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Short communication

Determination of differential activities of soluble and membrane-bound catechol-*O*-methyltransferase in tissues and erythrocytes

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Abstract

Catechol-*O*-methyltransferase (COMT) exists as two isoenzymes, a membrane-bound form (MB–COMT) and a soluble form (S–COMT), with different roles in the metabolism of catecholamines and other catechol compounds. This report documents an HPLC assay for separate estimation of S–COMT and MB–COMT activity and examines activities of the two isoezymes among different rat tissues and in human and rat erythrocytes. Activities of MB–COMT and S–COMT varied widely among tissues. There were higher activities of S–COMT than MB–COMT in all tissues except the adrenal medulla where MB–COMT was the predominant isoenzyme, consistent with the importance of this tissue and MB–COMT for the *O*-methylation of catecholamines. MB–COMT and S–COMT in rat and human erythrocytes showed divergent levels and patterns of activity. The assay represents a rapid and accurate method for quantifying MB–COMT and S–COMT in various tissues and examining the relative roles of COMT isoenzymes in the metabolism of catechol compounds in health and disease.

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

1. Introduction

Catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) catalyzes the conversion of the catecholamines to their *O*-methylated metabolites using S-adenosyl-L-methionine (SAM) as a methyl donor. There are two COMT isoenzymes: a membrane bound form (MB–COMT) and a soluble form (S–COMT). Investigations have mainly focused on the soluble form, present in most tissues at higher levels of activity than the membrane-bound enzyme [1]. Roth, however, noted that COMT activities are commonly determined as V_{max} values under saturating concentrations of substrate that favor S–COMT over MB–COMT [2]. Adjusting for the much higher affinity (i.e., lower K_m) for catecholamine substrate of MB–COMT than S–COMT, by dividing V_{max} values by respective K_m values, suggests that MB–COMT may be the more important isoenzyme involved in the metabolism of the low cytoplasmic concentrations of catecholamines at the extraneuronal sites where the enzyme is located.

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The importance of MB–COMT for metabolism of catecholamines in vivo is supported by recent findings that more than 90% of the high levels of circulating metanephrines found in patients with pheochromocytoma are derived from *O*-methylation of catecholamines within tumors and that these tissues have a greater abundance of MB–COMT over S–COMT [3]. These observations are consistent with other reports that the adrenals, not the liver, are the most important source of circulating metanephrines, responsible for over 90% of circulating metanephrine and about 25% of normetanephrine [4].

The above considerations highlight the need for reliable measurements of both MB–COMT and S–COMT. In this paper a rapid and accurate method, using HPLC with electrochemical detection, for measurement of COMT activity is described. The method was applied to examine the differential tissue distribution of S–COMT and MB–COMT in relation to known sites of metanephrine production. Since clinical studies of COMT typically rely on measurements in readily accessible red cells [5–7], the utility of the assay for measurements of erythrocyte S–COMT and MB–COMT activity was also examined.

2. Experimental

2.1. Reagents

S-adenosyl-L-methionine (SAM), pargyline, dihydroxybenzylamine (DHBA), and Triton X-100 were obtained from Sigma (St. Louis, MO, USA). Clorgyline and L-deprenyl were obtained from Research Biochemicals (Natick, MA, USA). All other reagents were analytical grade.

2.2. Preparation of tissue samples and erythrocytes

Cerebellum, cerebral cortex, salivary gland, lung, heart, spleen, liver, adrenals (cortex and medulla separated), kidney, vas deferens, and testes were harvested from male Sprague-Dawley rats (300–400 g) sacrificed by lethal injection of sodium pentobarbital. All tissues were immediately frozen on dry ice. Tissues were homogenized in a minimum of four volumes of ice-cold homogenizing buffer (50 *M* sodium phosphate buffer with 0.5 m*M* dithiothreitol, pH 7.5). The homogenates were centrifuged at 100 000 *g* for 30 min (2°C). The supernatant fraction, enriched with S–COMT, was stored at −80°C until assayed. The pellet, enriched with MB–COMT, was washed with 2 ml of homogenizing buffer and then resuspended in at least four volumes (v/w) of homogenizing buffer and also stored at −80°C.

