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Radiochemical high-performance liquid chromatographic assay for the determination of catechol *O*-methyltransferase activity towards various substrates

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Abstract

A new chromatographic catechol *O*-methyltransferase (COMT) assay based on S-adenosyl-L-[*methyl*-^{14}C]methionine and on-line radioactivity detection was developed. With minor modifications in the mobile phase composition the methylation velocities for 30 structurally diverse compounds including simple catechols, neurotransmitters, catecholestrogens and catecholic drugs could be measured using human and rat recombinant soluble COMT. The enzymes showed very similar substrate selectivities. The radiochemical method was validated using 3,4-dihydroxybenzoic acid as a model substrate and it was shown that accurate and reproducible methylation velocity values could be achieved for both of the catecholic hydroxyls. The method proved to be suited for determining the enzyme kinetic parameters and can probably be further used for gathering enzyme kinetic data on differentially substituted catechols in order to construct proper structure-activity relationships for COMT. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Catechol *O*-methyltransferase (EC 2.1.1.6, COMT) catalyses the transfer of the methyl group of S-adenosyl-L-methionine (AdoMet) to one of the catecholic hydroxyls of various endogenous and exogenous catechols including catecholamines, catecholestrogens and many drugs [1]. COMT appears in two forms, of which the soluble form (S-COMT) predominates over the membrane-bound form (MB-COMT) in most tissues. During the last decade, there has been a remarkable interest in COMT both as a possible drug target and regarding its role in drug metabolism and interactions. Recently, two COMT inhibitors, entacapone and tolcapone, have been introduced in order to enhance the L-dopa/dopa decarboxylase inhibitor therapy in the treatment of Parkinson's disease [2,3]

Although the structure-activity relationships of COMT inhibition has been determined for S-COMT isolated from rat liver [4] and brain [5], the substrate selectivity of COMT has not been studied extensive-

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ly. Information about the structure-activity relationships of COMT, and other catechol conjugation enzymes could, however, greatly benefit drug design in evaluating the metabolism and metabolic interactions of catecholic drugs and drug candidates. The two forms of human and rat COMT have been successfully cloned and expressed [6–12], and the recombinant proteins are very useful in gathering biological data for structure-activity analysis. However, analytical methods suitable for measuring the in vitro methylation velocity of structurally diverse compounds are needed. Catechols provide two methylation sites and when mechanisms underlying the regioselectivity are investigated, methods also capable of separating the possible regioisomeric products must be available. Earlier high-performance liquid chromatographic methods with different detection systems including UV- [13], electrochemical [14,15], fluorimetric [16–18] and radiochemical [19] detectors, have been developed to measure COMT activity using a specific substrate or to determine the methylation velocity for a small number of substrates. The use of ^{14}C- or ^{3}H-AdoMet in the reaction is a method of choice when the methylation velocities of many structurally diverse compounds have to be determined. However, methods commonly used in the separation of radioactively labelled AdoMet and the products, like liquid–liquid extraction and thin-layer chromatography [20–22], are time-consuming and do not allow the separation of the regioisomeric methylated products from each other.

The present paper describes a simple COMT assay based on HPLC with on-line radioactivity detection. The developed method was shown to be applicable for the screening of 30 structurally diverse catechols for their methylation velocity catalysed by recombinant human and rat soluble COMT. The method was validated using 3,4-dihydroxybenzoic acid as a model substrate and further shown to be suitable for enzyme kinetic measurements.

2. Experimental

2.1. Chemicals

The catechols tested and the other reagents used were purchased from Aldrich (Steinheim, Germany), Boehringer–Mannheim (Mannheim, Germany), Fluka (Buchs, Switzerland), ICN (Costa Mesa, CA, USA), Merck (Darmstadt, Germany), Rathburn Chemicals (Scotland, UK), Riedel-de Haën (Seelze, Germany), and Sigma (St. Louis, MO, USA), and were of the highest grade available. 3,5-Dinitrocatechol was kindly supplied from Orion Pharma (Espoo, Finland). S-adenosyl-L-[*methyl*-^{14}C]methionine was obtained from NEN (Du Pont, Boston, USA).

