CATECHOL-O-METHYLTRANSFERASE ACTIVITY: ASSAY, DISTRIBUTION AND PHARMACOLOGICAL MODIFICATION

by

Ilkka Reenilä

Institute of Biomedicine

Department of Pharmacology and Toxicology,

University of Helsinki, Finland

ACADEMIC DISSERTATION

To be presented, with the assent of the Medical Faculty of the University of Helsinki, for public examination in the small auditorium of the Institute of Biomedicine, University of Helsinki, Siltavuorenpenger 10 A, on December 3rd, at 12 noon

Helsinki 1999

Supervised by:

Professor Pekka T. Männistö, M.D.
Department of Pharmacology and Toxicology
University of Kuopio
Kuopio, Finland

and

Professor Raimo K. Tuominen, M.D.
Division of Pharmacology and Toxicology
University of Helsinki
Helsinki, Finland

Reviewed by:

Docent Erkki Nissinen, Ph.D.
Orion Pharma
Espoo, Finland

and

Docent Atso Raasmaja, Ph.D.
Department of Pharmacology and Toxicology
University of Kuopio
Kuopio, Finland

Opponent:

Docent Seppo Kaakkola, M.D.
Department of Neurology
Helsinki Central University Hospital
Helsinki, Finland

ISBN 951-45-8972-6 (PDF version) Helsingin yliopiston verkkojulkaisut Helsinki 1999

To Minna, Karla and Mitro

TABLE OF CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The present study is based on the following original publications which are referred to in the text by Roman numerals (I-V).

I Reenilä I, Tuomainen P, Männistö PT (1995): Improved assay of reaction products to quantitate catechol-O-methyltransferase activity by high-performance liquid chromatography with electrochemical detection. J. Chrom. B, **663**: 137-142

II Reenilä I, Tuomainen P, Tilgmann C, Männistö PT (1995): Opposite effect of ethanol on recombinant membrane-bound and soluble activities of catechol-O-methyltransferase. Pharmacol. Toxicol. **77**: 414-416

III Reenilä I, Tuomainen P, Soinila S, Männistö PT (1997): Increase of catechol-O-methyltransferase activity in rat brain microglia after intrastriatal infusion of fluorocitrate, a glial toxin. Neurosci. Lett **230**: 155-158

IV Reenilä I, Tuomainen P, Soinila S, Tuominen RK, Männistö PT (1999): Catechol-O-methyltransferase activity in primary neuronal and glial cell cultures and its inhibition by novel drugs. Neurosci. Res. Comm. **25**: 71-77

V Odlind C, Göransson V, Reenilä I, Hansell P (1999): Regulation of dopamine-induced natriuresis by the dopamine-metabolizing enzyme catechol-O-methyltransferase. Exp. Nephrol. **7**: 314-322

Also some unpublished data are presented.

ABBREVIATIONS

ALK-PDE	alkaline phosphodiesterase I
ANOVA	analysis of variance
COMT	catechol-O-methyltransferase
DA	dopamine
DDC	dopa decarboxylase
DHBAc	3,4-dihydroxybenzoic acid
DHBAlc	3,4-dihydroxybenzylalcohol
DHBAld	3,4-dihydroxybenzaldehyde
DHBAm	3,4-dihydoxybenzylamine
DMEM	Dulbecco's modified Eagle's medium
DNT	2-(3,4-dihydroxyphenyl)-naphtho-[1,2-*d*]thiazole
DOPAC	3,4-dihydroxyphenylacetic acid
E15-16	embryonal day 15-16
FCS	fetal calf serum
GFAP	glial fibrillary acidic protein
GFR	glomerular filtration rate
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HPLC-EC	high-performance liquid chromatography with electrochemical detection
HPLC-UV	HPLC with ultraviolet detection
HSOC	half saturating outside concentration
HVA	homovanillic acid
IC_{50}	the concentration which causes 50 % inhibition
IVA	isovanillic acid
IVA-CH_3-TFA	isovanillic acid methyl ester trifluoroacetate
IVAlc-TFA	isovanillylalcohol trifluoroacetate
Ivan-TFA	isovanillin trifluoroacetate
K_m	Michaelis constant
L-DOPA	3,4-dihydroxyphenyl alanine
MAO	monoamine oxidase
MAP	mean arterial pressure
3-MB	3-methoxy-4-hydroxybenzylamine
4-MB	4-methoxy-3-hydroxybenzylamine
MB-COMT	membrane-bound catechol-O-methyltransferase
MN	metanephrine
m-MNT	2-(3-methoxy-4-hydroxyphenyl)-naphtho[1,2-*d*]thiazole
P7	postnatal day 7
p-MNT	2-(4-methoxy-3-hydroxyphenyl)-naphtho[1,2-*d*]thiazole
3-MT	3-methoxytyramine
4-MT	4-methoxytyramine
NSE	neuron-specific enolase
3-OMD	3-O-methyldopa
PAH	para-aminohippuric acid
PBS	phosphate buffered saline
PCA	perchloric acid
RP-HPLC	reversed-phase HPLC
RSD	relative standard deviation
SAM	S-adenosyl-l-methionine
S-COMT	soluble catechol-O-methyltransferase
SD	standard deviation
SEM	standard error of mean

TH	tyrosine hydroxylase
VA	vanillic acid
VA-CH_3-TFA	vanillic acid methyl ester trifluoroacetate
VAlc-TFA	vanillylalcohol trifluoroacetate
V_{max}	maximal velocity
Van-TFA	vanillin trifluoroacetate

1. ABSTRACT

Catechol-O-methyltransferase (COMT) is an important enzyme in the metabolism of compounds which have a catechol structure. A high-sensitivity and reliable high-performance liquid chromatography (HPLC) with coulometric detection was developed to analyze the COMT reaction products, vanillic acid and isovanillic acid. Kinetic approximations, using the COMT activity assay with dihydroxybenzoic acid (DHBAc) as a substrate showed that activity was mainly mainly due to S-COMT present in rat brain homogenate. A novel method to analyze COMT activity in cultured cells was developed. By adding DHBAc directly to the viable cells without addition of the intracellular methyl donor S-adenosyl-l-methionine, the localization of COMT inside the cells was confirmed.

The localization of COMT in activated microglial cells was seen after intrastriatal administration of fluorocitrate, a glial toxin. This was shown by comparing COMT activity with microglia/macrophage and astroglial marker enzymes and defining microglial and astroglial cells immunohistochemically. COMT activity in neuronal cells was demonstrated in primary cultured rat brain cells. In regionally discrete cultures, the glial COMT activity was almost equally distributed and usually higher than neuronal COMT activity.

Ethanol (at 1000 mM concentration) inhibited *in vitro* recombinant MB-COMT activity with a mixed inhibition pattern while recombinant S-COMT activity tended to be increased. These minor changes in COMT activity were also seen in striatal homogenate. A trend of decreasing recombinant MB-COMT activity, however, was seen already at 100 mM concentration of ethanol, which is a clinically toxic concentration.

Inhibitors with a nitrocatechol structure, entacapone and tolcapone, decreased COMT activity in rat brain cell primary cultures at nanomolar concentrations while CGP 28014, a hydroxypyridine-type inhibitor of O-methylation *in vivo*, did not affect COMT activity at all. Due to its better permeability, tolcapone was generally a more potent COMT inhibitor than entacapone in glial cell containing cultures. In contrast, neuronal COMT appeared to be slightly more sensitive to inhibition by entacapone and tolcapone, which were equipotent COMT inhibitors in neuronal cell cultures.

In the rat kidney, COMT activity was decreased equally in the cortex, which contained the highest COMT activity, in outer medulla and in papilla *ex vivo* after entacapone administration. The local increase of dopamine by blocking its metabolism with entacapone induced a more profound D_1 receptor-sensitive natriuresis than could be obtained by administration of the precursor, L-DOPA.

2. INTRODUCTION

Catechol-O-methyltransferase (COMT, E.C.2.1.1.6.) was first characterized by Axelrod in 1958 (Axelrod and Tomchick 1958). COMT catalyses the formation of methoxylated products in the presence of Mg^{2+} and S-adenosyl-l-methionine (SAM) from the substrate which must contain a catechol moiety (Fig. 1). During the first two deacades much of the properties of COMT were described, e.g. distribution and biochemical data (Guldberg and Marsden 1975). In the beginning of the 80's, there was more interest to explore the properties of COMT since novel inhibitors of COMT were being developed (Männistö et al. 1992b, Kaakkola et al. 1994). Nowadays, the molecular biology of COMT has been studied, the enzyme has been crystallized and recombinant proteins constructed (Vidgren and Ovaska 1997). Also, the first COMT inhibitors have been introduced in to the clinic where they are used as adjuncts to L-DOPA in the therapy of Parkinson's disease (Dingemanse 1997; Männistö and Kaakkola 1999).

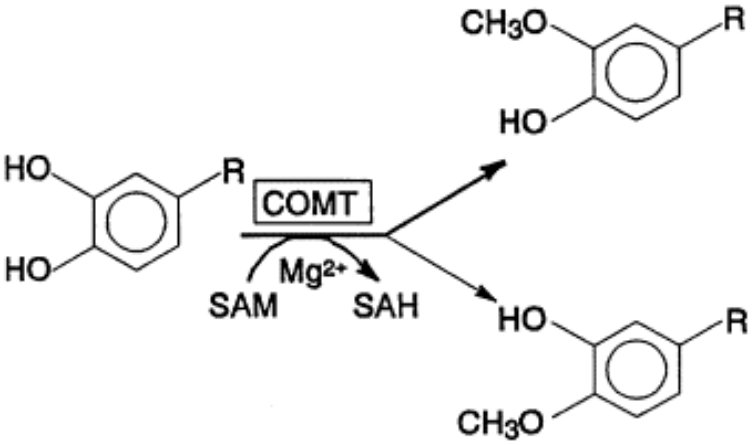

Figure 1. Reaction mechanism of COMT. For DHBAc R = -COOH, SAM = S-adenosyl-l-methionine and SAH = S-adenosyl-l-homocysteine.

3. REVIEW OF THE LITERATURE

3.1. Molecular characteristics of COMT proteins

The COMT gene is localized in chromosome 22q11.2 (Grossman et al. 1992; Winqvist et al. 1992). The COMT gene can produce two mRNA species. The P1 promoter guides the transcription of a shorter mRNA which is included in the longer mRNA sequence expressed by P2 promoter (Tenhunen and Ulmanen 1993; Tenhunen et al. 1994; Tenhunen 1996). The P2 promoter seems to be expressed ubiquitously while P1 is expressed in a differing amounts depending on the tissue (Tenhunen 1996). The nucleic acid sequence of the longer mRNA coding for membrane-bound (MB-) COMT protein contains the shorter mRNA coding for soluble (S-) COMT. The expression of the two mRNA forms does not correlate precisely with COMT protein levels since both MB-COMT and S-COMT proteins are produced from the longer mRNA while S-COMT is produced also from shorter the mRNA (Ulmanen and Lundström 1991; Tenhunen and Ulmanen 1993).

Two alleles of the COMT gene at a single autosomal locus produce S-COMT proteins with three to four-fold difference in activity, at least in human erythrocytes, liver and kidney (Weinshilboum 1988; Boudikova et al. 1990; Grossman et al. 1992). This is caused by a single amino acid substitution of valine to methionine (108 in S-COMT or 158 in MB-COMT) (Lotta et al. 1995; Lachman et al. 1996) which produces low, intermediate and high COMT activities in individuals. Recently, a knockout mouse strain, lacking COMT, has been developed (Gogos et al. 1998).

The recombinant COMT enzymes have been expressed in bacterial and eukaryotic cells. The S-COMT has been expressed in *E. coli* (Lundström et al. 1992), both MB-COMT and S-COMT in *E. coli*, human 293 cells (Malherbe et al. 1992) and in *Spodoptera frugiperda* (Sf9) cells (Tilgmann et al. 1992). MB-COMT contains the S-COMT amino acid sequence with an additional membrane anchor. The molecular weights of recombinant enzymes are 28 kDa and 29-30 kDA for rat and human MB-COMT, respectively, and 25 kDa and 26 kDa for rat and human S-COMT (Bertocci et al. 1991; Lundström et al. 1991; Ulmanen and Lundström 1991; Tilgmann et al. 1992). These values, as well as other properties, correspond well to the enzymes isolated from the natural tissues. With respect to the reported difference between species, the amino acid sequence of S-COMT shares 80 % similarity between rat and man (Lundström et al. 1991) and pigs and humans have 83 % identity as deduced by a partial amino acid sequence interpreted from cDNA (Bertocci et al. 1991). These recombinant COMT proteins have also been used for the crystallization studies (Vidgren et al. 1994).

3.2. COMT activity

3.2.1. Reaction mechanism and kinetics of COMT

Endogenous substrates of COMT include the catecholamine neurotransmitters, i.e. dopamine, noradrenaline and adrenaline, the amino acid L-DOPA and also catecholestrogens. Several exogenous substances and drugs such as benserazide, carbidopa, dobutamine, fenoldopam, isoprenaline, α-methyldopa, rimiterol (Guldberg and Marsden 1975; Männistö et al. 1992b; Kaakkola et al. 1994) and vitamin C (Kern and Bernards 1997) are also metabolized by COMT.

Reaction mechanism. Based on the three-dimensional structure of the active site of COMT, a shallow groove binds SAM, Mg^{2+} and molecules with a catechol structure (Vidgren et al. 1994). Inclusion of an electron-withdrawing group in position 5 of the catechol ring increases the affinity (Taskinen et al. 1989). If the substitution in position 5 is a nitro group, the ligand is not likely to be methylated (only tolcapone is O-methylated and even then only by about 3 %) (Funaki et al. 1994) and is very slowly released. This makes nitrocatechol molecules

very potent as COMT inhibitors. The side chain also affects the affinity. Modeling of the atomic structure has shown that the side chain affects the orientation of the molecule to the active site (Lotta et al. 1995; Vidgren and Ovaska 1997). Apolar and planar substituents in side chain, as in catecholestrogens, have a high affinity since they apparently bind also well to the hydrophobic part adjacent to the active site.

Preliminary kinetic studies with partially purified enzyme preparations have suggested many different reaction mechanisms including rapid equilibrium random-order (Coward and Wu 1973), ping-pong (Borchardt 1973) and sequentially ordered (Rivett and Roth 1982; Tunnicliff and Ngo 1983) reaction mechanisms. Also, a rapid equilibrium binding of Mg^{2+} before SAM and the substrate has been proposed (Jeffery and Roth 1987). However, in the active site of COMT, SAM is bound deeper than Mg^{2+} and the substrate in the hydrophobic pocket (Vidgren et al. 1994). Kinetic analysis with recombinant COMT enzymes have supported a sequential order reaction mechanism, i.e. SAM binds first to the enzyme then Mg^{2+} before the substrate is bound. The release of the molecules follows in the reverse order (Lotta et al. 1995). The reaction mechanism should be the same for both COMT isoforms.

Kinetics. MB-COMT has a higher affinity but lower O-methylation capacity for the catecholamines than S-COMT. The K_m values for the substrates are at least one order of magnitude higher for S-COMT than those for MB-COMT. For example, the K_m values for dopamine are 3.6 μM and 3.3 μM for the rat and human brain MB-COMT, respectively, while the corresponding K_m values for dopamine are 1000 μM and 280 μM for the rat and human brain S-COMT (Rivett et al. 1982; Rivett et al. 1983a). The affinities of recombinant COMT enzymes (15.1 μM and 207 μM for recombinant MB and S-COMT, respectively) are similar to the natural enzymes (Lotta et al. 1995). A widely used exogenic substrate, 3,4-dihydroxybenzoic acid (DHBAc), has similar K_m values, i.e. 30.0 μM and 38.9 μM for MB-COMT and S-COMT, respectively, with recombinant enzymes (Lotta et al. 1995).

There are more than ten fold difference in V_{max} values of natural catecholamines between MB-COMT and S-COMT. Usually, the COMT reaction is performed with substrate concentrations that saturate S-COMT. Thus, the importance of S-COMT on catecholamine metabolism is overestimated when the relative amount of O-methylation by different forms of COMT or by different preparations are predicted (Roth 1992). At lower, more physiologic substrate concentrations, the metabolism through MB-COMT increases due to its high affinity for the substrates. Based on *in vitro* kinetic modeling, at 50 μM concentration of dopamine, monoamine oxidase (MAO) metabolizes 75 %, whrereas MB-COMT and S-COMT both account for 10 % of dopamine metabolism in a human brain homogenate but at lower

concentrations the importance of MB-COMT in relative to other enzymes is emphasized. (Rivett et al. 1982). Rat striatal homogenate has been proposed to contain about 65 μM dopamine concentration (Männistö et al. 1992b; Kaakkola et al. 1994). Due to the high level of vesicular dopamine, only a minor fraction is apparently metabolized.

The importance of MB-COMT is also apparent when whole cells or tissue blocks have been used as an enzyme source at low substrate concentrations (Trendelenburg 1986, 1990). Extracellular concentrations of the substrates which are believed to saturate half of COMT activity are in the low micromolar range, below the saturating concentrations of uptake mechanisms (Guimaraes and Trendelenburg 1985; Trendelenburg 1986, 1990). For example, in cerebral cortex slices, the half saturating outside concentration (HSOC) of COMT for dopamine is 1.75 μM (Wilson et al. 1988). In lung perfusates, also lower HSOCs have been detected, i.e. 9.8 nM and 19.4 nM for noradrenaline and adrenaline, respectively (Bryan-Lluka 1994).

Another fact which must be considered, is the use of V_{max} values obtained from kinetic analyses. Usually the V_{max} values are expressed in terms of the amount of preparation (tissue weight or protein concentration) while the V_{max} values actually reflect the purity of the enzyme preparation (Männistö et al. 1992b) and the amount of enzyme in the analysis is not known. In some studies with COMT, this has been calculated by analyzing the molar concentration of COMT with tight-binding inhibitors in kinetic determinations (Schultz and Nissinen 1989; Lotta et al. 1995; Borges et al. 1997; Vieira-Coelho and Soares-da-Silva 1999).

Solubilization of MB-COMT with detergents seems to affect the kinetic values of COMT (Jeffery and Roth 1984). However, the proposed change in the properties so that it would resemble S-COMT has not been confirmed (Bonifacio et al. 1998).

Regioselectivity. COMT O-methylates either of the hydroxyl groups of the catechol containing molecule. The ratio of O-methylation of meta (3-) position and para (4-) site of the catechol ring (meta/para ratio) could be calculated from *in vitro* results (Creveling et al. 1970). These values are higher with MB-COMT than S-COMT. With rat brain COMT, dopamine has a meta/para ratio of 61.0 and 4.7 for MB-COMT and S-COMT, respectively (Nissinen 1984b). For DHBAc, these corresponding values are 23.7 and 5.1 for MB-COMT and S-COMT. The meta/para ratio is dependent on substrate and reaction conditions, for example being higher with lower concentrations of the substrates (Nissinen 1984b). Para-methylation is hardly ever found *in vivo* (Takahashi et al. 1978).

3.2.2. COMT activity analysis in vitro

COMT activity analyses have been made *in vitro* from purified enzymes, tissue homogenates, cells, tissue blocks and *ex vivo* from tissue samples after a pharmacological

treatment by incubation with a substrate (and possibly with additional Mg^{2+} and SAM). In most *in vitro* cases, COMT activity has been estimated from the amount of COMT derived metabolites after termination of the enzymatic reaction.

Earliest methods. COMT activity was originally analyzed by the decrease of the amount of substrate or formation of reaction product by native fluorescence (Axelrod and Tomchick 1958; Axelrod 1962). Later this fluorometric method was modified by improved derivatization and extraction of the products (Lin and Narasimhachari 1974; Okada et al. 1981). Early assay methods also included the analysis of the reaction product spectrophotometrically in the visible (Herblin 1973; Bade et al. 1974) or UV (Coward and Wu 1973; Borchardt 1974) wavelengths. A pulse polarographic method, a reaction product analysis with carbon paste electrode, was also introduced (Sternson et al. 1976). Gas chromatography has also been utilized in COMT activity analysis (Lin and Narasimhachari 1974; Koh et al. 1991). Radiochemical methods with several variations have also been used extensively. Originally, a radioactive substrate was incubated with the tissue and the isolated radioactive products were measured (Axelrod and Tomchick 1958). Subsequently the analysis performance improved with the use of labelled cofactor ([^{14}C]-SAM) (McCaman 1965; Parvez and Parvez 1972, 1973; Bade et al. 1974; Jonas and Gehrson 1974) which enabled the use of many kinds of substrates and better conditions to extract the labeled reaction product. The sensitivity and simplicity of radioactivity-based COMT activity analysis was additionally improved by the use of [^{3}H]-SAM (Gulliver and Tipton 1978). Later methods enabled the analysis of products from the reaction tube without requiring any separation methods other than the addition of scintillation fluid (Zürcher and DaPrada 1982).

