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Review

Separation methods for catechol *O*-methyltransferase activity assay: physiological and pathophysiological relevance

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Abstract

Catechol *O*-methyltransferase (COMT) transfers a methyl group from *S*-adenosyl-L-methionine to the catechol substrate in the presence of magnesium. After the characterisation of COMT more than four decades ago, a wide variety of COMT enzyme assays have been introduced. COMT activity analysis usually consists of the handling of the sample and incubation followed by separation and detection of the reaction products. Several of these assays are validated, reliable and sensitive. Besides the studies of the basic properties of COMT, the activity assay has also been applied to explore the relation of COMT to various disease states or disorders. In addition, COMT activity analysis has been applied clinically since COMT inhibitors have been introduced as adjuvant drugs in the treatment of Parkinson's disease.

Keywords: Reviews; Catechol *O*-methyltransferase; Enzymes

Contents

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1. Introduction

Catechol *O*-methyltransferase (EC 2.1.1.6, COMT) is an enzyme that catalyzes the transfer of a methyl group from *S*-adenosyl-L-methionine (AdoMet) to one of the hydroxyls of a catechol (Fig. 1). COMT was first described by Axelrod and Tomchick, who showed that it is the enzyme that is responsible for the 3-*O*-methylation of catecholamines [1]. Since that COMT has proved to play an important role not only in the inactivation of catecholamines but also in the metabolism of catecholestrogens, dietary catechols and catecholic drugs, such as L-dopa, carbidopa, benserazide, isoprenaline, and dobutamine. COMT can be found in many tissues, but the highest activities are found in liver, kidney and intestinal tract [2].

In the late 1980s, potent and selective COMT inhibitors with a nitrocatechol structure were introduced [3]. These compounds are used in combination with L-dopa and a dopadecarboxylase inhibitor in order to increase the bioavailability of L-dopa in the treatment of Parkinson's disease. The discovery of COMT as a drug target greatly increased the interest in this enzyme. Since that the soluble COMT (S-COMT) has been purified from rat liver [4] and human placenta [5] and subsequently cDNA cloned [6,7]. The membrane-bound COMT (MB-COMT) was cloned from the human hepatoma cell line G2 [8]. Recombinant COMT proteins have been produced in *Escherichia coli* [9,10] and in insect and mammalian cell lines [7–9,11].

S-COMT is located in the cytosol and MB-COMT is anchored to the rough endoplasmic reticulum [5,7,8,12]. Although the amino acid sequences of the two forms are identical, except a 50-amino-acid-long membrane anchoring signal sequence in the MB-form, the kinetic properties and in vitro regioselectivity of the enzymes are different [9,13,14]. Recently, also the presence of nuclear S-COMT has been suggested [12,15]. S-COMT is the predominant form in most tissues, but MB-COMT has a greater abundance in the human brain [16] and in pheochromocytoma [17]. COMT mRNA levels and protein levels have a poor correlation due to additional production of S-COMT from the longer, MB-COMT protein producing COMT transcript [18]. COMT can be visualised by immunohistochemical methods [19], but information of the relative amounts of COMT isoforms is missing since COMT antibodies do not distinguish between MB-COMT and S-COMT. Both isoforms, however, can be quantitated by protein blotting [20], but whether or not all expressed COMT protein is active is not known.

COMT appears in two polymorphic forms; a thermolabile low activity form contains Met-108

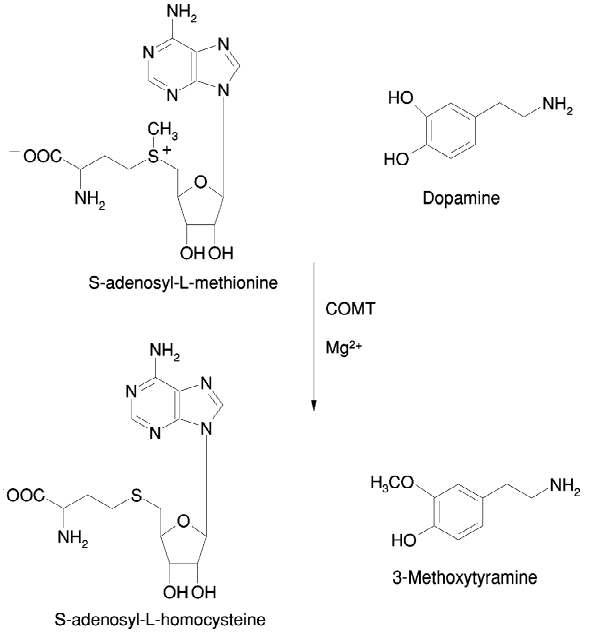

Fig. 1. COMT-catalysed methylation of dopamine.

(158 in MB-COMT) and a thermostable high activity form contains Val-108 (158 in MB-COMT) [13]. The two alleles result in homozygous individuals with high or low COMT activity and heterozygous individuals with intermediate activity [21]. Orientals and black Americans have been shown to exhibit a higher erythrocyte COMT activity than do Caucasians [22,23]. Recently, the association of COMT polymorphism with neurological and psychiatric disorders, such as schizophrenia, aggressiveness, and drug abuse as well as breast cancer risk has been suggested in many studies (for a review see Ref. [24]). However, contradicting findings have also been published [24].

