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Catechol-O-Methyltransferase Activity in Human Erythrocytes: Methodological Aspects

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ABSTRACT

Different methodological aspects on the assay of human erythrocyte catechol-O-methyltransferase (COMT) activity were studied. No temporal variations were found either over a 24 hour period or over one month. Erythrocytes from whole blood collected with any of the anticoagulants heparin, EDTA or citrate could be used as the enzyme source provided the cells were washed in saline. The COMT activity in lysed erythrocytes was rapidly lost when the lysate was stored at +4°C and -20°C. Intact erythrocytes could be stored up to one week in +4°C without considerable loss of activity. The COMT activity was stable for at least two years when storing the cells at -85°C. Freeze-thawing and hypotonic disruption of the erythrocytes resulted in the same activity and neither freeze-thawing nor sonication altered the apparent K_m for the substrate. Noradrenaline and 3,4-dihydroxybenozic acid (DBA) could both be used as substrates although DBA gave higher activity values and had a higher affinity to the enzyme. The COMT activity increased with increasing concentration of the methyl-donor S-adenosyl-l-methionine up to approximately 0.1 mM. Preincubation at 47°C decreased the COMT activity whereas the apparent K_m values remained unchanged. The present COMT assay was convenient and reproducible and could be used with small amounts of blood with different kinds of anticoagulants. Interactions with plasma factors were avoided by washing the erythrocytes with isotonic sodium chloride.

INTRODUCTION

Catechol-O-methyltransferase (EC 2.1.1.6; COMT) is the enzyme catalyzing the O-methylation of a wide range of catechol compounds (2, 3), including the neurotransmitters dopamine and noradrenaline. Since the discovery of COMT activity in human erythrocytes (1, 16) many attempts have been made to detect any possible involvement of a disturbed O-methylation in the etiology of several diseases by measuring COMT activity in red blood cells. However, consistent findings have not been reported. For example, erythrocyte COMT

activity has been found to be decreased, normal or elevated in affective disorders (5, 6, 12). This might at least partly be due to methodological differences. Several procedures for the determination of human erythrocyte COMT activity have been described (1, 4, 13, 15, 17, 21). Even though the general principle for COMT activity determinations is the same in the different reports, the mean activities for the populations vary about tenfold. Guldberg and Marsden (14) have pointed out the importance of standardizing the COMT assay for each kind of tissue (i.e. controlling factors such as temperature, pH, buffer, ionic strength and substrate concentrations) to make it possible to compare results from different laboratories.

As part of a more extensive study on human erythrocyte COMT we now report some methodological aspects on the enzyme assay. The possible interfering plasma components are removed by washing the red blood cells with isotonic sodium chloride. The washed cells are then lysed and the lysate is used directly for incubation with the substrates. The results obtained will be compared with earlier methodological results on human erythrocyte COMT activity.

MATERIALS

The unlabelled compounds noradrenaline (NA), 3,4-dihydroxybenzoic acid (DBA), S-adenosyl-l-methionine (SAM) and dithiothreitol (DTT) were purchased from Sigma Chemical Company. Radioactively labelled ^{14}C-methyl-SAM (sp.act. 46-59 mCi/mmol) was bought from New England Nuclear. All scintillation equipments were from Packard Instruments. Isoamylalcohol came from Fluka and all other chemicals were manufactured by Merck, West Germany and were of analytical grade.

METHODS

Unless otherwise specified the COMT assay procedure was as follows. Blood was collected into 10 ml heparinized Vacutainer tubes, 143 USP units of heparin/10 ml tube. The blood was centrifuged for 10 minutes at 1500 g and the plasma was removed with the upper layer of the packed cells. The cells were washed twice by adding double the volume of cold isotonic sodium chloride (9 g/l), mixing and centrifuging at 1500 g for 10 minutes. The cells were either used immediately or stored at -85°C until assayed. An aliquot of packed erythrocytes was added to four times the volume of cold distilled water and vigorously mixed. 150 µl of the lysate were added to 110 µl of 80 mM tris HCl buffer, pH 7.6, containing 1 mM $MgCl_2$, 0.15 M KCl and 0.5 mM DBA or 1 mM NA, (final concentrations). After preincubation for 5 minutes at 37°C the reaction was started by adding 10 µl of ^{14}C-SAM, 15 µM final concentration. The reaction was stopped after 15 minutes. When NA was used as substrate the reaction was

terminated by adding 100 µl 0.5 M borate-NaOH buffer, pH 10 and the product was extracted into 5 ml of water saturated isoamylalcohol by vortexing for 20 seconds. When DBA was used as substrate the reaction was stopped with 100 µl 1 M hydrochloric acid and the product was extracted into 5 ml of water saturated toluene/isoamylalcohol (7:3 v/v). Four ml of the organic phase were transferred into plastic vials containing 10 ml of Instagel (Packard Instr.). The radioactivity was measured in a Packard 3380 liquid scintillation counter. The blanks included all reagents except for the catechol substrate. The enzyme activity was expressed as nanomoles product formed/ml packed red blood cells/hour.