HPLC methods. In the late 1970's, HPLC techniques were introduced to separate the reaction products to take advantage of the high specificity of this technique in COMT activity analysis. The reaction products, separated with ion exchange (Borchardt et al. 1978), normal-phase (Nohta et al. 1984) or reversed-phase (Pennings and Van Kempen 1979) columns, have been detected by fluorometric (Pennings and Van Kempen 1979; Smit et al. 1990), fluorescence (Zaitsu et al. 1981; Nohta et al. 1984, 1986), electrochemical (amperometric) (Borchardt et al. 1978; Shoup et al. 1980; Koh et al. 1981; Nissinen and Männistö 1984; Ishimitsu et al. 1985; Schultz et al. 1989) and radiochemical (Nissinen 1985) detectors.

Modern methods. Table 1. shows some examples of the methods that have been used in the analysis of COMT activity. HPLC methods and radiochemical assays with a variety of substrates are most commonly used. The endogenous catecholamines are good substrates for

COMT. However, in some cases they need to be protected from deamination with MAO inhibitors. DHBAlc (alcohol), DHBAld (aldehyde), DHBAc (acid) (Koh et al. 1991) and a fluorogenic substrate DNT (2-(3,4-dihydroxyphenyl)-naphtho-[1,2-*d*]thiazole) (Nohta et al. 1984) are exogenous substrates which are not usually further metabolized under normal reaction conditions. The determination of meta/para ratio could be used to estimate the presence of each form of COMT in the sample preparation and, with the exception when catechol is used as the substrate, it is available with most of COMT analysis methods.

Handling of the samples after enzyme reaction increases the number of steps where the variation can be introduced into the analysis results. Usually, the reaction products are separated from proteins by centrifugation after addition of concentrated acid, which also protects the reaction products from oxidation. In most uncomplicated radiochemical assays, the scintillation liquid is added directly to the reaction tubes. For the gas chromatographic analyses and usually with fluorometric assays, derivatization of the products is needed. However, the present HPLC methods are usually quite simple and quick to perform (Table 1).

High sensitivity enables the use of low amounts of enzyme preparation or low substrate concentrations. The most sensitive analysis (0.04 pmol/20 µl injection) seems to be the HPLC separation with the use of radioactive substrates (Nissinen 1985). For the radiochemical assay (Zürcher and DaPrada 1982), no comparable value for sensitivity was given, but obviously picomolar concentrations of the reaction products could be analyzed. Also, HPLC linked to fluorescence detection is very sensitive, but requires the synthesis of a fluorogenic substrate (Nohta et al. 1984). With the HPLC-UV system, a more sensitive method utilizing 5,6-dihydroxyindole-2-carboxylic acid with a detection limit of 0.5 pmol/20 µl injection has been presented (Smit et al. 1990). The electrochemical detection systems are also sensitive and suitable for COMT analysis (Table 1).

Table 1. Examples of the different modern methods available for the analysis of COMT activity

Principle	Substrate (concentration)	Products	Handling	Detection limit	Reference
Gas chromato-graphic	DHBAlc	VAlc-TFA, IVAlc-TFA	extraction, evaporation, derivatization	20 pmol/5 µl	Koh et al. 1991
	DHBAld	Van-TFA, Ivan-TFA			
	DHBAc (1-2 mM)	VA-CH_3-TFA, IVA-CH_3-TFA			
Radio-chemical	Catechol (2.7 mM) + ^{3}H-SAM	^{3}H-guaiacol	add scintil-lation liquid	?	Zürcher and DaPrada 1982
HPLC-UV	DHBAc (250 µM)	VA, IVA	precipitation	20 pmol/20 µl	Smit et al. 1990
HPLC-Fluorescence	DNT (20 µM)	m-MNT, p-MNT	extraction	0.05 pmol/20 µl	Nohta et al. 1984
HPLC-Radio-chemical	DHBAc (10-40 µM) + ^{3}H-SAM	^{3}H-VA, ^{3}H-IVA	precipitation	0.45 pmol/20 µl	Nissinen 1984
	^{14}C-DA (10-200 µM)	^{14}C-3-MT, ^{14}C-4-MT		0.04 pmol/20 µl	
HPLC-Electro-chemical	DHBAm	3-MB, 4-MB	precipitation	1.0 pmol/20 µl	Nissinen and Männistö 1984
	DHBAc (400 µM)	VA, IVA			Schultz et al., 1989
HPLC-Electro-chemical	Adrenaline (5-500 µM)	MN	precipitation	0.35-0.5 pmol/20 µl	Vieira-Coelho and Soares-Da-Silva 1996

For abbreviations, see list of Abbreviations.

Due to differences in COMT enzyme sources, substrates, reaction conditions and polymorphism of S-COMT, the activity results vary between different reports. In addition to evaluate the precision and accuracy of the analysis, a validation of all its steps, as has been done with erythrocyte COMT assay (Tuomainen et al. 1996), can identify the possible sources of error.

COMT activity analysis is the most sensitive method to study the presence of COMT in a tissue. In addition, in some cases the activity of both isoforms could be analyzed from the same tissue homogenate or subcellular fractions (Rivett et al. 1982).

3.3. Distribution of COMT

COMT is found in invertebrates and vertebrates (Guldberg and Marsden 1975). In mammals, COMT is distributed in a variety of tissues (Guldberg and Marsden 1975; Roth 1992) which is related to their ability to metabolize the catecholamine neurotransmitters and catecholestrogens as well as xenobiotic compounds.

In catecholamine metabolic pathways, COMT and MAO are the primary enzymes. For example, the major route of dopamine metabolism is first the formation of 3,4-dihydroxyphenylacetic acid (DOPAC) which is further metabolized to homovanillic acid (HVA) (Fig. 2). A minor route is the O-methylation of dopamine to 3-methoxytyramine (3-MT) by COMT. MAO then metabolizes 3-MT to HVA. With certain limitations, 3-MT could be used as a marker of dopamine release (Wood and Altar 1988; Männistö et al. 1992b).

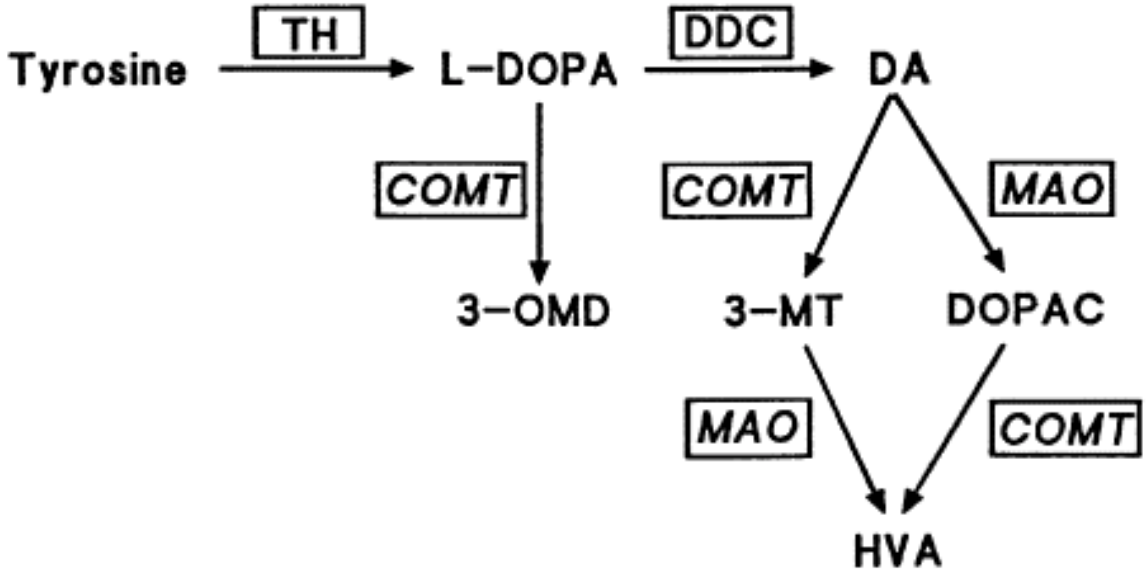

Figure 2. Biosynthesis and metabolism of dopamine. Tyrosine is obtained from dietary proteins. Additional metabolic routes include conjugation reactions and formation of noradrenaline and adrenaline from dopamine. For abbreviations see list of Abbreviations.

COMT activity becomes detectable at 13-15 days of gestation in cells collected from fetal rat brain (Fiszman et al. 1991) and the COMT mRNA is seen on prenatal day 18 in rat kidney (Meister et al. 1993). Thus, COMT seems to have importance already in fetal tissues. After birth, COMT activity is generally increased in various mammalian tissues during growth (Broch 1973; Parvez and Parvez 1973; Goldstein et al. 1980). In aggregate cultures, which contain all types of brain cells, specific COMT activity is also increased similarly as in mouse brain (Seeds 1975). During aging, rat kidney and liver COMT activities have been suggested to decrease (Vieira-Coelho and Soares-da-Silva 1996).

3.3.1. Peripheral COMT

Liver and kidney. The highest COMT activity has been found in liver and kidney. The highest amount of S-COMT in the liver provides the highest O-methylating capacity in the body (Ellingson et al. 1999). The liver is the most important site for the metabolism of circulating catechol containing molecules (Kopin 1985; Männistö and Kaakkola 1999). In addition to the high activity present in the small intestine (Nissinen et al. 1988b), dietary catechols are also metabolized in the liver before they enter in the circulation.

In the kidney, COMT activity (Guldberg and Marsden 1975; Männistö et al. 1992b; Roth 1992; Kaakkola et al. 1994) is related to the local metabolism of dopamine. Dopamine is synthetized from L-DOPA inside the kidney cells (Soares-da-Silva 1994). The highest amounts of dopa decarboxylase (DDC) in the kidney are found in proximal tubular cells. Dopamine is transported from these cells to other sites in the kidney to increase natriuresis via dopamine receptor stimulation (Eklöf et al. 1997) emphasizing the important role of dopamine in sodium homeostasis. Dopamine is preferentially metabolized by MAO (Soares-da-Silva 1994) (Fig. 2) but also COMT inhibitors enhance the local actions of dopamine in the kidney (Hansell et al. 1998). COMT mRNA (Meister et al. 1993) and protein (Kaplan et al. 1979; Karhunen et al. 1994; Weisz et al. 1998) have been detected in proximal tubules, the thick ascending limb of loop of Henle and the collecting duct. With respect to distribution within the kidney, the COMT activity (apparently S-COMT) has been suggested to be higher in cortex than in medulla (Goldstein et al. 1980).

Other sites. Other peripheral extraneuronal sites of COMT can also participate in the metabolism of circulating or local catechols. Several organs, glands, muscle tissues, adipose tissue, blood cells and other tissues contain COMT activity (Guldberg and Marsden 1975). In addition, COMT activity has been detected in other tissues, e.g. small intestine (Nissinen et al.

1988b), lymphocytes (Sladek-Chelgren and Weinshilboum 1981; Bidart et al. 1983), mononuclear cells (Allen and Myers 1992), skin fibroblasts (Breakefield et al. 1981) and melanocytes (Smit et al. 1990). Localization of COMT by immunohistochemical methods (Kaplan et al. 1979; Karhunen et al. 1994) corresponds to activity results and have extended the distribution, e.g. to tissue macrophages (Inoue and Creveling 1986; Inoue et al. 1991). Some of these results have also been confirmed by protein (Tenhunen et al. 1993, 1994; Weisz et al. 1998) or mRNA (Tenhunen and Ulmanen 1993; Tenhunen et al. 1993, 1994) blotting experiments from tissue homogenates.

The presence of peripheral neuronal COMT has been proposed. COMT protein is found in dorsal root ganglion neurons (Karhunen et al. 1996) and COMT activity has been found also in peripheral nerves (Axelrod et al. 1959; Jarrott 1971; Wooten and Coyle 1973) in addition to cultured neuroblastoma cell lines (Blume et al. 1970). However, the major site for metabolism by COMT is extraneuronal (Kopin 1985).

Catecholestrogens. COMT has a role in the metabolism of catecholestrogens, which are 2- and 4-hydroxylated products of estrogens. In principle, competition with catecholamines for the metabolism through COMT locally in tissues (e.g. breast, ovaries and uterus) could be possible, as has been noticed *in vitro* (Ball et al. 1972). Catecholestrogens seem to have importance at least in early pregnancy and in the initiation of some estrogen-dependent tumours (Männistö et al. 1992b; Cavalieri et al. 1997; Weisz et al. 1998; Zhu and Conney 1998). One mechanism could be the suggested regulation of COMT expression by estrogens (Xie et al. 1999). However, in animals, COMT inhibitors with a nitrocatechol structure have not been shown to have any effect on fertility or to be carcinogenic at clinically relevant doses (CPMP 1998). Also, knockout mice without the COMT gene have been reported to be fertile and apparently healthy (Gogos et al. 1998). The role of COMT and catecholestrogens *in vitro* and *in vivo* has not been clarified (Männistö and Kaakkola 1999).

3.3.2. Central COMT

Regional distribution. In the brain, the main function of COMT is to metabolize catecholamines which have escaped from neuronal reuptake after synaptic transmission. COMT activity (Guldberg and Marsden 1975; Roth 1992), protein (Tenhunen and Ulmanen 1993; Tenhunen et al. 1994) and mRNA (Tenhunen and Ulmanen 1993; Hong et al. 1998) are distributed quite evenly between different parts of the brain. Also in glial cell cultures and aggregating cultures, derived from different areas of the brain, the COMT activity did not differ

greatly (Honegger and Richelson 1977; Hansson 1984). The highest amounts of COMT have been found in cerebellum (Rivett et al. 1983b) and ependymal cells of the choroid plexus (Kaplan et al. 1979; Karhunen et al. 1994; Kastner et al. 1994). In spinal cord, neuronal COMT apparently metabolizes noradrenaline (Ekblom et al. 1993; Karhunen et al. 1996). COMT has also been detected in the cells which form the blood brain barrier (Lai and Spector 1978; Hardebo et al. 1980; Baranczyk-Kuzma et al. 1986; Spatz et al. 1986). These cells prevent the passage of catecholamines from the blood into the brain.

The mRNA levels of S-COMT and MB-COMT correlate poorly with the amounts of COMT isoform in brain tissue due to translation of S-COMT from the longer mRNA of COMT. Based on protein blotting data in humans and rats, the amount of MB-COMT is 70 % and 30 %, respectively, of total COMT protein (Tenhunen et al. 1994). However, no approximations of the amount of COMT protein compared to total proteins in the brain have been made.

Cellular localization. Immunohistochemical studies (Kaplan et al. 1979; Karhunen et al. 1995b) have indicated that COMT resides predominantly in glial cells while in neurons COMT is missing or present only at low amounts. COMT activity has also been detected *in vitro* in cultured glial cells, such as primary cultures of astrocytes (Pelton et al. 1981; Hansson 1984) and cell lines such as astrocytomas (Silberstein et al. 1972). In the striatum, the presence of COMT in neurons has been supported by studies with cell-specific toxins. Postsynaptic lesioning with kainic acid decrease the activity of MB-COMT in some studies, while S-COMT activity has been found to increase during the proliferation of astroglial cells (Rivett et al. 1983a; Kaakkola et al. 1987). Kainic acid treatment decreased the amount of 3-MT (Naudon et al. 1992) and increased the reduced HVA levels following the elevated DOPAC concentration in microdialysis experiments (Tokunaga and Ishikawa 1992). These results indicated that 3-MT is formed by MB-COMT in postsynaptic neurons and confirmed the presence of S-COMT in astroglia. The dopaminergic nigrostriatal cells do not seem to possess presynaptical COMT activity (Kaakkola et al. 1987; Karhunen et al. 1995a). In primary cultures of the brain cells (Karhunen et al. 1995b) and in brain tissue studied by immunoelectron microscopy (Kastner et al. 1994; Karhunen et al. 1995a) the immunofluorescence of striatal postsynaptic COMT has been detected. Also protein blotting studies of cultured brain cells have revealed the presence of both isoforms of COMT at about equal amounts in neuronal cells but higher levels of S-COMT than MB-COMT in glial cells (Karhunen et al. 1995b). However, the actual activity data indicating the presence of brain neuronal COMT have not been

demonstrated. Immunohistochemical studies also suggest the localization of COMT to oligodendrocytes (Kaplan et al. 1979; Karhunen et al. 1995b). The present view of the cellular localization of COMT in the striatum is presented in Fig. 3.

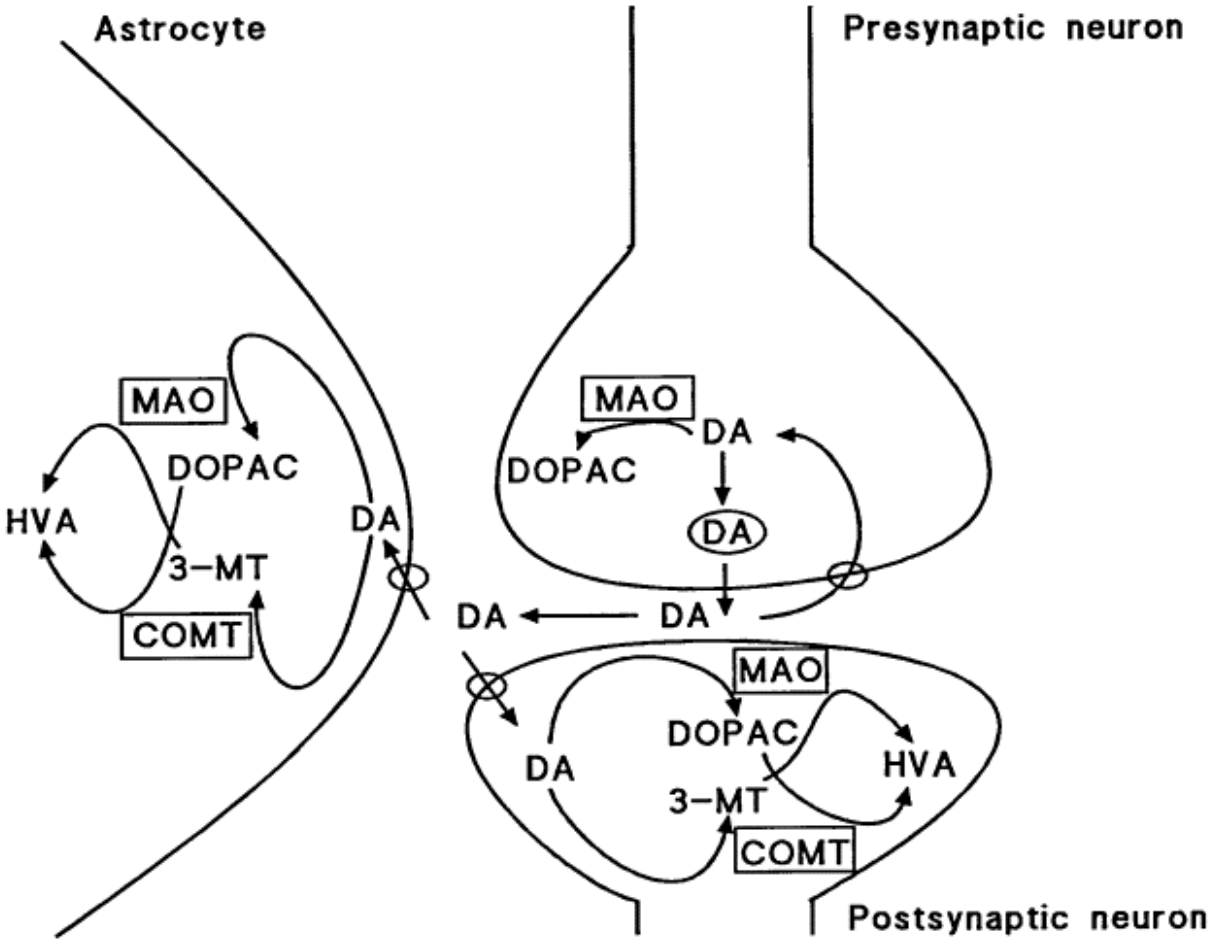

Figure 3. Simplified scheme of the striatal localization of COMT and MAO with respect to dopamine metabolism. Presynaptic neurons arise from substantia nigra and the postsynaptic neurons are intrastriatal neurons or striatal output neurons. The glial cells presumably contain more S-COMT than MB-COMT whereas in postsynaptic neurons both COMT isoforms are thought to be equally present. For abbreviations see list of Abbreviations.

