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Validation of assay of catechol-*O*-methyltransferase activity in human erythrocytes

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Abstract

The multistep assay of specific catechol-*O*-methyltransferase (COMT) activity in human erythrocytes was validated. Enzyme preparations from lysed erythrocytes were incubated with a substrate (3,4-dihydroxybenzoic acid) in the presence of Mg^{2+} and *S*-adenosylmethionine. The reaction products (vanillic acid and isovanillic acid) were analyzable by HPLC with electrochemical detection directly in the incubation medium after protein precipitation. The precision was calculated in order to define the random variability associated with the method by intra-assay and inter-assay relative standard deviations (RSDs) for the assays of both reaction products and protein. The intra-assay RDSs for the specific activities were between 4.8 and 11.9% ($n = 5–6$) at two levels of COMT activity. The inter-assay RSDs were between 6.4 and 14.2% ($n = 5–6$), respectively. The total variation was mostly caused by the protein assay and the HPLC assay, and contributions from the sample preparation and incubation steps were minor.

Some results from the clinical application of the erythrocyte COMT assay are also reported. For both normal volunteers and patients having Parkinson's disease, a single 400 mg dose of entacapone, a peripherally acting COMT inhibitor, decreased the erythrocyte COMT activity. The application demonstrates that the assay was able to detect differences between the subjects and the effect of COMT inhibition in the clinical study.

Keywords: Bioanalytical method validation; Reversed-phase HPLC; Electrochemical detection; Catechol-*O*-methyltransferase activity; Human erythrocytes

1. Introduction

Catechol-*O*-methyltransferase (COMT; EC 2.1.1.6) is an ubiquitous enzyme in nature. In mammals, COMT is distributed in all organs. The highest enzyme activities are in the liver and kidney. COMT also occurs in blood cells [1]. The "discovery" of COMT occurred in the late 1980s, when first potent and selective COMT inhibitors were developed [2], and then later the two forms of COMT, membrane-bound (MB) and soluble (S) enzymes, were characterized [3].

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The new COMT inhibitors are already undergoing clinical trials as adjunct drugs for the treatment of Parkinson's disease. Therefore, the assay of COMT activity, as a measure of the efficacy of the inhibitors, has become important. A particularly useful practice is to analyze the COMT activity in red blood cells of patients in order to confirm that the dose of the inhibitor has been adequate, since blood samples are easy to collect in clinical trials. Another way would be the analysis of plasma dopa or catecholamines and their metabolites.

The purpose of this study was to validate the previously developed assay of COMT activity in erythrocytes for use in the clinical study [4]. This assay has several phases, starting from the collection of blood and washing of the erythrocytes, then incubation of the erythrocytes with the substrate, analyzing the reaction products, and finally assaying the protein (or haemoglobin) content of the reaction mixture. All these phases have their own variability, which affects both the results and the actual results of the assay.

An attempt has been made to separate the assay procedure into smaller units and to estimate how much each phase contributes to the variation of the final results. In addition, the assay was applied to clinical studies, where entacapone, a COMT inhibitor, was given to healthy volunteers or to patients having Parkinson's disease.

2. Experimental

2.1. Materials

Ultrapure reagent-grade water was obtained with a Milli-Q system (Millipore/Waters, Milford, MA, USA). Vanillic acid (3-methoxy-4-hydroxybenzoic acid), isovanillic acid (3-hydroxy-4-methoxy benzoic acid), 3,4-dihydroxybenzoic acid (DHBA) and *S*-adenosyl-L-methionine iodide salt (AdoMet) were purchased from Sigma (St. Louis, MO, USA). Disodium hydrogen phosphate dihydrate, disodium edetate (EDTA), perchloric acid, magnesium chloride, sodium hydroxide, phosphoric acid, hydrochloric acid and dimethylsulfoxide were purchased from E. Merck (Darmstadt, Germany). The reagents and solvents were analytical grade and were used without further purification. HPLC-grade methanol was purchased from Rathburn (Walkerburn, UK).