2.2. Production of the recombinant COMT proteins

The expression vectors for the production of the human and rat recombinant S-COMT proteins in *Escherichia coli* have been described in detail earlier [10]. Three hours after induction with isopropyl-β-D-thiogalactopyranoside, the *E. coli* cells carrying the expression plasmids were harvested by centrifugation and disrupted by sonication in ice-cold phosphate buffer at pH 7.4. The lysates were clarified by centrifugation (10 000 *g*, 10 min) and the supernatants were divided into aliquots, frozen and kept at −70°C before being used for the enzyme assays. The total protein concentrations of the lysates were determined according to Bradford [23] using bovine serum albumin as a standard. Samples of the bacterial cultures were characterised with SDS–PAGE (10% acrylamide) [24] and Coomassie brilliant blue staining. The COMT specific proteins were also visualised by immunoblotting using a guinea pig polyclonal antiserum [9] and the ECL detection system (Amersham, UK).

2.3. Enzyme assays

The incubation mixtures for activity measurements contained $MgCl_2$ (5 m*M*), L-cysteine (20 m*M*), S-adenosyl-L-methionine (0.15 m*M*), and human or rat S-COMT bacterial lysate (12.5–100 μg protein) in 100 m*M* Na_2HPO_4/NaH_2PO_4 buffer (pH 7.4). For quantification purposes, 0.1 μCi ^{14}C-AdoMet was added in each reaction mixture. The samples were pre-incubated at 37°C for 5 min before the catechol substrate was added (0.5 m*M* final concentration). The total volume of the incubation mixture was 250 μl. After the 15–30 min incubation period the reaction was terminated by adding 25 μl of cold 4 *M*

perchloric acid. The samples were kept on an ice-bath for 10 min and centrifuged for 5 min (22 000 *g*). Since the 30 studied compounds may exhibit very different stability profiles, only fresh solutions were used and the samples were analysed immediately after preparation.

The methylated products were separated from S-adenosyl-L-methionine by HPLC (1090, Hewlett Packard, Germany, software HP Chemstation, version 2.1.5) and quantificated by a flow scintillation analyser (150TR, Packard, Meriden, CT, USA), which was fitted with a 300 μl flowcell packed with silanised cerium activated lithium glass as scintillant (Packard). The setting of the discriminator window was 0–156 KeV, and the update time in the integrate mode was 6 s. The signals from the radioactivity detector were converted to millivolts by an interface (HP 35900E) and the methylated products were quantitated by comparing their peak areas with the total area of radioactive peaks.

The columns used were Lichrospher 100 CH-18/2, 125×4 mm, 5 μm (Hibar, Merck, Darmstadt, Germany) and Hypersil BDS-C18 125×4 mm, 5 μm (Hewlett Packard, Germany). Injection volume was 50 μl. Oven temperature was set at 40°C and the mobile phase flow-rate in an isocratic system was 1.0 ml/min. The mobile phase consisted of a phosphate/citrate buffer (50 m*M* Na_2HPO_4, 20 m*M* citric acid, 0.15 m*M* Na_2EDTA, pH adjusted to 3.2 with *o*-phosphoric acid) and methanol. In the chromatographic system 2, 1-octanesulphonic acid (1.25 m*M*) was added to the above mentioned buffer. The amount of methanol in the mobile phase varied between 3 and 60% depending on the substrate (Table 1).

2.4. Validation of the method

The method was validated using 3,4-dihydroxybenzoic acid as a model substrate. The linear range of the reaction catalysed by human S-COMT with respect to protein concentration was determined by incubating 500 μ*M* 3,4-dihydroxybenzoic acid and 150 μ*M* AdoMet with different protein concentrations (1–40 μg) for 30 min. The linearity of the reaction with respect to incubation time was determined similarly by incubating the reaction mixture containing 8 μg protein for different times (10–60 min).