3.3.3. Subcellular localization

S-COMT has been found as a soluble enzyme in the cytoplasm of the cells while MB-COMT has been detected in plasma membrane or ER fractions in subcellular fractionation studies in peripheral tissues (Aprille and Malamud 1975; Raxworthy et al. 1982; Head et al. 1985) and in brain (Broch and Fonnum 1972; Tilgmann et al. 1992). Also, mitochondrial membranes could contain COMT (Grossman et al. 1985; Karhunen et al. 1995a). However, newer results with transfected cells have demonstrated the absence of MB-COMT in plasma membrane and the presence of S-COMT in nucleus (Ulmanen et al. 1997). Interestingly, the

amount of S-COMT protein, as detected with immunohistochemistry and protein blotting, is greatly increased in the nucleus in the hamster model of kidney cancer (Weisz et al. 1998).

3.4. COMT inhibitors

The earliest COMT inhibitors were quite nonspecific with low efficacy and rather high toxicity (Guldberg and Marsden 1975). At present, molecules containing a 5-nitrocatechol moiety, i.e. entacapone, nitecapone (Nissinen et al. 1988a), tolcapone and Ro 41-0960 (Zürcher et al. 1990), have been widely studied and shown to be effective inhibitors at nanomolar concentrations. The IC_{50} values, albeit uncomparable between different laboratories, of 2.2 - 160 nM for entacapone (Nissinen et al. 1992) and tolcapone (Zürcher et al. 1990) with liver and brain COMT have been presented. Nitecapone, entacapone and tolcapone are all tight-binding inhibitors of COMT (Schultz and Nissinen 1989; Lotta et al. 1995; Borges et al. 1997). Entacapone is a peripherally active inhibitor while tolcapone is able to cross the blood brain barrier to some extent (Männistö et al. 1992a). Entacapone and tolcapone are approximately equipotent and equieffective in animal studies *in vivo* (Männistö et al. 1992b; Kaakkola et al. 1994) and *in vitro* (i.e. about equal K_i values, Lotta et al. 1995), although a contradictory report has also been published (DeSanti et al. 1998). CGP 28014, a hydroxypyridine inhibitor of O-methylation, has also been studied (Waldmeier et al. 1990). It behaves quite similarly as tolcapone *in vivo*, but lacks any inhibitory action on COMT *in vitro*. Lately, also a dihydroxyvinyl-type COMT inhibitor has been developed (Perez et al. 1993).

Tolcapone has been claimed to be more selective against MB-COMT at low doses (Borges et al. 1997). When inhibition by tolcapone was tested with equal molar amounts of both COMT isoforms *in vitro*, the potency, i.e. IC_{50} values, were equal (Vieira-Coelho and Soares-da-Silva 1999) suggesting equal inhibition at the active site of both MB-COMT and S-COMT. However, after oral administration of tolcapone, the inhibitory efficacy and potency *ex vivo*, i.e. lower ED_{50} value, was higher with MB-COMT in similar reaction conditions. This suggests that low doses of tolcapone inhibits primarily against MB-COMT (Vieira-Coelho and Soares-da-Silva 1999).

Entacapone and tolcapone have been introduced to clinical use as adjuncts of drug treatment (L-DOPA + decarboxylation inhibitor) in Parkinson's disease to increase the efficacy and tolerability of L-DOPA (Männistö et al. 1992b; Kaakkola et al. 1994). At present, entacapone is available for clinical use. In Parkinson's disease, the number of dopaminergic neurons in substantia nigra is decreased. This leads to a decrease in dopamine levels in striatum which provokes the typical symptoms seen in Parkinson's disease. The inhibition of COMT

activity in the intestine, even exclusively (Nissinen et al. 1988a), and in liver prevents the metabolism of L-DOPA (Nissinen et al. 1992; Zürcher et al. 1990) to 3-O-methyldopa. This methylated metabolite may not simply be a drain on the L-DOPA dose, it may even be harmful (Dingemanse 1997). The increase of the bioavailability of L-DOPA leads to better distribution in the brain where L-DOPA is decarboxylated to dopamine. Therefore, the therapeutic response is improved and the L-DOPA dose can be decreased to reduce the side-effects (Dingemanse 1997).

4. AIMS OF THE STUDY

Although new data has been gathered of the localization of COMT, there are still some discrepancies, especially with respect to the cellular localization of COMT in the brain. Since the novel COMT inhibitors have now achieved clinical use, the properties of COMT still need to be established for safety reasons. Also, other possible agents, i.e. consumed ethanol, could have effects on COMT activity. Although the important role of dopamine in the regulation of sodium homeostasis has been demonstrated, the effects on COMT activity in the kidney in COMT inhibitor-induced natriuresis have not been sufficiently elucidated. The main aims of the present study were to examine the distribution of COMT and the effects of certain agents on COMT activity. In addition, the improvements in bioanalytics provide new possibilities to develop or modify COMT assay methods. More detailed aims were:

1. to characterize a sensitive and reliable method to analyze COMT activity from various enzyme sources, i.e. brain tissue homogenates and recombinant COMT enzymes *in vitro* (I, II, III), in primary cultured glial and neuronal cells *in vitro* (IV) and kidney homogenates *ex vivo* (V)

2. to examine the distribution of COMT utilizing a COMT activity analysis in brain tissue after *in vivo* lesions with a drug (III), *in vitro* in a primary cultured glial and neuronal cells obtained from various regions of the brain (IV) and in regions of the kidney tissue *in vitro* (V)

3. to investigate the effect of ethanol on COMT activity *in vitro* by using the most pure forms of COMT enzymes, the recombinant MB-COMT and S-COMT enzymes (II)

4. to compare the efficacy and potency of COMT inhibitors in primary cultured brain cells (IV)

5. to observe the consequences of COMT inhibition on COMT activity of dissected kidney homogenates and compare the COMT inhibitor-induced natriuretic effect with administration of dopamine precursor (V).

5. MATERIALS AND METHODS

5.1. COMT enzyme sources (I-V)

With the exception of the kidney studies (V), Wistar rats (Han/Kuo, Institute of Biomedicine, University of Helsinki) were used in the experiments (I-IV). The rats were housed in 12 h light and dark cycles (lights on at 7 a.m.). Normal laboratory pellets and tap water were available *ad libitum*. For the intrastriatal infusion studies (III) male rats weighing 200-250 g were used, otherwise both genders were used. As recombinant COMT enzymes (II), a 100 000 x g pellet from baculovirus-infected Sf9-cells (Tilgmann et al. 1992) and a lysate of *E. coli* (Lundström et al. 1992) for MB-COMT and S-COMT, respectively, were used. The glial cell cultures were obtained from one-week old (postnatal day 7, P7) rats and neuronal cultures from fetuses at 15-16 gestational day (embryonal day 15-16, E15-E16) (IV). For the kidney studies (V), regions of the kidneys and the whole brains were obtained from male WKY rats (265 ± 1.7 g, Möllergaard Breeding Center, Copenhagen, Denmark).

5.2. Methods

5.2.1. Handling of the COMT enzyme sources

The brains of the decapitated rats were cooled in liquid nitrogen, dissected and stored at -80^{o}C before enzyme analysis (I-III). The tissues were homogenized by sonication in 10 mM sodium phosphate or 3-(N-morpholino)-propanesulfonic acid (MOPS) buffer, pH 7.4, containing 0.5 mM dithiothreitol (DTT) and centrifuged 900 x g for 10 min. Supernatant, which contains both MB-COMT and S-COMT, was used as enzyme source. The two halves of the whole brains and pieces of the kidneys from each side of the rat were sliced with razor blade before homogenization (V). The suspension buffer for the MB-COMT pellet (II) contained additional 5 mM $MgCl_2$. Sucrose (0.32 M), occasionally included in homogenization buffer, did not affect the enzyme activity.

5.2.2. COMT reaction and activity analysis

The COMT reaction was based on a previous report (Nissinen and Männistö 1984) utilizing DHBAc as a substrate (Schultz et al. 1989) instead of dihydroxybenzylamine, which needed purification before enzyme reaction (Nissinen and Männistö 1984). DHBAc concentration (240 μM) used routinely in the COMT assay was 6 times higher than the K_m for MB-COMT and half-saturating for S-COMT preparations obtained from rat brain (Nissinen 1985). For calculational convenience, 200 μM concentration of DHBAc was used with recombinant enzymes (II). Double the amount of enzyme preparation was used to detect lower

amounts of COMT to be used in the other studies not presented here. Routinely, the enzyme preparation (100 µl) was incubated for 30 min at 37°C with 100 mM sodium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 200 µM SAM and DHBAc as a substrate in 250 µl of total volume. After incubation, the reaction was terminated with ice-cold perchloric acid (PCA, 4 M, 25 µl) and centrifuged for 5530 x g at 4°C for 10 min. The supernatants were injected to HPLC for vanillic acid (VA) and isovanillic acid (IVA) analysis. The kidney samples (V) and cell culture samples (IV) were filtered through 0.45 µm polyvinyldifluoride (PVDF) filter (Millipore, Japan) before HPLC analysis. Routinely, samples without enzyme and samples without substrate were run as blanks. Reaction with kidney tissues was made at the same protein level as brain homogenates, but due to the high activity, the reaction products were diluted (1:10-1:20) with homogenizing buffer before HPLC analysis.

Aliquots (usually 10 µl) of the samples were injected (Waters 712 Wisp autosampler with cooler) into a HPLC system which consisted of an isocratic pump (Waters Model 6000 A or Waters 510, Waters Association, Millford, MA, USA) and a LiChrospher 100 RP-18 column (5 µm, 125 x 4 mm, I.D., Merck, Darmstadt, Germany) with precolumn. The reaction products were detected with ESA coulometric detector 5100 A (gain 40 x 100, ESA Inc., Bedford, MA, USA) with analytical cell 5011, potential set to +0.10 V (detector 1), -0.30 V (detector 2) and a conditioning cell set to +0.40 V. The current response of detector 2 was recorded with a Hewlett Packard 3396 Series II integrator (Palo Alto, CA, USA). The mobile phase, 0.1 M Na_2HPO_4, pH 3.2, 0.15 mM EDTA and 15 % (vol/vol) methanol, was used at 1.0 ml/min flow rate.

5.2.3. Other biochemical analyses

Protein concentration. The protein content was analysed spectrophotometrically (Ultrospec III, Pharmacia LKB Biotechnology, Uppsala, Sweden) using the Bradford method (Bradford 1976) and bovine serum albumin (BSA) as a standard.

MAO B. MAO B activity (deamination of benzylamine to benzaldehyde) was used as a marker for astroglia (III) (Francis et al. 1985). The reaction was started by incubating 50 µl of the enzyme preparation with 140 mM sodium phosphate buffer, pH 7.2, and 200 µM benzylamine in total volume of 250 µl for 30 min at 37°C as described earlier (Nissinen 1984a). After addition of 4 M PCA (25 µl) and centrifugation the supernatant was analyzed with RP-HPLC (Hewlett-Packard 1084 B) equipped with LiChroCART 125-4 column (5 µm x 4 mm ID, Merck, Darmstadt, Germany). The reaction product benzaldehyde was detected with a variable

UV-detector at 245 nm built-in the HPLC system. The mobile phase was 50 mM Na_2HPO_4, pH 3.2, 1 mM heptanesulphonic acid and 40 % (vol/vol) methanol with a 1.2 ml/min flow rate. The limit of detection was 6 pmol/30 µl injection, the intra-assay and interassay variation was less than 15 % and less than 10 %, respectively.

TH. Tyrosine hydroxylase (TH) activity (hydroxylation of tyrosine to L-DOPA) was used as a marker for dopaminergic neurons (III). The enzyme reaction was based on a previous report (Naoi et al. 1988). The enzyme preparation (20 µl) was incubated with 100 mM sodium acetate buffer, pH 6.0, 10 mM $(NH_4)_2Fe(SO_4)$, 1 mM dl-6-methyl-5,6,7,8-tetrahydropteridine and 100 µM tyrosine in 250 µl of total volume for 10 min at 37°C. After addition of 4 M PCA (25 µl) and centrifugation, the reaction product L-DOPA was analyzed with the same RP-HPLC system as MAO B utilizing fluorescence spectrometer (Model LS-5, Perkin Elmer Ltd., Buckinghamshire, UK) at 281 nm excitation and 314 nm emission wavelength (Mandai et al. 1992). The mobile phase was 0.1 M H_3PO_4, pH 3.00, 20 mM citric acid, 0.15 mM Na_2EDTA, 1 mM octanesulphonic acid and 10 % (vol/vol) methanol with flow rate of 1.0 ml/min. The limit of detection was 6 pmol/30 µl injection, the intra-assay and interassay variation was less than 15 % and less than 20 %, respectively.

ALK-PDE. Alkaline phosphodiesterase I (alk-PDE) activity (formation of p-nitrophenol from p-nitrophenyl-thymidine-5'-phosphate) was used as a marker for macrophages/microglia (Morahan et al. 1980). The enzyme reaction was based on a previous report (Storrie and Madden 1990). The enzyme preparation (35 µl) was incubated with 200 mM Tris-HCl buffer, pH 9.0, 20 mM $MgCl_2$, and 5 mM p-nitrophenyl-thymidine-5'-phosphate in a total volume of 250 µl. After 10 min at 37°C, 0.5 M glycine-Na_2CO_3 was added (700 µl) and the reaction product p-nitrophenol was analyzed spectrophotometrically (Ultrospec III, Pharmacia LKB Biotechnology, Uppsala, Sweden).

5.2.4. Validation of the HPLC analysis of COMT reaction products (I)

The specificity, linearity, limit of detection, limit of determination, precision and accuracy for the determination of the reaction products were performed. For the enzyme reaction, the effects of protein concentration for the brain tissue and incubation time for the MB-COMT preparation were analyzed.

5.2.5. The effect of ethanol on COMT activity in vitro (II)

Ethanol (25-1000 mM) was incubated without preincubation with recombinant MB-COMT and S-COMT preparations and also in striatal homogenates. The effect of 1000 mM ethanol on the kinetic values (K_m and V_{max}) were determined with recombinant MB-COMT and S-COMT enzymes at DHBAc concentrations of 12.5-300 μM and 25-500 μM for recombinant MB-COMT and S-COMT, respectively.

5.2.6. Intrastriatal stereotaxic infusion (III)

The rats were anesthetized with chloral hydrate (350 mg/kg, i.p., 1.0 ml/kg) and placed in a David Kopf stereotaxic apparatus. Through a burr hole, an injection needle was lowered in the brain through a guide cannula to the final coordinates of +0.7 anterioposterior, ±3.0 lateral and -5.5 dorsoventral from bregma (Paxinos and Watson 1982). One or two μl of DL-fluorocitrate (right side of the striatum) and vehicle (left side) were infused bilaterally. After one, two or three days, COMT, MAO B, TH and alk-PDE activities were analyzed from the striatal homogenates. Immunohistochemical stainings with COMT, glial fibrillary acidic protein (GFAP, astroglial marker), TH (dopaminergic neuron marker) antiserums and OX-42 antibody (microglial marker) were carried out on days one and three.

5.2.7. Cell cultures and COMT enzyme reaction (IV)

The cultures were prepared as described previously (McMillian et al. 1997). The brain regions of P7 or E15-E16 rats were dissected and the cells were dissociated at ambient temperature by trituration in a Ca^{2+}-Mg^{2+} free buffer (145 mM NaCl, 5.4 mM KCl, 1 mM NaH_2PO_4, 11.2 mM glucose and 15 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) [HEPES] buffer pH 7.4 containing 133 U/ml penicillin and 133 μg/ml streptomycin). The cells, collected by centrifugation, were suspended in Dulbecco's modified Eagle's medium (DMEM)/F12 medium containing 10 % fetal calf serum (FCS), 0.12 % $NaHCO_3$, 100 U/ml penicillin, 20 μg/ml streptomycin, 71.3 μg/ml amikasin and phenol red. The cells were plated on 24-well cell culture plates (Greiner, Germany). The glial cells alone were grown for 35-41 days and the microglia were removed by shaking for 4 h before COMT assay. For neuron-enriched cultures, the culture wells were coated with polylysine (100 μg/ml) before plating at 100 000 neurons per well. The neuronal cultures were grown for 1 or 6-7 days or 7 days when plated on top of striatal or hypothalamic glial cells which were grown for 18-30 days. The growing media were changed weekly.

For the analysis of COMT activity, an artificial cerebrospinal fluid buffer (CSF) (Törnwall et al. 1994) was used. This Krebs-Ringer buffer contained 147 mM Na^+, 3.5 mM K^+, 1.0 mM Ca^{2+}, 1.2 mM Mg^{2+}, 129 mM Cl^-, 1.0 mM PO_4^{3-} and 25 mM HCO^{3-} supplemented with 1.25 g/l glucose and gassed with O_2/CO_2 (95%/5%) to pH 7.4. Based on pilot studies, the reaction conditions (60 min incubation at 37°C) and 400 µM substrate concentration were estimated to produce adequate COMT activity levels. Since 10 min preincubation of nitecapone in tissue homogenates produced a sufficient inhibitory effect (Schultz and Nissinen 1989), a 15 min preincubation time was chosen. The cells were washed twice with Krebs-Ringer buffer and preincubated with entacapone, tolcapone or CGP 28014. The substrate was added and after incubation the plates were moved on ice and the media were collected. To 200 µl sample of the medium, 20 µl of 4 M PCA was added and the sample was treated as with COMT enzyme reaction sample. The cells were scraped and collected with a plastic pipette in Krebs-Ringer buffer for protein analysis.

5.2.8. Immunohistochemistry (III, IV)

For the tissue immunohistochemical studies (III), anesthetized (sodium pentobarbital 45 mg/kg, i.p.) rats were perfused with 4 % paraformaldehyde (250 ml) and postfixed. Ten µm sections were air-dried and washed with phosphate bufferd saline (PBS). The specimens were incubated with non-immune swine serum before addition of the primary antibody against COMT (1:200 dilution), OX-42 (Graeber et al. 1989) (1:100), GFAP (1:50) or TH. After overnight incubation at 4°C, the specimens were incubated with secondary antibody (1:200 dilution) conjugated with rhodamine or fluorescein and examined with fluorescence microscope (Leitz Aristoplan).

Specimens of the cell cultures were plated on polylysine coated glass cover slips and grown on cell culture dishes analogously as cell cultures (III). The cultures on cover slips were fixed with 4 % paraformaldehyde in 100 mM PBS, pH 7.4, for 15 min at room temperature. The cover slips were rinsed and permeabilized with 0.1 % Triton X-100 in PBS and incubated with 5 % normal horse serum. After overnight incubation at 4°C with the primary antibody against GFAP (undiluted) or neuron specific enolase (NSE, 1:50), the secondary antibody (biotinylated rabbit anti-mouse IgG, 1:250 dilution) was added. After 1 h incubation with avidin-biotin-peroxidase complex, the slips were inverted on a drop of glycero-Na-veronal mixture on an object glass, and examined with a Leica DMLS microscope.