Blood samples, collected in heparinized tubes, were obtained from six rats (2–4 ml) under sodium pentobarbital anesthesia, and from 47 normal volunteers (10 ml) studied at the National Institutes of Health according to a protocol approved by the National Institute of Mental Health Institutional Review Board. Blood samples were centrifuged at 3000 *g* for 15 min (4°C). The plasma, white cells and platelets were separated and the packed red cells were washed with ice cold 0.9% saline solution three times and centrifuged at 3000 *g* for 10 min (4°C) between each wash. The washed erythrocytes were lysed with ice-cold distilled water and then centrifuged at 20 000 *g* for 20 min (4°C). The supernatant, enriched with S–COMT, was collected and stored at −80°C. The pellet was washed with four volumes of homogenizing buffer and recentrifuged at 20 000 *g* for 20 min (4°C). The supernatant was discarded and the pellet, enriched with MB–COMT, was resuspended in homogenizing buffer and stored at −80°C.

2.3. Reaction procedure

The reaction mixture, containing 100 μl of sample preparation in a total volume of 500 μl, included the following components: 50 m*M* sodium phosphate buffer (pH 7.5), 5 m*M* $MgCl_2$, 0.4 m*M* dihydroxybenzylamine (DHBA) substrate and 2 m*M* S-adenosyl-L-methionine. Reaction blanks for each sample consisted of identical components as described above but included 100 μl of homogenizing buffer instead of the tissue preparation.

Reactions were carried out for 60 min at 37°C in a shaking water bath and were initiated by addition of 50 μl of the DHBA substrate. Reactions were terminated by addition of 100 μl of 2 *M* perchloric acid. Mixtures were centrifuged at 3000 *g* for 15 min to remove denatured proteins and a 10 μl aliquot was directly injected onto the HPLC system.

2.4. HPLC with electrochemical detection

The HPLC apparatus included a Model 510 solvent delivery system and a model 717 refrigerated autosampler (both from Waters Associates, Milford, MA, USA). Chromatographic separation was achieved using a 5 μm particle size C_{18} ultrasphere reversed-phase analytical column (250×4.6 mm I.D.) obtained from Beckman Instruments (San Ramon, CA, USA). The column temperature was maintained at 18°C using a model 1166 refrigerated water circulator (PolyScience, Niles, IL, USA) that pumped a chilled equivolume mixture of water and ethylene glycol through a water jacket surrounding the column. The mobile phase (0.1 *M* NaH_2PO_4, 0.13 m*M* EDTA, 0.34 m*M* sodium octane sulfonate, 10% acetonitrile adjusted to pH 3.35 with phosphoric acid) was filtered through a 0.22 μm pore size filter, and pumped through the chromatographic system at 1.0 ml/min.

The 3-methoxy-4-hydroxybenzylamine (MHBA) produced in reaction mixtures from the DHBA substrate was quantified as it eluted off the analytical column using a Model 5100A coulometric detector with a triple electrode system from Environmental Sciences Associates (Bedford, MA, USA). The first electrode in the Model 5021 conditioning cell was set at a potential of +0.39 V. The second and third electrodes in the Model 5011 analytical cell were set at potentials off +0.15 V and −0.39 V. The output from the third electrode was recorded with a Macintosh SE computer (Apple Computer, Cupertino, CA, USA) and Dynamax Method Manager hardware and software package (version 1.4; Rainin Instruments, Woburn, MA, USA). COMT enzyme activities were calculated as pmoles of MHBA produced per min per mg of protein in the S–COMT or MB–COMT enriched tissue or erythrocyte preparations.

2.5. Assay validation

Validation of the assay included assessments of the linearity of MHBA production with respect to time of reaction and amount of COMT enzyme present in the reaction mixture. Intra-assay coefficients of variations were determined from repeated determinations of COMT activity in identical samples carried out within the same assay. Inter-assay coefficients of variation were determined from repeated determinations of COMT activity in stored aliquots of identical samples carried out in different assays. In order to optimize the measured activity of COMT, several different monoamine oxidase inhibitors (clorgyline, pargyline, L-deprenyl) added to the reaction mixture were examined for their effect on measured COMT activity.

3. Results

3.1. Assay validation

The assay was highly linear ($r=0.999$) with time of incubation and remained linear for up to 90 min incubation times (Fig. 1). The assay was also highly linear ($r=0.999$) with respect to a broad range of concentrations of COMT enzyme present in the incubation mixture. The intra-assay coefficient of variation determined from ten repeated determinations of the same sample in the same assay run was 4.7%. The inter-assay coefficient of variation determined from sixteen determinations of the same sample carried out in separate assay runs was 16.6%. No increase in recovery of MHBA product was observed in assay mixtures containing pargyline or a mixture of deprenyl and clorgyline mixture, so these inhibitors were not routinely included in the assay reaction mixture.