RESULTS

Temporal variation

Blood samples were collected from a healthy volunteer every 3 hours over a 24 hour-period. There was no variation in the COMT activity during this period. The mean value of the COMT activity was 14.83 $\pm$ 0.22 (SEM). In order to detect any temporal variation in COMT activity over a longer period blood was collected from 12 healthy volunteers (10 females and 2 males, age 24-40 years), twice weekly for 4 weeks. The mean individual deviation was 4.5 % $\pm$ 4.7 % (S.D., n = 92). These results indicate that within individuals, the variation in erythrocyte COMT activity is small over long time periods.

Anticoagulants

The influence of EDTA, heparin and citrate on COMT activity in whole blood is given in Table 1. The COMT activity in citrate and heparin blood was lower

Table 1. Human erythrocyte COMT activity in the presence of different anticoagulants in samples from one individual. Heparin tubes contained 143 USP units of sodium heparin per 10 ml tube. The EDTA concentration was 3.4 mM and the sodium citrate concentration was 13 mM. The addition of plasma to washed erythrocytes was made with equal volumes of red blood cells and plasma. Hematocrit values were determined on all samples before lysing the cells. The activity is expressed as nmoles of product formed/hour/ml packed red blood cells (RBC). Each value represents the mean of duplicates $\pm$ SEM.

	Heparin	EDTA	Citrate
Whole blood	7.6 $\pm$ 0.2	14.1 $\pm$ 0.2	8.6 $\pm$ 0.4
Unwashed RBC	8.9 $\pm$ 0.1	11.4 $\pm$ 0.1	8.8 $\pm$ 0.1
Washed RBC (WRBC)	10.1 $\pm$ 0.1	10.4 $\pm$ 0.1	10.3 $\pm$ 0.2
WRBC + heparin plasma	6.1 $\pm$ 0.2	6.3 $\pm$ 0.2	6.1 $\pm$ 0.2
WRBC + EDTA plasma	10.7 $\pm$ 0.3	10.4 $\pm$ 0.1	10.2 $\pm$ 0.1
WRBC + citrate plasma	6.7 $\pm$ 0.1	6.7 $\pm$ 0.1	6.6 $\pm$ 0.1

than in EDTA blood. This is likely due to the presence of inhibitory calcium ions in the heparin and citrate tubes. The concentration of EDTA used in Vacutainer tubes (3.9 mM) is high enough to chelate the calcium ions without interfering with the magnesium ions added in the assay. This is in agreement with the report of Bates et al. (4) who also found higher activity in EDTA whole blood (as well as in EGTA blood) than in heparin blood.

Storage of blood

Erythrocytes in whole blood could be stored for several days at +4°C without losing COMT activity, providing the cells remained intact (Fig. 1a). In room temperature the COMT activity decreased so that approximately 30 % remained after 6 days. The enzyme activity in lysed cells, however, was almost entirely lost after one week at +4°C, and storage at -20°C also resulted in a rapid decrease of enzyme activity (Fig. 1b). When stored at -85°C, however, there was only 6 % loss of activity after two years (Fig. 1c).

The stability of COMT activity in erythrocytes up to 6-10 days at +4°C was also found by Griffiths and Linklater (13) and Bates et al. (4). Gershon and Jonas (12) reported that COMT activity in red blood cells was stable for at least 12 months when stored at -80°C.