5.2.9. Effect of entacapone on kidney COMT activity and function (V)

For the distribution of COMT in kidney regions (cortex, outer medulla and papilla) COMT activity was analyzed *ex vivo* with or without entacapone treatment after 2 h and 3 h. The maximal natriuretic effect has been reached within 2 h after 30 mg/kg (i.p) entacapone dose (Hansell et al. 1998) and the inhibition of COMT activity has been suggested to last for 3-4 h after the same dose of nitecapone (administered by gavage) (Eklöf et al. 1997). To assess the possible role of brain COMT on natriuretic effect, the whole brain COMT activity was measured 1 h and 3 h after entacapone treatment. For the effects of dopamine on kidney function (Hansell et al. 1998) anesthetized rats were given *1)* vehicle, *2)* entacapone (30 mg/kg, i.p.), *3)* entacapone + SCH23390 (30 µg/kg/h, i.v.) *4)* entacapone + sulpiride (300 µg/kg/h, i.v.) , *5)* L-DOPA (60 µg/kg/h, i.v.) and *6)* L-DOPA + SCH23390. The urinary concentration of sodium, dopamine and DOPAC were analyzed. Mean arterial pressure (MAP), glomerular filtration (GFR) and renal plasma flow were also measured (V).

5.3. Reagents

Ethanol was from Alko Ltd. (Helsinki, Finland). Fluorocitrate, purchased from Sigma (St. Louis, MO, USA), was prepared as described earlier (Paulsen et al. 1987). Entacapone (OR-611, N,N-diethyl-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl) acrylamide), tolcapone (Ro 40-7592, 3,4-dihydroxy-4'-methyl-5-nitrobenzophenone), CGP 28014 (N-(2-pyridone-6-yl)-N',N'-di-n-propylformamidine), a gift from Orion Pharma (Espoo, Finland), were dissolved in a small amount of dimethylsulfoxide (DMSO) and diluted with water. Thiobutabarbital (5-ethyl-(1-methyl-propyl)-2-thio-barbiturate sodium, Inactin^{R}) was from Research Biochemicals International (Natick, MA, USA), [^{3}H]methoxyinulin and 4-aminohippuric acid (PAH) were obtained from Merck (Darmstadt, Germany). SCH23390 was purchased from Schering Corp. (Kenilworth, NJ, USA) and sulpiride from Ravizza (Milano, Italy). S-adenosyl-l-methionine iodide (SAM), 3,4-dihydroxybenzoic acid (DHBAc), vanillic acid (3-methoxy-4-hydroxybenzoic acid), isovanillic acid (4-methoxy-3-hydroxybenzoic acid), tyrosine and L-DOPA were from Sigma. Ultrapure reagent-grade water was obtained with a Milli-Q system (Millipore/Waters, Millford, MA, USA). Solvents (methanol) were HPLC-grade (Rathburn, Walkenburg, UK) and other HPLC chemicals were analytical-grade (Merck). DMEM/F12 medium, HEPES and additives in cell cultures were purchased from Sigma. FCS was from Boehringer Mannheim Biochemicals (Germany). OX-42 monoclonal antibody was obtained from Pharmingen (San Diego, CA, USA). Neuron specific enolase (NSE) was from Chemicon (Temecula, CA, USA), rabbit antimouse IgG was obtained from Vector. Benzaldehyde and benzylamine were from Fluka Chemie AG (Buchs, Switzerland).

5.4. Calibration and calculation

For each HPLC run, the method was calibrated with 7-8 calibration samples (COMT: 0.01-2.0 μM VA and IVA, MAO B: 0.2-50 μM benzaldehyde and TH: 0.2-50 μM L-DOPA). By using the calibration curve, obtained from linear regression of the peak heights of the calibration samples, the concentrations of the samples were calculated from the peak-height values of the samples (Quattro Pro, Borland International, Scott Valley, CA, USA).

5.5. Statistical analysis

The effects of ethanol (0-1000 mM) (II) or drugs (IV, V) were analyzed with one-way analysis of variance (ANOVA) followed by Tukey's test. Enzyme kinetic comparisons (II) and the effect of fluorocitrate treatment (III) were calculated with paired t-test (Systat Intelligent Software, Systat Inc., Evanston, IL, USA). Kinetic values (K_m and V_{max}) were computed using statistically weighed estimates with bilinear regression (Wilkinson 1961).

6. RESULTS

6.1. COMT activity analysis (I-V)

Vanillic and isovanillic acid were separated well with RP-HPLC using coulometric detection and no interfering peaks were seen. Due our excellent detection capabilities, both reaction products could be seen at low substrate concentrations (Fig. 4). Reproducibility of the analysis was tested for the reaction products. A summary of the characteristics is presented in Table 2. Compared to earlier method utilizing amperometric detection (Nissinen and Männistö 1984), the limit of detection was 10 times lower with only half of the injection volume. In the studied concentration range, the reaction product analysis was linear with less than 10 % variation in precision and accuracy. The precision of the analysis decreased when the same sample was analyzed on subsequent days and additionally when the reaction was made from the same homogenate pool and finally the lowest precision (RSD 37.8 %) was seen when different tissue samples were analyzed. The meta/para ratio calculated for the striatal homogenates was 6.3 (I) and 8.6 (II) suggesting preferential metabolism through S-COMT rather than MB-COMT since at the same reaction conditions the meta/para ratio was closer to that obtained with recombinant S-COMT than that of recombinant MB-COMT (II). In the WKY rats used in the kidney experiments (V) the specific

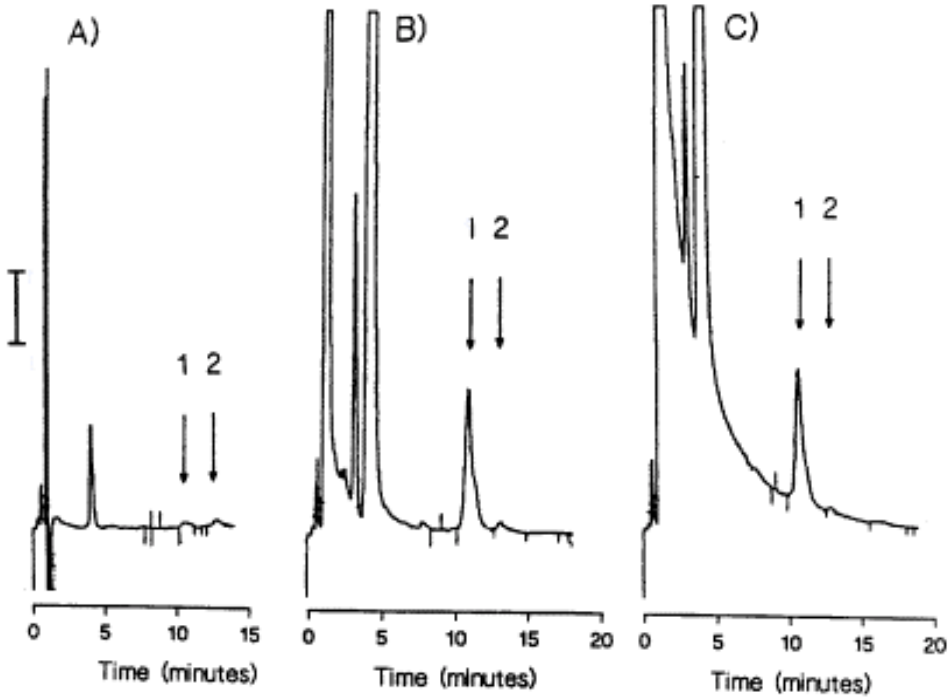

Figure 4. Chromatograms of A) 0.1 pmol calibration sample (10 µl injection), reaction products obtained from B) recombinant MB-COMT (5 µl injection) and C) recombinant MB-COMT assayed with 1000 mM concentration of ethanol (10 µl injection). The substrate (DHBAc) concentration was 12.5 µM. Peaks: 1=vanillic acid and 2=isovanillic acid. The bar at y-axis denotes 10 nA.

Table 2. Summary of the validation of COMT activity analysis by reversed phase high-performance liquid chromatography with coulometric detection (I). The results are mean ± SD.

	Vanillic acid	Isovanillic acid	(n)
Limit of detection	0.1 pmol/10 µl	0.1 pmol/10	
Linearity:			(13)
Slope	0.00945 ± 0.0032	0.00716 ± 0.0025	
Y-intercept	0.00206 ± 0.0090	0.00246 ± 0.0063	
Range	0.5 - 20 pmol/10 µl	0.5 - 20 pmol/10 µl	
Limit of quantitation	0.5 pmol/10 µl	0.5 pmol/10 µl	
Precision	0.28 - 6.6 %	0.58 - 9.9 % (9-14)	
Accuracy	-0.47 - 2.9 %	-0.92 - 2.0 % (10-14)	
Within-day:			(5-8)
Precision	0.65 %	2.8 %	
Accuracy	6.7 %	5.68 %	
Between-day-precision:			
Recombinant MB-COMT	10.4 %	14.9 %	(14)
Striatal sample	1.62 %	2.93 %	(8)
Striatal tissue pool	10.7 %	9.4 %	(4)
Striatal tissues	45.8 ± 17.3[a]	6.26 ± 2.90[a] (7)	

[a] pmol/min/mg

COMT activity in the whole brains was 8.52 ± 0.15 pmol/min/mg, which is about one fifth of that in striatal homogenates of the Wistar rats used in other studies (I-III). The brain and kidney specific COMT activities were lower than those of isolated S-COMT but higher than MB-COMT. For example, the specific activites of 86.6 pmol/min/mg protein and 16.5 pmol/min/mg protein for rat brain S-COMT and MB-COMT have been reported (Nissinen 1985). This was apparently due to the use of the lower substrate concentration and unpurified the COMT enzyme preparation. Meta/para ratios were about 2.5.

Kinetics. Kinetic values for the formation of vanillic acid were determined for the recombinant MB-COMT and S-COMT enzymes (II). Apparent K_m values were 27.2 ± 1.4 µM

and 136 ± 11 μM for recombinant MB-COMT and S-COMT, respectively. The corresponding V_{max} values, expressed as μM product formed in 30 min, were 1.8 ± 0.2 and 4.6 ± 1.4. These values agree well with the fact that recombinant MB-COMT has a higher affinity but lower methylation capacity than recombinant S-COMT. The meta/para ratios decreased non-significantly with recombinant MB-COMT from 19 to 13 with increasing substrate concentrations (12.5-300 μM of DHBAc concentration) and remained the same with recombinant S-COMT (from 5.2 to 5.5 with 25-500 μM of DHBAc concentration).

Cell cultures. The analysis of the COMT reaction products from cell culture studies (IV) was performed in a similar way. Artificial CSF with glucose supplement was used since the cell culture media produced background in the chromatograms. The COMT inhibitors did not interfere with the detection system. The reaction with increasing concentrations (12.5-400 uM) of DHBAc was in most cases linear with glial and cocultures (data not shown). Generally, the production of isovanillic acid was below the detection limit and could not be analyzed. A few meta/para ratios suggested a high value (more than 20) which could indicate that most of the metabolism was carried out by MB-COMT compared to S-COMT.

6.2. Distribution of COMT (III-V)

Lesion studies. Intrastriatal infusion of fluorocitrate, a glial toxin, at 4 nmol dose started to decrease insignificantly striatal COMT activity after 12 h (Fig. 5A) decreasing further at 24 h and 48 h (19 % and 24 %, respectively) (III). The two nmol dose followed insignificantly the same pattern. Surprisingly, after 72 h COMT activity increased with both 2 and 4 nmol doses of fluorocitrate infusion (62 % and 73 % respectively). The meta/para ratio was changed by +30 %, +4% and -7 % after 24 h, 48 h and 72 h, respectively, at 2 nmol dose of fluorocitrate while at 4 nmol dose of fluorocitrate the meta/para ratio was decreased by 3-8 % at the these timepoints. None of these changes were statistically significant. The control meta/para ratios (mean ± sem) with the 2 nmol dose of fluorocitrate were 9.4 ± 1.8, 11.1 ± 2.9 and 8.4 ± 0.91 for 24 h, 48 h and 72 h, respectively, and the control ratios (mean ± sem) with the 4 nmol dose of fluorocitrate were 8.3 ± 0.6, 7.5 ± 0.46 and 8.4 ± 0.45 for 24 h, 48 h and 72 h, respectively. MAO B activity, a marker for astroglia, remained below control levels more predictably with the 2 nmol dose of fluorocitrate throughout the studied period. Alk-PDE activity, a marker of macrophages/microglia, was increased significantly with the 4 nmol dose of fluorocitrate at 48 h and at 72 h with both doses of fluorocitrate. TH activity, a dopaminergic neuronal marker, gave

variable results and was not affected significantly by fluorocitrate during the study (Fig. 5B). The control values for specific TH activity were (mean ± sem) 336.8 ± 78.9, 388.7 ± 28.3 and 382.4 ± 54.7 pmol/min/mg protein at 24 h, 48 h and 72 h, respectively, with the 2 nmol dose of fluorocitrate and with the 4 nmol dose of fluorocitrate 508.6 ± 34.6, 615.3 ± 97.4 and 553.7 ± 47.7 pmol/min/mg protein at 24 h, 48 h and 72 h, respectively (n=5-20).

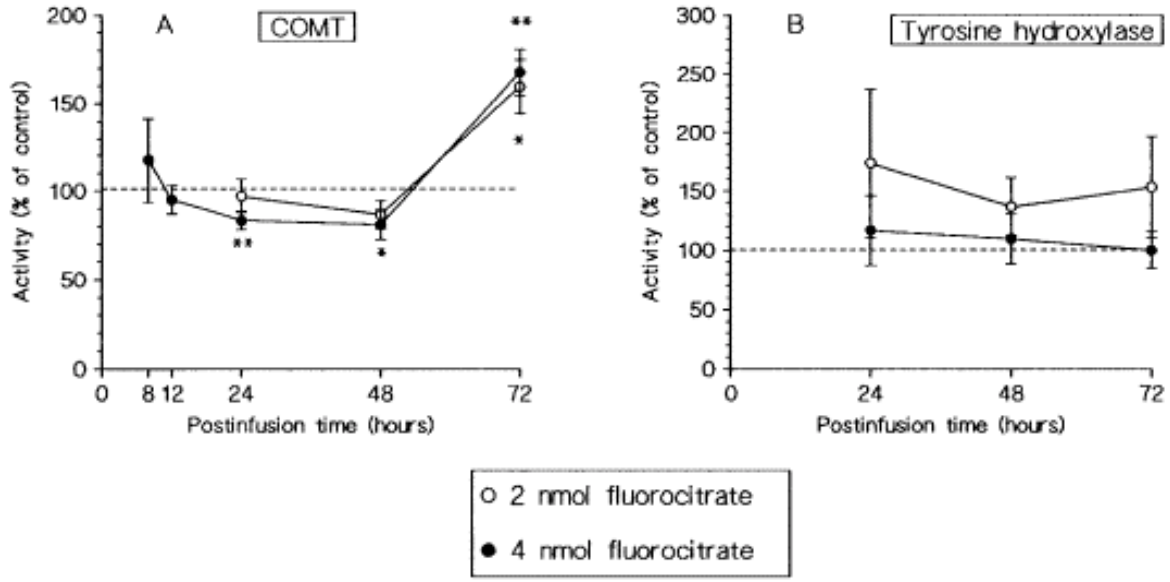

Figure 5. Time course of striatal enzyme activities after intrastriatal infusion of fluorocitrate. A) COMT activity (modified from Fig. 1, III) and B) tyrosine hydroxylase activity. Mean values and sems are presented. Individual specific activities were compared with control side and calculated with paired t-test, * $p<0.05$, ** $p<0.01$, n = 3-28.

Immmunohistochemical analysis of the toxin treated rat striata (III), revealed a distinct staining pattern by TH and GFAP (astroglial marker) antisera in control sides of the striata while COMT staining was low and inconclusive with respect to a definitive cellular localization. No OX-42 (microglial marker) immunoreactivity was observed. Fluorocitrate, especially 72 hours after the infusion, caused a decrease of TH and GFAP immunoreactivities in the injection region and an increase of distinguishable COMT reactivity which colocalized with OX-42 in double staining. Further away from the injection site, TH staining was increased while GFAP staining was comparable to control stainings.

Cell cultures. Primary brain cell cultures (IV) were partially characterized by using immunohistochemistry with antiserums against GFAP, an astroglial marker, and against NSE, a neuronal cell marker. The amount of immunopositive cells in a culture was classified and scored from 0 to 5. The ratios expressed in (IV) were calculated from the means of the results shown in Fig 6. All the glial cultures were immunoreactive with GFAP. In neuronal

cultures, 1-day basal forebrain was the most neuron-enriched. The number of GFAP positive cells increased during growth from 1 to 6-7 days indicating glial proliferation. In glial/neuronal cocultures, the immunoreactivity was so intense that no quantification could be done. Approximately half of cells were of glial and half were of neuronal origin.

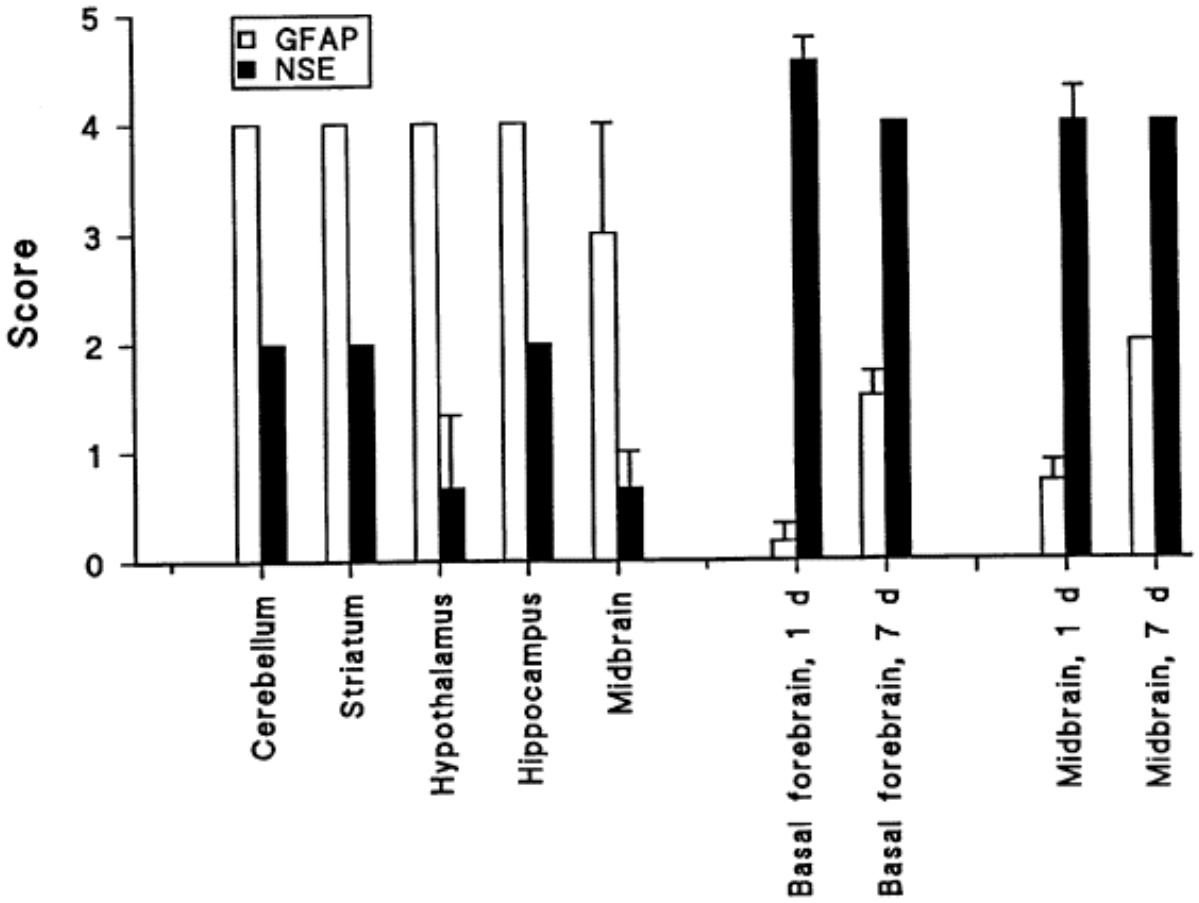

Figure 6. Immunohistochemical characterization of rat brain primary cultured cells. The number of GFAP or NSE stained cells were scored from 0 (no or low amount of stained cells) to 5 (all or almost all cells stained) and the mean + sem for each culture type was calculated, n = 1-7.

