism in drug-metabolising enzymes are mainly associated with the CYP isoforms.

However, many phase II metabolic enzymes are genetically polymorphic as well, the most familiar being N-acetyltransferase (NAT). Polymorphism of this enzyme was first discovered when isoniazid was used in the treatment of tuberculosis (Evans *et al*., 1960). The incidence of rapid and slow acetylators varies considerably between both ethnic groups and individuals in these groups, giving rise to different metabolic responses towards drugs metabolised *via* NAT (Evans, 1989). Other phase II metabolic enzymes exhibiting genetically polymorphic forms include GST (Laisney *et al*., 1984), COMT (Weinshilboum, 1984, 1988, Boudiková *et al*, 1990, Grossman *et al*., 1992), UGT (Clarke and Burchell, 1994) SULT (Coughtrie *et al*., 1999) and thiopurine methyltransferase (Weinshilboum and Sladek, 1980). Numerous studies suggesting their contribution to diseases and variations in drug responses have been carried out, but the clinical significance of most of the polymorphic phase II enzymes remains rather unclear.

External factors causing interindividual variations in drug metabolism include smoking, medication, nutrition and environmental chemicals (Pelkonen and Breimer, 1994). Binding of polycyclic aromatic hydrocarbons, abundant in tobacco smoke, to the corresponding receptor (Ah) has been found to induce not only certain CYP forms (Whitlock *et al*., 1996) but also some UGTs and GSTs (Bock *et al*., 1990). For example, heavy smoking has been reported to accelerate the glucuronidation of mexiletine and propranolol by 30 and 55%, respectively (Grech-Bélanger *et al.* 1985, Walle *et al*., 1987). Certain drugs are able to induce or inhibit some phase II metabolic enzymes thereby increasing or decreasing the plasma clearance of other drugs metabolised *via* the same enzymes. For instance, the anticonvulsant agents phenobarbitone, phenytoin and carbamazepine are known to enhance the glucuronidation of various drugs including oxazepam, paracetamol and valproic acid, (Scott *et al*., 1983, Miners *et al*., 1984, Panesar *et al*., 1989), while probenecid has been found to inhibit the glucuronidation of many drugs, especially those forming acyl glucuronides (Miners and Mackenzie, 1991).

Although metabolism normally inactivates a drug and transforms it to an easily excretable form, in some cases a metabolite has been even more potent than the parent compound. Two well-known examples of phase II metabolites exhibiting high pharmacological activity are the N-sulphate conjugate of minoxidil (Johnson *et al*., 1982), which, rather than minoxidil itself, causes the relaxation of smooth muscles (Kauffman *et al*., 1994), and morphine 6-*O*-glucuronide, which is a more potent mu-opioid receptor agonist than morphine (Pasternak *et al*., 1987, Paul *et al*., 1989, Frances *et al*., 1990). Entero-hepatic circulation, in which the formed glucuronide is excreted in the intestine, hydrolysed by bacterial β-glucuronidase, reabsorbed, and transported back to the liver, may markedly prolong the pharmacological action of a drug. Even though the most common reason for prodrug design is to enhance the absorption of hydrophilic drugs, also glucuronide conjugates have been developed as prodrug candidates. For example, a glucuronide conjugate of *p*-hydroxyaniline mustard, delivered in conjunction with the hydrolytic, tumour-targeted β-glucuronidase, has been tested for its ability to kill tumour cells (Cheng *et al.*, 1999).

In addition to prodrug discovery, drug designers have recently been able to take advantage of metabolic reactions using another approach. In Parkinson's disease, where the symptoms are caused by the lack of dopamine in the striatum, patients have generally been treated with L-dopa, a dopamine precursor capable of penetrating the blood-brain barrier. To decrease the decarboxylation of L-dopa to dopamine already in the periphery, it is normally administered in conjunction with a dopa decarboxylase (DDC) inhibitor (benserazide or carbidopa) (Männistö *et al*., 1992). However, when DDC is inhibited, the predominant metabolic pathway of L-dopa turns out to be the COMT-

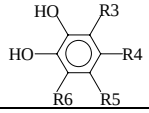
catalysed methylation of the hydroxyl in the 3 position. The formed 3-*O*-methyldopa may be harmful to the patients (Männistö *et al*., 1992), may compete with L-dopa for transport through the blood-brain barrier (Muenter *et al*., 1973, Wade and Katzman, 1975) and be a substrate of brain DDC, thereby competing with L-dopa for biotransformation to dopamine (Nutt and Fellman, 1984). Reduction of the formation of 3-*O*-methyldopa and enhancement of the bioavailability of L-dopa is, however, achieved by specifically inhibiting COMT. Recently, two COMT inhibitors, entacapone and tolcapone, have been introduced to the market as adjuncts to the combination therapy (L-dopa/DDC inhibitor) of Parkinson's disease.

2.2. Conjugation of catechols

A catecholic structure, two adjacent hydroxyls in a phenol ring, is found in many physiological compounds and in various drugs and drug candidates. Many catecholic drugs (Table 1) mediate their effects *via* the same receptors as physiological catechols. Consequently, catechol seems to be a central pharmacophore and will probably exist also in future drugs, especially in those developed for psychiatric disorders and neurological illnesses. In addition to the compounds in Table 1, catechols with pharmacological activity include noradrenaline (adrenergic α- and β-receptor agonist), salsolinol 1-carboxylic acid (naturally occurring amino acid), apomorphine (emetic, dopaminergic receptor agonist), dihydrexidine and SKF 38393 (dopaminergic 1 receptor agonists), rimiterol (adrenergic β_2-receptor agonist), capsazepine (vanilloid receptor antagonist), rosmarinic acid (anti-inflammatoric, C3-convertase inhibitor), tyrphostin (protein tyrosine kinase inhibitor) and catechin polyphenols (peroxynitrite scavengers). An important group of catecholic compounds occurring in the body are the phase I metabolites of catecholamines and estrogens.

The major metabolic routes of the clinically used catecholic drugs listed in Table 1 imply the central role of conjugation reactions in the deactivation and elimination of them. The most relevant reactions are methylation, glucuronidation and sulphation, and depending on the substrate and tissue one of them prevails. For example, the predominant conjugation pathway of catecholamines is methylation, although a considerable amount of dopamine sulphate is formed in the gastrointestinal tract, while glucuronidation seems to be of less importance (Boulton and Eisenhofer, 1998). Further, the DDC inhibitors carbidopa and benserazide are mainly methylated, while the most important elimination route of the COMT inhibitor entacapone is glucuronidation. Besides physico-chemical features, the conjugation pattern of a catechol is affected by for instance the administration route, interindividual variations and the relative capacity of the enzymes involved. For example, glucuronidation is known for a high capacity and thus often prevails over sulphation at high doses (Gibson and Skett, 1994).

Table 1. Clinically used catecholic drugs in Finland 1999 and their major metabolism routes.

Name		Pharmacological action	Major metabolism routes
Adrenaline	R4: $CH(OH)CH_2NHCH_3$	Adrenergic α- and β-receptor agonist	Oxidation, methylation
Benserazide	R3: OH R4: $CH_2NHNHCOCH(NH_2)CH_2OH$	Dopadecarboxylase inhibitor	Hydroxylation, methylation
Carbidopa	R4: $CH_2C(NHNH_2)(CH_3)COOH$	Dopadecarboxylase inhibitor	Methylation
Dobutamine	R4: $(CH_2)_2NHCH(CH_3)(CH_2)_2C_6H_4OH$	Adrenergic β_1-receptor agonist	Methylation
Dopamine	R4: $CH_2CH_2NH_2$	Adrenergic α-, β- and dopaminergic receptor agonist	Oxidation, methylation
Dopexamine	R4: $(CH_2)_2NH(CH_2)_6NH(CH_2)_2C_6H_5$	Adrenergic β_2- and dopaminergic receptor agonist	
Entacapone	R3: NO_2 R5: $CH=C(CN)CON(CH_2CH_3)_2$	COMT inhibitor	Glucuronidation
Isoprenaline	R4: $CH(OH)CH_2NHCH(CH_3)_2$	Adrenergic β_1- and β_2-receptor agonist	Methylation
L-dopa	R4: $CH_2CH(NH_2)COOH$	Dopamine precursor	Decarboxylation, methylation
α-Methyldopa	R4: $CH_2C(NH_2)(CH_3)COOH$	Antihypertensive with central mechanism	Sulphation

Even though the nitrocatecholic COMT inhibitor tolcapone has been implicated in fatal hepatotoxicity (e.g. Assal *et al.*, 1998) and was withdrawn from the market in European Union countries in 1998, its metabolism is interesting to compare with that of the structurally related entacapone. Both COMT inhibitors are almost completely metabolised before excretion in the urine and faeces (Wikberg *et al.*, 1993, Jorga *et al.*, 1999a). Consequently, their short elimination half-lives (0.3 h for entacapone and 2.3 h for tolcapone, Keränen *et al*., 1994, Dingemanse *et al*., 1995) may be partly due to extensive metabolism. Interestingly, despite the structural similarities with entacapone, tolcapone is eliminated at a 7-8 times lower rate. Over 95% of the urinary metabolites of entacapone have been reported to represent glucuronides of entacapone and its (Z)-isomer, whereas glucuronides of tolcapone account for 27% of the urinary metabolites (Wikberg *et al.*, 1993, Jorga *et al.*, 1999a). In humans, no methylation of entacapone has been detected, but minor amounts of 3-*O*-methyltolcapone have been found in plasma, urine and faeces.

The conjugation of catechols is catalysed by various forms of COMTs, UGTs and SULTs. Many of them have been cloned and expressed, and preliminary studies on their substrate acceptance have been carried out. However, research into the factors determining their substrate selectivities towards catecholic compounds is still in its infancy, and it is not possible to make predictions of the route and rate of conjugation from the

molecular structure. That would, however, be useful in assessing the capacity of catecholic drug candidates for interactions with physiological catechols, and other catecholic drugs, already at early stages of the drug development process.

2.1.1. Glucuronidation

A family of UDP-glucuronosyltransferases catalyses the conjugation of a nucleophilic O-, N-, S-, or C-atom with UDP-glucuronic acid (UDPGA) usually resulting in a more water-soluble glucuronide (Tephly and Burchell, 1990). The reaction mechanism is an S_N2-like nucleophilic substitution, in which the acceptor group attacks the C_1 of the pyranose acid ring of UDPGA (Fig 1).

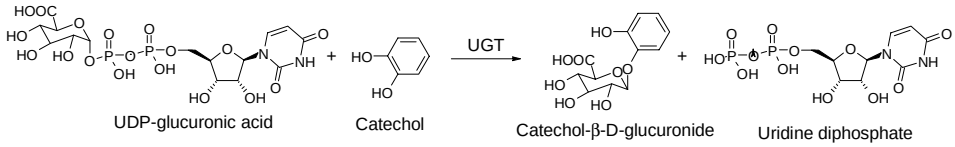


Fig. 1. Glucuronidation of catechol catalysed by UGT.

UGTs are bound to the endoplasmic reticulum, the substrate binding sites towards the lumen. Today, at least 20 members of the human UGT family have been identified on the basis of the amino acid sequences obtained from cDNAs and genomic clones (Clarke, 1998). The high sequence similarity between the isoforms in the carboxyterminal end seems to indicate the UDPGA binding site, while the more variable aminoterminal end probably determines the substrate specificity of the enzymes (Clarke and Burchell, 1994). The isoforms can be divided into two subfamilies with the criterion of greater than 60% sequence similarity within the subfamily (Burchell *et al*., 1995). Members of the UGT1 gene family are designated as phenol- and bilirubin-metabolising isoforms, and enzymes in the UGT2 family are known as steroid-metabolising isoforms. However, overlapping substrate acceptance has been detected both within and between the subfamilies making this classification sometimes inadequate. Because only few *in vitro* studies on the glucuronidation of catecholic compounds other than catechol estrogens have been carried out, the contribution of different UGT isoforms to the glucuronidation of this type of compound is exemplified with phenols (Table 2). A recommended nomenclature based on evolutionary divergence is applied to the isoforms (Mackenzie *et al*., 1997).