As interest in COMT has increased, more reliable, sensitive and rapid analytical COMT assays are needed, for example, for the measurement of activity of the recombinant proteins, for the testing of in vitro efficacy of new COMT inhibitor candidates, for the determination of structure–activity relationships of COMT, and for the measurement of COMT activity in various physiological and pathophysiological states. In addition, the efficacy of the COMT inhibitors in clinical studies is commonly determined from erythrocytes or mononuclear cells, because they exhibit COMT activity reflecting that in the liver [25–27] and they can be obtained relatively non-invasively. COMT activity is, however, low in these cells, and good sensitivity is required from the analytical method. Two regioisomeric products may be formed in the COMT-catalysed reaction. In cases where regioselectivity of the enzyme is studied, it is essential that the analytical method is capable of separating the regioisomeric O-methylated products from each other. As S-COMT and MB-COMT seem to exhibit different functions, the former being more relevant in the inactivation of xenobiotic catechols and the latter playing an important role in the termination of catecholaminergic neurotransmission [13,21,28], sensitive and reliable enzyme assay with separate MB-COMT and S-COMT fractions would provide considerable information about COMT.

This review is focused on analytical methods used in COMT activity assays, especially on the separation step in these methods. Incubation conditions and selection of the substrate and enzyme source, which are critical in COMT assays, are also discussed.

2. Variation of the basal COMT activity

In human tissues, basal COMT activity is distributed to three activity levels due to genetical polymorphism [29–31]. Low activity with thermolability is associated with a substitution of valine to methionine in amino acid 108 in S-COMT (or 158 in MB-COMT) peptide [13,32]. Individuals, who are homozygous for methionine have three to four times lower COMT activity than those who are homozygous for valine whereas heterozygotes have intermediate activity levels. By preheating the COMT samples before enzyme reaction the formation of catecholamines [13] and catecholestrogens [33] was reduced in low activity forms of COMT producing lower heated:control (H:C) ratio. Notably, rats and pigs have leucine at amino acid 108 [6]. The possible significance of the genetically determined COMT activity levels in various diseases and disorders has been studied recently. Although the correlation of COMT genotype with certain disorders is not strong and occasionally variable [24], some interesting positive correlations have been found, for example, with breast cancer [34] and alcoholism [35] (reviewed in Ref. [24]).

In general, basal COMT activity or expression is not greatly changed and drug treatments do not seem to affect greatly COMT expression [24]. Variations of basal COMT activity between different rat strains [36] and also gender differences [29] have been reported.

In some cases, which are related to disease states, COMT expression has been changed. Rat and hamster insulinomas have higher basal COMT activity [37], in human pheochromocytomas [17] and in estrogen-sensitive breast cancer the COMT expression has been increased [20]. At molecular level, the possible higher risk of developing a breast cancer by low COMT activity patients could be related to metabolism of catecholestrogens, the hydroxylated products of estrogen. Certain catecholestrogens are potentially mutagenic [15,38] and low COMT activity could be associated with reduced protection of the tissue against tumours [24,39]. Interestingly, estrogen has been claimed to reduce COMT expression in vitro [40], which could be one possible mechanism. In contrast, in hamster kidney S-COMT expression has been shown to be increased after

estrogen treatment [15], which could represent a protective response [24]. Finally, chemical lesioning of the brain by kainic acid [41,42] or glial toxin fluorocitrate [43] has been reported to change the COMT activity. Possibly an acute neuronal damage could induce the microglial activation leading to elevated COMT activity [43], which is followed by astroglial proliferation and prolonged higher level of COMT activation [41].

3. COMT catalysed reaction

To analyse enzyme activity, three factors must be included: (1) the detection method, which usually quantitates the reaction product derived from (2) the substrate, which has to be properly metabolised by (3) the enzyme source, the actual sample. In addition, incubation conditions have to be optimised. This comprises, for example, required supplements, pH of the incubation mixture, temperature, incubation time, and stability of the enzyme and substrates during reaction. Early COMT activity assays have been reviewed by Guldberg and Marsden [2].

3.1. Incubation conditions

Mg^{2+} ions are essential for COMT activity, since they are coordinated to both of the catecholic hydroxyls, to a water molecule and to three amino acid residues in the catalytic site of COMT [44], and are routinely added to reaction mixtures. Ca^{2+} ions inhibit the activation of COMT by Mg^{2+} ions [45], and therefore assays including removal of calcium and other divalent cations from erythrocyte lysates with chelating resin have been developed [46]. Although Ca^{2+} chelation was not necessary in a more recent red blood cell COMT assay [47], in other tissues addition of EGTA (ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid) in the reaction has also led to enhanced activity [48].

S-Adenosyl-homocysteine (AdoHcy), the demethylated end product of AdoMet, inhibits COMT [49]. In some assays adenosine deaminase has been added to the reaction mixture in order to convert formed AdoHcy to *S*-inosylhomocysteine [50–52]. The K_i value of AdoHcy for purified human liver S-COMT is 39 μ*M* [53], and in most assays its concentration is considered to be much lower than that and, consequently, the inhibitory effect to be negligible.

