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Application of an organic solvent extraction to the determination of catechol-O-methyltransferase activity by high-performance liquid chromatography in human mononuclear cells

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ABSTRACT

We report here a method for measuring mononuclear cell catechol-O-methyltransferase (COMT) activity which is ideally adapted to clinical studies. The method measures the O-methylation of dopamine to 3-methoxytyramine and 4-methoxy-3-hydroxyphenethylamine. Whole mononuclear cell sonicate is incubated with saturating concentrations of dopamine, S-adenosyl-L-methionine and magnesium chloride in sodium–potassium phosphate buffer at pH 7.3. An organic solvent extraction using ethyl acetate is then used for product separation, followed by high-performance liquid chromatography with electrochemical detection for product separation and quantification. This method allows both O-methylated products, 3-methoxytyramine and 4-methoxy-3-hydroxyphenethylamine, to be isolated and quantified separately. The apparent Michaelis constants for dopamine and S-adenosyl-L-methionine using this method are similar to values reported previously (0.51 and 14 μM, respectively). The optimal concentration of magnesium chloride is eight to ten times higher than previously reported. No endogenous inhibitors were apparent using this assay. The within-day coefficient of variation using this method is 7% when measuring 3-methoxytyramine and 5% when measuring 4-methoxy-3-hydroxyphenethylamine. The between-day coefficient of variation is 11%. Mononuclear cell COMT activity can be detected using protein concentrations as low as 0.75 mg/ml, corresponding to 2–3 ml of whole blood. The small amount of blood required per sample allows multiple sample analysis from a single patient, including infants.

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INTRODUCTION

In 1958, Axelrod and Tomchick [1] first described catechol-O-methyltransferase (COMT), an enzyme that catalyzes the ring O-methylation of dihydroxyphenolic compounds such as the catecholamine hormones and other catechol-containing drugs. Over the next 30 years, this enzymatic reaction was shown to be one of the three key pathways for metabolic degradation of catecholamines [2]. Norepinephrine, which diffuses from the synaptic cleft into the circulation, is metabolized rapidly by COMT [2] and endogenous epinephrine is excreted primarily as the methylated products, metanephrine and vanillylmandelic acid, in the urine of humans [2]. In addition, major portions of exogenously administered dopamine (DA) and isoproterenol are excreted as methylated products in urine [3,4]. Catechol-containing drugs such as L-DOPA and methyldopa are also metabolized extensively by COMT [5,6].

Population studies have shown a four- to five-fold inter-individual variation in COMT activity [7]. This variation in the relative level of COMT activity appears to be inherited in an autosomal codominant fashion [8]. Pedigree and twin studies suggest that COMT activity is regulated by two alleles at a single locus [8]. Therefore, individuals who are heterozygous display a wide range of intermediate COMT activity.

The relative activity of COMT has been shown to correlate with the degree of metabolic inactivation of the catechol drug L-DOPA [5]. Furthermore, these inter-individual differences in L-DOPA metabolism appear to have clinical implications as patients with relatively high COMT activity demonstrate a poorer response to L-DOPA [5]. Exogenous catecholamines such as DA, norepinephrine and epinephrine are frequently utilized today to augment cardiac output, blood pressure and urine output in critically ill patients [9]. The functional significance of the inter-individual variation in COMT activity in relation to the metabolism of these exogenous catecholamines, as well as endogenous catecholamines, is unknown.

COMT is distributed throughout many tissues in the body [10]. Clinical studies examing the role of COMT in exogenous catecholamine metabolism require the use of tissues that can be obtained relatively non-invasively. The activity in easily accessible tissues such as erythrocytes and mononuclear cells has been shown to correlate with that of other organs such as kidney and lung which have the highest specific enzyme activity [11,12]. Since critically ill patients are frequently transfused with erythrocytes, mononuclear cells appear to be the optimal tissue for the examination of COMT activity in these patients.

Previously reported methods for measuring mononuclear cell COMT activity require at least 7 ml of blood per sample and did not distinguish between the *m*- and *p*-methylated products [12]. We report here a simplified and sensitive procedure for the determination of COMT activity in human mononuclear cells. This method uses organic solvent extraction and high-performance liquid chromatography (HPLC) with electrochemical detection (ED).