Even though almost all the isoforms shown in Table 2 accept both catechol estrogens and other phenolic compounds, some of them play a quantitatively or qualitatively more important role in the glucuronidation of these types of compounds. UGT1A1 is a clinically relevant isoform catalysing the esterification of at least one of the two propionic acid side chains of the toxic haem breakdown product bilirubin (Bosma *et al*., 1994). Mutations in the UGT1 gene complex affecting all UGT1 enzymes cause potentially lethal hyperbilirubinemia known as Griggler-Najjar syndrome type I (Clarke *et al*., 1997). Griggler-Najjar syndrome type II is caused by less dramatic mutations or heterozygous expression of mutant and normal alleles. Gilbert's syndrome, associated with mutation in the promoter region of UGT1A1 exon, has been detected in about 6% of the population (Miners and Mackenzie, 1991). In addition to the raised serum bilirubin co n-

centrations, a decrease in the glucuronidation of for example acetaminophen has been observed in people suffering from this syndrome (De Morais *et al*., 1992). In addition to bilirubin, UGT1A1 catalyses the glucuronidation of catechol estrogens, phenols, anthraquinones and flavones with diverse structures (Senafi *et al.,* 1994). Interestingly, octylgallate, which has a pyrogallol structure, has appeared to be a better substrate of UGT1A1 than bilirubin itself. The important function of UGT1A1 in eliminating bilirubin in conjunction with the wide substrate acceptance and genetic polymorphism may imply possibilities for interactions between compounds glucuronidated *via* this isoform. On the other hand, UGT1A1 is expressed at higher levels in the liver compared with the other UGT1 isoforms and possibly exhibits a high capacity (Sutherland *et al*., 1992).

Table 2. Human UGT isoforms contributing to the metabolism of phenols and catechol estrogens.

Isoform	Major substrates	Phenols	Catechol estrogens	Reference
UGT1A1	Bilirubin	+	+	Senafi et al., 1994
UGT1A3	Scopoletin, norbuprenorphine	+	+	Mojarrabi et al., 1996 Green et al., 1998 Cheng et al., 1998a
UGT1A4	Amines	+	-	Green and Tephly, 1996
UGT1A6	Planar phenols	+	-	Harding et al., 1988 Ebner and Burchell, 1993 Wooster et al., 1993
UGT1A7[a]	Bulky phenols	+	+	Strassburg et al., 1998
UGT1A8[a]	Phenolic compounds	+	+	Mojarrabi and Mackenzie, 1998, Cheng et al., 1998b
UGT1A9	Bulky phenols	+	+	Ebner and Burchell, 1993 Wooster et al., 1993
UGT1A10[a]	Phenolic compounds	+	+	Mojarrabi and Mackenzie, 1998, Strassburg et al., 1998
UGT2A1	Phenolic compounds	+	+	Jedlitschky et al., 1999
UGT2B4	Hyodeoxycholic acid, catechol estrogens	+	+	Ritter et al., 1992
UGT2B7	3,4-Catechol estrogens, hyodeoxycholic acid, carboxylic acid drugs	+	+	Ritter et al., 1990 Ritter et al., 1992 Jin et al., 1993a
UGT2B8	Estriol	+	**?**	Irshaid and Tephly, 1987 Coffman et al., 1990
UGT2B11	Phenolic compounds	+	+	Jin et al., 1993b
UGT2B15	Dihydrotestosterone	+	+	Chen et al., 1993 Green et al., 1994
UGT2B17	Dihydrotestosterone	+	**?**	Beaulieu et al., 1996

[a]Not expressed in the liver

The main isoforms specialised in the glucuronidation of phenols are UGT1A6 and UGT1A9. Screening of over 100 compounds confirmed the earlier findings with the rat

orthologue (Jackson *et al*., 1988) showing that UGT1A6 preferentially catalyses the glucuronidation of small, planar phenols (Ebner and Burchell, 1993). In contrast, UGT1A9 was found to accept nonplanar phenols and anthraquinones, flavones, alcohols, aromatic carboxylic acids, steroids and drugs with diverse structures (e.g. propofol, propranolol, diflunisal, ethinylestradiol, furosemide, ibuprofen, ketoprofen and naproxen). There is evidence of polymorphism in UGT1A6; the genotype that contains two mutations exhibits a lowered activity towards 3-*O*-methyldopa, methylsalicylate and some β-blockers *in vitro*, but the clinical relevance of the polymorphism remains unclear (Ciotti *et al*., 1997). UGT1A9 may be the key enzyme in the detoxification of xenobiotics, since no special endogenous substrate, such as bilirubin for UGT1A1, has been identified and it shows substrate acceptance wider than any other UGT isoform. The importance of UGT1A7, UGT1A8, and UGT1A10, predominantly expressed in the gastrointestinal tract rather than in the liver, is still unclear, but their tissue localisation may refer to a special function in the metabolism of xenobiotics (Mojarrabi and Mackenzie, 1998). UGT1A4, the major isoform catalysing N-glucuronide formation, is suggested to have a minor role in the glucuronidation of phenolic hydroxyls (Green and Tephly, 1996). The recently cloned and characterised UGT2A1 appeared to be specific for olfactory tissue and catalyses the inactivation of odorants (Jedlitschky *et al*., 1999).

The major isoforms catalysing the glucuronidation of catechol estrogens are UGT2B7 and UGT1A1. UGT2B7 has shown a 100-, 30- to 90-, and 2- to 100-fold efficiency towards catechol estrogens compared with UGT2B4 (Ritter *et al*., 1992, Kim *et al*., 1997), UGT2B11 (Jin *et al*., 1993b) and UGT2B15 (Tephly *et al*., 1998), respectively. However, the activity is much higher towards 4-hydroxyestrogenic than 2-hydroxyestrogenic catechols. This is in contrast to UGT1A1, which shows high activity towards 2-hydroxyestrogens (Tephly *et al*., 1998). In theory, catecholamines may be endogenous inhibitors of the metabolism of catechol estrogens. However, the inability of noradrenaline to inhibit UGT1A1-, UGT1A3-, or UGT2B7-catalysed glucuronidation of catechol estrogens suggests that catecholamines are unlikely to interfere with their glucuronidation (Cheng *et al.*, 1998a). UGT2B7 is a clinically important isoform, since in addition to catechol estrogens, it catalyses the glucuronidation of many drugs including NSAIDs and morphine (Jin *et al*., 1993a, Coffman *et al*., 1997). The wide substrate acceptance (Green *et al*., 1994) and wide expression in many tissues (Levesque *et al*., 1997) of UGT2B15 suggest that also this isoform may exhibit a high contribution to the glucuronidation pathway. The significance of the polymorphic expression reported for UGT2B4 (Levesque *et al*., 1999), UGT2B7 (Coffman *et al*., 1998), and UGT2B15 (Levesque *et al*., 1997) remains to be determined.

In human liver microsomes both electron-donating and electron-withdrawing *para*-substituents have been shown to enhance the glucuronidation of phenols, whereas bulky *ortho*-substituents inhibit the reaction (Temellini *et al*., 1991). These general observations represent the sum effect of all contributing isoforms, yet basic knowledge of the substrate acceptance of the isoforms suggests that the various forms may obey very different rules. Despite the availability of recombinant enzymes, no systematic structure-activity analysis, that would take into account various substituent effects, has been carried out for any of the isoforms. The mechanisms of catalysis and the contributing amino acids have been investigated by chemical modification of UGT1A6 (Battaglia *et al*., 1994a, 1994b) and site-directed mutagenesis of UGT1A1 and UGT2B17 (Ciotti and Owens, 1996, Ciotti *et al*., 1998, Dubois *et al.*, 1999). However, since the three-dimensional structures are not known, models of the active sites remain inaccurate.

2.2.2 Methylation

COMT catalyses the transfer of a methyl group from S-adenosyl-L-methionine (AdoMet) to one of the catecholic hydroxyls (Männistö *et al.*, 1992) (Fig. 2). COMT accepts a wide range of structurally variable substrates with the only strict requirement that the substrate must have a catechol structure (Guldberg and Marsden, 1975).

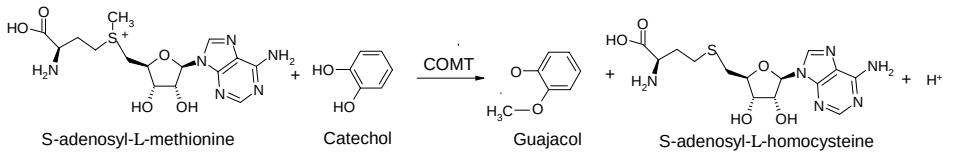


Fig. 2. Methyl transfer from AdoMet to catechol in the COMT-catalysed reaction.

COMT exists in two forms; the soluble form (S-COMT) is located in cytosol and the membrane-bound form (MB-COMT) is attached to the rough endoplasmic reticulum (Tilgmann and Kalkkinen, 1991, Bertocci *et al.*, 1991, Lundström *et al.*, 1991, Ulmanen *et al.*, 1997). The amino acid sequences of the two forms differ only by a 50-amino-acid-long extension in the MB-form, which is believed to represent the membrane anchoring signal sequence (Ulmanen and Lundström, 1991). Due to the structural similarities the two forms are thought to catalyse the methylation reaction with the same mechanism. The human brain MB-COMT is, however, reported to exhibit approximately 100 times lower K_m value for dopamine than the soluble form of the enzyme (Rivett and Roth, 1982, Nissinen, 1984). Studies with cloned and expressed COMT forms have confirmed the existing kinetic differences between the two forms (Malherbe *et al.*, 1992, Lotta *et al.*, 1995). They also show different regioselectivity towards catecholic hydroxyls *in vitro* (Nissinen, 1984, Lotta *et al.*, 1995). S-COMT is the predominant form in most tissues, but a Western blot analysis of the distribution of COMT has revealed that MB-COMT predominates in the human brain (Tenhunen *et al.*, 1994). This along with the higher affinity of catecholamines to MB-COMT at physiological concentrations indicate that MB-COMT may be more relevant in the termination of catecholaminergic neurotransmission, while S-COMT is likely to play a more important role in the inactivation of endogenous and xenobiotic catechols in other tissues (Roth, 1992, Lotta *et al.*, 1995, Bonifati and Meco, 1998).

In the 1980s COMT was discovered as a potential drug target. Peripheral COMT inhibition was desired in order to increase the bioavailability of L-dopa in the combination therapy (L-dopa/DDC inhibitor) of Parkinson's disease. Search for potent and selective inhibitors lead to the purification of S-COMT from rat liver (Tilgmann and Kalkkinen, 1990) and human placenta (Tilgmann and Kalkkinen, 1991) and subsequent cDNA cloning (Salminen *et al.*, 1990, Lundström *et al.*, 1991). MB-COMT was cloned from the human hepatoma cell line G2 (Bertocci *et al*, 1991). Recombinant COMT proteins have been produced in *Escherichia coli* (Lundström *et al*, 1992, Malherbe *et al.*, 1992), in mammalian cell lines (Lundström *et al.*, 1991, Bertocci *et al.*, 1991, Malherbe *et al.*, 1992, Tilgmann *et al.*, 1992) and in baculovirus-infected insect cells (Tilgmann *et al.*, 1992).

Advances in molecular biology have provided tools not only for studies on the structure, subcellular localisation, and tissue distribution of the COMT proteins and genes encoding them, but also for investigations on the mechanism of the COMT-catalysed reaction. Great progress in the field was made when Vidgren *et al.* succeeded in resolving the crystal structure of rat S-COMT (1994). The amino acid sequences of rat and human S-COMT exhibit 81% homology, and especially the active site is highly conserved (Vidgren and Ovaska, 1997). The active site consists of the AdoMet binding site and the catalytic site. The catalytic catechol-binding site is in a shallow groove on the surface of the protein (Fig. 3). Magnesium ion is essential for the catalytic activity. It is coordinated to a water molecule, to the side chain oxygens of three amino acid residues (Asp141, Asp169, Asn170) and to both of the catechol hydroxyls. Magnesium ion significantly lowers the pK_a of the catechol hydroxyls thereby making them more easily ionised. However, the negatively charged carboxyl acid group of Glu199 located a hydrogen bond from one of the hydroxyls stabilises the unionised form of this hydroxyl. The other hydroxyl donates the proton to the amino group of Lys144, and the ionised hydroxyl attacks the electron-deficient methyl group of AdoMet leading to the methyl transfer to the catechol hydroxyl. Lys144 acts as a catalytical base in this S_N2-like nucleophilic substitution reaction. Hydrophobic amino acids surrounding the catalytic site (especially Trp38 and Pro174) define the substrate selectivity of the enzyme towards different side chains of catecholic compounds.