COMT assays are normally performed under physiological conditions (pH around 7.4 and temperature +37 °C). The proportion of *p*-*O*-methylated metabolites of norepinephrine and its ring fluorinated derivatives have been shown to increase with pH of the incubation mixture as compared to the *m*-*O*-methylated metabolites [54,55]. This is suggested to be due to ionisation of the *p*-hydroxyl at higher pH values. The ratio of regioisomeric products of for example 2-hydroxyestradiol, dopamine and epinephrine also changes with pH of the incubation medium, but this phenomenon seems to be substrate-dependent [53,56]. Besides the effect on regioselectivity, pH has also been reported to affect the kinetic parameters of catecholamines [48]. The optimum temperature for the O-methylation of 2-hydroxyestradiol catalysed by purified human liver COMT was 42 °C [53]. Increase in temperature did not affect the percentage of the regioisomeric products formed.

3.2. Enzyme kinetics: the significance of the substrate

A wide selection of substrates are available for the COMT assay [57] since COMT has specificity only to a catechol structure. Some natural substrates, such as catecholamines and catecholestrogens, have enzymatic or non-enzymatic degradation, which could decrease the amount of the measured product. Dopamine auto-oxidation, for example, has been prevented by low pH and antioxidants, such as vitamin C, which is a substrate of COMT at millimolar concentrations [58]. In addition, metabolism of natural catecholamines by monoamine oxidase (MAO) has been inhibited by pargyline [48]. Some suitable exogenic substrates, which obviously are not degraded, include 3,4-dihydroxybenzoic acid and 3,4-dihydroxybenzaldehyde [59]. Some substrates do not follow the Michaelis–Menten kinetics and may show substrate inhibition, as shown for example for 2-hydroxyestradiol [53], and in these cases high substrate concentrations should be avoided.

MB-COMT has a higher affinity but lower reaction capacity than S-COMT for most of the

substrates. For example, dopamine has K_m values of 280 μ*M* and 1000 μ*M* for rat and human brain S-COMT, respectively, whereas the K_m values of 3.6 μ*M* and 3.3 μ*M* for rat and human brain MB-COMT, respectively, have been reported [41,60]. The recombinant COMT enzymes have similar K_m values (15.1 μ*M* and 207 μ*M*) as the native enzymes [13]. Since the enzyme activity assays have usually been performed in conditions several times higher than K_m values of S-COMT, the results may overestimate the importance of S-COMT in the metabolism of endogenous substrates [28]. Compared to extracellular dopamine concentrations, about 5 n*M* [61], endogenous dopamine and noradrenaline concentrations in the rat brain (homogenate) have been estimated to be 0.8–65 μ*M* [24]. This suggests that in the intracellular (vesicular and cytosolic) space, where COMT resides, the concentrations of catecholamines are at low micromolar range. Due to a higher affinity of MB-COMT for catecholamines, it is reasonable to assume that most of the endogenous catecholamine metabolism passes via MB-COMT. Indeed, it has been estimated, by enzyme kinetic calculations, that dopamine and noradrenaline are metabolised preferably by MB-COMT at low, physiological concentrations in human brain [28,60,62]. Furthermore, it has been suggested that both MB-COMT and S-COMT activity could be analysed from the same tissue sample by using low and high, respectively, concentration of the substrate [28,60,62]. However, by recent kinetic estimation, it was calculated that this might be done with human brain homogenates, but not with the rat brain samples due to lower amount of MB-COMT relative to S-COMT in rat brain [63]. Thus, to perform physiologically relevant enzyme activity analysis, such as studies of competition between catecholamines and catecholestrogens [64] enzyme kinetics should be considered.

The O-methylation of catechols shows regioselectivity. In vivo the O-methylation of catecholamines by COMT is almost exclusively to the 3-hydroxyl (*meta*) of the catechol ring whereas in vitro also 4-methoxy (*para*) product formation can be found [65]. Although the *m*/*p* ratio could be affected by the pH of the incubation solution [66] (see Section 3.1), with several substrates the formation of *para* product is usually higher (lower *meta*/*para* ratio) with S-COMT than with MB-COMT being about constant over a wide range of substrate concentration. However, by decreasing the amount of the substrate to the low micromolar concentrations the formation of *para* product is progressively decreasing with MB-COMT (high *meta*/*para* ratio) with partially purified [14] or recombinant [67] COMT resembling the situation of the in vivo product profile. This also supports the relative importance of MB-COMT over S-COMT in catecholamine metabolism.

3.3. The importance of COMT enzyme source

COMT enzyme activity in a certain tissue has usually been expressed as specific activity or V_{max} values, i.e. activity units (pmol/min) in mg protein, which is related to the amount of COMT in different tissue samples. As a matter of fact, these values express the purity of the enzyme. Therefore, the activities between differently prepared COMT enzyme sources are not necessarily comparable. By using a similar isolation method, specific activities of MB-COMT and S-COMT in different tissues could be compared, as has recently been shown [68]. Proper characterisation of the sample handling, isolation and purification process prevents a loss of an unknown amount of COMT, for example, due to poor homogenisation. In this case, the activity of nuclear S-COMT [12,15] could remain undetected. Notably, for the expression of specific activity, the variation derived from the protein assay should also be considered [69]. In further studies of the properties of the enzyme in a certain tissue, enzyme kinetic analyses have been performed. For kinetic experiments rather large amounts of enzymes are needed and preferably a highly purified protein (for a review of purification methods, see Ref. [70]) or recombinant enzymes [13] need to be used. These preparations have a high specific activity, which reduces the possible background noise. When purified COMT enzyme protein is used, stabilisation of the readily oxidised sulphydryl group(s) is needed [70] to maintain COMT activity. Reducing agents used for this purpose include L-cysteine, mercaptoethanol and dithiothreitol.