The basal COMT activities were similar as found in other studies with striatal tissues (I-III). Glial cells, prepared from various parts of the rat brain, displayed similar COMT activity indicating about equal distribution between different parts of the brain (Fig. 7). Cerebellar glial cultures, which had the highest COMT activity, differed from both 1-day neuron-enriched cultures and from both glial/neuronal cocultures. In other glial cultures, a partial glial dominance of COMT activity over neurons was also found compared to basal forebrain neuron-enriched cell cultures. COMT activity in striatal and hypothalamic glial cultures, which did not differ from each other, was higher than in 1-day basal forebrain neuron-enriched culture.

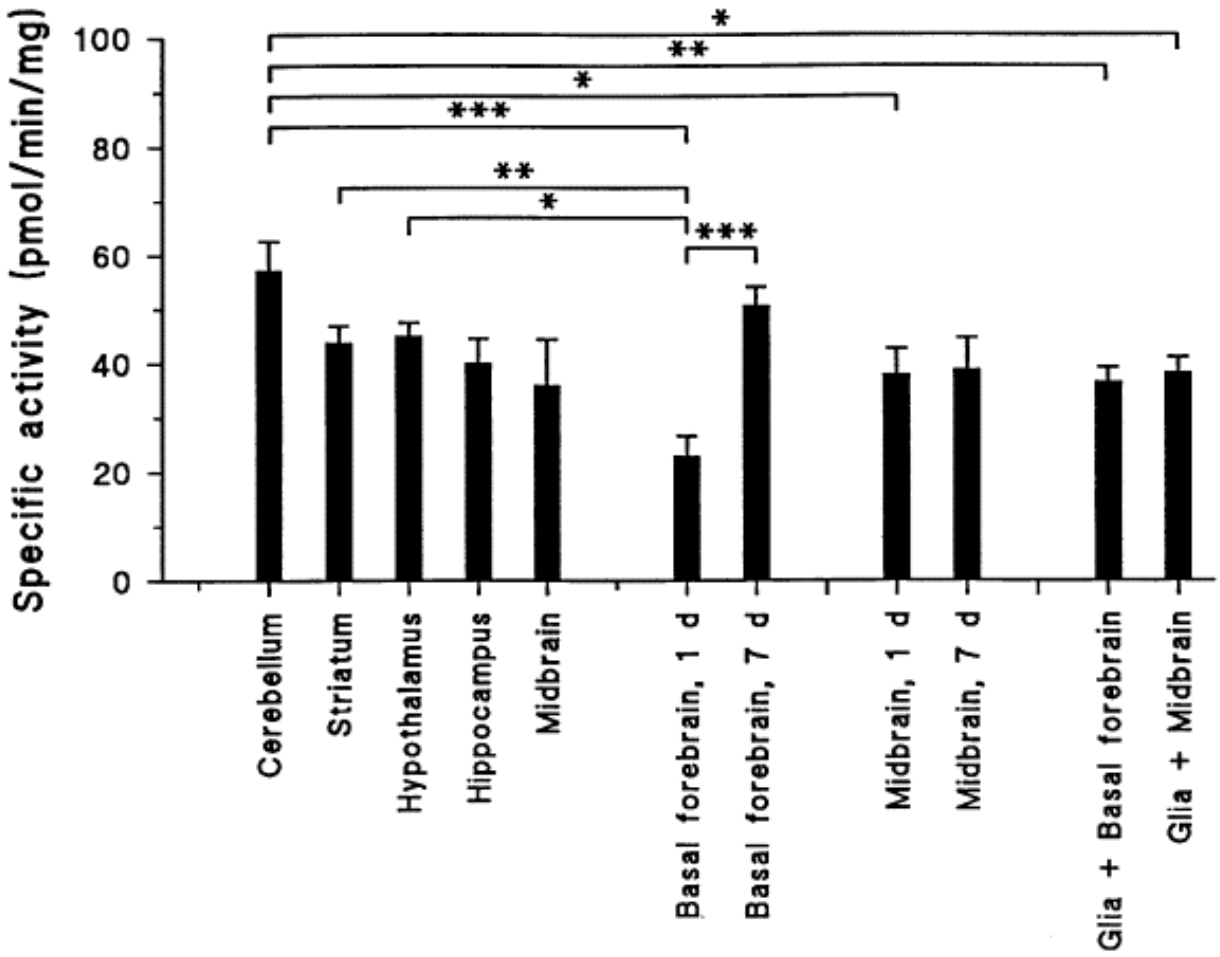

Figure 7. Basal specific COMT activities in primary cultures of the rat brain cells. Values are mean + sem, statistics: one-way ANOVA followed by Tukey's test, *: $p<0.001$, **: $p<0.01$, ***: $p<0.05$, n = 6-26.

Kidney. In rat kidney tissue homogenates (V), the specific COMT activity was highest in cortical sections (399 ± 104 pmol/min/mg) being about ten times higher than in striatal homogenates. In the outer medulla homogenates, the COMT activity (210 ± 48 pmol/min/mg) was approximately half of the cortical activity and nearly twice as high as in papillar tissues (123 ± 24 pmol/min/mg), which had the lowest activity. The meta/para ratios were approximately 5-9. These values resemble those of recombinant S-COMT (II) suggesting the primary response being attributable to S-COMT rather than MB-COMT.

6.3. Modification of COMT activity by various agents (II, IV, V)

Recombinant COMT. To test the effect of ethanol on COMT activity in vitro (II), the most pure enzyme preparations i.e. recombinant forms of COMT, were used. Ethanol did not affect the coulometric detection system (Fig. 4C). As the ethanol concentration increased, the

formation of both reaction products, i.e. vanillic acid and isovanillic acid, by recombinant MB-COMT tended to decrease and this fall reached statistical significance at 1000 mM ethanol concentration (51 % and 57 % decrease in the formation of vanillic acid and isovanillic acid, respectively). With recombinant S-COMT, only the formation of vanillic acid was affected by 1000 mM ethanol concentration. With both recombinant enzymes the meta/para ratio was increased at 1000 mM ethanol concentration (from 13.2 to 19.0 and from 5.3 to 7.7 with recombinant MB-COMT and recombinant S-COMT, respectively). Due to the opposite effect of ethanol on COMT activities, ethanol was not anticipated to interfere with the COMT assay. With striatal homogenate, 1000 mM concentration of ethanol decreased the formation of vanillic acid (10 %) and isovanillic acid (30 %, $p<0.001$) (Fig. 8).

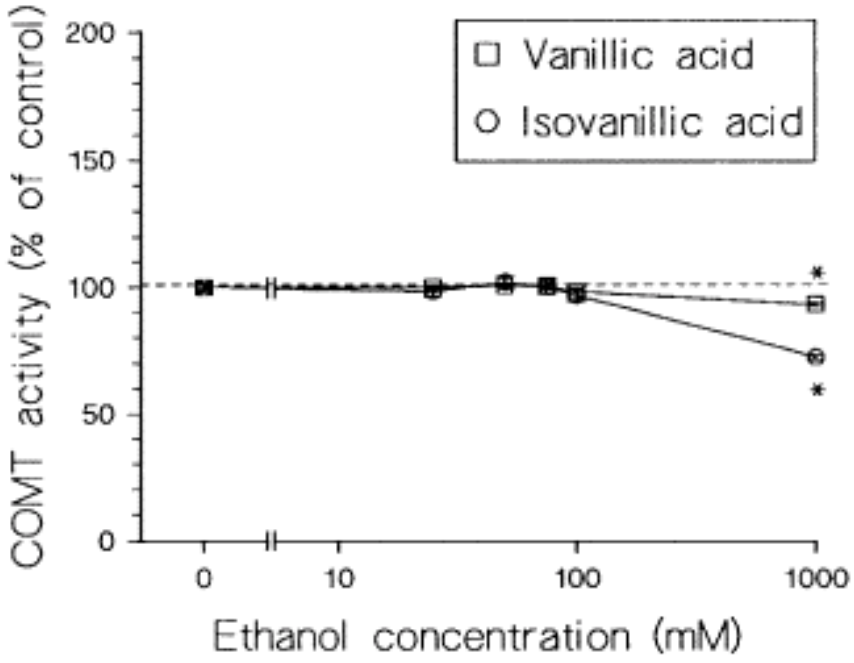

Figure 8. Effect of ethanol on striatal COMT activity *in vitro*. Values (mean ± sem) were obtained from three independent experiments. Statistics: one-way ANOVA followed by Tukey's test, *: $p<0.001$, n = 15-16.

The effect of 1000 mM concentration of ethanol to apparent kinetic values was tested with recombinant COMT enzymes. Ethanol decreased both K_m and V_{max} values of MB-COMT indicating a mixed type of inhibition mechanism (Table 3). With S-COMT, the V_{max} was increased. With increasing substrate concentrations, the meta/para ratio of recombinant MB-COMT was decreased (from 28 to 15) by 1000 mM ethanol concentration, which differed from the corresponding control value at the lowest concentration of DHBAc ($p<0.01$). With increasing concentrations of the substrate, 1000 mM concentration of ethanol did not affect the meta/para ratios (7.3-7.7) of recombinant S-COMT, while all meta/para values differed from

corresponding control values ($p<0.01$). In striatal tissue, only a decrease in the formation of isovanillic was observed.

Table 3. Effect of 1000 mM ethanol on the apparent kinetics of vanillic acid with recombinant COMT enzymes. Values are means ± sem, n = 3.

	K_m (μM)	V_{max} (μM)
MB-COMT		
Control	27.2 ± 1.4	1.8 ± 0.2
+ ethanol	43.4 ± 2.5[b]	0.8 ± 0.2[a]
S-COMT		
Control	136 ± 11	4.6 ± 1.4
+ ethanol	167 ± 45	9.8 ± 0.8[a]

DHBAc concentrations were 12.5-300 μM and 25-500 μM for the MB-COMT and the S-COMT, respectively, with 2-5 replicate samples. The values were obtained from the double reciprocal plots without or with 1000 mM ethanol in the assay. V_{max} is expressed as μmol/l product formed in 30 min. (10 μl of the sample analyzed with HPLC). [a]$p<0.05$, [b]$p< 0.01$ vs corresponding control (t-test).

Cell cultures. The effect of COMT inhibitors (IV) on COMT activity was tested in primary glial, neuronal and glial/neuronal co-cultures of brain cells. The inhibitors with a nitrocatechol structure, entacapone and tolcapone, decreased COMT activity in all glial, neuron-enriched and glial/neuronal cocultures (Fig. 9). In contrast, a pyridine derivative, CGP 28014, did not affect COMT activity in any of these cultures. Since the effect of nitrocatechol-type inhibitors was usually higher than 50 % inhibition of COMT activity, exact IC_{50} values were not obtained. The approximated concentrations of the nitrocatechol drugs inhibiting COMT activity in glial cell cultures by 50 % were 10-20 nM for tolcapone and 45-150 nM for entacapone. In glial/neuronal co-cultures the estimated 50 % inhibitory concentrations were 15 nM in both cultures for tolcapone and 45 nM in basal forebrain coculture and 100 nM in midbrain coculture for entacapone. This corresponds to about 3-7 times greater potency of tolcapone than entacapone. In neuron-enriched cultures, the efficacy was slightly better in 1-

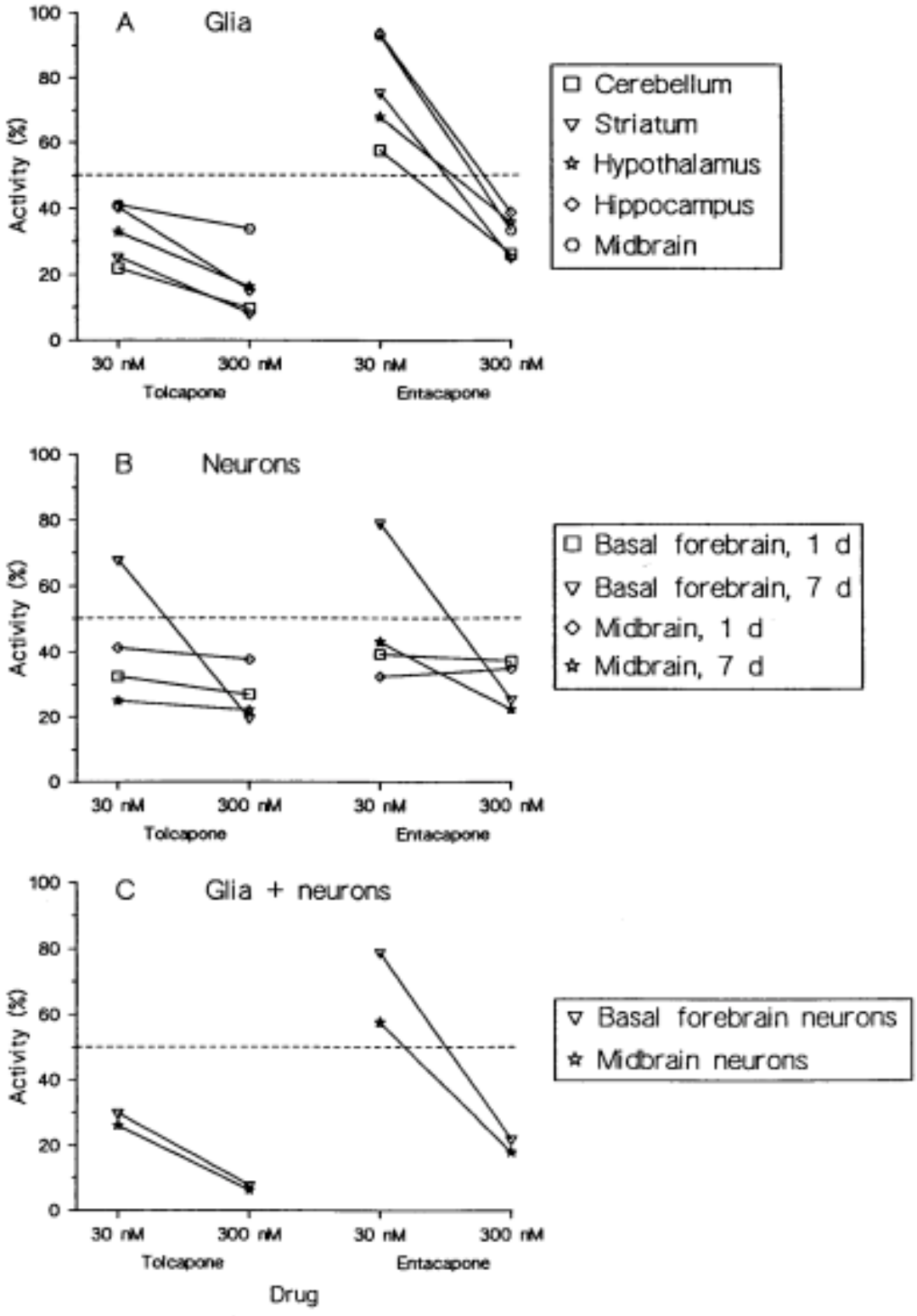

Figure 9. The efficacy of COMT inhibitors on specific COMT activity in primary cultures of rat brain cells. For the sake of clarity, only the results for 30 nM and 300 nM concentration of the drugs are shown. A) Glia. The cell cultures were grown for 35-41 days. B) Neurons. The cultures were grown for 1 day (1 d) or 6-7 days (7 d). C) Neuronal/glial coculture. The cocultures were grown for 7 days after plating of neurons on top of 18-36 day-old glia. With the exception of 30 nM concentration of entacapone in midbrain glial culture, the drugs decreased significantly COMT activity ($p<0.05$ at least).

day cultures than in 6-7 day cultures suggesting modest sensitivity of neuronal COMT to nitrocatechol drugs. The approximated 50 % inhibitory concentrations of the drugs in neuron-enriched cultures were 15-60 nM for tolcapone and 20-75 nM for entacapone, suggesting equal potency of both drugs. In all glial and neuronal/glial cocultures at 30 nM concentration tolcapone was more efficient than entacapone ($p<0.05$). This was true also at 150 nM concentration in striatal glial culture and 1-day basal forebrain cultures ($p<0.05$) in addition to glial/midbrain neuronal co-cultures ($p<0.05$) at 300 nM concentration of the drugs.

Kidney COMT activity. Peripheral actions of entacapone on COMT activity (V) were studied by analyzing COMT activity *ex vivo* in different regions of rat kidney. COMT activity decreased similarly in all sections of the kidney, i.e. cortex, outer medulla and papilla. Two and three hours after entacapone treatment (30 mg/kg, i.p.) the activity was decreased by nearly 100 %. To evaluate a possible correlation of central nervous system COMT activity to that of kidney, the whole brain COMT activity was analyzed after the same entacapone treament. The whole brain COMT activity was reduced by about 40 % one hour after entacapone treatment and had returned to its basal level three hours after entacapone administration.

Kidney function. The effect of certain compounds on rat kidney function (V) were also studied. Entacapone (30 mg/kg, i.p.) increased natriuresis by more than five-fold. The entacapone induced natriuretic effect was suppressed by dopamine receptor type 1 (D_1) antagonism with SCH23390 (30 μg/kg/h) by about 60 % whereas D_2 antagonism with sulpiride was without any effect on natriuresis. COMT inhibition with entacapone caused a trend towards transient increase of dopamine excretion while DOPAC excretion was increased by more than three-fold with or without antagonist treatments. Entacapone alone or with dopamine receptor antagonists did not affect the kidney hemodynamic responses (GFR, RPF or MAP). L-DOPA infusion (60 μg/kg/h) for one hour increased natriuresis by two-fold, and this could be blocked with D_1 antagonist treatment. Dopamine excretion increased by more than 17-fold and DOPAC excretion was elevated by two-fold.

7. DISCUSSION

7.1. COMT activity analysis

RP-HPLC is a non-radioactive method which does not involve many steps in processing the samples, making it easy to perform and automatize. The coulometric detection system proved to be a reliable method to analyze the COMT reaction products (I). This detection method means that the present set-up is the most sensitive of its kind used in the COMT activity analysis. Thus, it is suitable for the enzyme kinetic analyses and can detect lower COMT activites than previous methods. It allows the analysis of vanillic acid and isovanillic acid, and thus one can calculate the meta/para ratios, from several tissue sources when DHBAc is used as a substrate. Another COMT assay utilizing the described detection system has been developed to be used with dihydroxybenzylamine as a substrate (Ellingson et al. 1999). Since coulometric detection has been used for the analysis of catecholamines and their metabolites (Törnwall et al. 1994), the endogenous cateacholamines should also be applicable as substrates for COMT activity analysis.

The highest variation in COMT activity was found in repeated analysis of apparently similar pieces of tissues obtained from different individuals. The interindividual variation may be due to at least two sources, i.e. COMT assay and genetic differences. COMT assay includes the variation from analytical and sample preparation steps. Previously, we noticed that the variation in specific COMT activity in erythrocytes was mainly affected by the variation in HPLC and protein analysis while the handling of the samples produced less variation (Tuomainen et al. 1996). In the present assay the protein analysis introduces more variation than the enzyme reaction and the HPLC analysis. The genetical variation of COMT activity in humans could be observed by the thermolability of COMT enzyme (Weinshilboum et al. 1999). Although a different level of COMT activity between different inbred rat strains has been described, no thermolability of COMT enzyme has been noticed within a single rat strain (Goldstein et al. 1980; Lotta et al. 1995).

Kinetics. The K_m value for recombinant MB-COMT (27.2 μM) obtained (II) was comparable to that observed earlier (22.2 μM, Lotta et al. 1995). Also the higher affinity and lower methylation capacity of recombinant MB-COMT compared to that of recombinant S-COMT is in agreement with the observations with partially purified enzyme preparations. Meta/para ratios for DHBAc with recombinant COMT proteins were similar to those reported earlier (23.7 and 5.1 for MB-COMT and S-COMT, respectively, Nissinen 1984b). The increase of meta/para ratio of recombinant MB-COMT, but not recombinant S-COMT, was also found

with declining substrate concentrations as described previously (Nissinen 1984b). The high meta/para ratios with low substrate concentrations resemble the values encountered *in vivo*.