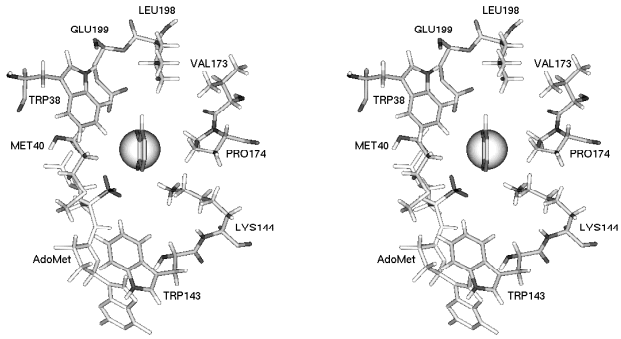


Fig 3. A stereoview of the catechol binding site adopted from the crystal structure of rat S-COMT (Vidgren et al.*, 1994). Bound AdoMet is shown left and magnesium complexed with two hydroxyls of pyrogallol in the middle.*

After early conflicting results on the kinetic mechanism of COMT that suggested a rapid-equilibrium random-order mechanism (Flohe and Schawabe, 1970, Coward *et al.*, 1973) and a ping-pong mechanism (Borchardt, 1973), Woodard *et al.* (1980) showed that the methylation reaction proceeds through an S_N2-like transition state. Subsequently a sequentially ordered mechanism was established (Rivett and Roth, 1982, Tunnicliff and Ngo, 1983). Lotta *et al.* (1995) demonstrated the following binding and dissociation order: AdoMet, magnesium, catechol substrate and methylated catechol product, magnesium and demethylated AdoMet (AdoHcy). The previously proposed order suggesting magnesium ions to bind first to the free enzyme in a rapid equilibrium reaction

(Jeffery and Roth, 1987) could be argued on the basis of the crystal structure of COMT; the AdoMet binding site is located behind the magnesium binding site thereby forcing AdoMet to bind before magnesium. Catecholamines are predominantly methylated at the *meta*-position, which in early studies was suggested to be due to the polar ionic side chain favouring that binding orientation (Creveling *et al.*, 1970, 1972). This interpretation was later verified by computer-aided modelling of one low-activity conformer of dopamine to the active site of COMT (Lotta *et al*., 1995). Modelling revealed that, in deed, binding in the orientation leading to *para*-methylation causes more unfavourable interactions with the hydrophobic residues surrounding the active site compared with the other binding mode. This might not, however, be the only explanation, because only one low-energy conformer was studied and the flexible side chain may adopt variable orientations. In addition to the hydrophobic interactions, the nature of substituents at various positions may affect the regioselectivity with other mechanisms as well. For e x-ample, in ring-fluorinated catecholamines variable preference to the hydroxyls has been explained by changes in nucleophilicity (Firnau *et al*., 1981, Creveling *et al*., 1981, Thakker *et al*., 1986).

COMT is usually thought to possess a very wide acceptance of structurally diverse catecholic compounds. The clinical importance of COMT inhibition has directed studies on the structure-activity relationships to the factors affecting inhibition potency rather than to the methylation reaction itself. Two research groups, both of which would introduce its own molecule to clinical use later, independently synthesised potent and selective COMT inhibitors with 3-nitrocatechol as the key structure (Borgulya *et al*., 1989, Bäckström *et al*., 1989). Further studies showed that inhibition activity is enhanced by electron-withdrawing substituents at positions 3 and 5 and that binding affinity is improved by a hydrophobic substituent at position 5 (Taskinen *et al.*, 1989, Lotta *et al*., 1992). Compounds containing electronegative groups, such as 3,5-dinitrocatechol, decrease the nucleophilicity of the ionised catechol hydroxyls and strongly stabilise the ionised catechol-COMT complex thus making the energy barrier for the methylation high (Vidgren and Ovaska, 1997, Ovaska and Yliniemelä, 1998). The few studies on the substrate selectivity of COMT have emphasised the relevance of hydrophobicity in the increment of affinity (Raxworthy and Gulliver, 1982, Youde *et al*., 1984). However, these conclusions have been derived on the basis of a small number of related compounds rather than a proper structure-activity analysis.

Existence of polymorphic COMT forms, a thermolabile low activity and a thermostabile high activity COMT, has been reported (Scanlon *et al*., 1979, Weinshilboum, 1984, 1988, Boudiková *et al*, 1990, Grossman *et al*., 1992). The molecular basis of the polymorphism is variation at the 108 amino acid residue (Val-108 being thermostabile and Met-108 thermolabile) (Lotta *et al*., 1995). The two alleles result in homozygous individuals with high or low COMT activity and heterozygous individuals with intermediate activity (Bonfati and Meco, 1999). A markedly higher frequency of the high activity allele has been reported among Chinese and Japanese people (~75%) than among Caucasians (50%) (Xie *et al*., 1997, Kunugi *et al*., 1997). Previous studies have also shown that Orientals and black Americans exhibit a higher COMT activity than do Caucasians (Rivera-Calimlim and Reilly, 1984, McLeod *et al*., 1994). In contrast, a lower COMT activity has been reported among the Saami population (Klemetsdal *et al*., 1994). Several studies have suggested the association of COMT polymorphism with neurological and psychiatric disorders (e.g. Kunugi *et al*., 1997, Strous *et al*., 1997a, Chen *et al*., 1997, Lachman *et al*., 1996) and with breast cancer risk (Lavigne *et al*., 1997, Thompson *et al*., 1998), but also contradicting findings have been published (e.g. Xie *et al*., 1997, Strous *et al*., 1997b, Ohara *et al*., 1998, Millikan *et al*., 1998).

2.2.3. Sulphation

The conjugation of 3'-phosphoadenosine 5'-phosphosulphate with an O-, N- or S- acceptor group is catalysed by cytosolic sulphotransferases. The SULT enzyme family includes enzymes that catalyse the sulphation of phenolic xenobiotics (P-PST), catecholamines (M-PST), estrogens (EST), and steroids (HST) (Coughtrie, 1998). Different isoforms exhibit overlapping substrate specificities, although many of them show preference towards some type of substrates. Structure-activity relationships have not been fully investigated for any of the isoforms. However, in human liver samples neutral substituents, usually rather in the *ortho*- than in the *para*-position, have been found to favour phenol sulphation (Temellini *et al*., 1991). SULT1A3 (M-PST), which is responsible for the sulphation of catecholamines, is predominantly expressed in the small intestinal mucosa and may have a special role in the detoxification of dietary xenobiotics (Coughtrie, 1998). As a result of the action of SULT1A3, circulating catecholamines appear mostly as sulphate conjugates thus facilitating their transportation to other tissues. At least SULT1A1 (P-PST) is genetically polymorphic, but further investigations on its significance are needed (Coughtrie *et al*., 1999).

2.3. *In vitro* studies on catechol conjugation

In vitro studies on conjugation reactions are usually performed by incubating the studied compound, in the presence of a cosubstrate, with the enzyme and by measuring the reaction velocity. The most common enzyme sources from different species are tissue homogenates, subcellular fractions and recombinant enzymes. Since following the disappearance of the substrate is insensitive and unspecific, reaction velocity is normally determined on the basis of formation of the products. In kinetic studies, the initial reaction velocity is determined as a function of substrate concentration at the saturating concentration of the cosubstrate (Cornish-Bowden, 1995). Most enzymes follow Michaelis-Menten kinetics; reaction velocity increases almost linearly at low substrate concentrations after which it starts to reach the maximum asymptotically. The enzyme kinetic parameters V_{max} and K_m, describing the capacity of the reaction and the affinity of the substrate to the enzyme, are derived by fitting the Michaelis-Menten equation (1) to the initial velocity values obtained. At low substrate concentrations, normally occurring *in vivo*, the reaction is best described by the ratio of V_{max} and K_m.

$$(1)\ V = (V_{max} \times [s]) / (K_m + [s])$$

V = reaction velocity
V_{max} = maximum reaction velocity
$[s]$ = substrate concentration
K_m = substrate concentration at half the maximum velocity

In addition to studies on substrate selectivity and reaction mechanisms, *in vitro* methods may be utilised in the selection of the animal model for toxicity studies and in the early assessment of metabolism and metabolic interactions in humans. Attempts to predict *in vivo* clearance based on *in vitro* data have been made almost exclusively utilising compounds that are metabolised by phase I enzymes (Houston and Carlile, 1997). However, a study on tolcapone shows that reasonably accurate predictions are possible also for compounds metabolised mainly by phase II enzymes (Lave *et al.*, 1996).

2.3.1. UGT assays

The major difficulty of *in vitro* studies on UGT is its latency, due to its subcellular localisation in the rough endoplasmic reticulum, the active site towards the lumen. Activity may be enhanced by damaging the membrane by detergents or sonication, which improves the access of the substrates to the active site. Optimum concentrations of detergents (e.g. digitonin, Lubrol WX, Brij 58, CHAPS, Tergitol NP-10 and Triton X-100) have been shown to increase the UGT activity by 2- to 5-fold (Winsnes, 1969, Magdalou *et al*., 1979, Thomassin *et al*., 1985, Lawrence *et al*., 1992, Lett *et al*., 1992, Martin and Black, 1994). However, detergents have been reported to alter not only the V_{max} but also the K_m values (Thomassin *et al*., 1985) and excessive detergent concentrations deactivate the enzyme. There is no direct evidence of the contribution of Mg^{2+} ion to the glucuronidation reaction, although magnesium has been found to increase the glucuronidation rate of for example morphine (Lawrence *et al*., 1992). β-Glucuronidase, which is located in the lumen of endoplasmic reticulum, as are UGTs, catalyses the hydrolysis of glucuronides, but it can be selectively inhibited by saccharolactone. In addition, the pH normally used in the glucuronidation studies (6-8) does not favour the action of β-glucuronidase that exhibits a pH optimum at 4-5 (Kauffman, 1994).

Assays for UGT include spectrometric and fluorometric methods, for instance continuous fluorometric monitoring of reaction products (Väisänen *et al*., 1983), but mostly common chromatographic techniques have been utilised. Most methods have been developed for specific substrates. However, a very widely used method, introduced by Bansal and Gessner (1980), is suited for structurally diverse compounds including simple phenols and hormones. The universality of the method is based on uridine diphospho[U-^{14}C]glucuronic acid (^{14}C-UDPGA) that is conjugated with the acceptor substrates forming ^{14}C-labelled products, which makes authentic reference standards unnecessary. In this method, the formed glucuronides are separated from unreacted UDPGA on preparative silica gel TLC plates with a mixture of *n*-butanol, water, acetone, glacial acetic acid and 30% ammonia (70:60:50:18:1.5). The glucuronide spots are identified by autoradiography and quantitated by liquid scintillation counting after scraping from the plate. The method with minor modifications has been especially useful in studies on substrate selectivity of UGTs. The tedious and time-consuming autoradiography and scraping with subsequent liquid scintillation counting have been replaced by quantitation of the radioactive glucuronides directly from the TLC plates by a digital autoradiograph or a radioanalytical imaging system (Ritter *et al*., 1990, Ebner and Burchell, 1993). The main disadvantage of the method is its inability to separate multiple glucuronides originating from the same parent compound. Therefore a general HPLC method, based on ^{14}C-UDPGA and on-line radioactivity detection (Coughtrie *et al*., 1986), recently published in an improved form (Ethell *et al*., 1998), may overcome the old TLC assay.

2.3.2. COMT assays

As explained previously, Mg^{2+} ions are essential for the COMT-catalysed methylation reaction. Some other divalent cations, such as Cd^{2+}, Hg^{2+}, Mn^{2+} and Cu^{2+}, have been found to promote methylation as well (Axelrod and Tomchick, 1958, Senoh *et al*., 1962, Flohe 1974, Boadi *et al*., 1991). In contrast, Ca^{2+} ions seem to inhibit COMT (Weinshilboum and Raymond, 1976). Purified human S-COMT has been shown to require cysteine as a reducing agent to maintain its activity (Tilgmann and Kalkkinen, 1991). Other agents capable of inhibiting the deactivation, probably caused by oxidation of the sul-

phydryl groups of the protein, include mercaptoethanol and dithiothreitol (Tilgmann and Ulmanen, 1996). Reducing agents in the reaction mixture may also protect the catecholic hydroxyls from oxidation during the reaction. AdoHcy, the demethylated end product of AdoMet, has been found to inhibit COMT (Coward *et al*., 1973), but at low substrate and enzyme and saturating AdoMet concentrations its effect becomes negligible.