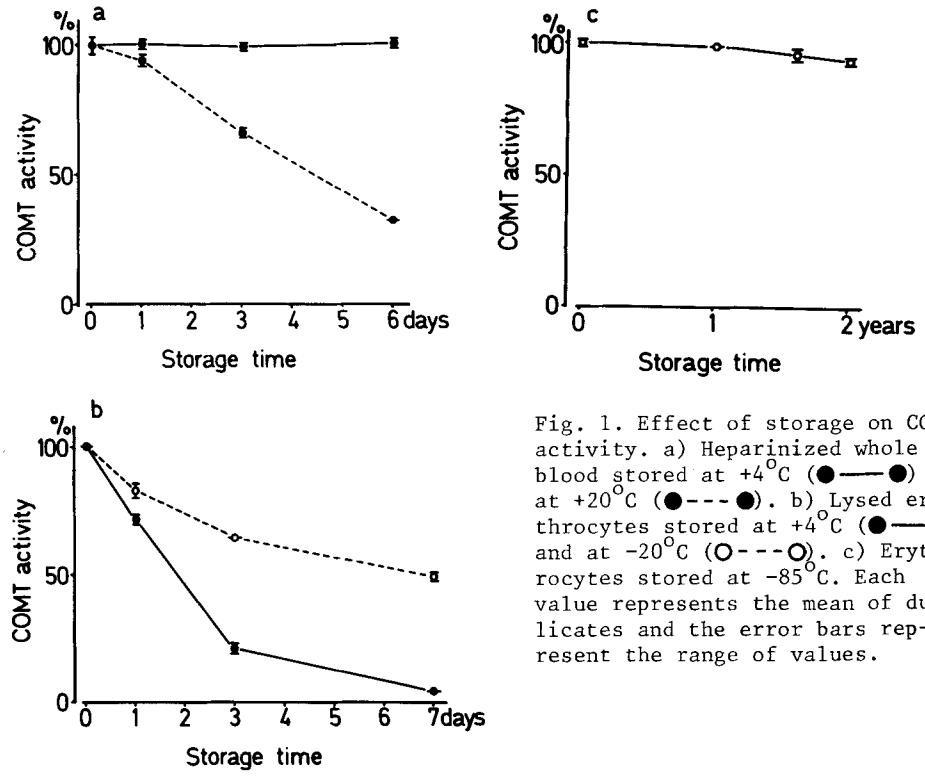

Fig. 1. Effect of storage on COMT activity. a) Heparinized whole blood stored at +4°C (●——●) and at +20°C (●---●). b) Lysed erythrocytes stored at +4°C (●——●) and at -20°C (○---○). c) Erythrocytes stored at -85°C. Each value represents the mean of duplicates and the error bars represent the range of values.

Disruption of cells

Since the transmembraneous transport of SAM and the catechol substrates is rather slow, the COMT activity measured in intact erythrocytes is low, approximately 3-4 % of the activity in lysed cells when using 15 /uM SAM. Therefore the cells should be disrupted prior to assay. Hypotonic treatment with distilled water (4 times in excess) gave a high activity which could only be slightly increased (5-10 %) by freeze-thawing in acetone/dry ice or sonication for one minute with a Braun sonicator at maximum output effect. The apparent affinity for DBA as substrate was not affected by the method of disruption. Freeze-thawing alone also gave maximal activity and in our further studies (7, 8, 10) we have used cells that were disrupted by freeze-thawing (storage in -85°C) and lysed with distilled water.

Dependence on pH, magnesium and substrates

The optimal pH for COMT activity was between 7.4 and 7.6. The optimal magnesium concentration was between 0.5-1 mM Mg^{2+} in a tris HCl buffer. Higher magnesium concentrations inhibited the enzyme. The relation between COMT activity and the concentration of the catechol substrates noradrenaline and 3,4-dihydroxybenzoic acid is shown in Fig. 2a. Both substrates inhibited the enzyme at high concentrations. Fig. 2b shows the relation between COMT activity and the concentration of the methyl donor SAM. The saturating concentration of SAM was about 0.1 mM.

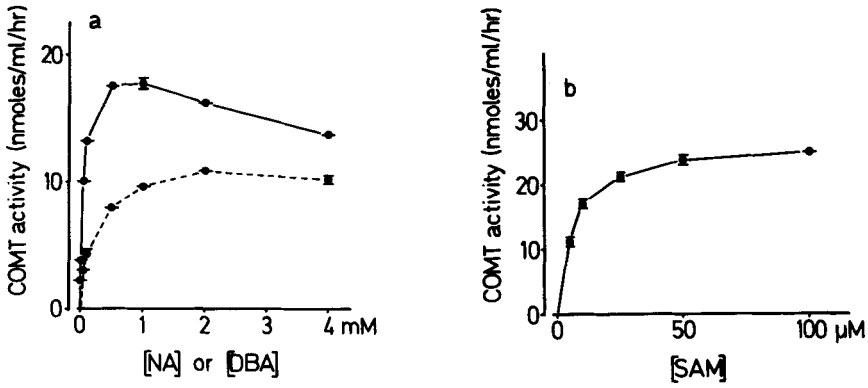

Fig. 2. a) The influence of increasing concentrations of substrate, DBA (●——●) and NA (●---●) on the COMT activity. Apparent K_m was calculated to 116 /uM for NA and 34 /uM for DBA in this experiment.
b) The influence of increasing concentrations of SAM on COMT activity.
Each value represents the mean of duplicates and the error bars represent the range of values.