DHBAc offers some advantages over most endogenic substrates. It is not metabolized further and it is not easily oxidized. The high sensitivity of the detection of reaction products means that one can use a non-saturating concentration of DHBAc. This was achieved in 900 x g supernatant which contains both COMT isoforms. Previously, the activity of the two enzyme forms, MB-COMT and S-COMT, has been analyzed in the same homogenate sample of human brain with a low concentration of dopamine for MB-COMT and a high concentration for S-COMT (Rivett et al. 1983a). Dopamine differs in its affinity and reaction velocity between MB-COMT and S-COMT. Using DHBAc in rat tissues, however, the difference is not so great.

The sum of metabolism of DHBAc through MB-COMT and S-COMT could also be calculated by adding the reaction velocities of both isoenzymes (Rivett et al. 1983a). Kinetic constants determined for recombinant human COMT enzyme forms (Lotta et al. 1995) could be assumed to represent the values of pure natural enzymes. Since the V_{max} values were given as catalytic numbers (k_{cat} in 1/min) they could be converted to V_{max} values (expressed as μmol/min of product formed) when total enzyme concentrations (E_{tot} in nM) are known. These calculations are shown in Table 5. These V_{max} values could be interpreted so that 64 nmol of recombinant MB-COMT produces maximally 1.41 μmol/min of the reaction product and 32 nM of recombinant S-COMT produces maximally 1.39 μmol/min of the reaction product. The molar ratio of 64 nM : 32 nM of recombinant COMT isoforms is close to the relative ratio of 70 % : 30 % (MB-COMT : S-COMT) obtained by COMT protein blotting in human brain (Tenhunen et al. 1994). By using the analyzed K_m values and the corresponding calculated V_{max} values, the approximation of total metabolism of DHBAc in a hypothetical human brain homogenate containing these amounts of COMT enzymes were computed. It is assumed that SAM is present at saturating concentrations and does not affect the kinetic values. At 10-400 μM DHBAc concentration range, 51-55 % of the metabolism is account for MB-COMT by our hypothetical human brain homogenate (Fig. 10A). In the rat brain, the ratio of COMT protein isoforms is 30 % : 70 % (MB-COMT : S-COMT) (Tenhunen et al. 1994). Assuming that the molar amount of MB-COMT is half of the corresponding amount of human brain (32 nM) and the amount of S-COMT is twice as high as in human brain (64 nM), the V_{max} values

TABLE 5. Calculated kinetic values for DHBAc.

Enzyme	K_m (μM)	k_{cat} (1/min)	E_{tot} (nM)	V_{max} (μmol/min)
MB-COMT	30[a]	22.2[a]	64[a]	1.41
			32	0.705
S-COMT	38.9[a]	43.4[a]	32[a]	1.39
			64	2.78

$V_{max} = k_{cat} \times E_{tot}$, k_{cat} = catalytic number, E_{tot} = concentration of the enzyme in the assay.
[a] determined with recombinant human MB-COMT and recombinant human S-COMT (Lotta et al. 1995).

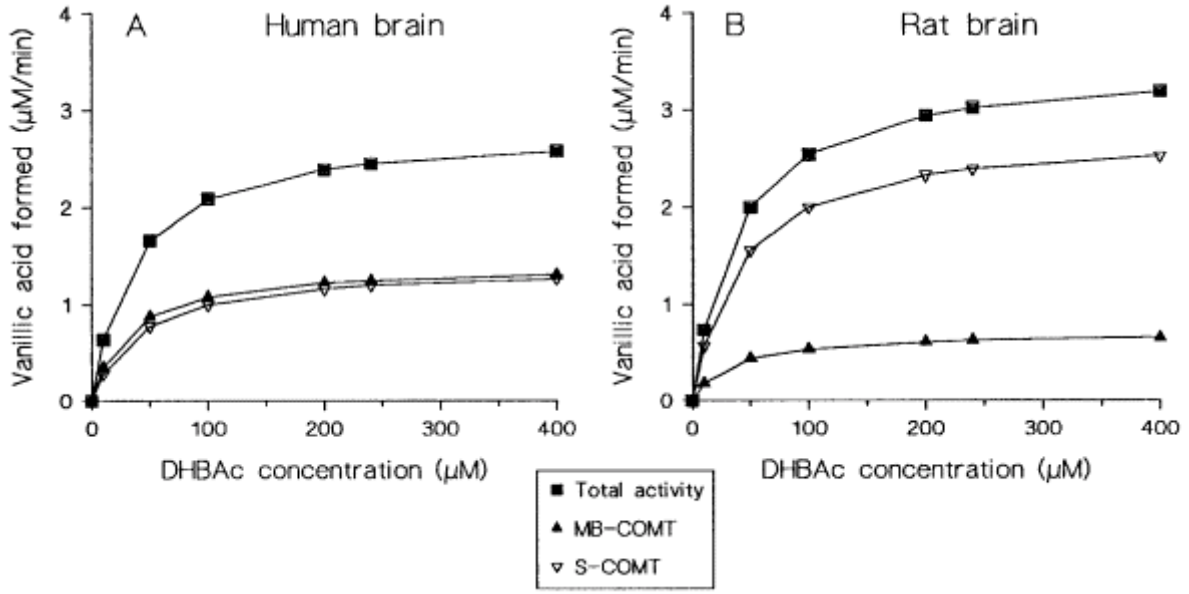

Figure 10. Calculated kinetics for brain COMT using DHBAc as a substrate. The K_m and V_{max} values from Table 5. were substituted to enzyme kinetic equation ($v = V_{max,MB}$ x substrate concentration/($K_{m,MB}$ + substrate concentration) + $V_{max,S}$ x substrate concentration/($K_{m,S}$ + substrate concentration) (Rivett et al., 1983a). Based on the relative amount of COMT isoforms present in the brain (Tenhunen et al., 1994), the V_{max} values corresponding to 63 nM and 32 nM for MB-COMT and S-COMT, respectively, were used for the human brain (A) while V_{max} values corresponding to 32 nM and 64 nM for MB-COMT and S-COMT, respectively, were used for the rat brain (B).

were calculated to be half and twice for MB-COMT and S-COMT, respectively. By substituting these V_{max} values to the corresponding equations for reaction velocities, the estimation of DHBAc metabolism in rat brain homogenate was computed (Fig. 10B). It revealed that only 21-24 % of 10-400 μM DHBAc is metabolized via MB-COMT. Since the reported K_m value for S-

COMT is lower than that of rat brain COMT (Nissinen 1985), these calculations slightly underestimate the metabolism through S-COMT. Nonetheless, with DHBAc as a substrate, it seems that half of the metabolism is via MB-COMT in human brain tissue homogenate independently of substrate concentration. Also, the present analysis (at 240 μM concentration of DHBAc, I) of rat brain tissue homogenates appears to assay preferentially the S-COMT activity.

Based on analogous calculations for endogenous substrates (within 10-400 μM concentrations), L-DOPA behaves similarly as DHBAc while dopamine and noradrenaline are metabolized primarily (50-90 %) via MB-COMT in hypothetical human brain homogenate (data not shown). In a hypothetical rat brain homogenate, L-DOPA again acts like DHBAc as a substrate while dopamine and noradrenaline are metabolized primarily via MB-COMT only at lower substrate concentrations (less than 100 μM and 50 μM for dopamine and noradrenaline, respectively, data not shown). These approximations represent a situation when other metabolism is blocked and the reaction is made at saturating concentrations of SAM. Also, in rat brain, the affinities of catecholamines for S-COMT are higher than with recombinant S-COMT (Lotta et al. 1995; Nissinen 1985). Altogether, these approximations are in line with earlier calculations (Rivett et al. 1982) supporting the importance of MB-COMT in the metabolism of endogenous catecholamines *in vivo*.

Amount of COMT. Although the actual amount of COMT proteins with respect to total protein content in the brain is not known, a rough estimation of the amount of COMT enzyme in the present COMT assay (I) could be calculated. Recombinant COMT, derived from rat liver S-COMT sequence, has been purified to near homogeneity (Lundström et al. 1992). Up to 98 % of purity (Vidgren et al. 1991) has been reported for the enzyme (denoted as fraction b by Lundström et al. 1992) in crystallization studies. This preparation has a specific activity of 500 nmol/min/mg protein (Lundström et al. 1992) with 400 μM concentration of DHBAc. If it is assumed that this preparation is 100 % active and pure soluble COMT, then 1 mg of COMT protein produces 500 nmol/min of the reaction product as a maximal capacity of methylation. Since 1 mg protein of striatal homogenate produces 45.8 pmol/min of the reaction product (note: with 240 μM concentration of DHBAc, I), only 91.6 ng of pure S-COMT protein is needed to produce that activity. This amount of COMT represents 0.09 % of the total protein in the rat brain homogenate. Since the molecular weight of recombinant S-COMT is 25 kD (Lundström et al. 1992), 9.1 ng of S-COMT protein corresponds to about 3.7 pmol of S-COMT in 1 mg of total protein in rat brain homogenate. In the present assay, approximately 400 μg of total protein has

been normally used in the COMT reaction. This corresponds to 1.5 pmol of COMT protein, which is close to the calculated molar range of recombinant COMT proteins (16 pmol and 8 pmol for recombinant MB-COMT and S-COMT, respectively) used in kinetic analyses (Lotta et al. 1995). In another study, 400 μM concentration of DHBAc produced about 0.15 nmol/min of the reaction product in 1 mg of total rat brain homogenate protein (Tilgmann et al. 1992). By analogous calculations, this homogenate contains 30 ng or 1.2 pmol (0.03 %) of S-COMT protein in 1 mg of total protein. These approximations, however, are likely to overestimate the amount of S-COMT since the specific activities obtained in these studies also contain the activity derived by MB-COMT (about 20 %, Fig. 10). On the other hand, since the present assay utilizes the 900 x g supernatant, the loss of COMT activity (approximately 10 %) in the pellet is clear due mostly to loss of S-COMT (Ulmanen et al. 1997). Assuming that COMT activity in 100 000 x g supernatant is derived only from S-COMT, which has a V_{max} value of 186 pmol/min/total protein (Nissinen 1985), the amount of COMT in this preparation is 1.57 ng or 63 pmol (about 0.16 %) in 1 mg of the total protein in this fraction. Altogether, these calculations suggest that the amount of COMT protein is less than 1 % of the total protein of the rat brain homogenate. Also, this molar amount of COMT enzyme in the assay could be physiologically relevant e.g. in kinetic analyses.

Cell cultures. Earlier, the COMT activity in brain cell cultures has been analyzed after homogenization of the collected cells (Hansson 1984). In our studies (IV), COMT activity was analyzed by adding the substrate directly to the viable cell cultures without addition of SAM, which penetrates poorly through cell membranes (Baldessarini 1987). Thus, the product formation must have been occurred inside the cells confirming the intracellular localization of COMT (Trendelenburg 1990; Männistö et al. 1992b; Kaakkola et al. 1994; Ulmanen et al. 1997). Although preliminary kinetic data suggest non-saturating conditions with the current concentration of the substrate, it cannot be excluded that the saturation occurred in certain cultures (data not shown). Also, the penetration of DHBAc inside the cell and the low micromolar concentration of intracellular SAM (Baldessarini 1988) affect the overall pace of the reaction. In peripheral tissues, addition of substrate in low concentrations seems to saturate COMT, below the level of uptake saturation (Trendelenburg 1986, 1990; Wilson et al. 1988). It can be speculated that MB-COMT, which has a higher affinity than S-COMT, is responsible for the methylation at low micromolar concentrations of substrates. Also, apparently high meta/para ratios in culture studies (IV) support the primary metabolism through MB-COMT over S-COMT.

7.2. Distribution of COMT in the brain and in the kidney

Brain regions. The present results with cultured cells from discrete regions of the brain (IV) agree well with concept that COMT is widely distributed throughout the rat brain (Kaplan et al. 1979; Hansson 1984; Roth 1992; Karhunen et al. 1994). Also, the highest COMT activity was found in cerebellar cultures (IV), which corresponds to COMT staining of Bergmann glia (Kaplan et al. 1979; Karhunen et al. 1994). The basal COMT activities found here in brain cell cultures were comparable with earlier reports of primary glial cultures (Hansson 1984). In addition, in the present cultures (IV), the COMT activities are at the same level as in striatal (I,II), hypothalamic and hippocampal homogenates from rat brain.

Brain cells. Cell cultures studies (IV) confirm the presence of COMT in neurons and glia (Karhunen et al. 1994, 1995b). For the first time, the COMT activity was demonstrated in primary cultures of brain neurons. Cultured fetal basal forebrain and midbrain neurons have commonly been used as a model of striatal and nigral neurons, respectively (McMillian et al. 1995, 1997). For basal forebrain neurons, neuronal COMT is probably located in site postsynaptical to nigrostriatal dopaminergic neurons. Midbrain neuronal cultures confirm the observations of nigral COMT activity (Guldberg and Marsden 1975; Rivett et al. 1983a). Only a weak COMT immunoreactivity has been observed in dopaminergic neurons in human substantia nigra (Kastner et al. 1994). Also, a nigral lesion does not change the COMT activity in striatum (Kaakkola et al. 1987). Thus, COMT must be located in neurons other than the dopaminergic neurons in the substantia nigra.

Some of the COMT activity data supported the predominant role of COMT in glia, especially in basal forebrain neurons. First, the lowest COMT activity was found in the most pure neuronal cultures. Second, when neurons were grown on top of striatal and hypothalamic glial cells, the specific COMT activity was not changed. Indeed, when increasing amounts of neurons were plated on top of glia (12 500 - 200 000 neurons/plate) the total COMT activity increased while specific activity did not (data not shown). Third, in basal forebrain neuron enriched cultures, the increase of COMT activity corresponded to glial proliferation, which was indicated by increased GFAP staining.

Microglia. A novel finding was the presence of COMT in the microglial cells in the striatum (III). This was demonstrated three days after fluorocitrate infusion by the increased COMT activity and colocalization with double stained activated microglial cells. The

astrogliosis, which takes place about one week after the toxin lesion (Rivett et al. 1983a; Kaakkola et al. 1987) can be excluded since MAO B activity, which is present in astroglia and absent in microglia (Ekblom et al. 1994), was decreased and GFAP staining was low at the injection site throughout the study. The appearance of activated microglia at the lesion site was demonstrated by increased alk-PDE activity, a marker for activated peripheral macrophages (Morahan et al. 1980), and high OX-42 staining, a marker for microglia (Graeber et al. 1989). The localization of COMT in macrophages has earlier been speculated as a cause of the transient increase of COMT activity in virus-infected brain (Guchhait and Monjan 1980). In addition, COMT immunoreactivity has been detected in peripheral macrophages (Inoue and Creveling 1986; Inoue et al. 1991). Since the meta/para ratios were not greatly changed, the dominance of either COMT isoform in microglia could not be determined. One function of increased COMT activity may be to metabolize the catecholamines which are leaking out of damaged cells. Due to reduced COMT and MAO B activity at 1-2 days after glial damage, the amount of extracellular dopamine could increase and represent as a possible factor for microglial activation. Depending on whether the effect of increased COMT activity in microglia/macrophages is regenerative (or degenerative) on brain tissue, the brain penetrating COMT inhibitors could be either clinically harmful (or useful) in pathological situations.

Although fluorocitrate is taken up by astrocytes (Clarke et al. 1970), the selectivity of this toxin is reduced at higher doses. This has been shown by decreased activity of a cholinergic neuronal marker after intrastriatal infusion of fluorocitrate (Paulsen et al. 1987). However, the major effect of fluorocitrate on glial cells was seen as a decrease of MAO B activity and by a decrease of COMT activity for two days after fluorocitrate infusion evidence of the presence of COMT in glial cells. A trend towards increased meta/para ratio 24 h after the 2 nmol dose of fluorocitrate suggested that the reduction of total COMT activity is due to decreased glial S-COMT activity. The higher dose seems to reduce almost equally the activity of both COMT isoforms. Apparently fluorocitrate affected also dopaminergic neurons, since staining of TH at the site of lesion was decreased and increased outside the lesion suggesting induction of compensatory dopaminergic tone. Differential staining of TH also explains the inconsistent results in TH activity. No studies are available concerning the long term effects of fluorocitrate.

Kidney. In kidney COMT activity is concentrated in cortex but the deeper parts also have a considerable COMT activity (V). Little is known about the distribution of COMT activity in the different regions of kidney tissue. COMT activity in kidney cortex has been

reported to be higher than in medulla (Goldstein et al. 1980). The COMT mRNA (Meister et al. 1993) and protein (Kaplan et al. 1979; Karhunen et al. 1994) have been demonstrated to reside in proximal and distal tubules in addition to the collecting duct and ureter in rat kidney. The present results on the distribution of COMT activity suggest that metabolism via COMT could take place throughout the kidney but the primary site is in cortical areas, e.g. proximal tubular cells.

7.3. Modification of COMT activity

Recombinant COMT. The direct effect of ethanol on COMT activity was studied *in vitro* with recombinant COMT enzymes (II). Ethanol inhibited recombinant MB-COMT activity but this was significant only at 1000 mM concentration. At this ethanol concentration, recombinant MB-COMT activity was inhibited with a mixed type of inhibition. With recombinant S-COMT only the formation of vanillic acid was affected by 1000 mM ethanol concentration, which increased the V_{max} value of recombinant S-COMT. These opposite effects of ethanol on MB-COMT and S-COMT seemed to cancel each other out, producing mainly a decrease of the formation of isovanillic acid in striatal tissue homogenates. As expected, all these changes were reflected in the meta/para ratios.

There was a trend towards reduction of MB-COMT activity already at 100 mM ethanol concentration (II). Previously, no evidence for the inhibition of (apparently S-) COMT by ethanol at concentrations up to 90 mM have been found *in vitro* (Lahti and Majchrowicz 1974; Giovine et al. 1977; Hoffman et al. 1981). Due to the opposite effect of ethanol on COMT isoforms these changes have not been detected earlier. However, an acute *in vivo* administration of ethanol (1 g/kg, i.p., which produced 25 mM concentration of ethanol in the blood) on rats has decreased the 3-MT concentration in the nucleus accumbens of non-alcohol prefering rats and (pargyline treated) Wistar rats (Honkanen et al. 1994). Also, in the mouse striatum, a decrease of 3-MT by ethanol (3.5 g/kg, i.p.) has been noticed (Milio and Hadfield 1992). In contrast, ethanol and other reinforcing drugs, rather increase the release of dopamine in the brain, especially in nucleus accumbens. Thus, it is tempting to speculate that decreased 3-MT formation is caused by selective inhibition of postsynaptic MB-COMT, possibly leading to elevated dopaminergic tone. The present experiments give only faint support for the inhibition of MB-COMT by 100 mM concentration of ethanol, which is a clinically intoxicating blood concentration (4.6 g/l). Thus, a slight potentiation of COMT inhibitor toxicity could only be possible at very high concentrations of ethanol.

Cell cultures. In primary cultures of rat brain cells, COMT activity was effectively decreased by the nitrocatechol-type inhibitors with tolcapone being slightly more potent than entacapone in cultures containing glial cells (IV). The approximated 50 % inhibition of COMT activity was achieved with 10-150 nM concentration of entacapone and tolcapone. This is in agreement with the reported IC_{50} values of entacapone and tolcapone (2.2-160 nM for brain and liver COMT) (Zürcher et al. 1990; Nissinen et al. 1992). Based on K_i values determined with recombinant human COMT enzymes, tolcapone is slightly more potent against MB-COMT (2.0 nM and 0.3 nM for entacapone and tolcapone, respectively), while the K_i values of both drugs do not differ for S-COMT (Lotta et al. 1995). At the catalytic site, the binding should be similar suggesting equal affinity for both drugs (Lotta et al. 1995; Vidgren and Ovaska 1997). The apparent difference in potency between entacapone and tolcapone is probably due to their membrane penetrating ability. Since tolcapone is a brain penetrating drug (Männistö et al. 1992a) it could reach the enzyme slightly more effectively.

Examination of the approximated 50 % inhibitory concentrations of the nitrocatechol-type drugs revealed a trend for more sensitive inhibition of neuronal COMT than glial COMT. This was supported by the reduction of COMT inhibitor efficacy after the proliferation of glial cells in neuron-enriched cultures. Both entacapone and tolcapone were equipotent in these neuronal cultures. Since tolcapone has been claimed to be more effective against MB-COMT than S-COMT (Borges et al. 1997; Vieira-Coelho and Soares-da-Silva 1999) and primary neuronal cultures apparently contain about the same amount of MB-COMT and S-COMT protein (Karhunen et al. 1995b), a slight sensitivity of neuronal COMT to inhibition by both entacapone and tolcapone could possibly explain these results.