The accuracy of quantitation based on radioactivity was determined by connecting an UV-detector to the HPLC system and analysing samples containing different initial concentrations of 3,4-dihydroxybenzoic acid simultaneously by radioactivity and UV-detector. Reference standards containing the 3-*O*-methylated product of 3,4-dihydroxybenzoic acid, vanillic acid, (2–20 μ*M*) were prepared in the sample matrix for the quantitation by the UV-detector. The wavelength used in the UV-detector was 260 nm.

The recovery was determined by comparing the sum of AdoMet, vanillic and isovanillic acid peak areas in the radioactivity detector with the peak area of unincubated AdoMet ($n=6$). The limit of quantitation and the limit of detection were estimated for vanillic acid using the criteria of signal-to-noise ratio of ten and three, respectively.

The reproducibility of the method was tested by incubating six different initial concentrations of 3,4-dihydroxybenzoic acid (5–300 μ*M*) in duplicate at a constant AdoMet concentration (150 μ*M*) three times. Four μg protein was added to the reaction mixtures and the incubation time after the 5-min-long pre-incubation period was 15 min. To increase the sensitivity of the assay, the total volume of the reaction mixture was reduced to 100 μl, and after the termination of the reactions, the whole reaction mixture was injected to the HPLC system equipped with a 100 μl injector loop. The results of these 3,4-dihydroxybenzoic acid series were used to investigate the applicability of the method for determining the apparent enzyme kinetic parameters and the reproducibility of that determination. The Michaelis–Menten equation was fitted to the data using the Leonora Steady-state Enzyme Kinetics program version 1.0 by A. Cornish-Bowden (Oxford University Press, UK, 1995).

3. Results and discussion

3.1. Chromatographic method

A new COMT assay was developed, which is based on measurement of the products formed utilising ^{14}C-labelled co-substrate S-adenosyl-L-methionine and HPLC with an on-line radioactivity detector. The great advantage of the method com-

Table 1
Mobile phase composition and retention times for *O*-methylated products of different catecholic compounds[a]

Compound	Mobile phase buffer[b]–methanol	Retention time(s) of the metabolite(s) (min)
A. Chromatographic system 1		
3,4-Dihydroxyphenylglycol	97:3	6.3, 10.7
3,4-Dihydroxymandelic acid	97:3	3.9
Pyrogallol	97:3	12.5
Gallic acid	97:3	14.0, 16.0
3,4-Dihydroxybenzoic acid	9:1	12.9, 15.7
Catechol	8:2	11.2
4-Methylcatechol	8:2	26.3, 28.9
4-Nitrocatechol	8:2	14.9, 16.1
2,3-Dihydroxybenzoic acid	8:2	ND[c]
2,3-Dihydroxybenzaldehyde	8:2	6.6
Catechin	8:2	6.0, 8.5
Epicatechin	8:2	13.1
3,4-Dihydroxyacetic acid	83:17	7.6, 9.4
Epigallocatechin	83:17	8.4, 9.3
Epigallocatechin Gallate	83:17	11.3
Epicatechin Gallate	77:23	13.0
Rosmarinic acid	7:3	15.1, 17.2
3,5-Dinitrocatechol	6:4	ND
2-Hydroxyestrone	45:55	12.8, 14.4
2-Hydroxyestradiol	4:6	9.4, 12.3
4-Hydroxyestradiol	4:6	8.7, 13.0

Compound	Mobile phase buffer[d]–methanol	Retention time of AdoMet (min)	Retention time(s) of the metabolite(s) (min)
B. Chromatographic system 2			
Apomorphine	5:5	1.7	ND
5-Hydroxydopamine	85:15	3.3	6.0
6-Hydroxydopamine	85:15	3.3	5.6
Dopamine	85:15	3.2	6.7, 8.8
Adrenaline	9:1	5.2	7.2, 11.2
Noradrenaline	95:5	17.7	10.6
S(-)-Carbidopa[e]	85:15	3.2	7.2, 8.1
Benserazide[e]	9:1	3.0	6.4
L-dopa[e]	95:5	13.6	10.4