2.2. Collection and washing of the samples

Blood samples were taken in tubes containing EDTA. Plasma was separated by centrifugation at +4°C at 1500*g*. The red blood cells were washed with three times the cell volume of cold 0.9% NaCl. The procedure was repeated three times. The washed cells were stored at −80°C.

2.3. Preparation of samples

2.3.1. Calibration samples

Calibration samples (0.2, 0.5, 1.0 and 2.0 μM) were prepared daily from stock solutions of vanillic acid and isovanillic acid (1 mM in 1% dimethylsulfoxide in 0.1 M sodium phosphate buffer (pH 7.8). The stock solutions could be stored at −20°C for several months.

2.3.2. Quality control samples

Quality control blood samples of different levels of COMT activity (after different doses of the COMT inhibitor) were taken from healthy volunteers and used in each analysis batch. One pool was taken from a person who had undergone no treatment, one was obtained from a person before, and 45 min after, dosage with 200 mg of entacapone, and one pool was taken from a third person before, and 45 min after, dosage with 800 mg of entacapone. The pools were divided after centrifugation and washing (see above), before freezing at −80°C. Two of the quality control samples were analyzed in one assay at different levels of COMT activity.

The samples were also used for the validation of the method. Moreover, the pools taken from a single person after different doses of entacapone (0, 200 and 400 mg) were used to measure the between-day variation of the COMT activity.

Control samples of vanillic acid were prepared from the compound. The sample was diluted with buffer to a concentration of 1 μM, divided into small aliquots, and stored at −20°C. During each

HPLC run, every eighth sample was taken from this pool.

2.3.3. Red blood cell samples

After melting, the washed cell samples were haemolyzed with four times the cell volume of cold 1 mM sodium phosphate buffer (pH 7.4), then vortex-mixed. The samples were then left to stand in an ice bath for 10 min, before centrifugation for 20 min at +4°C at 20000*g* to separate MB-COMT from S-COMT. The supernatant was used immediately for the measurement of soluble COMT enzyme activity. An aliquot of the supernatant was diluted 1:100 with 0.9% NaCl and stored at −20°C before measurement of the protein content.

2.4. COMT assay

2.4.1. Incubation

The COMT assay was performed essentially according to the method of Schultz et al. [4] with a minor modification in the incubation volume and with centrifugation, in order to be more compatible with our instrumentation and to obtain complete precipitation, respectively. Duplicate samples were incubated in 0.1 M Na_2HPO_4 (pH 7.8), 2.0 mM $MgCl_2$+, 0.2 mM AdoMet, 300 μl of the 20000*g* supernatant and 400 μM DHBA as the enzyme substrate. The final volume was 750 μl. The samples were incubated in a water bath at +37°C for 60 min. The tubes were transferred to an ice bath and the reaction was stopped by adding 75 μl of ice-cold 4 M perchloric acid. After 10 min, the samples were centrifuged at +4°C (10 min; 5400*g*). The supernatants were filtered through a 0.45 μm membrane filter and collected in autosampler vials for determination of the 3-O- and 4-O-methylated products. The samples were stored at −80°C before the assay.

2.4.2. HPLC

The reaction products in the supernatant were analyzed by HPLC with electrochemical detection. The chromatographic system consisted of an isocratic Minipump VS (Milton Roy Company, Shannon, Ireland), an SPH 125 autosampler equipped with cooler (Spark Holland, Emmen, The Netherlands), a Hewlett Packard 3396A integrator (Palo Alto, CA, USA), and an Ultratechsphere 5 ODS column (5 μm, 150 × 4 mm i.d.; HPLC Technology, Cheshire, UK) with an Ultratechsphere ODS precolumn (10 mm × 4 mm i.d.). An LC-4B electrochemical detector from Bioanalytical System (West-Lafayette, IN, USA) with a glassy carbon TL-5 electrode was used. A glassy carbon working electrode was set at 0.90 V versus an Ag/AgCl reference electrode. The sensitivity was set at 10 nA (or 20 nA depending on the concentration of the vanillic acid formed). The mobile phase consisted of the buffer disodium hydrogen phosphate (0.1 M) and EDTA (0.15 mM)–methanol (78:22, v/v) (the pH was adjusted to 3.2 with phosphoric acid). The flow rate was 0.9 ml min^{-1}. The injection volume of the standard solution and the filtered supernatant was 20 μl.