CGP 28014 did not affect COMT activity in any of the cultures (IV). Also previous experiments with tissue homogenates (Waldmeier et al. 1990) and with aggregate cultures (Wiese et al. 1993) demonstrated that CGP 28014 does not inhibit COMT *in vitro*. Thus, CGP 28014 is not metabolized to an active COMT inhibitor by brain cells. Since the main metabolite of CGP 28014 was also ineffective *in vitro*, inhibition of glial $uptake_2$ was suggested (Waldmeier et al. 1990). However, in our laboratory CGP 28014 did not reduce the uptake of [^{3}H]-dopamine into glial cells (M. Törnwall 1994, unpublished results). Thus, the mechanism of the inhibition of O-methylation CGP 28014 remains unknown.

Kidney. In kidney (V), increased natriuresis by entacapone was demonstrated to be caused by inhibition of COMT activity. Furthermore, the inhibition of COMT activity by entacapone was seen to be equal in all regions of the kidney. Previously, the inhibition of COMT

activity with nitecapone has been noted, but this was not fully demonstrated (Eklöf et al. 1997). The natriuretic effect was produced by local COMT inhibition in the kidney since only a transient effect of entacapone on brain COMT was observed. At this dose entacapone affects briefly also brain COMT activity (Kaakkola and Wurtman 1992). However, due to the shorter duration of COMT inhibition in brain than in kidney, a local effect on kidney function predominates.

Both entacapone and L-DOPA induced natriuresis, which was mediated by D_1 receptors, as has been reported before (Eklöf et al. 1997; Hansell et al. 1998). Nitecapone and gamma-L-glutamyl-DOPA, a kidney specific dopamine precursor, have additive effect on natriuresis (Eklöf et al. 1997). This also emphasizes the important role of kidney dopamine in the regulation of salt balance.

In addition to the natriuretic effect, the inhibition of COMT was accompanied by a slight increase of dopamine levels and over three-fold increase of DOPAC levels in the urine whereas L-DOPA produced a smaller natriuretic effect but greater efflux of dopamine and less increase in DOPAC excretion. This suggests that L-DOPA is metabolized first to dopamine outside of the kidney while entacapone acts more locally to elevate kidney dopamine concentrations providing more dopamine to stimulate its receptors within the kidney.

8. CONCLUSIONS

RP-HPLC using coulometric detection with improved sensitivity was developed for the analysis of COMT activity. It is a reliable and sensitive method and applicable for COMT activity analysis from various sources . It is suitable for distribution studies and studies of the effects of various agents on COMT activity. Based on kinetic calculations, the metabolism of DHBAc, at saturating concentrations of SAM, in rat brain homogenate *in vitro* is proposed to be preferentially mediated by S-COMT irrespective of the DHBAc concentration. In contrast, endogenous catecholamines has been calculated to be metabolized preferentially or exclusively by MB-COMT. It was estimated from kinetic data that COMT protein represents less than 1 % of all the proteins present in rat brain.

The localization of COMT to activated microglial cells was demonstrated. The function of COMT in microglia, though demonstration if this is a general phenomenon during microglial activation, remains to be established. The presence of COMT activity in neuronal cells was presented. However, in general a slightly higher COMT activity resides in glial cells. COMT was found to be almost equally distributed in glia throughout the rat brain. In rat kidney, the COMT activity is highest in cortical areas with less activity found deeper in the tissue, indicating that the proximal tubular cells are the principal site of COMT in proximal tubular cells.

A small reduction of MB-COMT activity by ethanol was noticed *in vitro*. There was a coincidental ethanol-induced increase of S-COMT activity counteracting any possible reduction of catecholamine metabolism. However, these effects were achieved only at intoxicating concentrations of ethanol. Therefore, it is unlikely that COMT inhibitors would have any major interaction with ethanol.

COMT activity was effectively decreased in cultured brain cells by nanomolar concentrations of nitrocatechol-type inhibitors *in vitro*. In general, tolcapone was a slightly more potent inhibitor of glial COMT this being due to its better permeability through membranes. In contrast, CGP 28014 did not affect COMT activity excluding the possibility that it is metabolized to an active COMT inhibitor in brain cells. These effects could be demonstrated by analyzing COMT activity in viable cells grown in culture.

COMT activity was demonstrated to be reduced effectively by entacapone in all regions of rat kidney *ex vivo*. The increase of the dopamine concentration *in vivo* following administration of the precursor, L-DOPA, or blocking dopamine metabolism by entacapone, produced a D_1-receptor sensitive natriuretic effect in the kidney. Entacapone and L-DOPA

exhibited quantitatively different effect on natriuresis and excretion of dopamine and its metabolites.

9. ACKNOWLEDGEMENTS

This study was carried out in the Department of Pharmacology and Toxicology, Institute of Biomedicine, University of Helsinki, during the years 1991-1999.

I want to thank Professor Heikki Vapaatalo, MD, and Professor Matti K. Paasonen, MD, the heads of the Department during these years, for providing me with the facilities in the Department and constructive criticism and encouragement.

I sincerely wish to thank my supervisor, Professor Pekka T. Männistö, M.D., one of the world's leading experts on COMT. Professor Männistö has provided me with background knowledge, encouragement as well as enthusiasm, being a living example of how to do scientific research. I sincerely thank also my other supervisor, Professor Raimo K. Tuominen, M.D., for his professional attitude, unflagging positive attitude and confidence in my work.

My respectful thanks are due to Docent Erkki Nissinen, Ph.D., and Docent Atso Raasmaja, Ph.D., the official referees of the dissertation for valuable criticisms and comments.

I extend my thanks to Doctor Päivi Tuomainen, Ph.D., a maestro of HPLC techniques, Docent Seppo Soinila, M.D., for his immunohistochemical contribution, Professor Peter Hansell, Ph.D, and Cecilia Odlind, M.Sci., for introducing me to the interesting field of kidney physiology. I also want to thank Michael K. McMillian, Ph.D., and Docent Pekka Rauhala, M.D., for their excellent technical collaboration and comments. I am greatful that Docent Pekka Rauhala has accepted to act as a custos of my dissertation. Further thanks go to Orion Pharma for providing me with entacapone, tolcapone and CGP 28014 for the scientific work. Without them the studies could not have been done. I am greatful to Docent Ismo Ulmanen, Ph.D. and Carola Tilgmann, Ph.D., for the other essential tools. I want to thank also Professor Jyrki Taskinen, Pharm.D, for his comments on the kinetic calculations. I also want to express my thanks to members of our neurobiology group, Martti Törnwall, M.D., Petri J. Vainio, Med.Lic., Docent Jussi Idänpään-Heikkilä Jr, M.D., Vesa Kontinen, M.D., Xu Mei, Ph.D., and all the others who have worked in our group during these years.

It is my pleasure to thank Mrs Nada Bechara-Hirvonen, Ms Satu Mäkinen, Mrs Vuokko Pahlsten, Mrs Lahja Eurajoki and Ants Kask, M.Sc., for their splendid help in the laboratory during this work. I thank Mrs Tiina Kellosalo and Ms Mervi Tikkanen and the other

staff of the departmental office. Mr Esa Oja in the library, Mr Erkka Solatunturi and Mr Eino Ruusula are gratefully acknowledged for skillful technical help.

I am grateful to Ewen MacDonald, Pharm.D., for linguistic revision of this thesis and other manuscripts.

I express my sincere gratitude to Professor Mauri J. Mattila, M.D., Professor Ilari Paakkari, M.D., and Professor Heikki Karppanen, M.D., as well as colleagues and other personnel for creating a pleasant atmosphere in the department.

I am thankful to all my noble and good friends for the unforgettable and even the forgotten moments I have spent with them.

I owe my gratitude to my mother and father for all of the support and encouragement during these years.

Finally, my dearest thanks go to my family, my wife Minna, my daughter Karla and my son Mitro for providing me with their everlasting love, confidence and stimulating atmosphere.

This study was supported by grants from Sigrid Juselius Foundation, Helsinki, and Academy of Finland, Helsinki.

I am grateful to Pharmacal Research Foundation, Finland, for financial support.

Helsinki, November 1999

Ilkka Reenilä

REFERENCES

Allen E, Myers C (1992) Application of an organic solvent extraction to the determination of catechol-O-methyltransferase activity by high-performance liquid chromatography in human mononuclear cells. J Chromatogr 578:175-188

Aprille J, Malamud D (1975) Catechol-O-methyl transferase in mouse liver plasma membranes. Biochem Biophys Res Commun 64:1293-1309

Axelrod J (1962) Catechol-O-methyltransferase from rat liver. Meth Enzymol 5:748-751

Axelrod J (1966) J. Methylation reactions in the formation and metabolism of catecholamines and other biogenic amines. Pharmacol Rev 18:95-113

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

Axelrod J, Alberts W, Clemente CD (1959) Distribution of catechol-O-methyl transferase in the nervous system and other tissues. J Neurochem 5:68-72

Bade P, Christ W, Rakow D (1974) Catechol-O-methyltransferase measurement using spectrophotometric and radiometric procedures. Naunyn-Schmiedeberg's Arch Pharm 282,Suppl.:R3

Baldessarini RJ (1987) Neuropharmacology of S-adenosyl-L-methionine. Am J Med 83, Suppl 5A:95-103

Ball P, Knuppen R, Haupt M, Breuer H (1972) Interactions between estrogens and catecholamines III. Studies on the methylation of catechol estrogens, catechol amines and other catechols by the catechol-O-methyltransferase of human liver. J Clin Endocrinol Metab 34:736-746

Baranczyk-Kuzma A, Audus KL, Borchardt RT (1986) Catecholamine-metabolizing enzymes of bovine brain microvessel endothelial cell monolayers. J Neurochem 46:1956-1960

Bertocci B, Miggiano V, DaPrada M, Dembic Z, Lahm HW, Malherbe P (1991) Human catechol-O-methyltransferase cloning and expression of the membrane-bound form. Proc Natl Acad Sci USA 88:1416-1420

Bidart JM, Motte P, Assicott M, Bohuon C, Bellet D (1983) Catechol-O-methyltransferase activity and aminergic binding sites distribution in human peripheral blood lymphocyte subpopulation. Clin Immunol Immunopathol 26:1-9

Blume A, Gilbert F, Wilson S, Farber J, Rosenberg R, Nirenberg M (1970) Regulation of acetylcholinesterase in neuroblastoma cells. Proc Natl Acad Sci USA 67:786-792

Bonifacio M, Vieira-Coelho MA, Borges N, Soares-da-Silva P (1998) Kinetics of membrane-bound, solubilized membrane-bound and soluble forms of rat liver and brain catechol-O-methyltransferase. Naunyn-Schmiedeberg's Arch Pharm 358,Suppl.2:P6.54

Borchardt R (1973) Catechol-O-methyltransferase 3. Mechanism of pyridoxal 5'-phosphate inhibition. J Med Chem 16:387-391

Borchardt RT (1974) A rapid spectrophotometric assay for catechol-O-methyltransferase. Anal Biochem 58:382-389

Borchardt RT, Hegazi MF, Schowen RL (1978) Determination of O-methylated metabolites of catecholamines using high-performance liquid chromatography and electrochemical detection. Anal Biochem 58:382-389

Borges N, Vieira-Coelho MA, Parada A, Soares-da-Silva (1997) Studies on the tight-binding nature of tolcapone inhibition of soluble and membrane-bound rat brain catechol-O-methyltransferase. J Pharmacol Exp Ther 282:812-817

Boudikova B, Szumlanski C, Maidak B, Weinshilboum R (1990) Human liver catechol-O-methyltransferase pharmacogenetics. Clin Pharmacol Ther 48:381-389

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248-254

Breakefield XO, Braveman M, Riker DK, Giller ELJ (1981) Catechol-O-methyltransferase activity in cultures human skin fibroblasts from controls and patients with dystonia musculorum deformans. J Neurosci Res 6:349-369

Broch OJ (1973) The postnatal development of catechol-O-methyl transferase in the rat brain. J Neurochem 20:847-852

Broch OJ, Fonnum F (1972) The regional and subcellular distribution of catechol-O-methyltransferase in the rat brain. J Neurochem 19:2049-2055

Bryan-Lluka LJ (1994) Evidence for saturation of catechol-O-methyltraansferase by low concentrations of noradrenaline in perfused lungs of rat. Naunyn-Schmiedeberg's Arch Pharm 351:408-416

Cavalieri EL, Stack DE, Devanesan PD, Todorovic R, Dwivey I, Higginbotham S, Johansson SL, Patil KD, Gross ML, Gooden JK, Ramanathan R, Cerny RL, Rogan EG (1997) Molecular origin of cancer: Catechol estrogen-3,4-quinones as endogenous tumor initiators. Proc Natl Acad Sci USA 94:10937-10942

Clarke DD, Nicklas WJ, Berl S (1970) Tricarboxylic acid-cycle metabolism in brain: Effect of fluorocitrate on the labelling of glutamate, aspartate, glutamine and gamma-aminobutyrate. Biochem J 120:345-351

Coward JK, Wu FY (1973) A continuous spectrophotometric assay for catechol-O-methyltransferase. Anal Biochem 55:406-410

CPMP (Committee for Propietary Medicinal Products) (1998) EPAR (European Public Assessment Report) COMTESS. EMEA (European Agency for the Evaluation of Medicinal Products), 16 December 1998, CPMP/1249/98, http://www.eudra.com/emea

Creveling G, Dalgard N, Shimizu N, Daly J (1970) Catechol-O-methyltransferase.III. m- and p-O-methylation of catecholamines and their metabolites. Mol Pharmacol 6:691-696

DeSanti C, Giulianotti PC, Pietrabissa A, Mosca F, Pacifici GM (1998) Catechol-O-methyltransferase: variation in enzyme activity and inhibition by entacapone and tolcapone. Eur J Clin Pharmacol 54:215-219

Dingemanse J (1997) Catechol-O-methyltransferase inhibitors: Clinical potential in the treatment of Parkinson's disease. Drug Develop Res 42:1-25

Ekblom J, Aquilonius S-M, Jossan S (1993) Differential increases in catecholamine metabolizing enzymes in amyotrophic lateral sclerosis. Exp Neurol 123:289-294

Ekblom J, Jossan SS, Oreland L, Walum E, Aquilonius S-M (1994) Reactive gliosis and monoamine oxidase B. J Neur Transm Suppl 41:253-258

Eklöf AC, Holtbäck U, Sundelöf M, Chen S, Aperia A (1997) Inhibition of COMT induces dopamine-dependent natriuresis and inhibition of proximal tubular Na+,K+-ATPase. Kidney Int 52:742-747

Ellingson T, Duddempudi S, Greenberg BD, Hooper D, Eisenhofer G (1999) Determination of differential activities of soluble and membrane-bound catechol-O-methyltransferase in tissues and erythrocytes. J. Chrom. B 729:347-353

Fiszman ML, Zuddas A, Masana MI, Barker JL, DiPorzio U (1991) Dopamine synthesis precedes dopamine uptake in embryonic rat mesencephalic neurons. J Neurochem 56:392-399

Francis A, Pearce LB, Roth JA (1985) Cellular localization of MAO A and B in brain: evidence from kainic acid lesions in striatum. Brain Res 334:59-64

Funaki T, Onodera H, Ushiyama N, Tsukamoto Y, Tagami C, Fukazawa H, Kuruma I (1993) The disposition of the tolcapone 3-O-methylated metabolite is affecet by the route of administration in rats. J Pharm Pharmacol 46:571-574

Giovine A, Renis M, Bertolino A (1977) In vivo studies of catechol-O-methyl transferase activity following intoxication with ethanol, L-DOPA, and dopamine derived alkaloids. Pharmacol Res Comm 9:203-214

Gogos JA, Morgan M, Luine V, Santha M, Ogawa S, Pfaff D, Karayiorgou M (1998) Catechol-O-methyltransferase-deficient mice exhibit sexually dimorphic changes in catecholamine levels and behaviour. Proc Natl Acad Sci USA 95:9991-9996

Goldstein DJ, Weinshilboum RM, Dunnette JH, Creveling CR (1980) Developmental patterns of catechol-O-methyltransferase in genetically different rat strains: enzymatic and immunochemical studies. J Neurochem 34:153-162

Graeber MB, Streit WJ, Kreutzberg GW (1989) Identity of ED2-positive perivascular cells in rat brain. J Neurosci Res 22:103-106

Grossman M, Creveling C, Rybczynski R, Braverman M, Isersky C, Breakefield X (1985) Soluble and particulate forms of rat catechol-O-methyltransferase distinguished by gel electrophoresis and immune fixation. J Neurochem 44:421-432

Grossman MH, Emanuel BS, Budarf ML (1992) Chromosomal mapping of the human catechol-O-methyltransferase gene to 22q11.1-q11.2. Genomics 12:822-825

Guchhait RB, Monjan AA (1980) Lymphocytic choriomeningitis virus-induced modification of catecholamine metabolism in developing rat brain. Neuroscience 5:1105-1111

Guimaraes S, Trendelenburg U (1985) Deviation supersensitivity and inhibition of saturable sites of loss. Trends Pharmacol Sci 6:371-374

Guldberg HC, Marsden CA (1975) Catechol-O-methyl transferase: Pharmacological aspects and physiological role. Pharmacol Rev 27:135-206

Gulliver PA, Tipton KF (1978) Direct extraction radioassay for catechol-O-methyltransferase activity. Biochem Pharmacol 27:773-775

Hansell P, Odlind C, Männistö PT (1998) Different renal effects of two inhibitors of catechol-O-methylation in the rat: entacapone and CGP 28104. Acta Physiol Scand 162:489-494

Hansson E (1984) Enzyme activities of monoamine oxidase, catechol-O-methyltransferase and τ-aminobutyric acid transaminase in primary astroglial cultures and adult rat brain from different brain regions. Neurochem Res 9:45-57

Hardebo JE, Emson PC, Falck B, Owman C, Rosengren E (1980) Enzymes related to monoamine transmitter metabolism in brain microvessels. J Neurochem 35:1388-1393

Head RJ, Irvine J, Barone S, Stitzel RE, DelaLande IS (1985) Nonintracellular, cell-associated O-methyltaion of isoproterenol in the isolated rabbit thoracic aorta. J Pharmacol Exp Ther 234:184-189

Herblin WF (1973) A simple colorimetric assay for catechol-O-methyltransferase. Anal Biochem 51:19-22

Hoffman AB, Majchrowicz E, Poth MA, Paul SM (1981) Ethanol reduces hepatic estrogen-2-hydroxylase activity in the male rat. Life Sci 29:789-794

Honegger P, Richelson E (1977) Biochemical differentiation of aggregating cell cultures in different fetal brain regions. Brain Res 133:329-339

Hong J, Shu-Leong H, Tao X, Lap-Ping Y (1998) Distribution of catechol-O-methyltransferase expression in human central nervous system. Neuroreport 12:2861-2864

Honkanen A, Chrapusta SJ, Karoum F, Korpi ER (1994) Alterations in dopamine metabolism by intraperitoneal ethanol in rats selected for high and low ethanol preference: a 3-methoxytyramine study. Alcohol 11:323-328

Inoue K, Creveling CR (1986) Immunocytochemical localization of catechol-O-methyltransferase in the oviduct and in macrophages in corpora lutea of rat. Cell Tissue Res 245:623-628

Inoue K, Nishino T, Creveling CR (1991) Immunocytochemical evidence for the site of O-methylation in rat dental pulp. J Dent Res 70:966-969

Ishimitsu T, Hirose T, Creveling CR (1985) Determination of m- and p-O-methylated products of L-3,4-dihydroxyphenylalanine using high-performance liquid chromatography and electrochemical detection. Anal Biochem 150:300-308

Jarrott B (1971) Occurrence and properties of catechol-O-methyltransferase in adrenergic neurons. J Neurochem 18:17-27

Jeffery DR, Roth JA (1984) Characterization of membrane-bound and soluble catechol-O-methyltransferase from human frontal cortex. J Neurochem 42:826-832

Jeffery DR, Roth JA (1987) Kinetic reaction mechanism for magnesium binding to membrane-bound and soluble catechol-O-methyltransferase. Biochemistry 26:2955-2958