Traditionally, the linearity of an enzyme assay is usually characterised for a certain range of the tissue protein. However, instead of the V_{max} values, the k_{cat}

values, i.e. catalytic values (in mol substrate metabolised in time unit), are sometimes more useful since k_{cat} describes the enzyme activity independently of the enzyme purity. Catalytic values have been determined by analysing the actual molar amount of the enzyme by tight-binding COMT inhibitors [13,71–73]. Alternatively, protein blotting [20] and ICELISA [33] using purified COMT proteins as references, have been utilised to estimate the actual amount of COMT, which is needed for the calculations of k_{cat}. As an example, if the same amount of protein from brain and liver in an assay was used, the IC_{50} values of a COMT inhibitor between brain and liver differed, while if the molar amount of COMT enzyme from both sources was the same in an assay, the IC_{50} values did not differ [73]. This is due to the fact that the samples from different tissues do not contain the same amount of COMT since there are no data suggesting different properties of COMT derived from different tissues in an individual. In addition, in primary cultured cells of the rat brain, for example, the greater potency of COMT inhibitors for neuronal than glial cultures is obviously due to a lower amount of COMT in neuronal cultures [74]. Due to similar structure of the catalytic site of both isoforms of COMT, the K_i values for entacapone and tolcapone are similar [13]. Thus, the possible differences between the potencies of COMT inhibitors in vivo or ex vivo are related to the distribution properties of the COMT inhibitors, e.g. lipophilicity [74]. Anyway, by using the same molar concentration of enzyme in an assay, COMT activity analysis is suitable for ED_{50} studies [73], to compare, for example, the different doses of COMT inhibitors to achieve a suitable inhibition in certain tissues.

COMT activity has also been analysed from a sample which contains both isoforms of COMT. This approach has been used utilising COMT homogenates [75], cell cultures [74], brain slices [76], tissue blocks [77–79] and lung perfusion [80]. By using two different substrate concentrations in a single homogenate, both forms of COMT have been analysed [28,60,62]. Also, by reduction of the substrate concentration near the K_m value of S-COMT it was possible to reveal the change of COMT activity ex vivo after pharmacological treatment [43]. Due to differences in substrate regioselectivity, i.e. methylation to *meta* or *para* position [13,14,65], the *meta*/*para* ratio could be useful to estimate the relative proportion of COMT isoforms in a sample [63].

4. Analytical methods in COMT assays

There is a great variation in analytical methods of COMT assays. The most commonly used methods are shown in Fig. 2 and are discussed in the following.

4.1. Direct methods

Although most assays for COMT activity include a separation step in which O-methylated products are separated physically from the substrate and/or co-substrate, some early methods excluded that step. Some of these assays were based on color reactions that were characteristics for the parent compounds, but did not apply to the O-methylated metabolites [81,82]. Determination of, for example, the COMT-catalysed methylation of catecholamines was based on their complex with hydroxylamine, which forms a colored complex with ferric chloride reagent [81]. Quantitation was performed by following the disappearance of the catechol substrate colorimetrically. O-Methylation of nitrocatechol could also be determined by measuring its disappearance, since it exhibits a cherry-red color when both of the hydroxyls are ionised in strong alkali [82]. Although the reported repeatability values for these assays were reasonable (RSD = 4–9%, $n = 5–10$) [81,82], sensitivity was not very high and stability of the color complexes might have caused a problem. In addition, measurement of the products of an enzyme-catalysed reaction is generally more preferable than following the disappearance of the substrate, since other reactions than the targeted may also take place affecting the disappearance of the substrate. In a spectrophotometric assay the O-methylated products of 3,4-dihydroxyacetophenone could be quantitated without separation from the parent compound due to the shift of their absorption maxima to longer wavelengths as compared to the parent compound [83]. The method required knowledge of the relative amounts of the regioisomeric products formed from 3,4-dihydroxyacetophenone.