2.5. Protein measurement

The proteins were measured using a Coomassie Protein Assay Reagent (Pierce, Rockford, IL, USA) kit [5] and an Ultrospec III spectrophotometer (Pharmacia LKB Biotechnology, Uppsala, Sweden). Bovine serum albumin (BSA) included in the kit was diluted with 0.9% NaCl and used as the standards and calibration control samples.

The quality control samples were diluted (1:100) from 20000*g* supernatant, and small aliquots were stored at −20°C. Two quality control samples were measured in every assay.

2.6. Quantification and calculations

The concentrations of both vanillic acid and isovanillic acid in the samples were always in the range of our standards (0.2–2.0 μM).The standard curves were calculated by linear regression of the peak heights of the samples with Quattro Pro software (Borland International, Scotts Valley, CA, USA). The concentrations of the reaction products were calculated from their peak heights using the equation. Only vanillic acid was used for the final calculations of COMT activity. Isovanillic acid was used as an internal control to monitor the meta/para methylation ratio.

The specific activity of COMT was expressed as the vanillic acid (pmol) formed during 1 min, per milligram of protein in the sample.

The concentrations of the protein standards used daily were between 100 and 1500 μg ml^{-1} and polynomial curve fitting was used in the calculation.

The precision was evaluated by calculating the intra-assay and inter-assay relative standard deviations (RSDs). The accuracy was evaluated by calculating the relative error at each concentration.

3. Results

3.1. HPLC assay of analytes

3.1.1. Linearity and limit of quantitation

A good linearity between the vanillic acid and isovanillic acid concentrations of the standards and the peak heights was observed between 0.2 and 2 μM. The method was linear over a range wide enough to determine the COMT activities in clinical samples before and after dosage with different COMT inhibitors. The slopes for vanillic acid and isovanillic acid were $(50.00 \pm 4.10) \times 10^{-8}$ and $(53.7 \pm 5.18) \times 10^{-8}$ relative height μM^{-1} (mean $\pm$ standard deviation (SD); $n = 19$), respectively, with mean ($\pm$SD) Y intercepts of 0.01173 ± 0.01692 and -0.00307 ± 0.01690, respectively ($n = 19$).

The limit of detection, determined under the present chromatographic conditions, was 0.5 pmol per injection for both pure analytes at a signal-to-noise ratio of 3. The limit of quantitation was set at 0.2 μM taking into consideration the precision of the method.

3.1.2. Precision and accuracy

Under the present chromatographic conditions, the accuracy and precision of the calibration samples were good. In the range 0.2–2.0 μM, the precision of the calibration for both vanillic acid and isovanillic acid was maximally 4.8%, and the accuracy for vanillic acid and isovanillic acid was maximally 0.86% and 0.25% (expressed as the percentage error; $n = 19$), respectively (see Tables 1 and 2).

Table 1
The precision and accuracy of concentrations of the vanillic acid calibration samples

Added (μM)	Found $\pm$ SD (μM)	RSD (%)	Error (%)	n
0.2	0.209 ± 0.010	4.8	0.86	19
0.5	0.495 ± 0.012	2.4	−0.55	19
1.0	0.993 ± 0.016	1.6	−0.69	19
2.0	2.004 ± 0.006	0.3	0.35	19

The intra-assay RSD, evaluated by measuring the same quality control sample of vanillic acid (1 μM), was 2.27% ($n = 6$), whereas the inter-assay RSD was 4.00% ($n = 5$).

3.2. Protein assay

In the range 100–1500 μg ml^{-1}, the precision of the protein calibration samples was below 5.42%, and the accuracy was between −2.33 and 0.38% (expressed as the percentage error; $n = 10$) (see Table 3).

The final calculated concentrations of protein were 74.67 ± 2.12 and 67.58 ± 2.34 mg ml^{-1} (mean $\pm$ SD) in two quality control samples. The samples were diluted from pools from two different people. The intra-assay and inter-assay variations in the protein assay are shown in Table 4.