3.2. COMT activities in rat tissues

There was considerable variation in COMT V_{max} activities among the different tissues, with liver showing by far the highest activity for S–COMT and heart showing the lowest activity for both S–COMT and MB–COMT (Fig. 2). All tissues, except the adrenal medulla, showed higher activities of S–COMT than MB–COMT, in agreement with previous reports showing that V_{max} activities of S–COMT predominate over those of MB–COMT [2]. In liver, the activity of S–COMT was almost 30 times that of MB–COMT. The adrenal medulla was the only tissue where V_{max} activities of MB–COMT were higher than those of S–COMT. Activities of MB–COMT in the adrenal medulla were similar to activities of MB–COMT in kidneys and vas deferens

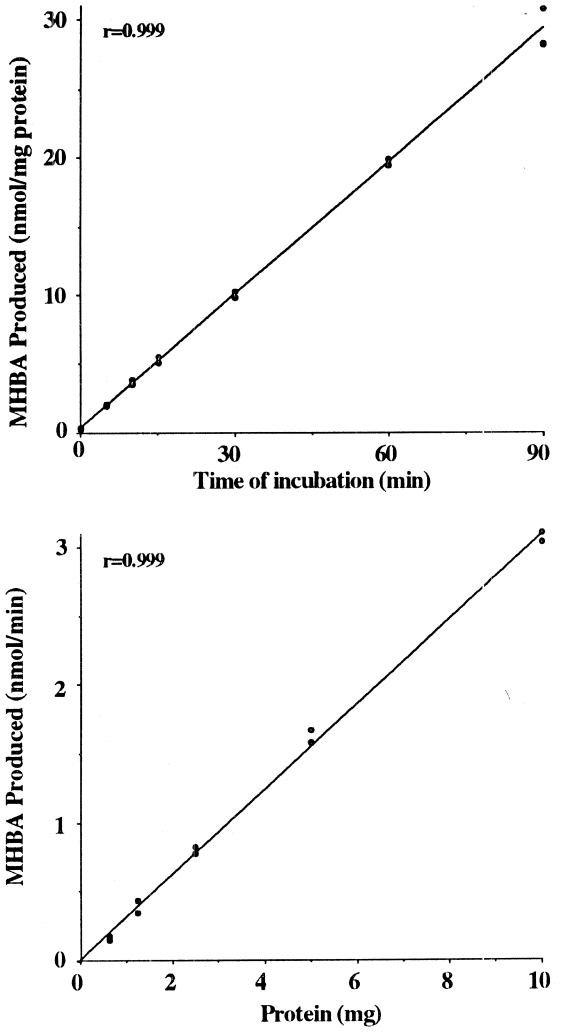

Fig. 1. Relationship of MHBA production with time of incubation (top) or amount of enzyme (bottom). The relation with time represents the accumulated total production of MHBA product at each time point ($n=2$ for each time point) per mg protein and indicates a highly linear relationship with incubations up to 90 min. The relationship with amount of enzyme was established from variable dilutions of a rat liver S–COMT preparation ($n=2$ for each dilution).

and higher in all three of these tissues than any of the other tissues examined. Although the V_{max} activity of S–COMT in liver (388±24 pmol/mg/min) was 10-fold higher than that of MB–COMT in adrenal medulla (37±8 pmol/mg/min), dividing these V_{max} values by previously established K_m values of S–COMT (369 μmol/l) and MB–COMT (24.1 μmol/l) for norepinephrine [8] yielded V_{max}/K_m values for MB–COMT in adrenal medulla that were slightly higher than those for S–COMT in liver (of $1.5\pm0.3\times10^{-3}$ versus $1.1\pm0.1\times10^{-3}$ ml/mg/min).

3.3. Erythrocyte COMT activity

Comparisons of human and rat erythrocyte COMT activities indicated markedly divergent levels of activity and differences in activities of MB–COMT relative to S–COMT (Table 1). Levels of S–COMT activity in human erythrocytes varied from 0.25 to 0.76 pmol/mg/min, a similar range to that reported by Syvanen et al. [7]. These investigators found mean (±SD) values in patients heterozygote for a common functional COMT polymorphism (0.53±0.17 pmol/mg/min) almost identical to those estimated here in our population of subjects with mixed COMT genotypes (0.52±0.14 pmol/mg/min). The much higher activities of S–COMT in rat than human erythrocytes are also in agreement with a report by Schultz et al. [9], who found S–COMT activity in rat erythrocytes of 33.7±4.8 pmol/mg/min close to the values reported here and considerably higher than activities of human erythrocyte S–COMT. Higher activities of S–COMT than MB–COMT in rat erythrocytes contrasted with higher activities of MB–COMT than S–COMT in human erythrocytes, a pattern in agreement with one other single study of S–COMT and MB–COMT in rat and human erythrocytes [10].