Many early COMT assays relied on fluorometric (e.g. Axelrod and Tomchick, 1958), spectrophotometric (e.g. Coward and Wu, 1973, Borchardt, 1974) or most commonly radiochemical methods (e.g. Jonas and Gershon, 1974, Raymond and Weinshilboum, 1975, Gulliver and Tipton, 1978, Bates *et al*., 1979, Zürcher and Da Prada, 1982). Adding a radioactively labelled substrate or cosubstrate (S-adenosyl-L-[*methyl*-^{14}C]methionine or S-adenosyl-L-[*methyl*-^{3}H]methionine) to the reaction mixture resulted in radioactive end products that could be separated from the parent compounds by liquid-liquid extraction or thin-layer chromatography, and that could be subsequently quantitated in a liquid scintillation counter. Radiochemical methods are simple and sensitive, applicable for various catechol substrates and require no reference standards. However, impurities in radiochemicals and variable recovery in the extraction procedure impair their reliability. In addition, regioisomeric *O*-methylated metabolites, produced from many compounds *in vitro*, cannot be quantitated separately. Development of gas chromatographic (Creveling *et al*., 1972, Lin and Narasimhachari, 1974, Koh *et al*., 1991) and liquid chromatographic COMT assays has, however, enabled separation of the regioisomeric products. High-performance liquid chromatography has been coupled with various detection devices including UV (Pennings and Van Kempen, 1979), electrochemical (Borchardt *et al*., 1978, Shoup *et al*., 1980, Koh *et al.*, 1981, Nissinen and Männistö, 1984), fluorometric (Zaitsu *et al*., 1981, Nohta *et al*., 1984, Smit *et al*., 1990, Zürcher *et al*., 1996) and radiochemical detectors (Nissinen, 1985). Most of the assays are intended for the measurement of COMT activity in different tissues including those with a low level of COMT expression, such as brain or erythrocytes. Especially assays utilising electrochemical, radioactivity and fluorescence detectors are specific and sensitive (respective limits of detection 0.5, 0.04 pmol and 11 fmol reaction product per injection) (Reenilä *et al*., 1995, Tuomainen *et al*., 1996, Nissinen, 1985, Zürcher *et al*., 1996). These methods use a specific substrate and do not allow determination of COMT activity towards catechols with diverse structures.

3. Aims of the study

The primary aims of this study were 1) to evaluate the susceptibility of nitrocatecholic COMT inhibitors to glucuronidation *in vitro* and 2) to characterise the structural features of catecholic compounds that determine their properties as S-COMT substrates.

The specific aims were:

- to develop and validate analytical methods for the *in vitro* studies on the glucuronidation and methylation of catechols
- to evaluate the glucuronidation of various nitrocatechols in rat liver microsomes
- to characterise the human UGT isoforms that are mainly responsible for the glucuronidation of the COMT inhibitors entacapone and tolcapone
- to compare the glucuronidation kinetics of entacapone and tolcapone in rat and human liver microsomes as well as by human UGT isoforms
- to compare the substrate selectivity of rat and human S-COMT
- to construct predictive models for the methylation of catechols by human S-COMT

4. Materials and methods

The original publications contain more detailed descriptions of the materials and methods utilised.

4.1. Chemicals

Most of the 57 catecholic compounds were purchased from commercial sources and were of the highest grade available. Nitrocatechol derivatives 3-nitrocatechol, 3,5-dinitrocatechol, entacapone, entacapone(Z)-isomer, nitecapone and tolcapone were kindly supplied by Orion Pharma (Espoo, Finland). UDPGA was obtained from Sigma Chemical Company (St. Louis, Missouri, USA) or Boehringer-Mannheim (Mannheim, Germany), AdoMet from Boehringer-Mannheim and ^{14}C-UDPGA and ^{14}C-AdoMet from NEN Du Pont (Boston, USA). 4-Nitrophenyl-β-D-glucuronide (4NPG) was purchased from Sigma Chemical Company and vanillic acid from Aldrich (Sigma-Aldrich Chemie, Steinheim, Germany). Reference standards of the 3-*O*-glucuronides of entacapone and tolcapone were synthesised at the Department of Pharmaceutical Chemistry, University of Helsinki, Finland (Luukkanen *et al*., 1999).

4.2. Enzyme sources

Rat liver microsomes were prepared from liver homogenates of male Wistar rats by differential centrifugation at the Finnish Institute of Occupational Health, Helsinki (I, II). The rats were pre-treated with creosote (200 mg in 4 ml olive oil/kg) or did not receive any pre-treatment. Protein concentrations of the microsomal suspensions were determined by the method of Lowry *et al*. (1951). Human liver microsomes were purchased from Human Biologics Inc. (Arizona, USA) (III). Microsomes were stored at -70°C before being used.

Recombinant V79 cell lines expressing human UGT isoforms were grown up and maintained at the Department of Molecular and Cellular Pathology, Ninewells Hospital and Medical School, Dundee, Scotland, as described previously (Ethell *et al*, 1998) (III). The harvested cells were stored at -70°C and, before use, disrupted by sonication. The protein concentrations were determined using bovine serum albumin as a standard (Lowry *et al*., 1951). Recombinant rat and human S-COMT proteins were produced in *E. coli* at the Department of Molecular Biology and Target Protein Research, Orion Pharma, Finland, as described in detail earlier (Lundström *et al.*, 1992) (IV, V). The harvested cells were disrupted by sonication and kept at -70°C before being used for the enzyme assays. The total protein concentrations of the lysates were determined according to Bradford (1976).

4.3. Reaction mixtures

In the UGT assays the reaction mixture contained $MgCl_2$ (5 mM), UDPGA (2-5 mM), substrate (0.010-2.5 mM), and the UGT source (microsomes or lysates from cells expressing UGT isoforms) in 50-100 mM phosphate or Tris/maleate buffer (pH 7.4) (I-III). When radioactivity was utilised in the quantitation, 0.1 μCi ^{14}C-UDPGA was added to the reaction mixture (II, III). The samples were incubated at 37°C for 15-60 minutes before the reactions were terminated by adding organic solvent (methanol or acetonitrile) or 4 M perchloric acid. The precipitated proteins were removed by centrifugation.

The COMT assays were performed in 100 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4) containing $MgCl_2$ (5 mM), L-cysteine (20 mM), AdoMet (150 μM), ^{14}C-AdoMet (0.1 μCi), a catechol substrate (0.25-3000 μM) and human or rat S-COMT bacterial lysate (IV, V). In kinetic studies, the amount of enzyme was chosen separately for each substrate on the basis of the concentration range used in order to maintain appropriate conditions for Michaelis-Menten kinetics. The samples were pre-incubated at 37°C for 5 min before the reactions were started by adding the catechol substrate or the AdoMet/^{14}C-AdoMet mixture. After the 15- to 30-min-long incubation period the reactions were terminated by adding cold 4 M perchloric acid. The samples were centrifuged before the HPLC analysis.

4.4. Analytical methods

4.4.1. Thin-layer chromatography (I-II)

The glucuronides of nitrocatechols were separated from the parent compounds and UDPGA on RP-18 HPTLC plates using a horizontal development mode. The eluent consisted of 50 mM NaH_2PO_4 (pH 2.2) and acetonitrile (6:4 v/v). The sample application was performed utilising the Linomat IV spray-on technique (Camag, Muttenz, Switzerland). The glucuronides were quantitated with the aid of one sample in which ^{14}C-UDPGA had been added before incubation. Four different volumes of this sample were applied to the plate in order to obtain a calibration curve. After development, the air-dried plates were scanned with a Camag TLC Scanner II, controlled by the Camag Cats program version 3.17, at wavelengths specific for each nitrocatechol. Three of the radioactivity-containing glucuronide spots were scraped from the plates and, after addition of liquid scintillation cocktail (Optiphase Hisafe 2, FSA Laboratory Supplies, Loughborough, UK), quantitated in a liquid scintillation counter (Wallac 1410, Turku, Finland). The amounts of the glucuronides in the standard spots, calculated from the mean value derived from the radioactivity measurements, were fed into the Cats program and the glucuronides in the other samples were quantitated on the basis of the densitometric analysis.

4.4.2. High-performance liquid chromatography (II-V)

The UGT assays were performed according to published HPLC methods or by making minor modifications to them (Ethell *et al.*, 1998, Wikberg *et al*., 1993). The fluorometric COMT assay with 6,7-dihydroxycoumarin as the substrate was previously developed at the Department of Pharmaceutical Chemistry, University of Helsinki, Finland (Nummila, 1999), while the radiochemical assay for the determination of COMT activity towards various substrates (IV) was developed during this study. The experimental conditions of the five HPLC methods utilised are summarised in Table 3.

Table 3. Chromatographic conditions of the HPLC methods utilised in the UGT and COMT assays. The mobile phase flow rate was 1.0 ml/min in all methods.

UGT/ COMT	HPLC equipment	Column	Oven °C	Mobile phase composition	Detection
UGT (II)	1090, Hewlett-Packard, Waldbronn, Germany	RP-18, LiChrosorb, 125 x 4 mm, 5 µm (Hibar, Merck, Darmstadt, Germany)	35	25 mM NaH_2PO_4 (pH 2.2), acetonitrile (7:3[a], 6:4[b], v/v)	UV (PU4020, Pye Unicam) 305 nm[a], 278 nm[b]
UGT (III)	Schimadzu (LC-6A pumps), Kyoto, Japan	Techspere ODS2, 250 x 46 mm, 5 µm (HPLC Technology, Macclesfield, UK)	-	Binary gradient: 0-100% acetonitrile in 0.05 M ammoniumacetate in 15 min	Radioactivity[c], (9701, Reeve Analytical, Glasgow, Scotland)
UGT (III)	1100, Hewlett-Packard, Waldbronn, Germany	Hypersil BDS-C18, 250 x 4 mm, 5 µm (Hewlett-Packard, Waldbronn, Germany)	40	25 mM NaH_2PO_4, 10 mM citric acid (pH 2.2), methanol (52:48[a], 42:58[b], v/v)	UV, (Hewlett-Packard, Waldbronn, Germany) 305 nm[a], 278 nm[b]
COMT (IV, V)	1090, Hewlett-Packard, Waldbronn, Germany	RP-18, LiChrosorb (Hibar, Merck) and Hypersil BDS-C18, 125 x 4 mm, 5 µm (Hewlett-Packard)	40	50 mM Na_2HPO_4, 20 mM citric acid, 0.15 mM Na_2-EDTA (pH 3.2)[d], 3-60% methanol	Radioactivity[e], (150TR, Packard, Meriden, CT, USA)
COMT (V)	1100, Hewlett-Packard Waldbronn, Germany	Hypersil BDS-C18, 250 x 4 mm, 5 µm (Hewlett-Packard Waldbronn, Germany)	40	50 mM Na_2HPO_4 (pH 3.0), methanol (27:73 v/v)	Fluorescence, (Shimadzu RF-535, Kyoto, Japan) excitation 335 nm, emission 455 nm

[a]for entacapone

[b]for tolcapone

[c]a 200-µl cell with cerium-activated lithium glass as scintillant (Reeve Analytical)

[d]1.25 mM 1-octanesulphonic acid was added when basic substrates were analysed

[e]a 300-µl cell with cerium-activated lithium glass as scintillant (Packard) or a 500-µl cell receiving scintillation liquid (Monoflow 3, National Diagnostics, Atlanta, USA) 3 ml/min

4.4.3. Method validation

The HPTLC method applied in the investigation of the glucuronidation of nitrocatechols by rat liver microsomes was validated with 4-nitrophenol as the model substrate (I). The validation procedure comprised specificity, limit of quantitation, repeatability of sample application (n=7), recovery (3 concentrations, n=6), reproducibility of the method (6 concentrations, n=6), and reproducibility of the determination of the enzyme kinetic parameters (n=6). The reliability of quantitation based on the method combining densitometry and radioactivity measurement was tested by comparing the results obtained from the same samples by this method and by the densitometric method that utilises 4NPG as the reference standard (6 concentrations).

3,4-Dihydroxybenzoic acid (DHBA) was used as the model substrate in the validation of the new radiochemical HPLC method developed for the assay of COMT activity towards various substrates (IV). In this method the methylated products were quantitated by comparing their peak areas in the radioactivity detector with the total area of radioactive peaks. The accuracy of this quantitation method was investigated by analysing samples containing different initial concentrations of DHBA simultaneously by radioactivity and UV detection. In the UV detection, the 3-*O*-methylated product of DHBA, vanillic acid, was used as the reference standard. Recovery was determined by comparing the sum of AdoMet, vanillic and isovanillic acid peak areas in the radioactivity detector with the peak area of unincubated AdoMet (n=6). The limit of quantitation and the limit of detection were estimated for vanillic acid by using the criteria for signal to noise ratio of ten and three. The same samples containing six initial concentrations of DHBA (n=6) were used for investigating the reproducibility of the method and the reproducibility of the determination of the enzyme kinetic parameters.