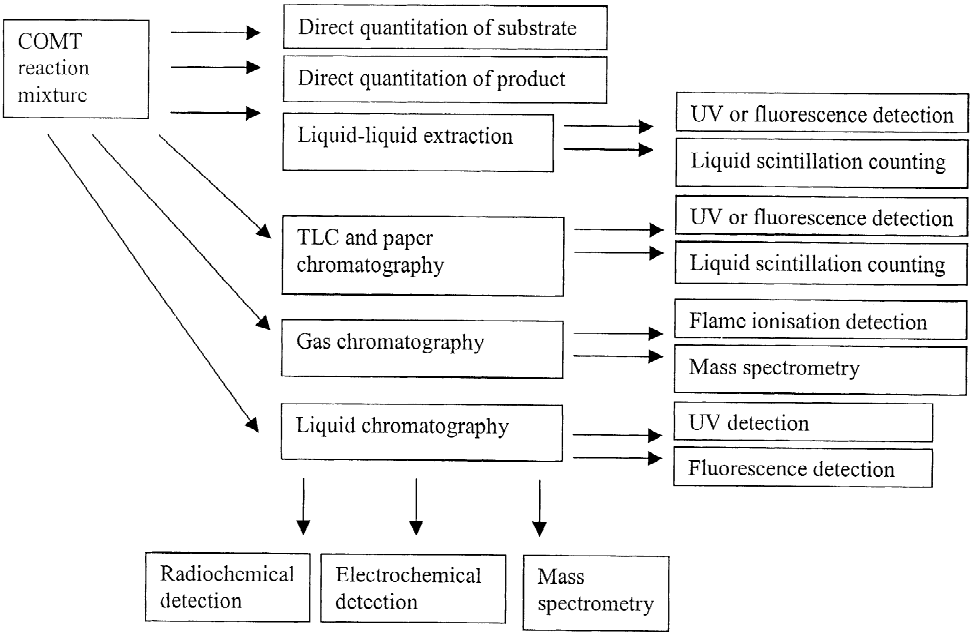

Fig. 2. Analytical methods applied to COMT activity analysis.

In the COMT-catalysed reaction, transfer of the methyl group from AdoMet to a catecholic hydroxyl results in the formation of not only methoxy derivatives but also in the formation of AdoHcy (Fig. 1). The continuous spectrophotometric COMT assay of Coward and Wu was based on difference in the ultraviolet absorption spectra of AdoHcy and *S*-inosylhomocysteine, which was formed from the former when adenosine deaminase was added to the reaction mixture [50]. Besides offering a detection system, this coupled enzyme assay continuously removed AdoHcy, which has been shown to inhibit COMT [49]. The main disadvantage of the method was high background detected at the analytical wavelength used (265 nm).

4.2. Liquid–liquid extraction

The first methods to separate an O-methylated metabolite from the parent compound included liquid–liquid extraction with organic solvents. Choice of the solvent was dependent on the substrate used, but commonly used organic solvents were isoamyl alcohol [84,85], toluene [46], a mixture of isoamyl alcohol and toluene [51,86–88], ethyl acetate [66,89–93], ethyl ether [94], a mixture of ethyl ether and chloroform [95] and chloroform [59]. After extraction, the organic phase was usually evaporated and the products quantitated from the evaporation residue with different detection systems. In some cases liquid–liquid extraction was combined with another separation method, such as TLC or paper chromatography [46,90,91,94–96], HPLC [66,97–99] or GC [59,100], prior to detection.

4.2.1. Fluorometric detection

In an early work, metanephrine, the 3-*O*-methylated metabolite of epinephrine, was quantitated fluorometrically after extraction from borate buffer (pH 10) with organic solvents and re-extraction to acidic aqueous phase with 0.1 N HCl [1]. To control the recovery of metanephrine in this two-step extraction, a synthetic reference standard was carried through the procedure. Possible interference of the fluorometric detection by, for example, the parent compound was not discussed.

4.2.2. Radiochemical detection

The introduction of methods relying on radioactivity measurement improved the sensitivity of

COMT assays. The radioactive label may be in the substrate or in the methyl group of the co-substrate AdoMet. A disadvantage in using labelled co-substrate is that it can act as a co-substrate for other enzymes as well, which can lead to erroneous results when using crude homogenates [2]. On the other hand, use of labelled co-substrate enables free choice of the catechol substrate and does not require synthesis of labelled catecholic compounds. In general, labelled AdoMet was easier to separate from the O-methylated product than the parent compound, due to higher hydrophobicity difference between the two molecules. As COMT was first discovered as an enzyme involved in the inactivation of catecholamines, these endogenous compounds were also the first compounds used as the substrate in the radiochemical in vitro assays for COMT [84–87,90]. Other substrates, for example 3,4-dihydroxyphenethanol [93], 3,4-dihydroxybenzoic acid [46,88,89], 3,4-dihydroxyacetic acid [51,92], 2-hydroxyestrone [94] and catechol [52], have been used to improve the sensitivity of the assay, since these compounds have revealed higher affinity to COMT than catecholamines themselves.

Evaporation of the organic solvent has been suggested to be a critical step in the COMT assays utilising radioactive compounds, because high amounts of [^{14}C]methanol were formed enzymatically from [^{14}C]AdoMet during incubation in human erythrocytes [84]. Later, however, Jonas and Gershon showed that [^{14}C]methanol did not interfere with their erythrocyte COMT assay utilising [^{14}C]AdoMet and 3,4-dihydroxyphenyl acetic acid [92]. The evaporation step has been omitted by extracting the analytes directly with scintillation fluid [52]. Physical separation of the water and organic solvent layers before liquid scintillation counting could be avoided on the basis of an assumption that water quenches the emission of [^{3}H]AdoMet remaining in the water phase [51].