4. Discussion

This report describes and validates an assay for separate measurements of S–COMT and MB–COMT and documents considerable variations in absolute and relative activities of the two COMT isoezymes among different tissues. Importantly, this study establishes that compared to other tissues, the adrenal medulla shows a reverse of the normal pattern of higher activity of S–COMT compared with MB–COMT. Previous findings in humans [4] and dogs [11] indicated that the adrenals represent an important source of circulating *O*-methylated metabolites of catecholamines. This combined with the much lower affinity of MB–COMT than S–COMT

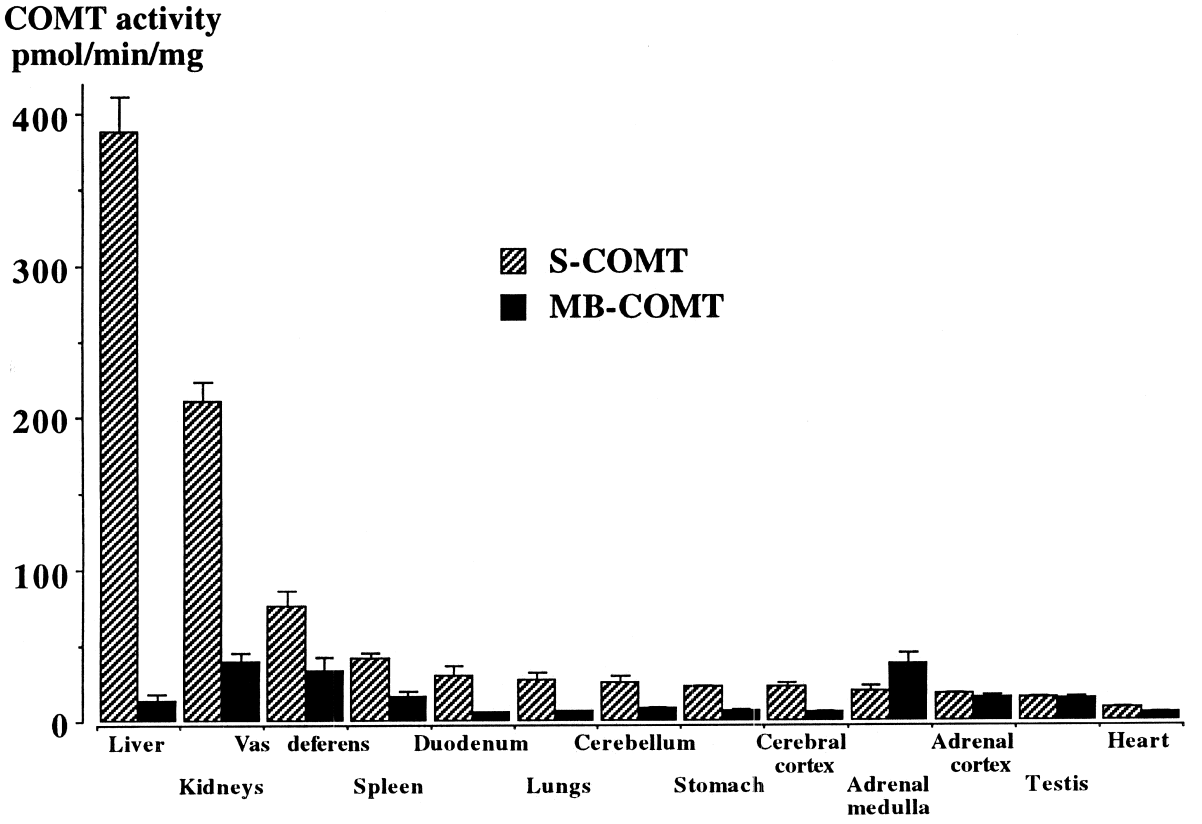

Fig. 2. Activities of S–COMT (cross-hatched bars) and MB–COMT (solid bars) in samples of rat liver, kidney, vas deferens, spleen, duodenum, lungs, cerebellum, stomach, cerebral cortex, adrenal medulla, adrenal cortex, testes and heart with the order established according to the level of activity of S–COMT for each tissue. Results are mean±SEM values in units of pmol of MHBA produced per min per mg protein (n=5–9 per tissue).

for catecholamines [2,8] and the present findings of higher levels of activity of MB–COMT than soluble COMT in the adrenal medulla are consistent with the dawning realization that MB–COMT is the more important isoenzyme responsible for the *O*-methylation of catecholamines.