4.5. Enzyme kinetic analysis (I-V)

The apparent enzyme kinetic parameters V_{max} and K_m were determined by measuring the initial reaction velocity as a function of catechol concentration at fixed cosubstrate concentration. The Michaelis-Menten equation was fitted to the initial velocity values using the Leonora Steady-state Enzyme Kinetics program version 1.0 by A. Cornish-Bowden (1994). In some cases equations for substrate inhibition and competitive or mixed inhibition were used.

4.6. QSAR and molecular modelling (V)

The octanol-water distribution coefficients were calculated using the LOGKOW method (Meylan and Howard, 1995), and the SPSS 8.0.1 program (SPSS Inc., Chicago, IL, USA) was utilised in statistical analyses. The catechols were modelled by the Spartan 5.0 program (Wavefunction Inc., Irvine, CA, USA) and optimised by using the semiempirical AM1 method. The molecular electrostatic potentials (MEP) were computed by single point *ab initio* calculations at the 3-21G(*) level. The Insight II program (Molecular Simulations Inc., San Diego, CA, USA) was utilised in superimposing the catechols on 3,5-dinitrocatechol in the active site model of rat S-COMT (Brookhaven Protein Data Bank entry 1VID).

5. Results and discussion

5.1. Development and validation of enzyme assays

5.1.1. Thin-layer chromatographic UGT assay for the determination of nitrocatechol glucuronidation (I, II)

An HPTLC method was developed for the assay of UGT activity towards nitrocatecholic compounds using 4-nitrophenol as the substrate. This compound is a generally used, structurally relevant UGT substrate and the respective glucuronide, 4NPG, is commercially available. 4NPG could be separated from the parent compound and from UDPGA on RP-18 HPTLC plates with a mixture of 50 mM NaH_2PO_4 (pH 2.2) and acetonitrile (6/4, v/v). The same chromatographic conditions were shown to be applicable for the analysis of certain nitrocatechols as well (Fig. 4). All glucuronides were well separated from the respective parent compounds and UDPGA. Good separation from UDPGA was especially important in the analyses where radioactive spots were scraped from the plate.

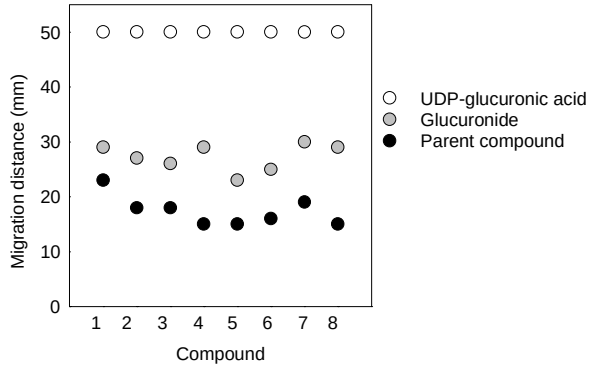


Fig. 4. Separation of glucuronides from parent compounds and UDPGA on RP-18 HPTLC plates developed with 50 mM NaH_2PO_4 (pH 2.2) / acetonitrile (6/4, v/v) to 50 mm. 1; 4-nitrophenol, 2; 4-nitrocatechol, 3; 3-nitrocatechol, 4; 3,5-dinitrocatechol, 5; entacapone, 6; entacapone(Z)-isomer, 7; nitecapone, 8; tolcapone.

The developed UGT assay was validated with 4-nitrophenol as the substrate. Good correlations between the *in situ* spectra measured 0.5 mm before and after the 4NPG peak centre (r=0.9805 and 0.9847, respectively) in conjunction with symmetrical peak shape ($T_{0.05}$=1.0) demonstrated that the sample matrix did not interfere with the analysis. The repeatability of sample application, often a critical stage in quantitative analysis, proved

to be satisfactory (RSD=2.2%, 50 μM 4NPG, n=7). The limit of quantitation, 15 pmol 4NPG on the plate, representing the lowest concentration at which the RSD of sample application (n=3) did not exceed 5% (Ferenczi-Fodor *et al*., 1993), enabled reaction velocity measurements at low 4NP concentrations. The recoveries of various 4NPG concentrations (12.5, 50 and 75 μM) spiked in the sample matrix were rather consistent (95.2±3.4, 102.8±2.5 and 102.0±2.2%, respectively, n=6) and showed a mean value of 100%. The relatively low RSD values (between 4.1 and 11.7%, n=6) achieved at six initial concentrations of 4-nitrophenol suggested that the within-laboratory reproducibility of the method was at a tolerable level. The good reproducibility of the determination of the enzyme kinetic parameters K_m and V_{max} (RSD 6.1 and 6.3%, respectively, n=6) indicated that the method was suitable for enzyme kinetic studies.

Lack of reference standards of the different nitrocatechol glucuronides necessitated the utilisation of ^{14}C-labelled UDPGA in quantitation. However, to decrease the costs and risks of the analysis, the radioactive cosubstrate was added to just one of the samples in each kinetic series. The concentration level of the glucuronides was obtained with this sample and the glucuronides in the other samples were quantitated on the basis of densitometric analysis. The good correlation (r=0.9920) between the results obtained for the same 4-nitrophenol samples by this method and by the method utilising 4NPG demonstrated that reliable results could be obtained in a wide range of glucuronide concentrations. In general, the benefits of TLC include low solvent consumption, off-line character and possibility to analyse several samples simultaneously. Compared with the traditional UGT assay developed by Bansal and Gessner (1980), the new method was cost-effective and did not require time-consuming exposure to x-ray films. Although it is only applicable for compounds exhibiting a reasonably high molecular absorptivity, all the nitrocatecholic compounds could be analysed under the same conditions. The main disadvantage of the method, that multiple glucuronides could not be separated, is shared by all TLC-based UGT assays. However, the idea of a using radioactively labelled cosubstrate only for calibrating the product level is applicable for HPLC as well. This approach was used in a study on chrysene and benzo(a)pyrene phenols in which, after HPLC separation, quantitation was based on the linear relationship observed between radioactivity, fluorescence and UV absorption (Bock *et al*., 1992). Improvement in sensitivity by combining UV or fluorescence detection with radioactivity measurement may be desired in kinetic studies where a high UDPGA concentration is required, which decreases the formation of radioactively labelled glucuronides.

5.1.2. Radiochemical high-performance liquid chromatographic COMT assay (IV, V)

A radiochemical HPLC method for the assay of COMT towards various substrates was developed with 3,4-dihydroxybenzoic acid (DHBA) as the substrate. The method could be applied in studying the *in vitro* methylation of 55 catecholic compounds including physiological catechols, drugs and drug candidates, and structurally simple catechols. The mobile phase used for acidic and neutral compounds consisted of a phosphate/citrate buffer (pH 3.2) and a varying amount of methanol (3-60%). To increase the retention of compounds containing basic groups, an ion-pair reagent, 1-octanesulphonic acid, was added to the buffer. Examples of chromatograms obtained from different catechol substrates and mobile phase compositions are shown in Fig. 5. The chromatograms illustrate that the method allowed detection of regioisomeric *O*-methylated products formed from many compounds.

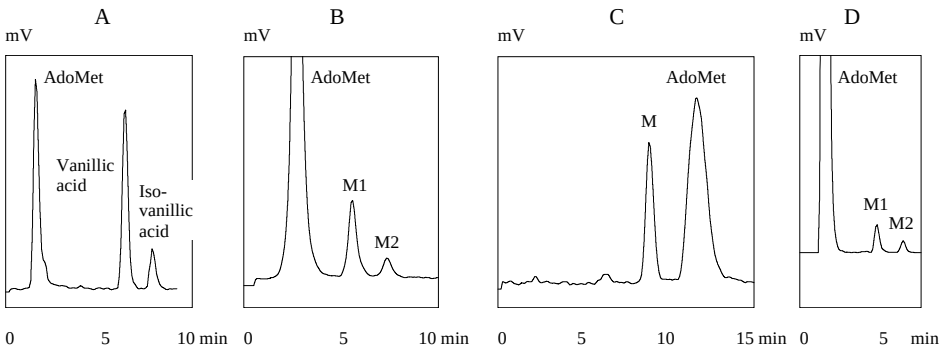


Fig. 5. Chromatograms in the radioactivity detector obtained from samples in which AdoMet/^{14}C-AdoMet and recombinant human S-COMT were incubated with a) 3,4-dihydroxybenzoic acid b) dopamine c) L-dopa d) 2-hydroxyestradiol. The column used was a Hypersil BDS-C18, 125x4 mm, 5 µm, and the proportion of phosphate/citrate buffer (pH 3.2) and methanol was a) 90:10 b) 85 (including 1.25 mM 1-octanesulphonic acid) :15 c) 95 (including 1.25 mM 1-octanesulphonic acid) :5 d) 40:60.

The COMT assay was validated with DHBA as the substrate and recombinant human S-COMT as the enzyme source. The *O*-methylated metabolites were quantitated by comparing their peak areas in the radioactivity detector with the total area of radioactive peaks. The results of the validation experiments based on the quantitation of vanillic acid are summarised in Table 4. The quantitation based on comparison of the area of radioactive peaks correlated well with the method relying on UV detection. The recovery and the repeatability of the method were good, and the good repeatability of the determination of the enzyme kinetic parameters showed that the method was well suited for its intended use. When applying the method to other compounds, however, more variation in for example recovery and stability may be expected. Fortunately, no loss of total radioactivity, represented by the sum of peak areas in the radioactivity detector, could be detected among the compounds studied. Stability problems were minimised by using fresh solutions only, adding antioxidant (L-cysteine) to the reaction mixture and always analysing the samples immediately after preparation. Earlier published K_m values for DHBA, dopamine, noradrenaline and L-dopa determined by using HPLC with electrochemical detection are well comparable with those obtained by this method (Lotta *et al*., 1995).

Most of the compounds could be analysed with a solid scintillant in the radioactivity detector. A solid scintillant, earlier utilised in UGT assay (Ethell *et al.*, 1998), has some advantages compared with on-line liquid scintillation counting. No scintillation liquid or additional pump is needed, obviating the need to optimise the scintillant/mobile phase flow rate. The use of a solid scintillant not only reduces costs but also markedly decreases the production of waste. However, compounds exhibiting low K_m values required improved sensitivity. The limit of detection could be lowered from 9 to 1 pmol/injection by substituting the 300-µl cerium-activated lithium glass cell by a 500-µl flow cell that received scintillation liquid 3 ml/min. With this modification the sensitivity level (0.45 pmol) of an earlier COMT assay utilising S-adenosyl-L-[*methyl*-^{3}H]methionine and on-line radiochemical detection (Nissinen, 1985) could be reached. Considerably lower metabolite levels, however, are detectable by electrochemical and fluorescence detectors (Reenilä *et al*., 1995, Tuomainen *et al*., 1996, Zürcher *et al*.,

1996), and assays relying on them are methods of choice when COMT activity has to be measured in erythrocytes or other tissues with low COMT expression. Nevertheless, the new method utilising ^{14}C-AdoMet and on-line radiochemical detection is suitable for studies on structure-activity relationships and mechanisms of regioselectivity in which structurally diverse catecholic compounds are included. The method provides also a rapid screening assay for the *in vitro* methylation of for instance new catecholic drug candidates.

Table 4. Validation of the radiochemical HPLC method for the assay of COMT with 3,4-dihydroxybenzoic acid as the substrate.

Validation parameter	Result
Accuracy of quantitation (5-300 µM DHBA)	compared to UV-detection, r^2=0.9988 (n=6)
Recovery (300 µM DHBA)	97.5 ± 2.5%, (n=6)
Repeatability	
-at different DHBA concentrations (5-300 µM)	RSD= 3.30-7.30% (6 concentrations, n=6)
-determination of kinetic parameters	K_m: RSD=7.94%, V_{max}: RSD =3.57% (n=3)
Limit of detection	
-solid cell	9 pmol/injection at signal/noise = 3
-liquid cell	1 pmol/injection at signal/noise = 3

5.2. Glucuronidation of nitrocatechols

5.2.1. Glucuronidation of nitrocatechols by rat liver microsomes (II)

In order to assess the properties of nitrocatechol-type compounds as UGT substrates, enzyme kinetic parameters were determined for a nitrocatechol series and the model substrate 4-nitrophenol in rat liver microsomes (Table 5). Microsomes were derived from male Wistar rats treated with creosote, which, similar to 3-methylcholantherene, has been found to cause a two-fold induction of 4-nitrophenol glucuronidation (Luukkanen *et al*., 1997). In this study creosote was found to increase the glucuronidation rate of entacapone and tolcapone, by approximately two-fold.