EXPERIMENTAL

Chemicals

S-Adenosyl-L-methionine (SAM), adenosine deaminase (EC 3.5.4.4, bovine spleen, 78 U/mg of protein), 3-methoxytyramine (3MT), 3-methoxy-4-hydroxybenzylamine (MHBA) and dopaminehydrochloride (DA) were obtained from Sigma (St. Louis, MO, USA).

Dopamine was purified prior to use by adsorption to acid-washed alumina at pH 8.7, washed three times with water, followed by elution of the compound with 0.1 *M* perchloric acid [13]. Dopamine prepared in this fashion showed no deterioration over seven days when stored at 4°C. Therefore, stock solutions of DA for use in the assay were prepared weekly.

4-Methoxy-3-hydroxyphenethylamine (4MT) was purchased from Regis (Chicago, IL, USA). Sodium octylsulfate was purchased from Eastman Kodak (Rochester, NY, USA). Ficoll was supplied by Pharmacia Fine Chemicals (Piscataway, NJ, USA). Partially purified catechol-O-methyltransferase (EC 2.1.1.6, porcine liver 2200

U/mg of protein) was purchased from Calbiochem (La Jolla, CA, USA). HPLC-grade acetonitrile was obtained from Burdick & Jackson (Muskegon, MI, USA). All other chemicals were reagent quality.

Apparatus

An LC-400 liquid chromatograph equipped with a carbon/carbon electrode (Bioanalytical Systems, West Lafayette, IN, USA) was interfaced with a Varian Instruments (Sunnydale, CA, USA) Model 2510 pump. The potential of the working electrode was maintained at +750 mV *versus* an Ag^+/AgCl reference electrode.

Separations were performed on a 100 mm × 3.2 mm I.D. stainless-steel column prepacked with 3 μm reversed-phase material (Phase II ODS, Bioanalytical Systems).

Mobile phase

The mobile phase consisted of 0.15 *M* monochloroacetic acid with 108.5 mg of sodium octylsulfate, 750 mg of Na_2EDTA and 16.5 ml of acetonitrile added to each liter. The pH was adjusted to 3.0 with 5 *M* NaOH prior to adding the acetonitrile. The flow-rate was maintained at 1.0 ml/min.

Mononuclear cell preparation

Mononuclear cells used to develop the assay were obtained from fifteen healthy white adult volunteers. None of the subjects had a chronic illness. None had received any medication for the two weeks prior to phlebotomy. Blood was collected by venipuncture in Vacutainer tubes that contained 10.5 mg Na_2EDTA. These tubes were kept at room temperature for a maximum of 3 h prior to mononuclear cell isolation.

Platelets have been shown to contain COMT activity [14]. In order to minimize contamination of the mononuclear cell preparation with platelets, blood samples were initially centrifuged at 200 *g* for 10 min at room temperature. This produced a platelet-rich plasma supernatant which was removed and discarded.

To isolate the mononuclear cells, the remaining loosely packed cell pellet was diluted 2:1 (v/v) with 0.27 *M* sucrose and layered over Ficoll–Hypaque (specific gravity 1.077) in a 50-ml conical-bottom polypropylene centrifuge tube. The samples were centrifuged at 250 *g* for 40 min at room temperature in a Beckman Model TJ-6 swinging-bucket centrifuge. The mononuclear layer was subsequently withdrawn and placed in a 15-ml conical-bottom polystyrene centifuge tube. The mononuclear cells were then washed with 10 ml of Hanks balanced salt solution (HBSS) containing 350 mg of $NaHCO_3$ and 500 mg of Na_2EDTA in each liter. The cells were centrifuged at 625 *g* for 20 min at 4°C. The mononuclear cells were washed a second time with 7 ml of the Na_2EDTA- and $NaHCO_3$-enriched HBSS solution plus 3 ml of 0.2% NaCl in order to lyse the erythrocytes [15]. They were then centrifuged at 625 *g* for 10 min at 4°C. The mononuclear cells underwent a third and final wash with 10 ml of the Na_2ED-