Buffers and ionic strength

In order to increase the ionic strength of the tris HCl buffer (9) 0.15 M KCl was added to the reaction incubate. Tris HCl buffer was used instead of phosphate buffer because of the appearance of insoluble magnesiumphosphate in the incubate (9, 17).

Linearity with time and enzyme concentration

The time course of COMT activity was linear for at least 30 minutes with both NA and DBA as substrate. Dilution of the enzyme did not alter the activity per volume of red cells. These results confirm earlier reports (4, 13, 17).

Temperature dependence

The COMT activity increased with temperature and reached a maximum around 42°C. At 60°C the activity was completely abolished (Fig. 3). Figure 3 also shows the increasing blank values at higher temperatures, probably due to decomposition of SAM to a product containing the radioactive methyl group which is extractable into the organic solvent. This emphasizes the importance of using blanks including SAM but without the catechol substrate.

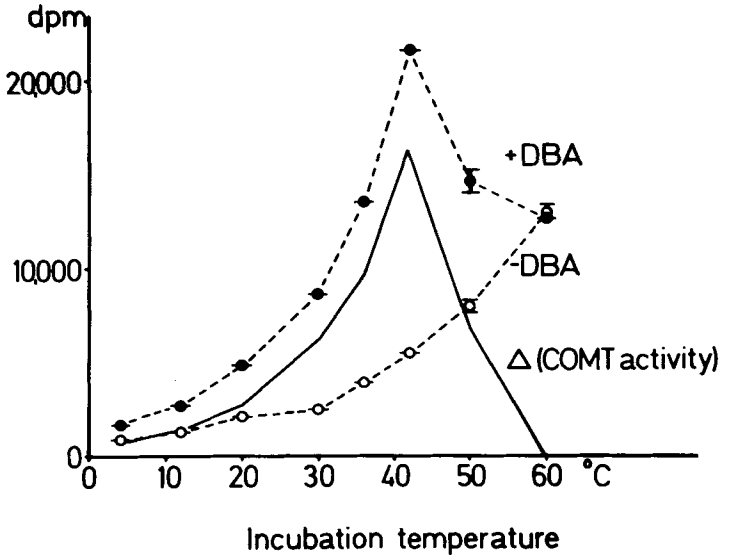

Fig. 3. The activity of COMT with DBA as substrate when incubated at different temperatures for 15 minutes (————). (●---●) = dpm without subtracting the blank values. Blanks (○---○) were made with all reagents except for DBA. Each value represents the mean of duplicates and the error bars represent the range of values.

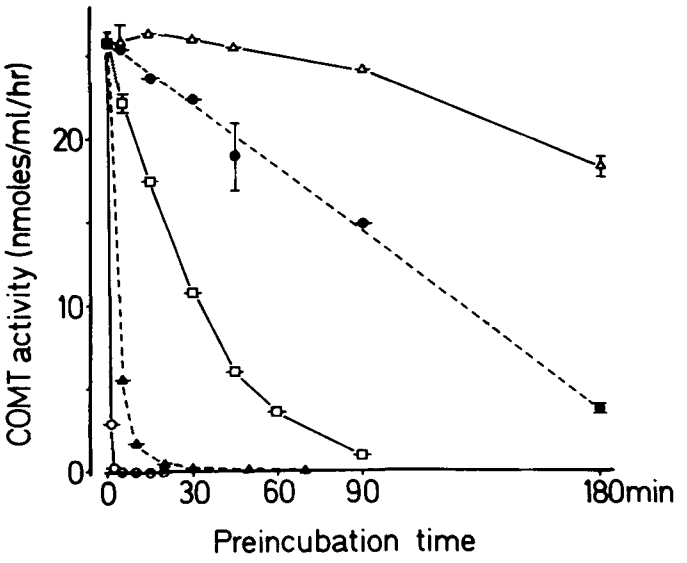

Fig. 4. The thermostability of COMT. The enzyme activity was measured at +37°C, after different times of preincubation at various temperatures, +37°C (Δ——Δ), +42°C (●---●), +47°C (□——□), +52°C (▲---▲) and +57°C (○——○). Each value represents the mean of duplicates and the error bars represent the range of values.