The main disadvantages in liquid–liquid extraction have been poor, and in some cases variable recovery, and poor selectivity of the extraction. Poor recovery directly affects the sensitivity of the method. For example in a method with [^{14}C]adrenaline as the substrate, recovery of extraction, tested with unlabelled metanephrine, was only 42% [87]. Results on COMT activity were then corrected by using this percentage. This can lead to erroneous results if the recovery is variable. A variable recovery (between 45 and 85%) was reported for 2-methoxyestrone, but the recovery was controlled by spiking ^{14}C-labelled 2-methoxyestrone in each sample before extraction [94]. Recoveries exceeding 90% have been reported for O-methylated products of 3,4-dihydroxyphenethanol, *N*-acetyldopamine, and 3,4-dihydroxyphenylacetic acid [92,93]. TLC has been used to reveal how much of the extracted radioactivity actually represents the compound that should be analysed [46,52]. Regarding poor selectivity, at least products originating from labelled AdoMet could also be found from the organic layer [2] and blank samples omitting enzyme or substrate (when labelled AdoMet was used) were essential to prepare. Despite the problems discussed above, the few repeatability values reported for radiochemical assays based on liquid–liquid extraction show relative standard deviations less than 2% ($n = 20$) [52,87]. Especially the method of Raymond and Weinshilboum, which utilises [^{14}C]AdoMet and 3,4-dihydroxybenzoic acid, as such or with minor modifications, has been widely used in studies relating to variation of erythrocyte COMT activity between individuals and ethnic groups [22,23,29,101,102].

4.3. Paper and thin layer chromatography

Paper or thin layer chromatography has been used to identify the O-methylated products, extracted from COMT incubation mixtures, by comparing the *Rf* values with those of authentic reference compounds [1,46,52,84,85,90]. The spots were visualised by colour reagents or under ultraviolet light. A scanner capable of detecting radioactivity directly from TLC plates or paper has also been used in analysing the samples [90,95].

TLC has provided another separation step after liquid–liquid extraction. However, after chromatographic separation the spots representing the O-methylated metabolites had to be eluted from the plate and could be quantitated, for example, fluorometrically [91] or with liquid scintillation counting [56,65,94]. Lin and Naraschimhachari were able to quantitate 4-*O*-methyldopamine fluorometrically directly from a silica gel plate after a post-separation derivatization with isothiocyanate [96]. A disadvan-

tage of TLC and paper chromatography is that in many cases the regioisomeric products could not be separated from each other. For example, separation of 3- and 4-*O*-methyldopa was achieved only after extracting the co-eluted compounds from the paper and applying the mixture to a resin column [56]. However, the products of 2-hydroxyestradiol could be separated from each other with paper chromatography and even quantitated directly on the paper by a paper-strip scanner [95]. In more recent COMT assays other chromatographic methods, mainly HPLC, have overtaken TLC as a separation method.

4.4. Gas chromatography

With gas chromatography regioisomeric O-methylated products are relatively easily separated from each other. Gas chromatography has been used to separate esters of homovanillic and iso-homovanillic acid and O-methylated products of dopamine [96], 3,4-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzyl alcohol [59] and 4-methyl- and 4-ethylcatechol [56]. The mean relative standard deviations ($n=5$) for O-methylated products of 3,4-dihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde and 3,4-dihydroxybenzyl alcohol after separation with a deactivated metal column followed by a flame ionisation detector varied from 2.8% to 5.4% [59]. In early works mass spectrometric detection was mainly focused on identification of the products, rather than quantitative determination [96]. However, in recent COMT assays O-methylated metabolites of catechol estrogens [33] and dopamine [100] have been quantitated by gas chromatography–mass spectrometry. Gas chromatographic methods usually require derivatisation of the products and use of internal standard.

4.5. High-performance liquid chromatography

High-performance liquid chromatography has been the most common separation technique in COMT assays. Many of these methods have also been validated more properly than, for example, methods based on liquid–liquid extraction and LSC [69,97,103,104]. In addition, regioisomeric O-methylated products formed from catecholic compounds can be easily separated from each other. Reversed phase columns, in most cases octadecylsilane, have been most widely used, although assays utilising cation-exchange resin [105] and columns packed with silica gel particles [97,98,103] have also been developed. Detection systems have been chosen on the basis of the substrate properties and required sensitivity.

4.5.1. UV detection

There are only a few COMT assays using HPLC with UV detection [106,107]. The reasons for that may be poor selectivity and moderate sensitivity of this detection method. However, for the O-methylated products of 3,4-dihydroxybenzoic acid a detection limit of 1.8 pmol/injection was achieved with partially purified rat liver COMT [106]. HPLC with UV detection has also been applied in testing the potency of bifunctional polyhydroxybenzamides as inhibitors of COMT from porcine liver using 3,4,5-trihydroxybenzoate (methyl gallate) as substrate [107].

4.5.2. Fluorescence detection

The O-methylated compounds may be analysed directly with fluorescence detector, if they exhibit native fluorescence themselves or they can be derivatised with fluorogenic reagents. In general, fluorescence detection may be very sensitive. In the assay for the natural COMT substrates, catecholamines, a detection limit of 20 pg (~0.1 pmol) was achieved utilising the native fluorescence of the O-methylated metabolites [108]. A similar detection limit (0.2 pmol/injection) was obtained after post-reaction derivatisation of O-methylated products of 3,4-dihydroxybenzoic acid [97]. For measurement of COMT activity in tissues with low activity, such as erythrocytes, further increase in sensitivity was desired, and therefore specific fluorogenic COMT substrates have been introduced [98,103,109]. With 4-(naphtho[1,2-d]thiazol-2-yl) pyrocatechol as the substrate a detection limit as low as 11 fmol per injection has been reported [103]. This method has been commonly applied in assessing the inhibition of erythrocyte COMT caused by tolcapone in clinical studies [110–112].