Disubstituted COMT inhibitors showed approximately one order of magnitude lower V_{max}/K_m values compared with 4-nitrophenol and 4-nitrocatechol. This may be due to UGT1*06, an isoform known to be induced by 3-methylcholantherene (and possibly by creosote) and reported to exhibit a restricted specificity towards small and planar phenols (Jackson *et al*., 1988). Consequently, other isoforms are likely to contribute to the glucuronidation of the compounds with bulkier substituents. Existence of a parabolic dependence between glucuronidation rate and lipophilicity has been suggested, with an optimum value of logP=2.25 for phenolic compounds in rats (Kim, 1991). Although the glucuronidation rates of 4-*n*-propylphenol and 4-*tert*-butylphenol (respective estimated $logP_{ow}$=3.04 and 3.42) have been reported to be higher than that of 4-nitrophenol ($logP_{ow}$

=1.91) in rat liver microsomes (Jackson *et al*., 1988), in this study entacapone and tolcapone (respective $\log P_{ow}$=2.22 and 3.13) exhibited approximately five times lower V_{max} values than 4-nitrophenol. Apparently other structural features, not identified in this study, affected the glucuronidation of these COMT inhibitors. Nevertheless, the more lipophilic nature of tolcapone compared with entacapone and especially nitecapone ($\log P_{ow}$=1.04) may explain its higher affinity towards rat UGTs. The especially high K_m value obtained for nitecapone may indicate a contribution of a distinct low-affinity isoform to the glucuronidation of this compound.

Table 5. Apparent enzyme kinetic parameters for the glucuronidation of 4-nitrophenol and some nitrocatechols by liver microsomes from creosote-treated rats. The values represent mean ± SD of three or four independent experiments.

Compound	V_{max} (nmol min^{-1} mg^{-1})	K_m (mM)	V_{max}/K_m (ml min^{-1} mg^{-1})
4-Nitrophenol	68.8±3.8	0.11±0.02	0.63
4-Nitrocatechol	153.0±26.4	0.19±0.05	0.79
3-Nitrocatechol	127.7±15.5	0.72±0.11	0.18
3,5-Dinitrocatechol	36.2±7.7	1.04±0.27	0.035
Entacapone	13.4±2.1	0.75±0.27	0.018
Entacapone(Z)-isomer	15.5±3.4	0.40±0.11	0.039
Nitecapone	52.9±5.9	2.40±0.19	0.022
Tolcapone	11.0±1.5	0.29±0.06	0.038

4-nitrocatechol was a better substrate than 4-nitrophenol, which demonstrates that an *ortho*-hydroxyl does not interfere with the binding to the enzyme, and that catechols may be excellent substrates of UGTs. Substituents in the benzene ring, however, markedly influence the properties catechols exhibit as substrates of UGTs. The four-fold increase in the K_m value observed with 3-nitrocatechol compared with 4-nitrocatechol may be due to steric hindrance. Accordingly, bulky *ortho*-substituents have been found to decrease the tendency of phenol glucuronidation in rat and human liver microsomes (Boutin *et al*., 1985, Temellini *et al.*, 1991). The even higher K_m value of 3,5-dinitrocatechol compared with 3-nitrocatechol may be caused by the increased nucleophilicity of the catecholic hydroxyls. Conclusions about the electronic effects of substituents cannot, however, be drawn on the basis of this study in which only nitrocatechols were included. In general, contradicting results have been published concerning the electronic effects of substituents (Magdalou *et al.*, 1982, Jackson *et al*., 1988, Temellini *et al*., 1991, Kim, 1991, Yin *et al*., 1994) and proper structure-activity analyses with individual UGT isoforms are needed to explain them.

The V_{max}/K_m value, which describes the reaction at low substrate concentration, was approximately two times higher for tolcapone than the respective value for entacapone. Consequently, the longer elimination half-life of tolcapone observed in humans *in vivo* could not be explained by their glucuronidation kinetics in rat liver microsomes.

5.2.2. Glucuronidation of entacapone and tolcapone by human liver microsomes and recombinant UGT isoforms (III)

To characterise the human UGT isoforms involved in the glucuronidation of entacapone and tolcapone, the COMT inhibitors were incubated with cell lysates containing a representative set of recombinant UGT isoforms: UGT1A1, UGT1A6, UGT1A9, UGT2B7, and UGT2B15 (Fig. 6). Results of the screening revealed that UGT1A9 was the most important isoform contributing to the glucuronidation of both entacapone and tolcapone. Tolcapone was glucuronidated at the same rate by this isoform as the widely used standard substrate, propofol, whereas entacapone showed almost two-fold velocity compared with them.

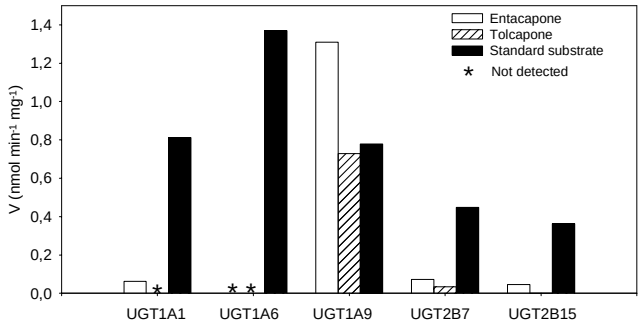


Fig. 6. Glucuronidation velocity of 500 µM entacapone and tolcapone by recombinant human UGT isoforms. Respective standard substrates for UGT1A1, 1A6, 1A9, 2B7, and 2B15 were octylgallate, 1-naphthol, propofol, 4-hydroxyestrone, and 8-hydroxyquinoline.

Tolcapone seemed not to be a substrate of the bilirubin isoform UGT1A1, while entacapone was glucuronidated at a low rate by it. According to the known restricted substrate specificity of UGT1A6 towards small planar phenols (Ebner and Burchell, 1995), neither entacapone nor tolcapone was glucuronidated by this isoform. Both COMT inhibitors were glucuronidated at low velocities by the representative members of the UGT2B family, UGT2B7 and UGT2B15.

To compare the kinetic properties of entacapone and tolcapone, apparent enzyme kinetic parameters were determined for them using recombinant human UGT isoforms and human liver microsomes (Table 6). Studies with UGT2B7 revealed K_m values in the millimolar range, which prevented the proper kinetic analysis with this isoform. The COMT inhibitors seemed to be very similar substrates of UGT2B15, while a qualitatively considerable difference was detected with UGT1A1. Tolcapone was not glucuronidated by this isoform, whereas it was the only isoform that catalysed the formation of two glucuronides of entacapone. The very similar chromatographic behaviour of the second glucuronide to that of entacapone 3-*O*-glucuronide (rt 8.4 and 8.9 min, respectively) suggests that it was the 4-*O*-glucuronide conjugate. The contribution of UGT1A1 to the glucuronidation of entacapone seems, however, to be minor compared with that of UGT1A9, which is supported by the fact that only 3-*O*-glucuronides of entacapone have been found in human urine (Wikberg *et al.*, 1993). Therefore, possibili-

ties for interactions with the most important physiological UGT1A1 substrate, bilirubin, are likely to be only theoretical.

*Table 6. Apparent kinetic parameters for the formation of 3-*O*-glucuronides of entacapone and tolcapone by human liver microsomes (HLM) and recombinant UGT isoforms. The values represent mean ± SD of two to five determinations.*

UGT source	Entacapone			Tolcapone		
	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/K_m (ml min^{-1} mg^{-1} x 10^{-3})	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/K_m (ml min^{-1} mg^{-1} x 10^{-3})
HLM	6.8±2.0	47±5	144	2.1±1.1	201±102	10.3
UGT1A1	0.004 0.005[a]	31 93	0.13 0.05	ND[b]	-	-
UGT1A9	1.8±0.6	10.0±1.9	182	0.48±0.15	66±12.0	7.3
UGT2B7[c]	0.007	1800	0.004	0.013	640	0.020
UGT2B15	0.024	322	0.08	0.024	429	0.06

[a]Another glucuronide of entacapone detected, most probably 4-*O*-glucuronide

[b]ND=not detected

[c]No proper kinetic analysis could be performed due to low activity and high substrate concentration needed

Comparable variation in the kinetic parameters of entacapone and tolcapone by UGT1A9 and human liver microsomes (3- to 4-fold V_{max} and 4-6 times lower K_m for entacapone) suggest that this particular isoform might be mainly responsible for the differences in the overall glucuronidation of these compounds (Fig. 7, Table 6). Particularly strong involvement of UGT1A9 was further supported by the high glucuronidation rates measured in kidney microsomes (2.31 and 0.763 nmol min^{-1} mg^{-1} for entacapone and tolcapone, respectively), since this isoform is richly expressed in the kidney (Sutherland *et al*., 1993, McGurk *et al.*, 1998). The finding is also consistent with the previous observations on the wide substrate acceptance of UGT1A9 including phenolic and carboxyl-acid containing drugs (Ebner and Burchell, 1993, Wooster *et al.*, 1993). No obvious reason for the reduction of the reaction velocity by UGT1A9 at high entacapone concentration (Fig. 7) could be discovered, yet fitting of the equation for substrate inhibition resulted in the V_{max}/K_m value close to that derived from the Michaelis-Menten equation (156 and 144 ml min^{-1} mg^{-1}). Nevertheless, this phenomenon was not observed with liver microsomes and should exhibit no significance at physiological concentrations. The finding that UGT1A9 is probably the most relevant isoform in the glucuronidation of entacapone and tolcapone may be, although no metabolic interactions with entacapone nor tolcapone have been reported, of great help in assessing possibilities for them.

Compared with tolcapone, entacapone exhibited more than three-fold V_{max} and four times lower K_m value in human liver microsomes, leading to about 14-fold V_{max}/K_m value. The differences in *in vitro* glucuronidation kinetics relate to *in vivo* observations showing that entacapone is almost exclusively metabolised by glucuronidation (Wikberg *et al.*, 1993), while, although glucuronidation is the main pathway, also other routes significantly contribute to the metabolism of tolcapone (Jorga *et al.*, 1999a). Be-

cause both compounds are almost completely eliminated by metabolism, glucuronidation kinetics may also partly explain the shorter elimination half-life of entacapone compared with tolcapone. Evaluation of the structural features determining the variation in the glucuronidation ability of these closely related compounds is, however, hindered by lack of knowledge on the structure-activity relationships of UGTs.

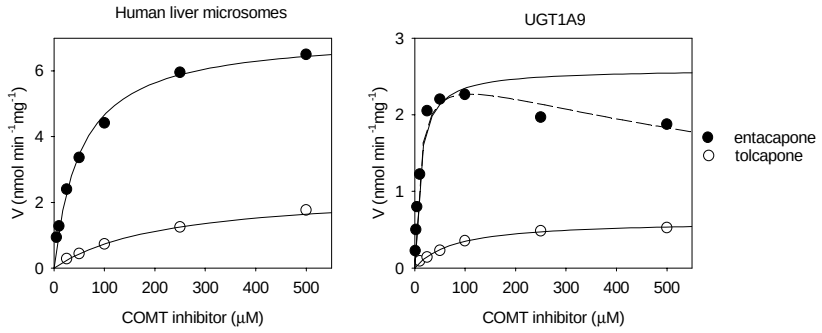


Fig. 7. Michaelis-Menten plots for the glucuronidation of entacapone and tolcapone by human liver microsomes and by human UGT1A9. The dashed line represents fitting of the equation for substrate inhibition.

Although *in vitro*/*in vivo* correlation was not a subject of this study, it is interesting to note that the microsomal clearance (V_{max}/K_m) of entacapone, scaled for the whole liver with the following assumptions: 77 mg/microsomal protein/g liver, 1800 g liver/human, liver blood flow 1.45 l/min (Pelkonen 1999, Davies *et al*, 1993), gives a hepatic clearance value of 1.35 l/min. Conversion of the *in vivo* plasma clearance value reported for entacapone (750 ml/min) (Keränen *et al*., 1993) to blood clearance value, by dividing it with the blood/plasma partitioning value of 0.6, gives 1.25 l/min. The good correspondence of these values, although calculated from insufficient data (for example the clearance by extrahepatic tissues and other metabolism routes were excluded and a microsome pool from six individuals only was used), indicate that in the case of entacapone the *in vitro* results may at least be suggestive of the *in vivo* situation.