[a] Chromatographic conditions are described in Section 2.
[b] 50 m*M* Na_2HPO_4, 20 m*M* citric acid, 0.15 m*M* Na_2EDTA, pH 3.2.
[c] ND=not detectable.
[d] 1.25 m*M* 1-octanesulphonic acid was added to the buffer used in system 1.
[e] The column used was Hypersil BDS-C_{18} 125×4 mm, 5 μm (Hewlett Packard, Germany).

pared to previous methods is that the methylation velocity of structurally diverse catechols catalysed by S-COMT could be determined only with simple modifications in the mobile phase. The set of 30 studied compounds included catecholamines, catecholestrogens and catecholic drugs. Due to the great variation in the chemical characters of the compounds, two chromatographic systems were used. The mobile phase used for acidic and neutral compounds consisted of a phosphate/citrate buffer (pH 3.2) and methanol (system 1). When compounds containing basic groups were analysed, 1-octane-

sulphonic acid was added to the buffer (system 2). The amount of methanol in the two mobile phases varied depending on the lipophilicity of the catechol (Table 1).

AdoMet was rapidly eluted from the column by all the mobile phase mixtures used in the HPLC system 1 with the retention times between 1.6 and 2.2 min. However, addition of the ion-pair reagent in system 2 affected the retention of not only the *O*-methylated products but also of AdoMet that contains various groups capable of acting as the cation in an ion-pair. The low concentration of 1-octanesulphonic acid used resulted in only partial ion-pairing of AdoMet and its retention time could be kept short; only at a buffer–methanol composition of 95:5 its retention was dramatically increased (Table 1). In chromatographic system 2 to some extent broader peaks had to be accepted compared with the peaks obtained using system 1. A representative chromatogram illustrating the methylation of 3,4-dihydroxybenzoic acid, which was used as a model substrate in the validation experiments, and a chromatogram from a blank sample incubated without the catechol substrate are shown in Fig. 1. The peak widths for ^{14}C-AdoMet and the *O*-methylated products of 3,4-dihydroxybenzoic acid, vanillic and isovanillic acid, were 2.2, 1.8 and 0.9 min, respectively. The resolution between the regioisomeric products was 1.4 allowing for quantitation of both products. No interfering peaks were detected in the blank chromatogram.

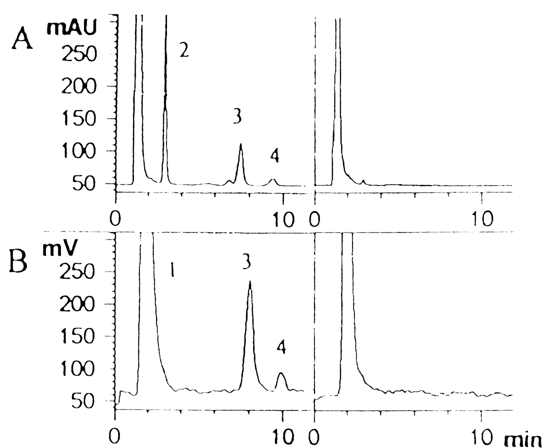

Fig. 1. Chromatograms obtained with UV-detector (A) and radiochemical detector (B) from an assay with 3,4-dihydroxybenzoic acid (50 μM) and cloned and expressed human S-COMT (left) and from an blank assay without the catechol substrate (right). The peaks are 1, AdoMet; 2, 3,4-dihydroxybenzoic acid; 3, vanillic acid and 4, isovanillic acid.