Jonas WZ, Gehrson ES (1974) A method for determination of catechol-O-methyltransferase in red blood cells. Clin Chim Acta 54:391-394

Kaakkola S, Wurtman RJ (1992) Effects of COMT inhibitors on striatal dopamine metabolism: a microdialysis study. Brain Res 587:241-249

Kaakkola S, Männistö PT, Nissinen E (1987) Striatal membrane-bound and soluble catechol-O-methyl-transferase after selective neuronal lesions in the rat. J Neural Transm-Gen Sect 69:221-228

Kaakkola S, Gordin A, Männistö PT (1994) General properties and clinical possibilities of new selective inhibitors of catechol O-methyltransferase. Gen Pharmacol 25:813-824

Kaplan GP, Hartman BK, Creveling CR (1979) Immunohistochemical demonstration of catechol-O-methyltransferase in mammalian brain. Brain Res 167:241-250

Karhunen T, Tilgmann C, Ulmanen I, Julkunen I, Panula P (1994) Distribution of catechol-O-methyltransferase enzyme in rat tissues. J Histochem Cytochem 42:1079-1090

Karhunen T, Tilgmann C, Ulmanen I, Panula P (1995a) Catechol-O-methyltransferase (COMT) in rat brain: immunoelectron microscopic study with an antiserum against rat recombinant COMT protein. Neurosci Lett 187:57-60

Karhunen T, Tilgmann C, Ulmanen I, Panula P (1995b) Neuronal and non-neuronal catechol-O-methyltransferase in primary cultures of rat brain cells. Int J Devel Neurosci 13:825-834

Karhunen T, Ulmanen I, Panula P (1996) Catechol-O-methyltransferase in rat sensory ganglia and spinal cord. Neuroscience 73:267-276

Kastner A, Anglade P, Bounaix C, Javoy-Agid F, Bromet N, Agid Y, Hirsch EC (1994) Immunohistochemical study of catechol-O-methyltransferase in the human mesostriatal system. Neuroscience 62:449-457

Kern C, Bernards CM (1997) Ascorbic acid inhibits spinal meningeal catechol-O-methyl transferase *in vitro*, markedly increasing epinephrine bioavailability. Anesthesiology 86:405-409

Koh S, Arai M, Kawai S, Okamoto M (1981) Assay of catechol O-methyltransferase activity by high-performance liquid chromatography with electrochemical detection. J Chromatogr 226:461-465

Koh S, Urayama K, Kawai S (1991) Application of a metal capillary column in gas chromatographic determination of catechol-O-methyltransferase activity. J Chromatogr 549:434-439

Kopin IJ (1985) Catecholamine metabolism: Basic aspects and clinical significance. Pharm. Rev. 37:333-365

Lachman HM, Papolos DF, Saito T, Yu Y-M, Szumlanski CL, Weinshilboum RM (1996) Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. Pharmacogenetics 6:243-250

Lahti RA, Majchrowicz E (1974) Ethanol and acetaldehyde effects on metabolism and binding of biogenic amines. Q J Stud Alcohol 35:1-14

Lai FM, Spector S (1978) Studies on the monoamine oxidase and catechol-O-methyltransferase of the rat cerebral microvessels. Arch Int Pharmacodyn Ther 233:227-234

Lin RL, Narasimhachari N (1974) Specific tlc, gc, and gc-ms methods for kinetic studies with COMT. Anal Biochem 57:46-58

Lotta T, Vidgren J, Tilgmann C, Ulmanen I, Melen K, Julkunen I, Taskinen J (1995) Kinetics of human soluble and membrane-bound catechol O-methyltransferase: A revised mechanism and description of the thermolabile variant of the enzyme. Biochemistry 34:4202-4210

Lundström K, Salminen M, Jalanko A, Savolainen R, Ulmanen I (1991) Cloning and characterization of human placental catechol-O-methyltransferase. DNA Cell Biol 10:181-189

Lundström K, Tilgmann C, Peränen J, Kalkkinen N, Ulmanen I (1992) Expression of enzymatically active rat liver and human placental catechol-O-methyltransferase in Eschericia coli; purification and partial characterization of the enzyme. Biochim Biophys Acta 1128:149-154

Männistö PT, Tuomainen P, Tuominen RK (1992a) Different in vivo properties of three new inhibitors of catechol-O-methyltransferase in the rat. Br J Pharmacol 105:569-574

Männistö PT, Ulmanen I, Lundström K, Taskinen J, Tenhunen J, Tilgmann C, Kaakkola S (1992b) Characteristics of catechol O-methyltransferase (COMT) and properties of selective COMT inhibitors. Prog Drug Res 39:291-350

Männistö PT and Kaakkola S (1999) Catechol-O-methyltransferase (COMT): Biochemistry and molecular biology, and pharmacology and clinical efficacy of new selective COMT inhibitors. Pharm Rev. In press

Malherbe P, Bertocci B, Caspers P, Zürcher G, Da Prada M (1992) Expression of functional membrane-bound and soluble catechol-O-methyltransferase in Escherichia coli and a mammalian cell line. J Neurochem 58:1782-1789

Mandai M, Iwaki M, Honda Y (1992) Simple and accurate assay for tyrosine hydroxylase from bovine retina by high-performance liquid chromatography with fluorescence detection. Ophth Res 24:228-233

McCaman RE (1965) Microdetermination of catechol-O-methyltransferase in brain. Life Sci 4:2353-2359

McMillian MK, Kong L-Y, Sawin SM, Wilson B, Das K, Hudson P, Hong J-S, Bing G (1995) Selective killing of cholinergic neurons by microglial activation in basal forebrain mixed neuronal/glial cultures. Biochem Biophys Res Commun 215:572-577

McMillian M, Vainio PJ, Tuominen RK (1997) Role of protein kinase C in microglia-induced neurotoxicity in mesencephalic cultures. J Neuropathol Exp Neurol 56:301-307

Meister B, Bean AJ, Aperia A (1993) Catechol-O-methyltransferase mRNA in the kidney and its appearance during ontogeny. Kidney Int 44:726-733

Milio C, Hadfield MG (1992) Ethanol alters monoamines in specific mouse brain regions. Brain Res Bull 29:599-603

Morahan PS, Edelson PJ, Gass K (1980) Changes in macrophage ectoenzymes associated with anti-tumor activity. J Immunol 125:1312-1317

Naoi M (1988) Simple assay procedure for tyrosine hydroxylase activity by high-performance liquid chromatography employing coulometric detection with minimal sample preparation. J Chrom 427:229-238

Naudon L, Dourmap N, Leroux-Nicollet I, Costentin J (1992) Kainic acid lesion of the striatum increases dopamine release but reduces 3-methoxytyramine level. Brain Res 572:247-249

Nissinen E (1984a) Determination of monoamine oxidase B activity by high-performance liquid chromatography. J Chromatogr - Biomed Appl 309:156-159

Nissinen E (1984b) The site of O-methylation by membrane-bound catechol-O-methyltransferase. Biochem Pharmacol 33:3105-3108

Nissinen E (1985) Determination of catechol-O-methyltransferase activity in brain tissue by high-performance liquid chromatography with on-line radiochemical detection. Anal Biochem 144:247-252

Nissinen E, Männistö PT (1984) Determination of catechol-O-methyltransferase activity by high-performance liquid chromatography with electrochemical detection. Anal Biochem 137:69-73

Nissinen E, Linden I-B, Schultz E, Kaakkola S, Männistö PT, Pohto P (1988a) Inhibition of catechol-O-methyltransferase activity by two novel disubstitued catechols in the rat. Eur J Pharmacol 153:263-269
Nissinen E, Tuominen R, Perhoniemi V, Kaakkola S (1988b) Catechol-O-methyltransferase activity in human and rat small intestine. Life Sci 42:2609-2614

Nissinen E, Linden I-B, Schultz E, Pohto P (1992) Biochemical and pharmacological properties of a peripherally acting catechol-O-methyltransferase inhibitor entacapone. Naunyn-Schmiedeberg's Arch Pharm 346:262-266

Nohta H, Noma S, Ohkura Y, Yoo B (1984) Assay for catechol-O-methyltransferase in erythrocytes using a new fluorogenic substrate, 2-(3,4-dihydroxyphenyl)naphthol[1,2-d]thiazole. J Chromatogr 308:93-100

Nohta H, Noma S, Ohkura Y (1986) High-performance liquid chromatographic assay for catechol-O-methyltransferase in human and rat erythrocyte membrane and soluble fractions, and tissues. Chem Pharm Bull 34:4687-4693

Okada Y, Zaitsu K, Ohtsubo K, Nohta H, Ohkura Y (1981) Fluorimetric assay for catechol-O-methyltransferase. Chem Pharm Bull 29:1670-1673

Parvez H, Parvez S (1972) Simplified and precise method for the assay of enzyme catechol-O-methyltransferase without high speed fractionation. Clin Chim Acta 39:253-254

Parvez H, Parvez S (1973) Micro radioisotopic determination of enzymes catechol-O-methyltransferase, phenylethanolamine-N-methyltransferase and monoamine oxidase in a single concentration of tissue homogenate. Clin Chim Acta 46:85-90

Paulsen RE, Contestabile A, Villani L, Fonnum F (1987) An in vivo model for studying function of brain tissue temporarily devoid of glial cell metabolism: the use of fluorocitrate. J Neurochem 48:1377-1385

Paxinos G, Watson C (eds) (1982) The rat brain in stereotaxic coordinates, 1st edn. Academic Press, Sydney

Pelton EW, Kimelberg HK, Shipherd SV, Bourke RS (1981) Dopamine and norepinephrine uptake and metabolism by astroglial cells in culture. Life Sci 28:1655-1663

Pennings EJM, Van Kempen GMJ (1979) Assay of catechol O-methyltransferase by determination of the m- and o-methylated products using high-performance liquid chromatography. Anal Biochem 98:452-454

Perez RA, Fernandez-Alvarez E, Nieto O, Piedrafita FJ (1993) Inhibition of catechol-O-methyltransferase by 1-vinyl derivatives of nitrocatechols and nitroguaiacols. Kinetics of the irreversible inhibition by 3-(3-hydroxy-4-methoxy-5-nitrobenzylidene)-2,4-pentadione. Biochem Pharmacol 45:1973-1981

Raxworthy MJ, Gulliver PA, Hughes PJ (1982) The cellular location of catechol-O-methyltransferase in rat liver. Naunyn-Schmiedeberg's Arch Pharm 320:182-188

Rivett J, Roth JA (1982) Kinetic studies on the O-methylation of dopamine by human brain membrane-bound catechol-O-methyltransferase. Biochemistry 21:1740-1742

Rivett J, Eddy B, Roth J (1982) Contribution of sulfate conjugation, deamination, and O-methylation to metabolism of dopamine and norepinephrine in human brain. J Neurochem 39:1009-1016

Rivett J, Francis A, Roth JA (1983a) Distinct cellular localization of membrane-bound and soluble forms of catechol-O-methyltransferase in brain. J Neurochem 40:215-219

Rivett J, Francis A, Roth JA (1983b) Localization of membrane-bound catechol-O-methyltransferase. J Neurochem 40:1494-1496

Roth JA (1992) Membrane-bound catechol-O-methyltransferase: a reevaluation of its role in the O-methylation of catecholamine neurotransmitters. Rev Physiol Biochem Pharmacol 120:1-29

Schultz E, Nissinen E (1989) Inhibition of rat liver and duodenum catechol-ortho-methyltransferase by a tight-binding inhibitor OR-462. Biochem Pharmacol 38:3953-3956

Schultz E, Nissinen E, Kaakkola S (1989) Determination of catechol-O-methyltransferase activity in erythrocytes by high performance liquid chromatography with electrochemical detection. Biomedical Chromatography 3:64-67

Seeds NW (1975) Expression of differentiated activities in reaggregated brain cell cultures. J Biol Chem 250:5455-5458

Shoup RE, Davis GC, Kissinger PT (1980) Determination of catechol-O-methyltransferase activity in various tissues by liquid chromatography. Anal Biochem 52:483-487

Silberstein SD, Shein HM, Berv KR (1972) Catechol-O-methyltransferase and monoamine oxidase activity in cultured rodent astrocytoma cells. Brain Res 41:245-248

Sladek-Chelgren S, Weinshilboum RM (1981) Catechol-O-methyltransferase biochemical genetics: human lymphocyte enzyme. Biochem Genet 19:1037-1053

Smit NPM, Pavel S, Kammeyer A, Westerhof W (1990) Determination of catechol-O-methyltransferase activity in relation to melanin metabolism using high-performance liquid chromatography with fluorimetric detection. Anal Biochem 190:286-291

Soares-da-Silva P (1994) Source and handling of renal dopamine: its physiological importance. News Physiol Sci, 9:128-134

Spatz M, Kaneda N, Sumi C, Creveling CR, Nagatsu T (1986) The presence of catechol-O-methyltransferase activity in separately cultured cerebromicrovascular and smooth muscle cells. Brain Res 381:363-367

Sternson LA, Sternson AW, Bannister SJ (1976) A differential pulse polarographic assay for O-methylation of catechols by catechol-O-methyltransferase. Anal Biochem 75:142-152

Storrie B, Madden EA (1990) Isolation of subcellular organelles. Meth Enzymol 182:203-225

Taskinen J, Vidgren J, Ovaska M, Bäckström R, Pippuri A, Nissinen E (1989) QSAR and binding model for inhibition of rat liver catechol-O-methyltransferase by 1,5-substituted-3,4-dihydroxybenzenes. Quant Struct-Act Relat 8:210-213

Takahashi S, Yoshioka M, Yoshie S, Tamura Z (1978) Mass fragmentographic determination of vanilmandelic acid, homovanillic acid and isohomovanillic acid in human body fluids. J Chrom. 145:1-9

Tenhunen J (1996) Characterization of the rat catechol-O-methyltransferase gene proximal promoter: Identification of nuclear protein-DNA interaction that contributes to the tissue-specific regulation. DNA Cell Biol 15:461-473

Tenhunen J, Ulmanen I (1993) Production of rat soluble and membrane-bound catechol O-methyltransferase forms from bifunctional mRNAs. Biochem J 296:595-600

Tenhunen J, Salminen M, Jalanko A, Ukkonen S, Ulmanen I (1993) Structure of the rat catechol-o-methyltransferase gene - separate promoters are used to produce messenger RNAs for soluble and membrane-bound forms of the enzyme. DNA Cell Biol 12:253-263

Tenhunen J, Salminen M, Lundström K, Kiviluoto T, Savolainen R, Ulmanen I (1994) Genomic organization of the human catechol-O-methyltransferase gene and its expression from two distinct promoters. Eur J Biochem 223:1049-1059

Tilgmann C, Melen K, Lundström K, Jalanko A, Julkunen I, Kalkkinen N, Ulmanen I (1992) Expression of recombinant soluble and membrane-bound catechol-O-methyltransferase in eukaryotic cells and identification of the respective enzymes in rat brain. Eur J Biochem 207:813-821

Törnwall M, Kaakkola S, Tuomainen P, Kask A, Männistö PT (1994) Comparison of two new inhibitors of catechol O-methylation on striatal dopamine metabolism: a microdialysis study in rats. Br J Pharmacol 112:13-18

Tokunaga K, Ishikawa K (1992) Evidence for different sites of monoamine oxidase and catechol-O-methyltransferase in the striatum. Brain Res 575:164-166

Trendelenburg U (1986) The metabolizing systems involved in the inactivation of catecholamines. Naunyn-Schmiedeberg's Arch Pharm 332:201-207

Trendelenburg U (1990) The interaction of transport mechanisms and intracellular enzymes in metabolizing systems. J Neur Transm Suppl 32:3-18

Tunnicliff G, Ngo TT (1983) Kinetics of rat brain soluble catechol-O-methyltransferase and its inhibition by substrate analogues. Int J Biochem 15:733-738

Tuomainen P, Reenilä I, Männistö PT (1996) Validation of the assay of catechol-O-methyltransferase activity in human erythrocytes. J Pharmaceut Biomed Anal 14:515-523

Ulmanen I, Lundström K (1991) Cell-free synthesis of rat and human catechol-O-methyltransferase. Eur J Biochem 202:1013-1020

Ulmanen I, Peränen J, Tenhunen J, Tilgmann C, Karhunen T, Panula P (1997) Expression and intracellular localization of catechol-O-methyltransferase in transfected mammalian cells. Eur J Biochem 243:452-459

Vidgren J, Ovaska M (1997) Structural aspects in the inhibitor design of catechol O-methyltransferase. In: Veerapandian P (ed) Structure-based drug design, 1st edn. Marcel Dekker, Inc, New York

Vidgren J, Tilgmann C, Lundström K, Liljas A (1991) Crystallization and preliminary X-ray investigation of a recombinant form of rat catechol-O-methyltransferase. Proteins 11:233-236

Vidgren J, Svensson LA, Liljas A (1994) Crystal structure of catechol O-methyltransferase. Nature 368:354-358

Vieira-Coelho MA, Soares-da-Silva P (1996) Ontogenic aspects of liver and kidney catechol-O-methyltransferase sensitivity to tolcapone in developing rats. Br J Pharmacol 117:516-520

Vieira-Coelho MA, Soares-da-Silva P (1999) Effects of tolcapone upon soluble and membrane-bound brain and liver catechol-O-methyltransferase. Brain Res 821:69-78

Waldmeier PC, Baumann PA, Feldtrauer J-J, Hauser K, Bittiger H, Bischoff S, Von Sprecher G (1990) CGP 28014, a new inhibitor of cerebral catechol-O-methylation with a non-catechol structure. Naunyn-Schmiedeberg's Arch Pharm 342:305-311

Weinshilboum RM (1988) Pharmacogenetics of methylation: Relationship to drug metabolism. Clin Biochem 21:201-210

Weinshilboum R, Otterness DM, Szumlanski CL (1999) Methylation pharmacogenetics: Catechol-O-methyltransferase, thiopurine methyltransferase, and histamine N-methyltransferase. Annu Rev Pharmacol Toxicol 39:19-52

Weisz J, Fritz-Wolz G, Clawson GA, Benedict CM, Abenroth C, Creveling CR (1998) Induction of nuclear catechol-O-methyltransferase by estrogens in hamster kidney: Implications for estrogen-induced renal cancer. Carcinogenesis 19:1307-1312

Wiese C, Cogoli-Greuter M, Weinreich R, Winterhalter KH (1993) COMT inhibitors and metabolism of fluorodopa enantiomers in aggregating cell cultures. Naunyn-Schmiedeberg's Arch Pharm 348:582-585

Wilkinson GN (1961) Statistical estimations in enzyme kinetics. Biochem J 80:324-332

Wilson VG, Grohmaan M, Trendelenburg U (1988) The uptake and O-methylation of 3H-(+-)isoprenaline in rat cerebral cortex slices. Naunyn-Schmiedeberg's Arch Pharm 337:397-405

Winqvist R, Lundström K, Salminen M, Laatikainen M, Ulmanen I (1992) The human catechol-O-methyltransferase (COMT) gene maps to band q11.2 of chromosome 22 and shows a frequent RFLP with Bg/I. Cytogenet Cell Genet 59:253-257

Wood PL, Altar A (1988) Dopamine release in vivo from nigrostriatal, mesolimbic, and mesocortical neurons: Utility of 3-methoxytyramine measurements. Pharmacol Rev 40:163-187

Wooten GF, Coyle JT (1973) Axonal transport of catecholamine synthesizing and metabolizing enzymes. J Neurochem 20:1361-1371

Xie T, Ho S-L, Ramsden D (1999) Characterization and implications of estrogenic down-regulation of human catechol-O-methyltransferase gene transcription. Mol Pharmacol 56:31-38

Zaitsu K, Okada Y, Nohta H, Kohashi K, Ohkura Y (1981) Assay for catechol-O-methyltransferase by high-performance liquid chromatography with fluorescence detection. J Chromatogr 211:129-134

Zhu BT, Conney AH (1998) Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis 19:1-27

Zürcher G, DaPrada M (1982) Rapid and sensitive single-step radiochemical assay for catechol-O-methyltransferase. J Neurochem 38:191-195

Zürcher G, Colzi A, Da Prada M (1990) Ro 40-7592: Inhibition of COMT in rat brain and extracerebral tissue. J Neural Transm Suppl 32:375-380