5.2.3. Species differences

An obvious discrepancy in the glucuronidation kinetics of entacapone and tolcapone was detected between the results obtained from rat and human liver microsomes. The most relevant enzyme kinetic parameter V_{max}/K_m was two times higher for tolcapone than for entacapone in rat liver microsomes, while in human liver microsomes entacapone, with a 14-fold V_{max}/K_m value, was demonstrated to be superior over tolcapone. Species differences have also appeared in the *in vivo* metabolism of entacapone; in humans over 95% of the metabolites excreted in the urine 0-2 h after administration were glucuronides of entacapone and its (Z)-isomer, whereas in the rat only approximately 65% of the metabolites represented glucuronide conjugates and almost 10% were sul-

phates (Wikberg *et al*, 1993). Therefore, based on both *in vivo* and *in vitro* data, rat seems not to be a good animal model for predicting the glucuronidation of this type of compound in humans. This may concern all the compounds glucuronidated mainly by UGT1A9, since predominant *in vivo* excretion as sulphate conjugates and low glucuronidation activity in liver microsomes have been reported for propofol in the rat (Le Guellec *et al.*, 1995, Simons *et al*., 1991), while in humans its main metabolic pathway is glucuronidation, which is also supported by the high *in vitro* glucuronidation rate measured in liver microsomes (Simons *et al*., 1988, Le Guellec *et al.*, 1995).

5.3. Substrate selectivity of rat and human S-COMT (IV, V)

The three dimensional structure of rat S-COMT has been solved by crystallography (Vidgren *et al*., 1994). In order to evaluate its applicability for modelling human S-COMT, enzyme kinetic parameters of 17 catecholic compounds were determined using recombinant rat and human S-COMT. For the first time substrate selectivity of rat and human S-COMT was determined under the same experimental conditions thus allowing a proper comparison. The enzymes exhibited very similar regioselectivities and both the K_m and V_{max} values, determined for the same catechols, were highly correlated (r^2=0.988 and r^2=0.927, respectively, n=25, including regioisomers). This is in agreement with the high sequence homology (81%) of the enzymes and the almost completely conserved amino-acid residues in the active site (Vidgren and Ovaska, 1997). The absolute V_{max} values obtained using rat and human S-COMT were not comparable, since concentrations of active enzyme were not known, but the K_m values showed 3-4 times higher figures for the rat enzyme, on average. It is possible that the rat enzyme, although expressing similar mechanisms for substrate selectivity, possesses a somehow looser active site structure thereby decreasing the affinity of substrate binding.

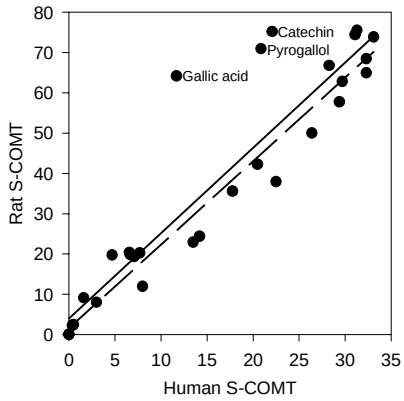


Fig. 8. Correlation of reaction velocities (nmol min^{-1} mg^{-1}) between human and rat S-COMT (r^2=0.858, n=30). The dashed line represents the correlation without gallic acid, pyrogallol and catechin (r^2=0.960, n=27).

When rat and human S-COMT activity was screened at one relatively high concentration (IV, 500 μM) discarding three outliers (gallic acid, pyrogallol and catechin) from the analysis improved correlation from r^2=0.858 (n=30) to r^2=0.960 (n=27) (Fig. 8). Pyrogallol derivatives are low-affinity inhibitors of COMT and at the high concentration used they inhibited their own methylation. The higher affinity towards the human enzyme appeared as a more pronounced inhibition of the human enzyme than of the rat enzyme, which probably explains the deviation from the correlation. Despite the kinetic difference, rat S-COMT proved to be an excellent model for the human enzyme thereby validating its use as a template in computer-aided molecular modelling.

5.4. Methylation of structurally diverse compounds by human S-COMT (V)

5.4.1. Enzyme kinetic parameters

Methylation of 46 structurally diverse catechols was investigated using recombinant human S-COMT to obtain data for structure-activity analysis. The enzyme kinetic parameters could be determined for 41 compounds including simple substituted and physiological catechols and catecholic drugs and drug candidates (Tables 7-8).

Under the conditions used, no methylation of 3,5-dinitrocatechol, 2,3-dihydroxybenzoic acid, tolcapone, entacapone, or apomorphine was observed. Two regioisomeric products could be detected from many of the compounds, yet the analytical method utilising ^{14}C-AdoMet did not allow elucidation of the metabolite structures. However, the enzyme kinetic parameters were determined separately for both isomers when possible and useful experimental data about regioselectivity, that supported observations derived from molecular modelling, could be gathered. Pyrogallol and catechol showed the highest V_{max} values (53.2 and 48.9 nmol min^{-1} mg^{-1}), while the lowest measurable V_{max} was 0.15 nmol min^{-1} mg^{-1} for 3-nitrocatechol. Among the series of substituted catechols in Table 7, catechol exhibited the highest K_m value (50 μM). This value was more than three orders of magnitude higher than the lowest value obtained (29 nM, for tetrachlorocatechol). Nevertheless, many endogenous and other catechols with pharmacological activity exhibited several-fold higher K_m values than catechol (Table 8). The V_{max}/K_m values were highest for tetrachlorocatechol and 2,3-dihydroxynaphthalene.

Among the endogenous catechols 2- and 4-hydroxyestradiols were superior COMT substrates; they exhibited high V_{max} values and low K_m values leading to, for instance, approximately 60- and 10-fold V_{max}/K_m values compared with dopamine, respectively. Among drugs used in the treatment of Parkinson's disease, entacapone and tolcapone were not methylated *in vitro* and L-dopa and carbidopa were poor substrates with respective V_{max}/K_m values of 0.053 and 0.024 ml min^{-1} mg^{-1}, while the other DDC inhibitor, benserazide, showed V_{max} and K_m values equal to those of catechol. Differences found between carbidopa and benserazide are in agreement with the previous *in vitro* results obtained with COMT purified from pig liver (Hagan *et al*., 1980, Gordonsmith *et al*., 1982). *In vitro* results also support the *in vivo* observations concerning the effect of COMT inhibition by tolcapone on the pharmacokinetics of these compounds; no interactions were detected with carbidopa, whereas the bioavailability of benserazide was enhanced by tolcapone at large L-dopa/benserazide doses (Sedek *et al*., 1993, Jorga *et*

al., 1999b, Jorga *et al*., 1999c). Among the other clinically used drugs dobutamine was a relatively good and isoprenaline a moderately good COMT substrate, whereas α-methyldopa showed the highest K_m value (1.8 mM) among all 41 compounds. These findings are in agreement with the knowledge on their preferential metabolism routes *in vivo* (Table 1). Even though apomorphine has been reported to be a good substrate of rat liver COMT *in vitro* (Cannon *et al*., 1972), and its elimination half-life in rats is increased by tolcapone (Coudore *et al*., 1997), no methylation was detected under the experimental conditions applied. *In vitro* observations may not always correspond to the *in vivo* situation, as exemplified by tolcapone that, despite showing no implications for methylation in our study, has been reported to undergo biotransformation to 3-*O*-tolcapone *in vivo* (e.g. Dingemanse *et al*., 1995). On the other hand, in a study on the metabolism of intravenously administered apomorphine in a Parkinsonian patient, no methylated products were found in plasma or urine (van der Geest *et al*., 1997). In our study, apomorphine was demonstrated to inhibit the methylation of DHBA (K_i=240 μM for competitive inhibition) and thus shown to be capable of binding to the enzyme.

Table 7. Apparent enzyme kinetic parameters for the methylation of substituted catechols catalysed by recombinant human S-COMT.

Compound	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/K_m (ml min^{-1} mg^{-1})
Catechol	48.9±0.9	49.7±1.6	0.98
4-Methylcatechol	24.4±0.9	29.1±3.9	0.84
	23.3±1.1	36.7±3.3	0.63
4-Isopropylcatechol	36.3±1.7	26.1±3.0	1.39
	6.9±1.5	40.5±27.4	0.17
4-*tert*-Butylcatechol	41.9±2.7	31.9±8.0	1.31
4-Chlorocatechol	36.0±0.1	11.7±0.5	3.08
3,4-Dihydroxyacetophenone	9.8±0.7	1.4±0.3	7.00
3,4-Dihydroxybenzoic acid ethyl ester	7.2±1.7	0.66±0.17	10.9
	3.5±1.1	0.66±0.17	5.30
4-Nitrocatechol	0.77±0.09	0.12±0.02	6.42
	0.78±0.05	0.12±0.02	6.50
4-*tert*-Butyl-5-methoxycatechol	32.5±2.4	18.5±2.3	1.76
Pyrogallol	53.2±1.5	10.4±0.6	5.12
3-Nitrocatechol	0.15±0.02	0.020±0.001	7.50
3-Fluorocatechol	30.7±1.8	7.8±1.0	3.94
3-Methoxycatechol	39.8±1.8	24.5±2.8	1.62
3-Methoxy-5-bromocatechol	17.3±0.9	2.5±0.4	6.92
Methylgallate	11.7±0.4	1.0±0.1	11.7
3,5-Dinitrocatechol	ND		
3,4-Dihydroxybenzoic acid	35.6±0.9	19.9±2.0	1.79
	7.7±0.1	28.3±0.4	0.27
2,3-Dihydroxybenzoic acid	ND		
Caffeic acid	25.9±1.9	3.2±0.7	8.09
	15.5±0.2	2.8±0.1	5.54
Hydrocaffeic acid	31.5±1.7	25.4±4.5	1.24
	12.1±0.03	45.1±0.3	0.27
Tetrachlorocatechol	0.62±0.03	0.029±0.002	21.4
2,3-Dihydroxynaphthalene	30.8±1.6	1.5±0.3	20.5
6,7-Dihydroxycoumarin	3.3±0.03	0.30±0.01	11.0

ND = not detected

Table 8. Apparent enzyme kinetic parameters for the methylation of some endogenous catechols, catecholic drugs and drug candidates catalysed by recombinant human S-COMT.

Compound	V_{max} (nmol min^{-1} mg^{-1})	K_m (μM)	V_{max}/K_m (ml min^{-1} mg^{-1})
L-Noradrenaline	33.2±0.8	256±20	0.13
3,4-Dihydroxymandelic acid	43.7±2.0	132±11	0.33
3,4-Dihydroxyphenylglycol	37.2±1.4	46.4±4.7	0.80
L-Adrenaline	44.0±1.0	132±13	0.33
Dopamine	35.9±1.4	188±16	0.19
5-Hydroxydopamine	46.7±1.1	61.6±4.1	0.76
6-Hydroxydopamine	25.3±2.0	244±32	0.10
3,4-Dihydroxyphenyl acetic acid	34.0±2.2 4.0±0.1	68.8±10.8 53.7±4.0	0.49 0.074
2-Hydroxyestradiol	32.4±2.6 13.7±1.8	3.7±1.1 4.2±2.2	8.76 3.26
4-Hydroxyestradiol	31.5±2.5	12.4±2.6	2.54
α-Methyldopa	21.4±0.7 2.6±0.3	1789±108 1170±309	0.012 0.002
L-dopa methylester	44.2±0.7	51.1±2.9	0.86
L-dopa	29.8±0.9	564±28	0.053
S(-)-Carbidopa	14.3±0.8	599±63	0.024
Benserazide	41.8±3.6	50.2±7.2	0.83
Tolcapone	ND		
Entacapone	ND		
Isoprenaline	25.8±0.6 3.1±0.3	145±8.5 170±31	0.18 0.018
Dobutamine	29.0±0.9 6.9±0.5	23.7±3.1 28.6±7.5	1.22 0.24
1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline	18.3±0.9 10.6±0.1	29.4±3.7 36.2±0.2	0.62 0.29
SKF 38393	5.0±0.04 10.5±0.5	67.2±1.2 73.6±8.3	0.074 0.14
Dihydrexidine	10.1±2.3 10.5±6.2	25.0±14.5 53.1±50.6	0.40 0.20
Apomorphine	ND		

ND = not detected

5.4.2. Effect of molecular structure on binding affinity and reactivity

Combining experimental data, molecular modelling and calculated data on substituent physico-chemical properties, mechanisms affecting binding affinity and reactivity could be elucidated. The most important substituent effects identified in this study were the electronic effect on catecholic hydroxyls, interactions with the surroundings of the catechol binding groove, steric hindrance, especially by *ortho*-substituents, and some other *ortho*-effects.