Use of a solid scintillant in the radioactivity detector, which has earlier been applied to determining UDP-glucuronosyltransferase activity [25], decreases the production of waste compared to on-line liquid scintillation counting. In addition, no scintillation pump nor optimisation of the scintillant/mobile phase flow-rate are needed. The limit of detection determined under the present chromatographic conditions was 2 μM vanillic acid at a signal-to-noise ratio of 3, which corresponds to 9 pmol of ^{14}C-labelled metabolite per injection (100 μl). However, the sensitivity of our method can be increased by the use of a 500 μl flow-cell and a liquid scintillator in the detector. In an earlier HPLC method with on-line radiochemical detection, using S-adenosyl-L-[*methyl*-^{3}H]methionine as the methyl donor and a liquid cell, 0.45 pmol ^{3}H-labelled product could be detected [19]. Improvement of sensitivity may be required when kinetic parameters are determined for MB-COMT since this form has been reported to exhibit over a ten times lower K_m value for example for dopamine compared to S-COMT [26].

Because of the large volume of the radioactivity cell (300 μl), a better separation between the peaks eluted from the HPLC column is required compared to, for example, UV-detection where the cells are normally 5–10 μl. The effect of peak broadening can be demonstrated by the decrease of resolution between vanillic and isovanillic acid from 2.0 in UV-detector to 1.4 in radioactivity detector (Fig. 1). It is possible that overlapping elution of regioisomers occurred in the case of some compounds for which only one peak was detected. However, this is unlikely, since baseline separation could usually be achieved within fifteen min for compounds showing regioisomeric products (Table 1). Most of the compounds were predominantly methylated to one of the hydroxyls. It was not determined which hydroxyl was preferentially methylated, but it is known that for instance catecholamines are mostly methylated in the *meta*-hydroxyl [1]. However, the nature and position of the substituents can greatly affect the preferential methylation site, as shown by Thakker et

al. in their study on the site of methylation of ring-fluorinated norepinephrines [27].

3.2. Validation of the method

The linearity of the methylation reaction by human S-COMT with respect to time and protein concentration was studied using 3,4-dihydroxybenzoic acid as the substrate. At a fixed concentration of COMT, the formation rates of vanillic and isovanillic acid were linear functions of time up to 60 min (Fig. 2A). Linear curves were also obtained by varying the protein concentration at a fixed incubation period of 30 min (Fig. 2B). The accuracy of quantitation based on radioactivity was studied by measuring the same samples containing different initial concentrations of 3,4-dihydroxybenzoic acid by UV-detection utilising vanillic acid as the reference standard and by radioactivity detection. The good correlation between the results obtained by the two detection systems (r^2=0.9988, Fig. 3) suggests that accurate results can be achieved by the radioactivity detector at a wide concentration range of metabolites.

The recovery of total radioactivity after incubation of 3,4-dihydroxybenzoic acid and ^{14}C-AdoMet/AdoMet with human S-COMT was 97.5±2.5% (mean±SD, n=6). The recovery of ^{14}C-AdoMet/AdoMet incubated alone with the enzyme was 100.7±1.6% (n=6). Although recoveries were not determined for other studied compounds, calculation of the sum of peak areas in the radioactivity detector for the quantification purposes, also gave information of the recovery. No loss of recovery could be detected among the structurally diverse compounds studied. The limit of quantitation (signal-to-noise ratio 10) and the limit of detection were (signal-to-noise ratio 3) correspond to 5 μ*M* and 2 μ*M* vanillic acid in the reaction mixture, respectively. The detection limit is somehow higher than in the previous HPLC methods with other detectors [13–18], but may be improved by increasing the share of ^{14}C-AdoMet compared to cold AdoMet. However, the sensitivity of the method was good enough for the screening of COMT activity by recombinant rat and human S-COMT.