Preincubation of the erythrocyte lysate at various temperatures resulted in a decrease of enzyme activity with a half life of 0.5, 3, 35 and 105 minutes at 57, 52, 47 and 42°C, respectively (Fig. 4). At 37°C the activity remained constant for approximately 30 minutes but then decreased so that 70 % of the activity remained after 180 minutes of preincubation. The apparent K_m for DBA remained constant after 45 minutes of preincubation at 47°C while the corresponding V_{max} value had decreased to 1/5 of the original value.

A significant correlation (r = 0.84) was observed between the COMT activity values of twelve individuals before and after heat inactivation of the lysate for 5 minutes at 52°C (Fig. 5). There was also a positive correlation (r = 0.69) between the original activity and the ratio of the activity in heated/unheated samples. This is in accordance with Scanlon et al. (18) who reported on the presence of a thermolabile COMT in individuals homozygous for low COMT activity. When a lysate was incubated at 47°C for variable periods of time, COMT was inactivated more rapidly in subjects with low erythrocyte activity than in lysates from high activity subjects.

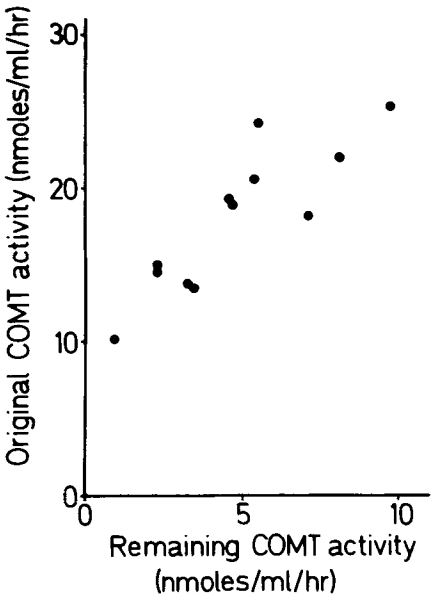

Fig. 5. COMT activity in erythrocytes from twelve subjects before and after heat inactivation at +52°C for five minutes. The correlation coefficient r = 0.84.

DISCUSSION

COMT determinations in human blood are usually made in order to detect any possible disturbances in catecholamine metabolism among different groups of patients. Since many patients are treated with drugs it is advantageous to remove all plasma residues from the erythrocytes to avoid drug interaction in the enzyme assay. Human plasma alone contains calcium which inhibits COMT (11, 20) and one way of removing calcium is by treating the erythrocyte lysate with a chelating resin which binds free calcium and magnesium ions (17). The chelating resin is removed from the lysate by centrifugation. Washing the intact erythrocytes with isotonic sodium chloride, as in this report, removes the inhibitory calcium together with all other plasma components. The total calcium concentration in the incubate was below 10 µM as measured by atomic absorption spectrophotometry. Furthermore, the results presented in Table 1 show that identical COMT activities were obtained with different antiacoagulants if the erythrocytes were washed.

It has been reported that the addition of dithiothreitol (DTT) can be used to stabilize COMT and its activity (20). We have not been able to detect any stabilizing effect of 0.5 mM DTT on erythrocyte COMT activity in our storage experiments at +20°C, +4°C, -20°C or -85°C.

In routine assays DBA was used as catechol substrate, instead of the endogenous substrate NA, because of its higher affinity to the enzyme as well as

the higher activity values achieved with DBA (Fig. 2a). There is a high correlation between COMT activities obtained with the two substrates (17). The concentration of SAM used in routine assays (appr. 15/uM) is below the saturating concentration of appr. 100/uM (Fig. 2b). To reach the saturating concentration unlabelled SAM must be added to the radioactive compound. This may present metodological problems since commercially available batches of unlabelled SAM may contain inhibiting impurities. A substrate concentration in the incubate of 3 times the K_m value has been recommended in order to avoid substrate and product inhibition of the reaction (19). In our system apparent K_m value for SAM with 0.5 mM DBA was calculated to 5/uM and consequently 15/uM SAM would be an adequate concentration. This concentration was obtained by adding undiluted ^{14}C-SAM directly to the reaction tubes. The use of a standard in the assay is necessary to correct for concentration differences in the ^{14}C-SAM batches.

The results reported here summarize some methodological aspects on the assay of human erythrocyte COMT activity. Many of the results confirm earlier findings on other tissues and species. We have found the present assay to be reproducible and convenient to use in routine COMT activity determinations since only small amounts of blood are used. The washing of the erythrocytes allows the use of different kinds of anticoagulants with minimal interaction of plasma factors.

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