In this study, the most important factor affecting reactivity appeared to be the electron-withdrawing effect of substituents. The electronic effect of substituents is best demonstrated by a set of small 4-substituted compounds that exhibit a minimum amount of direct interactions with the amino acid residues in the binding site (the first eight compounds in Table 7). Their V_{max} values were highly correlated with parameters describing electronic effects of the substituents. Hammet σ^- value, which quantitates the effect of a 4-substituent on the ionisation of phenol, explained 94% of the variation in the V_{max} values, and even better correlation (r^2=0.98) was achieved when the lower molecular electrostatic potentials (MEP) were calculated for the monoanionic forms of the compounds. A semiempirical study on COMT inhibition has suggested that the reason for lowered reactivity by electronegative substituents is stabilisation of the catecholate anion, formed *via* proton transfer to the amino group of Lys 144 (Ovaska and Yliniemelä, 1998). The catecholate anion-enzyme complex, rather than the transition state complex, is stabilised leading to a high energy barrier for methylation. As expected on the basis of QSAR studies on COMT inhibition (Taskinen *et al.*, 1989, Lotta *et al*., 1992), results of this study showed that binding affinity was increased by electronegative substituents as well. The Hammet σ^- value and MEP explained 94 and 97%, respectively, of the variation in $\log(1/K_m)$ values among the eight 4-substituted catechols. Direct interactions of the catechols with the surroundings of the binding site were elucidated by superimposing the compounds on 3,5-dinitrocatechol in the crystal structure of rat S-COMT. Favourable and unfavourable interactions with the hydrophobic amino acid residues lining the catechol binding site could be observed. In general, hydrophobic substituents increased the affinity, as exemplified by the lowered K_m values of 4-alkylsubstituted catechols and 2,3-dihydroxynaphathlene compared with catechol. The most favourable interactions were formed with 2-hydroxyestradiol that has a flat fused ring structure filling optimally the binding pocket (Fig. 9). The higher K_m values obtained for other compounds with a fused ring structure compared with hydroxyestradiols were obviously due to the unfavourable interactions caused by the ionised amino group. In apomorphine the amino group is in such an orientation that it should not affect the binding. However, its bulky *ortho*-substituent overlaps severely one of the surrounding residues (Trp143). The methyl group of AdoMet is also overlapped, which may cause the observed loss of reactivity. Inhibition of the COMT-catalysed methylation of DHBA by apomorphine revealed that binding of apomorphine is possible, yet conformational changes of the enzyme are required.

The positively charged side chain of catecholamines (e.g. dopamine) has been suggested to interact unfavourably with the hydrophobic amino acids in the active site thereby lowering the affinity (Lotta *et al*., 1995). However, the side chain is flexible and may be in an orientation in which interactions are avoided. In this study, hydrocaffeic acid exhibited almost one order of magnitude lower K_m compared with that of dopamine, although the flexibility of the oppositely charged side chains of these compounds should be similar. Fitting of 27 low energy conformers of dopamine to the active site

revealed that most of them had the amino group overlapping or in contact with the active site residues. In the case of hydrocaffeic acid, however, most of the 31 low energy conformations had the carboxyl group pointing out from the binding site or at least in orientations with fewer contacts with the binding site. Consequently, the allowed or favoured conformations seemed to determine the affinity of compounds with flexible ionised side chains towards S-COMT. The high K_m values of L-dopa and α-methyldopa (Table 8) suggest that compounds that have a crowded side chain with two polar groups exhibit especially unfavourable interactions with the binding site residues.

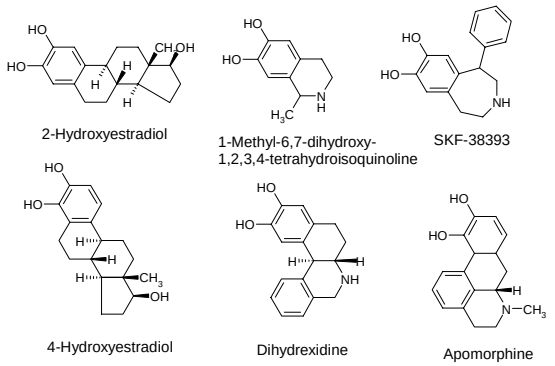


Fig. 9. Structural formulae of compounds with a fused ring system.

A nitro group in 3-nitrocatechol has been reported to increase the binding affinity even more than expected from its strong electronic effect (Taskinen *et al.*, 1989). Accordingly, a six times lower K_m value was obtained for 3-nitrocatechol than for 4-nitrocatechol in this study (0.02 and 0.12 μM, respectively). A nitro group at the 3-position probably exhibits favourable hydrophobic and van der Waal interactions with the binding site. Another *ortho*-substituent that improved affinity was a hydroxyl group; pyrogallol showed a five times lower K_m compared with catechol and the K_m of 5-hydroxydopamine was four times lower than that of 6-hydroxydopamine. Since no group capable of hydrogen bonding is located in the contact distance with the *ortho*-hydroxyl, a plausible reason for the increased affinity may be an exceptionally favourable van der Waals interaction. A carboxyl acid group in the *ortho*-position decreased the affinity by one order of magnitude as exemplified by the K_i and K_m values of 2,3-, and 3,4-dihydroxybenzoic acid (380 and 20 μM, respectively). The carboxylate group fits sterically to the binding site, but the impaired affinity is obviously due to the unfavourable interactions caused by the ionised group. The carboxylate anion may also interfere with the catalytic machinery of the reaction and thereby cause the complete loss of reactivity.

Although the structures of the methylated compounds were not confirmed, suggestion of the preferential methylation site could be derived from the sterical fit of the two alternative binding modes. Tetrachlorocatechol was methylated at a low rate and, in conjunction with 2-methoxy-3-fluorophenol, a small amount of 2-fluoro-6-methoxyphenol was formed from 3-fluorocatechol, which suggests that small substituents can be accommodated in the R6 position. However, *ortho*-substitution seems to direct a catechol preferentially to the binding orientation that results in the methylation of the hydroxyl next to the substituent. Consistently, only one major product was de-

tected for pyrogallol derivatives and other 3-substituted catechols. One metabolite was formed also from 4-hydroxyestradiol, while two metabolites with similar affinity were detected for 2-hydroxyestradiol. The relatively bulky *ortho*-substituent in 4-hydroxyestradiol seems to direct the binding to the orientation that leads to the methylation of the hydroxyl next to it. Different kinds of interactions of the side chain with the binding site residues at different binding modes can explain the regioselectivity of many compounds. For example, catecholamines obviously bind predominantly in the orientation resulting in the *m*-methylation because of less unfavourable interactions in this orientation, as previously suggested by Lotta *et al.* (1995). Compared with the steric factors, the electronic effects of the substituents seem to have a minor contribution to regioselectivity. In studies on ring-fluorinated catecholamines, *ortho*-fluorine has been suggested to facilitate methylation of the adjacent hydroxyl by increasing its ionisation (Firnau *et al.*, 1981, Creveling *et al.*, 1981, Thakker *et al.*, 1986). However, it is now known that electronegative substituents conversely decrease reactivity, and the reason for the altered regioselectivity may be the steric *ortho*-effect discussed above.

5.4.3. Predictive models

The most decisive factor determining the reactivity of a compound was the electronic effect of the substituents on the catechol hydroxyls. The volume of an isoenergy MEP was used to describe the electronic effects, because this parameter can be readily calculated for any substrate. In the case of neutral compounds (n=20), MEP, calculated for the catecholate monoanion exhibiting the lower minimum, could explain over 93% of the variation in the V_{max} values. The correlation clearly deteriorated when compounds containing ionisable side chains were included (r^2=0.705, n=38). However, ionisable groups are expected to affect the acidity of the catechol hydrogens to a small extent only, and the V_{max} of this kind of compound can be predicted to be near as that of unsubstituted catechol. Deterioration of correlation between MEP and V_{max} was probably caused by the poor steric fit of some compounds to the active site. Therefore, for predicting V_{max} both calculation of the electronic effect and modelling of the compound to the active site are required.

Besides the electronic effect of the substituents, hydrophobicity of a catechol appeared to be an important factor in predicting affinity. $LogK_{ow}$ values were calculated by the LOGKOW method, because the method is rapid and has been shown to correlate well with experimental values (Meylan and Howard, 1995). In the two parameters' regression model (2), the $logK_{ow}$ values were calculated for the compounds with their side chain in ionised form.

$$(2)\ \log(1/K_m) = -0.30(\pm 0.03)\ MEP_{vol} + 0.21(\pm 0.03)\ \log K_{ow} + 6.99(\pm 0.23)$$

The correlation between the calculated and observed $\log(1/K_m)$ values was r^2=0.833 (n=38), and the leave-five-out cross-validation gave r^2=0.782. Although the model excludes for example the pronounced unfavourable effects of polar groups caused by steric crowding (e.g. α-methyldopa) and the increased affinity caused by an *ortho*-hydroxyl, the affinity of a catechol may be predicted with reasonable accuracy. Moreover, most steric and conformational effects may be evaluated by fitting the compound to the crystal structure of rat S-COMT.

The predictive models constructed in this study, supported by modelling of the active site, may be utilised in evaluating the interactions between endogenous and exogenous

catechols (e.g. dietary compounds and drugs) and in designing new catecholic drugs with controlled metabolic methylation.

Conclusions

An HPTLC method combining densitometry and radioactivity measurement was developed for the study of the *in vitro* glucuronidation of nitrocatechols. These types of compounds may be excellent substrates of rat liver UGTs, but the position and nature of substituents greatly affect their glucuronidation. Nitrocatechols with large substituents, such as entacapone and tolcapone, exhibited one order of magnitude lower V_{max}/K_m values compared with smaller ones in microsomes from creosote-treated rats. Tolcapone exhibited a two-fold V_{max}/K_m value compared with entacapone. In contrast, entacapone was shown to be a clearly better UGT substrate in human liver microsomes suggesting that the rat is a poor model for predicting the glucuronidation of these compounds in humans. Since both entacapone and tolcapone are rapidly glucuronidated *in vivo*, the 14-fold V_{max}/K_m for entacapone in human liver microsomes may explain some of its approximately seven times shorter elimination half-life. Both compounds, but particularly entacapone, appeared to be excellent substrates of UGT1A9, knowledge which may be utilised in assessing the risks for metabolic interactions. More knowledge on the structure-activity relationships of individual UGT isoforms is required for the evaluation of the structural features turning these COMT inhibitors to such good UGT substrates and causing the differences between them.

A radiochemical HPLC method for the assay of COMT activity towards structurally diverse catechols was developed. Enzyme kinetic parameters were determined for 41 catechols using human S-COMT. Of all the compounds, tetrachlorocatechol and 2,3-dihydroxynaphthalene exhibited the highest V_{max}/K_m values, while hydroxyestrogens were the most specific physiological substrates. Catecholic drugs showed variable methylation ability. For instance among drugs used in the treatment of Parkinson's disease, entacapone and tolcapone were not detectably methylated, L-dopa and carbidopa showed low affinity towards COMT, while benserazide appeared to be a good substrate of COMT. Electronic factors were found to be the most decisive substituent effect determining the reactivity of the catecholic hydroxyls. Electron-withdrawing substituents not only decreased reactivity but also increased affinity. The affinity was improved by hydrophobic substituents as well, whereas hydrophilic side chains deteriorated it. In the case of substrates with flexible side chains the favoured conformations seemed to determine the affinity. As demonstrated with apomorphine, bulky *ortho*-substituents decreased the affinity and reactivity. In contrast, an *ortho*-hydroxyl enhanced the methylation. An *ortho*-substituent generally directed the methylation to the hydroxyl next to it. Predictive models were constructed for reactivity, comprising the electronic effect on catechol hydroxyls, and for affinity, including also a factor describing hydrophobicity. The models may be utilised, supported by modelling of the active site, in evaluating interactions between catecholic compounds or in designing catecholic drugs with controlled metabolic methylation.

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