4.5.3. Radiochemical detection

Radiochemical HPLC assays for COMT activity have involved separation of labelled co-substrate [57,113] or labelled substrate [113] from the products. On-line radiochemical detector coupled with HPLC apparatus has the advantage over liquid–liquid extraction that the regioisomeric products may be separated from each other. In addition, because of on-line detection, sample handling is more straightforward. Because quantitation is based on radioactivity measurement, synthetic reference standards are not needed. If regioisomeric products are formed, however, reference standards or additional spectroscopic methods are required for structural characterisation of the products. Regarding sensitivity, HPLC with on-line radiochemical detection is competitive with HPLC methods with fluorescence or electrochemical detection (limit of detection 0.04 pmol/injection) [113]. However, because other methods are available, use of radioactive material in COMT assays is often restricted to special cases. For example, enzyme kinetic parameters for methylation of more than 40 structurally diverse catechols catalysed by human recombinant S-COMT could be determined with a radiochemical HPLC assay utilising [^{14}C]AdoMet and, consequently, many structural features that affect the affinity and reactivity of catechol O-methylation were revealed [114]. Examples of radiochemical chromatograms are shown in Fig. 3. A radiochemical HPLC method provides also a screening assay for methylation velocity of new catecholic drug candidates [57].

4.5.4. Electrochemical detection

Catechols, and phenolic hydroxyls of the O-methylated products, are easily oxidised, which allows electrochemical detection in the COMT assays. Electrochemical detection is not suitable for O-dimethylated compounds. However, it is known that dimethylated products are not formed in the COMT-catalysed reactions, since the active site requires a catechol structure [2].

Substrates used in electrochemical HPLC assays for COMT activity include catecholamines [66,105,115], L-dopa [116], 3,4-dihydroxybenzylamine [68,117] and 3,4-dihydroxybenzoic acid [47,69,104]. The potential normally set against Ag/AgCl reference electrode was +0.75–0.9 V. With this set-up a detection limit of 0.5 pmol per injection was achieved [69]. In order to reduce the noise in the analytical cell, a guard cell has been installed prior to the analytical cell [68,104,116]. Contaminants in the mobile phase were peroxidised by applying a high voltage (+0.4 V) in the guard cell. By using two analytical cells set at different potentials, interfering compounds may be oxidised or reduced before the cell that is recorded thereby improving the selectivity [116]. As compared to the electrochemical detection

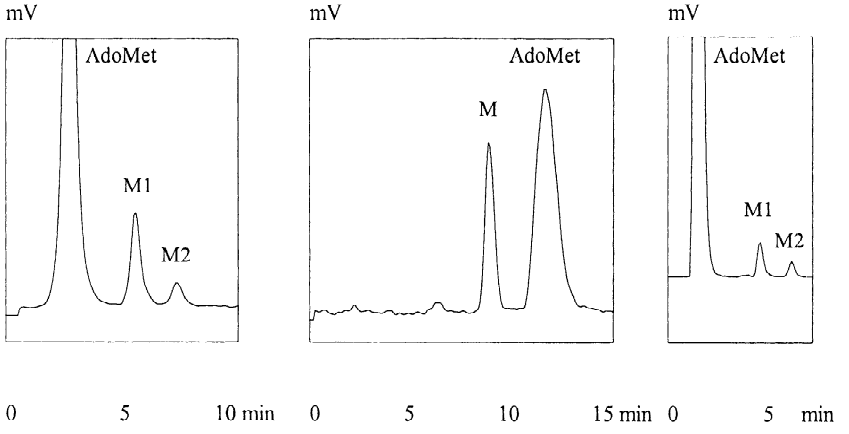

Fig. 3. Radiochemical chromatograms obtained from samples in which AdoMet/[^{14}C]AdoMet and recombinant human S-COMT were incubated with (from the left) dopamine, L-dopa and 2-hydroxyestradiol. A Hypersil BDS-C_{18}, 125×4 mm, 5 μm, column was used. The proportions of phosphate–citrate buffer (pH 3.2, including 1.25 m*M* 1-octanesulphonic acid) and methanol were 85:15, 95:5 and 40:60 (1-octanesulphonic acid omitted), respectively. M1 and M2 refer to regioisomeric O-methylated metabolites formed.

with one working electrode [117], the detection limit could be lowered by 10-fold with coulometric detection (three electrodes in series) [104]. In clinical studies, HPLC with electrochemical detection has been applied to determination of the inhibitory effect of entacapone and nitecapone on COMT in erythrocytes [118,119].

4.5.5. Other detection systems

A COMT assay using LC–APCI–MS in the quantitation of the O-methylated products of catechol estrogens was introduced recently [99]. In this assay LC–MS–MS was used in identifying the compounds, but in the future, sensitivity of COMT assays will probably be enhanced by this technique. A limit of detection as low as 5 fmol of normetanephrine per injection was achieved with another new COMT assay [120]. A peroxyoxalate chemiluminescence reaction detection was coupled with HPLC in order to measure the MB-COMT activity in rat erythrocytes.