Six initial concentrations of 3,4-dihydroxybenzoic acid were incubated in duplicate three times to investigate the reproducibility of the method. The

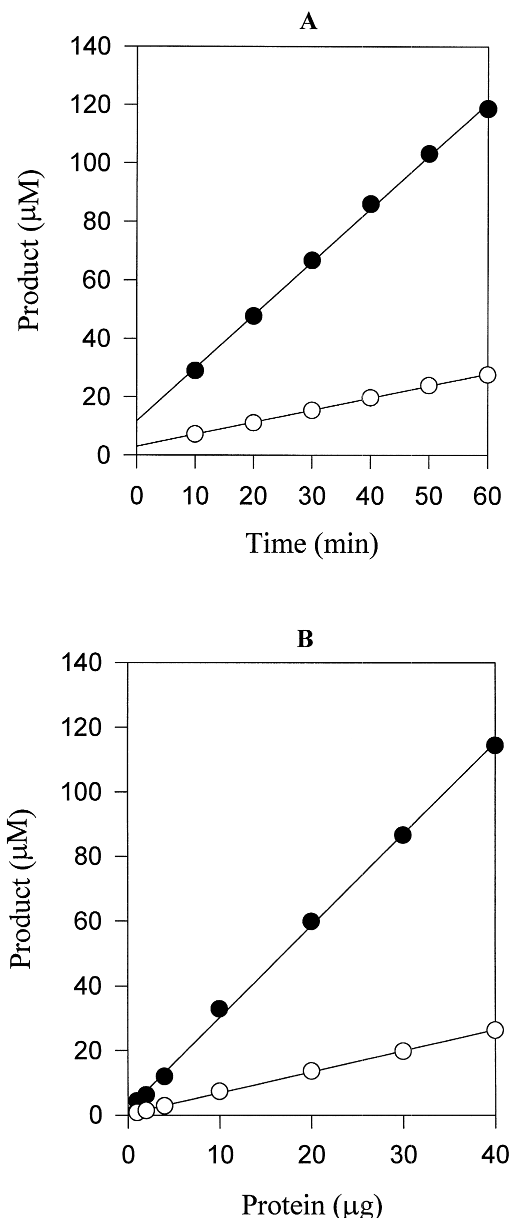

Fig. 2. Formation of vanillic acid (●) and isovanillic acid (○) from 500 μ*M* 3,4-dihydroxybenzoic acid catalysed by human S-COMT at different incubation times (A) and protein concentrations (B). The amount of protein used in (A) was 8 μg and the incubation time in (B) 30 min. The r^2 values of the lines determined by linear regression were 0.9986 and 0.9988 for vanillic acid and 0.9993 and 0.9992 for isovanillic acid.

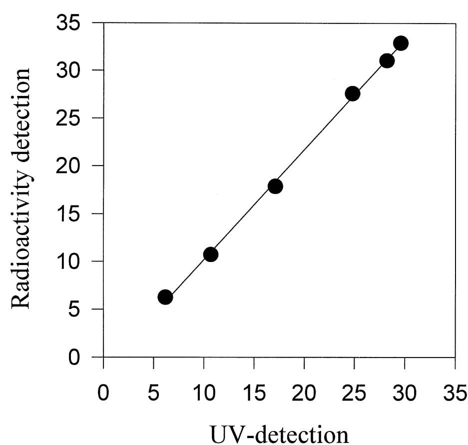

Fig. 3. Formation velocity of vanillic acid (nmol min^{-1} mg^{-1}) at different initial concentrations of 3,4-dihydroxybenzoic acid assayed by UV-detection utilising vanillic acid as the reference standard and by radiochemical detector utilising ^{14}C-AdoMet (r^2=0.9988).