Apart from catecholamines, COMT also acts on many other catechol substrates including the following compounds: (1) L-DOPA, the immediate precursor of catecholamines; (2) 3,4-dihydroxyphenylglycol (DHPG) and 3,4-dihydroxyphenylacetic acid (DOPAC), the deaminated metabolites of catecholamines; and (3) catecholestrogens, steroid compounds involved in tumorogenesis [12,13]. It seems likely that S–COMT may be important for the *O*-methylation of these other catechol compounds. Support for this notion is reinforced when the extraneuronal location of COMT is considered together with the fact that most metabolism of catecholamine neurotransmitters occurs intraneuronally by deamination and that there is considerably more *O*-methylation of the much higher circulating concentrations of deaminated catecholamine metabolites than of the precursor catecholamines [14–16]. In particular, the liver and kidneys

Table 1
Activities for S–COMT and MB–COMT in soluble and particulate fractions from human and rat erythrocyte preparations[a]

	S–COMT	MB–COMT
Human (n=47)	0.5±0.0	6.8±0.2
Rat (n=6)	28.9±1.9	18.5±3.0

[a] Activities are shown as mean±SEM rates in units of picomoles per min per mg of protein.

extract and metabolize considerably more circulating L-DOPA, DHPG and DOPAC than catecholamines [14–16], and this likely reflects the function of the considerably higher amounts of S–COMT than MB–COMT present in these and other tissues. The above considerations imply that the differing relative activities of MB–COMT and S–COMT reflect different functions of the isoenzymes among different tissues and illustrate the need to consider both COMT isoenzymes.

In clinical studies, COMT activity is most conveniently determined in erythrocytes. Applications include use in assessing the effectiveness of COMT inhibitors introduced as adjuncts to L-DOPA therapy in the treatment of Parkinson's disease [6,17,18] and role of COMT in clinical disorders such as schizophrenia [19,20], depression [21,22] and cancer [12,13,23]. The potential importance of COMT in psychiatric conditions has been fueled by advances in molecular genetics establishing that COMT deficient mice exhibit emotional and aggressive behavioral abnormalities [24]. Clinical studies have also indicated an association of polymorphisms affecting COMT activity with aggressiveness and violence [25], drug abuse [26] and obsessive compulsive behavior [27].

There are a considerable number of previously described assays of COMT activity [6,9,10,28–33] and the present assay is based on some of these [9,33]. Almost all modern methods for measuring COMT activity utilize HPLC methods, but some involve an extraction step subsequent to the reaction procedure [10,29] or radioisotope analysis [28,31] and others are described for assay of only S–COMT activity [10,28–30] or activity specifically in red cells [6,9] or peripheral tissues [29–33]. The present non-isotopic method involves direct injection of perchloric acid reaction mixture supernatants and represents a rapid procedure for reliable assay of both MB–COMT and S–COMT activity in both tissues and red cell preparations.

A limitation of the present and other procedures for assaying MB–COMT and S–COMT activity is that no method is available that provides complete separation of both isoenzymes during sample preparation. Thus, the S–COMT and MB–COMT are not completely separated and the preparations should be regarded as those enriched with either S–COMT or MB–COMT. As shown by Tilgmann et al. [34], this may be particularly problematic for the larger membrane-bound isoenzyme, a reasonable proportion of which is not actually anchored to membranes and is recovered in the soluble fraction. Thus, estimates of MB–COMT activity probably represent underestimates and those of S–COMT activity overestimates. This may pose little problem to tissues such as the liver, where the soluble isoenzyme far predominates over the membrane-bound form. However, for tissues where the membrane-bound form predominates, such as the adrenal medulla, relative activities of MB–COMT to S–COMT may be substantially underestimated.

In summary, the present study lends support to the emerging realization of the importance of MB–COMT in the metabolism of catecholamines and suggests that the considerable differences in absolute and relative activities of MB–COMT and S–COMT among different tissues might reflect differing roles of the isoenzymes in metabolism of catecholamines, catechol precursors, catechol metabolites, catecholestrogens and other catechol substrates. The rapid and reliable assay procedure described here for measurement of both S–COMT and MB–COMT activity in tissues and erythrocytes provides a useful method for examination of the relative roles of COMT isoenzymes in metabolism of catechol compounds and involvement in clinical conditions associated with alterations in COMT activity.

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