3.3. COMT assay

3.3.1. COMT activity

For the measurement of the intra-assay variation in the COMT activity, expressed as reaction product (vanillic acid) per sample for the 60 min incubation, pools of washed red blood cells

Table 2
The precision and accuracy of the concentrations of the isovanillic acid calibration samples

Added (μM)	Found $\pm$ SD (μM)	RSD (%)	Error (%)	n
0.2	0.203 ± 0.010	4.8	0.25	19
0.5	0.499 ± 0.011	2.2	−0.08	19
1.0	0.997 ± 0.013	1.3	−0.29	19
2.0	2.001 ± 0.006	0.3	0.12	19

Table 3
The precision and accuracy of the protein calibration samples

Added (μg ml^{-1})	Found $\pm$ SD (μg ml^{-1})	RSD (%)	Error (%)	n
100	97.67 $\pm$ 5.30	5.42	−2.33	10
250	252.9 $\pm$ 7.01	2.77	+1.15	10
500	501 $\pm$ 8.06	1.61	+0.20	10
750	746.2 $\pm$ 7.45	1.00	−0.51	10
1000	1004 $\pm$ 4.91	0.49	+0.38	10
1500	1498 $\pm$ 1.97	0.13	−0.14	10

(RBCs) were used. The pools were taken from different people. The mean ($\pm$ SD) activities were 2.021 $\pm$ 0.100 μM ($n = 6$) and 1.143 $\pm$ 0.041 μM ($n = 5$) vanillic acid, depending on the entacapone dose, and intra-assay RSDs were 4.95% ($n = 6$) and 3.58% ($n = 5$), respectively. The RSD for the duplicates was calculated according to Abraham et al. [6] and was 1.45% and 4.95% ($n = 5$–6), respectively.

The inter-assay variation of COMT activity was measured from the pools taken from a single person after two doses of COMT inhibitor. The RSDs were 6.73 and 9.21% ($n = 6$) (Table 4).

3.3.2. Specific activity of COMT

The specific COMT activity was calculated as vanillic acid concentration against the protein content per minute in the sample. The intra-assay RSDs for the specific activities were between 4.8 ($n = 6$) and 11.9% ($n = 5$). The mean ($\pm$ SD) specific activities of the quality control samples from the same person without and with treatment with 800 mg of entacapone were 1.054 $\pm$ 0.073 pmol mg^{-1} of protein min^{-1} and 0.726 $\pm$ 0.086 pmol mg^{-1} of protein min^{-1}, respectively.

Inter-assay RSDs were 6.38 ($n = 3$) and 14.2% ($n = 12$) at two levels of COMT activity (Table 4).

3.3.3. Selectivity and recovery

Typical chromatograms obtained from standard and RBC samples before and after enzyme inhibition are shown in Fig. 1. The vanillic acid and isovanillic acid eluted with retention times of 8.6 min and 9.7 min, respectively (Fig. 1). The retention times did not differ in the standard samples and the biological matrix samples. Incubation with 0.4 mM DHBA induced the formation of reaction products with the same retention times as vanillic acid and isovanillic acid. The incubation of supernatant without substrate or incubation of substrate without the enzyme preparation did not induce any interfering peaks near the vanillic acid or isovanillic acid peaks. An interfering peak in the chromatogram was detected when the COMT assay was performed using human red blood cells and when the running time of the chromatogram was less than 20 min. The interference resulted from a late-eluting compound affecting the chromatogram of the next injected sample. This peak was not related to any treatment.

The recovery in the spiked sample of the biological matrix was 98.7%. When standards of two different concentration levels were added to the biological matrix, and determined following the described incubation procedure without substrate, the mean ($\pm$ SD) recoveries were 71.5 $\pm$ 1.5 and 70.0 $\pm$ 1.3% ($n = 5$).

3.3.4. The O-methylation ratio of products

The ratio of 3- to 4-methylated products (meta/para ratio) in the control samples of the study was 4.50 $\pm$ 0.21 ($n = 76$) for S-COMT. The ratio was 4.55 $\pm$ 0.42 (mean $\pm$ SD) ($n = 205$) for all the samples of the study, including the quality control samples.