4.6. Validation of the analytical methods

Table 1 summarises the most commonly reported validation results of some selected COMT assays representing different separation methods used in the assays. In assays utilising liquid–liquid extraction and liquid scintillation counting [52,87], specificity of the extraction could be limited and blank incubations omitting the co-substrate or the enzyme were necessary in order to correct the results. In chromatographic methods interfering peaks were shown to be absent from chromatograms of blank samples. Recovery of the analytes has been determined in most COMT assays. This has been especially important in methods utilising liquid–liquid extraction, since in those the recovery was often poor and sometimes variable. In addition, quantitation was based on absolute radioactivity, rather than a calibration curve, and the results had to be corrected according to the recovery. When quantitation has been based on external calibration with a calibration curve, linearity and range have been reported in a few cases only [59,100]. Measurement of COMT activity in tissues with low expression has required improved sensitivity from the analytical methods. Therefore the limit of detection has been determined in most COMT assays. The highest sensitivity has been achieved by HPLC with fluorescence and electrochemical detection [69,97,103,104]. Accuracy of COMT assays is rarely reported [100,104], but precision, especially intra-day precision, has commonly been investigated. There seem not to be considerable differences in precision values between methods with different separation techniques.

Table 1
Validation results of some selected COMT assays representing different separation methods

Method	Recovery	Limit of detection	Precision	Ref.
LLE + LSC	42%	NR	Intra-assay, $n = 20$, RSD = 1.7%	[87]
LLE + LSC	NR	NR	Intra-assay, $n = 20$, RSD = 0.7%	[52]
LLE + TLC + fluorescence	>90%	5 ng/spot	NR	[96]
LLE + GC + flame ionisation	76.2–100.4%	20 pmol/injection	Intra-assay, $n = 5$, RSD = 2.8–5.4%	[59]
LLE + GC–MS	34.5–40.6%	<0.5 pmol/injection	Intra-day, $n = 5$, RSD = 4.6–11.9% Inter-day, $n = 5$, RSD = 10.3–13.8%	[100]
HPLC + UV	96–97%	1.8 pmol/injection	NR	[106]
HPLC + fluorescence	99%	0.2–0.3 pmol/injection	Intra-assay, $n = 15$, RSD = 1.7%	[97]
HPLC + fluorescence	NR	11–16 fmol/injection	Intra-assay, $n = 16$, RSD = 2.8–3.0% Inter-assay, $n = 8$, RSD = 4.2–5.3%	[103]
HPLC + radiochemical	97.5%	9 pmol/injection	Inter-day, $n = 3$, RSD = 3.6–7.3%	[57]
HPLC + electrochemical	NR	0.1 pmol/injection	Intra-day, $n = 5$–8, RSD = 0.7–6.7% Inter-day, $n = 14$, RSD = 10.4%	[104]
HPLC + electrochemical	98.7%	0.5 pmol/injection	Intra-assay, $n = 5$–6, RSD = 3.6–5.0% Inter-assay, $n = 6$, RSD = 6.7–9.2%	[69]

LLE, liquid–liquid extraction; NR, not reported.

5. Conclusions

Discovery of COMT as a drug target and revelation of polymorphic COMT forms have increased the demand for reliable, fast and sensitive assays for COMT activity. However, the old method of Raymond and Weinshilboum relying on [^{14}C]AdoMet and liquid–liquid extraction with subsequent liquid scintillation counting, has still been widely used, especially in studies on variation of erythrocyte COMT activity between individuals and ethnic groups [22,23,29,101,102]. The method is fast and removes calcium that inhibits COMT, but requires use of radiolabelled compounds. In addition, liquid–liquid extraction is not a very selective separation method, as discussed above. In assessment of the clinical efficacy of the nitrocatechol COMT inhibitors, new sensitive HPLC methods have been utilised. The inhibition of COMT in human erythrocytes caused by tolcapone has usually been determined by HPLC with fluorometric detection utilising 4-(naphtho[1,2-d]thiazol-2-yl) pyrocatechol as the substrate [98]. The inhibitory effect of entacapone and nitecapone in human erythrocytes has been determined by HPLC with electrochemical detection [47]. These methods are validated and high sensitivity is achieved. Another type of COMT assay was needed when the structure–activity relationships for recombinant human S-COMT were studied [114]. With minor modification of the HPLC eluent, enzyme kinetic parameters could be determined for more than 40 structurally diverse catechols with HPLC coupled with on-line radiochemical detector. For the separate measurement of MB-COMT and S-COMT activity different approaches have been used. These include separation of the two forms by differential centrifugation [68] and use of two different substrate concentrations for the same homogenate [28,60,62]. Recently, a very sensitive peroxyoxalate chemiluminescence reaction detection was introduced for the measurement of MB-COMT activity in rat erythrocytes [120]. In principle, it is still possible to increase the sensitivity of COMT assay since novel methods for the detection of very low amounts of catecholamines have been introduced [121].

In conclusion, reliable and sensitive COMT assays have been developed for current needs, for instance for the measurement of variation of erythrocyte COMT activity after drug treatment or to study the involvement of COMT in tissue injury, cancer pathogenesis or as a tumor marker in small tissue samples.

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