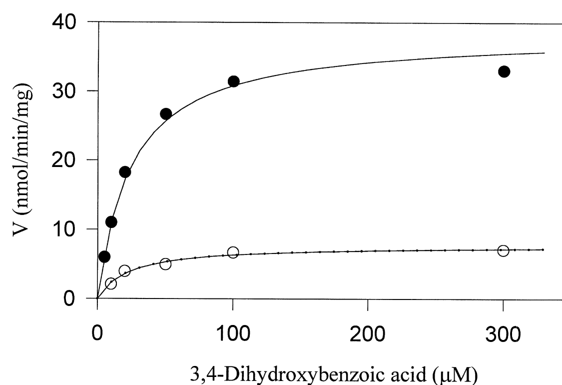

Fig. 4. Michaelis–Menten plot for the methylation of 3,4-dihydroxybenzoic acid to form vanillic (●) and isovanillic acid (○) by human S-COMT. V_{max} values were 38.5±1.9 and 7.7±0.4 nmol min^{-1} mg^{-1} and K_m values 24.6±2.6 and 22.7±4.6 μ*M*, respectively.

relative standard deviations (RSD) for the formation rate of vanillic acid at different 3,4-dihydroxybenzoic acid concentrations were very good in all cases, but at the lowest substrate concentrations the RSD values for isovanillic acid exceeded 20% (Table 2). To test the suitability of the developed method to kinetic measurements, the Michaelis–Menten equation was fitted to the results obtained from the 3,4-dihydroxybenzoic acid series. A representative Michaelis–Menten plot is shown in Fig. 4. Despite the relatively high deviations obtained for some isovanillic acid samples, determination of the kinetic parameters showed good reproducibility for both regioisomers (Table 2). The K_m values for the formation of vanillic and isovanillic acid achieved were very comparable with those previously determined (38.9±4.1 and 35.1±1.4 μ*M*, respectively) by an HPLC method with electrochemical detection

Table 2
Reproducibility of the formation rate of vanillic and isovanillic acid catalysed by human S-COMT at different initial concentrations of 3,4-dihydroxybenzoic acid (*n*=6) and reproducibility of the determination of the enzyme kinetic parameters (*n*=3)

3,4-Dihydroxybenzoic acid (μ*M*)	Vanillic acid		Isovanillic acid	
	mean±SD (nmol min^{-1} mg^{-1})	RSD (%)	mean±SD (nmol min^{-1} mg^{-1})	RSD (%)
5	5.96±0.43	7.30	–	
10	11.01±0.62	5.64	2.13±0.71	33.3
20	18.23±0.69	3.76	3.96±0.95	24.1
50	26.70±0.97	3.65	4.94±0.47	9.46
100	31.46±1.04	3.30	6.64±0.82	12.3
300	33.05±1.20	3.64	7.06±0.84	11.8
Apparent kinetic parameter				
K_m (μ*M*)	23.16±1.84	7.94	26.87±2.76	10.2
V_{max} (nmol min^{-1} mg^{-1})	37.90±1.35	3.57	7.94±0.71	8.88

1 3,4-Dihydroxyphenylglycol
2 3,4-Dihydroxymandelic acid
3 Pyrogallol
4 Gallic acid
5 3,4-Dihydroxybenzoic acid
6 Catechol
7 4-Methylcatechol
8 4-Nitrocatechol
9 2,3-Dihydroxybenzoic acid
10 2,3-Dihydroxybenzaldehyde
11 Catechin
12 Epicatechin
13 Epigallocatechin
15 Epigallocatechin Gallate
16 Epicatechin Gallate
17 Rosmarinic acid
18 3,5-Dinitrocatechol
19 2-Hydroxyestrone
20 2-Hydroxyestradiol
21 4-Hydroxyestradiol
22 Apomorphine
23 5-Hydroxydopamine
24 6-Hydroxydopamine
25 Dopamine
26 Adrenaline
27 Noradrenaline
28 S(-)-Carbidopa
29 Benserazide
30 L-dopa