3.4. Stability of samples

The stock substrate solution (1 mM DHBA) could be stored for several weeks at −20°C and at least 2 weeks also at +4°C without any losses.

The prepared samples kept overnight in the cooled autosampler did not lose their vanillic acid content, as was shown by the intra-assay variation, with an RSD of 2.27% ($n = 6$) for vanillic acid and the inter-assay variation, with an RSD of 4.00% ($n = 5$).

The stability of COMT activity in the frozen RBCs was followed using the quality control samples. There appeared to be no loss of COMT activity during the 14 month storage at −80°C (Fig. 2).

Table 4
Intra-assay and inter-assay RSDs of the steps in the COMT assay in human erythrocytes

Assay step	Intra-assay RSD (%)	n^a	Inter-assay RSD (%)	n
HPLC vanillic acid	2.27	6	4.00	5
Protein assay[b]	3.16[c]	10	2.84[c]	7
	2.08[d]	10	3.46[c]	7
COMT activity	4.95[c]	6	9.21[c]	6
	3.58[d]	5	6.73[d]	6
Specific COMT activity	4.80[c]	6	6.38[c]	3
	11.89[d]	5	14.2[d]	12

[a]Number of samples in each assay.
[b]In the protein assays, diluted human blood batches were used.
[c]COMT activity and specific COMT activity were determined at two different levels in blood batches of people not treated with entacapone.
[d]COMT activity and specific COMT activity were determined at two different levels in blood batches of people treated with entacapone.

3.5. Application

The method was applied to the analysis of the COMT activity in the red blood cells of healthy volunteers and patients having Parkinson's disease. In the healthy volunteers, the mean specific activity of COMT before intake of entacapone was 0.727 ± 0.259 (mean $\pm$ SD) pmol mg^{-1} of protein min^{-1} ($n = 12$) with a range from 0.41 to 1.18 pmol mg^{-1} of protein min^{-1}. The activity 1 h after the intake of 400 mg was 0.316 ± 0.115 pmol mg^{-1} of protein min^{-1} ($n = 12$) (see Fig. 3A).

In the group of patients with Parkinson's disease, the baseline activity of S-COMT was 0.617 ± 0.260 pmol mg^{-1} of protein min^{-1} ($n = 22$), with a range from 0.223 to 1.112 pmol mg^{-1} of protein min^{-1}. The activity 1 h after the intake of 400 mg of entacapone was 0.317 ± 0.151 pmol mg^{-1} of protein min^{-1} ($n = 22$) (Fig. 3B).

4. Discussion

Validation of bioanalytical procedures is carried out to demonstrate the performance and reliability of the method. Two phases can be distinguished in the validation process: a method development phase before the clinical study, and an application phase during the study [7,8]. In this paper we are dealing with the former phase, but give some examples of the application phase also.

The assay of COMT activity in human erythrocytes has several steps (Table 4). The collecting of the blood samples and the initial washing of the erythrocytes were carried out in hospital. Written instructions were provided and a practical demonstration of the procedure was also given. Variations caused by the procedure could have been important. It seems, however, that the procedure was very well standardized and contributed only a little to the total variation (see below).

The HPLC assay of the reaction products, notably that of vanillic acid, was carefully explored. Both intra- and inter-assay variations (as the relative standard deviations) were in the range 2–5%, which is quite normal for HPLC assays. Of course, the variation was higher at low levels of vanillic acid. Almost similar RSDs were seen in the protein assay. The colour-binding protein assay was not completely satisfactory, since the standard curve lost its linearity at levels exceeding 1000 μg ml^{-1}. However, our samples were always of concentrations below this. On the whole, however, the biochemical assays were easily controlled and their contribution to the total variation (up to 10% altogether) was at an acceptable level.

Considering the variation caused by the enzyme incubation is possible only when combined with the assay of vanillic acid. Intra-assay and inter-as-

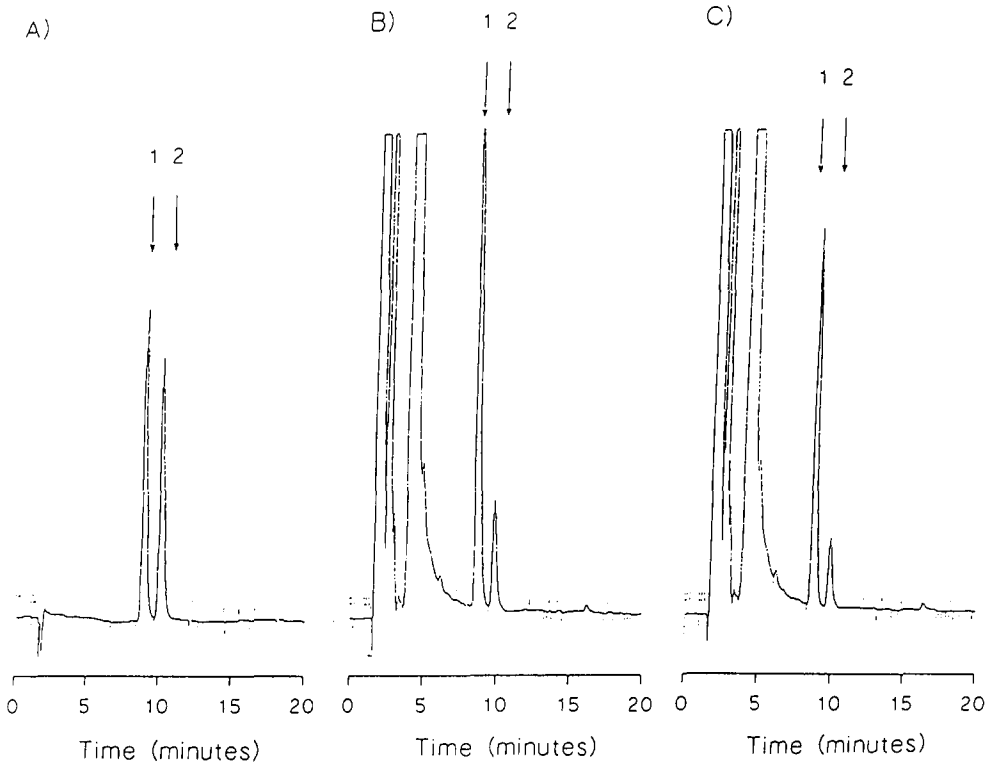

Fig. 1. Chromatograms obtained from the samples. (A) Standard sample containing 1 μM vanillic acid (peak 1) and isovanillic acid (peak 2); (B) assayed reaction product of a sample of human RBC, without treatment; (C) assayed reaction product of a sample of human RBC after treatment with 800 mg of the COMT inhibitor entacapone.

say variations of the analyzed COMT activity were 3–5% and 6.7–9.2%, respectively. This may be interpreted to show that enzyme incubation as such added to the intra-assay variation only slightly, if not at all, and to the inter-assay variation by 3.7–4.2%.

Finally, when the variation of the protein assay is also taken into consideration, the RSDs in the specific COMT activity were 4.8–11.9% (intra-assay) and 6.4–14.2% (inter-assay). These RDSs are smaller than those obtained by counting back from the variations from the various analytical steps. Even when the variations due to blood collecting and washing of the erythrocytes are excluded, these summary variations are somewhat higher (7.9–13.5%, intra-assay; 13.6–16.8%, inter-assay) than those of the specific COMT assay including all the analytical phases, but at least are of the same magnitude. The effect of washing includes the variation of the protein assay in the red blood sample. Indirectly these calculations demonstrate that the variation from sample handling is quite small.

The sensitivity of the erythrocyte COMT activity assay was 0.2 μM. In this assay, DHBA is used as a substrate. The reaction products, vanillic acid and isovanillic acid, are analyzable by HPLC with amperometric detection directly in the incubation medium after protein precipitation without any extraction. The selectivity of the assay was assessed on incubating, in the presence of Mg^{2+} and *S*-AdoMet, the lysed erythrocyte supernatant without substrate and the substrate alone. No interfering compounds at the retention times of vanillic acid and isovanillic acid were observed. One interfering compound in the chromatogram could be detected only if the running time was less than 20 min. Even this peak was in no way related to the treatment of people with entacapone.