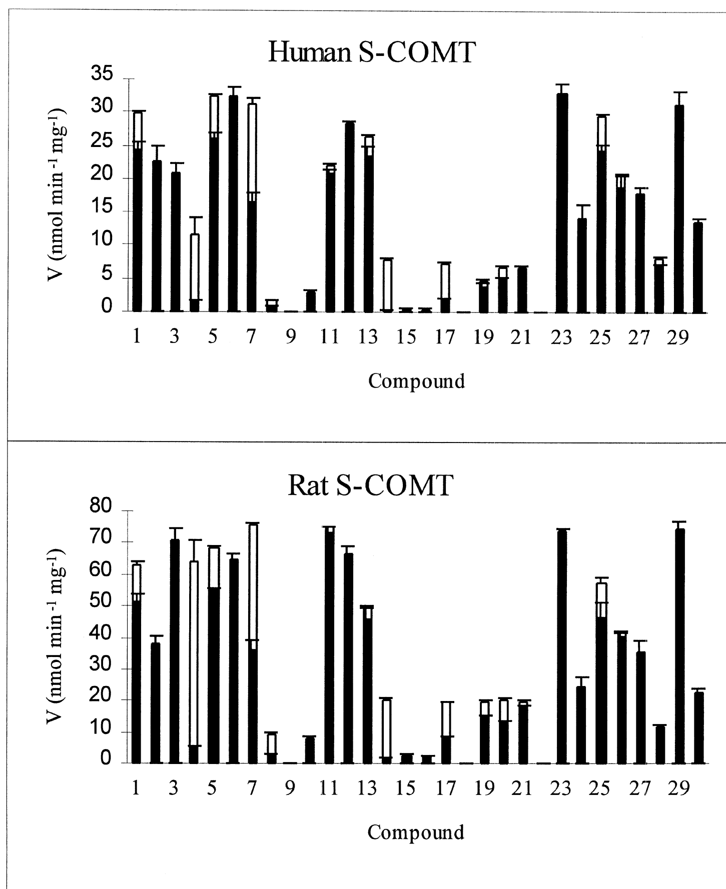

Fig. 5. Methylation velocities (mean±SD, $n=3$) of different catechols (500 μ*M*) catalysed by human and rat S-COMT. Black and white parts in the columns represent the amounts of regioisomeric products detected.

[26]. These results suggest that the method can be applied to determining the enzyme kinetic parameters for other catechols as well.

3.3. Substrate selectivity of human and rat S-COMT

Although the actual methylation velocity values by human and rat S-COMT from different cell batches are not directly comparable with each other, it can clearly be seen from the selectivity profiles that the substrate selectivities of these enzymes are very similar (Fig. 5). The enzymes exhibit very similar regioselectivities as well. Plotting of the methylation velocities obtained using rat S-COMT against the values obtained using human S-COMT showed a rather good correspondence with an r^2 value of 0.8578. Disregarding three clear outliers from the correlation analysis (gallic acid, pyrogallol and catechin) a very good correlation ($r^2=0.9604$) could be achieved. The high similarities we found in the substrate selectivities of these enzymes are in agreement with the similarities in their structure; it has been reported that the amino acid sequences of rat and human S-COMT exhibit 81% homology and in the active site only two amino-acid residues are different [28]. Interestingly, two of the outliers in the correlation analysis, pyrogallol and gallic acid, are known to be low-affinity inhibitors of COMT [29]. The differences in their methylation velocity catalysed by human and rat S-COMT at the relatively high concentration used in the screening may indi-

cate different affinity with the enzymes. Therefore, for a proper comparison of the substrate selectivity of human and rat S-COMT, enzyme kinetic data on structurally diverse compounds is needed.

4. Conclusions

An accurate and reproducible HPLC method with on-line radioactivity detection for the assay of COMT was developed. The method was proved to be suited for the determination of methylation velocity for 30 structurally diverse catechols. The method was also shown to be applicable for the determination of enzyme kinetic parameters for the reaction of each catecholic hydroxyl. Using this method it will be probably possible to determine enzyme kinetic parameters for a large variety of substrates laying the basis for structure-activity analysis for COMT.

Acknowledgements

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