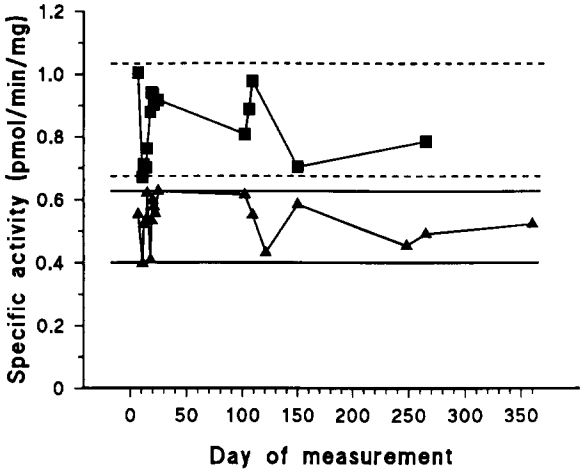

Fig. 2. The stability of specific activity in human erythrocytes (■) before treatment with entacapone and (▲) 45 min after treatment with 800 mg of entacapone, during storage at −80°C.

The stability of S-COMT activity in the frozen and washed erythrocytes was good over a 1 year follow-up time (Fig. 2). It is noteworthy, however, that after osmotic lysing, the COMT reaction must be performed immediately, and no storage is possible without a loss of activity [11]. After enzyme incubation, the reaction products can again be stored in the frozen state without loss.

Some results of the clinical application of the erythrocyte COMT assay have also been reported. In both normal volunteers and in patients having Parkinson's disease, a single 400 mg dose of entacapone, a peripherally acting COMT inhibitor [3,12,13] decreased the erythrocyte COMT activity in every subject. The variation from person to person was more than threefold, both in the case of basal COMT activity and in the case of COMT activity after entacapone. This application demonstrates that the assay is able to detect differences between the subjects and also the effect of COMT inhibition.

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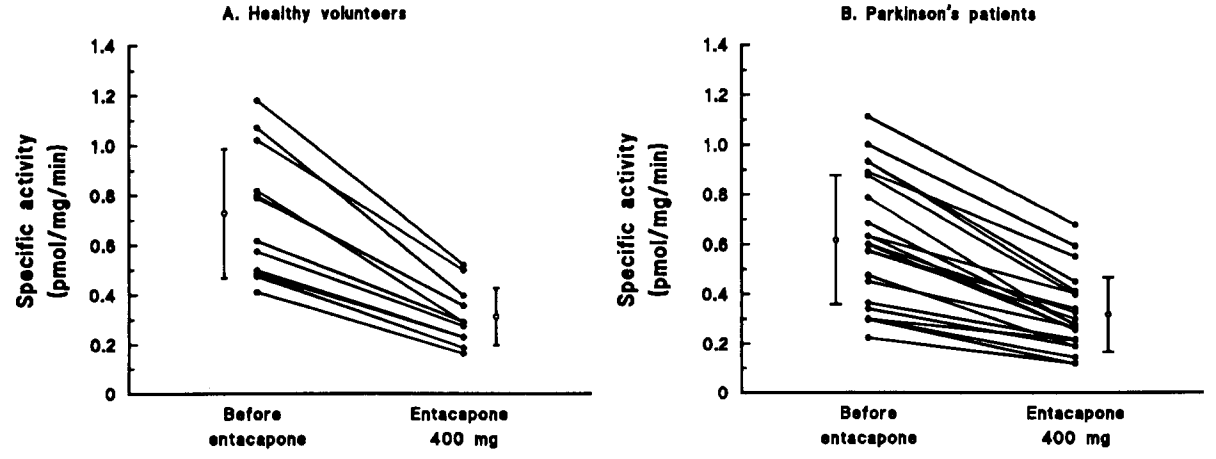

Fig. 3. The specific activity of COMT in erythrocytes of (A) healthy volunteers and (B) Parkinson's disease patients before and after dosage with 400 mg of entacapone. Individual values (●) and mean ± SD (○) are given.

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