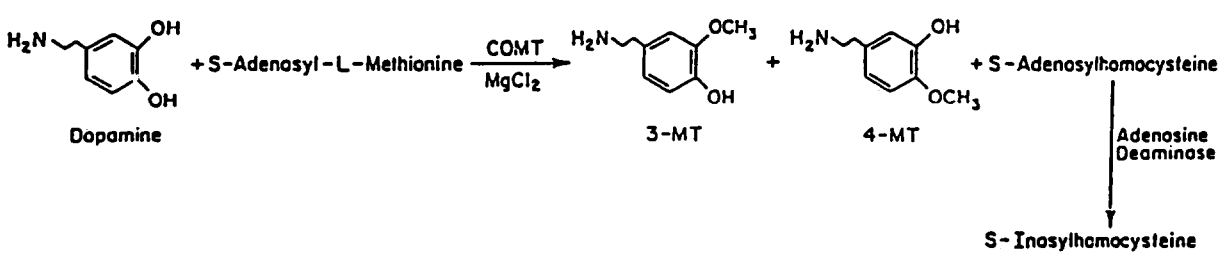

Fig. 1. Methylation of dopamine to 3-methoxytyramine (3MT) and 4-methoxy-3-hydroxyphenethylamine (4MT) via COMT. The reported ratio of *m/p* methylation of dopamine using purified COMT varies from 10.2 at pH 7.0 to 3.5 at pH 9.1 [17]. S-Adenosyl-L-methionine, functioning as a methyl donor, is converted to S-adenosylhomocysteine during the reaction. Adenosine deaminase is added to convert S-adenosylhomocysteine, which is an inhibitor of COMT, to S-inosylhomocysteine. $MgCl_2$ is a cofactor necessary for COMT activity.

TA- and $NaHCO_3$-enriched HBSS solution, followed by centrifugation at 625 *g* for 10 min at 4°C.

After the final wash, the supernatant was aspirated completely. The tube containing the mononuclear cell pellet was purged with nitrogen for 30 s, then placed immediately at −70°C. Mononuclear cells stored in this fashion retained 100% of their COMT activity for at least fourteen days (250 U on day 1 compared to 264 U on day 14). All cells used for development of the assay procedure were used within ten days of freezing.

COMT assay

The assay is based on the conversion of DA to its methylated products (Fig. 1). The assay procedure employed is a modification of that reported by Shoup *et al.* [13] to measure COMT activity in rat liver, brain and human erythrocytes. The assay conditions were optimized for use with human mononuclear cells, and an organic solvent extraction step was substituted for the ion-exchange columns usually employed for separation of substrate from products.

The mononuclear cell pellets were thawed on ice. A 250-μl volume of 0.5 *M* sodium–potassium phosphate buffer, pH 7.3, was added to each pellet. The cell suspension was then sonicated on ice for 15 *s* using a Sonifier cell disrupter (Heat Systems, Ultrasonics).

The reaction mixture consisted of 50 μl of 120 m*M* $MgCl_2$, 200 μl of 600 μ*M* SAM, 200 μl of cell sonicate and 50 μl of adenosine deaminase. The adenosine deaminase used in the reaction mixture was diluted from the stock enzyme 1:3 (v/v) in phosphate buffer so that 50 μl equaled 3 U. The reaction mixture was preincubated for 5 min in a shaking water bath at 37°C. It was then initiated by the addition of 50 μl of 50 m*M* DA, bringing the final volume of the reaction mixture up to 550 μl. Following the addition of DA, the tube was vortex-mixed gently and replaced in the shaking water bath for 10 min. The reaction was terminated by placing the tube into an ice bath at 0°C. A 50-μl volume of internal standard, MHBA, was added to each tube, followed by vigorous vortex-mixing.

The reaction mixture was alkalinized with borate prior to organic extraction by adding 0.5 ml of 1.33 *M* borate, pH 11, containing 1% (w/v) Na_2EDTA to each tube. Cold ethyl acetate (4 ml) was added immediately after the borate buffer. Each tube was vortex-mixed vigorously for 30 s and the aqueous layer containing the majority of the DA substrate was discarded. The upper organic layer was removed and evaporated to dryness. The methylated products were redissolved in 200 μl of 0.1 *M* perchloric acid, filtered through a microfilter (0.2 μm pore size) and 100 μl were injected onto the HPLC column. Fig. 2 depicts a representative chromatogram.

A blank reaction mixture was included with each set of samples. The blank consisted of the complete reaction mixture with buffer substituted for mononuclear cell sonicate. An external standard was also run with each set of samples in order to calibrate the HPLC system since the sensitivity of the detector varied daily. This external standard consisted of a complete reaction mixture with 12.8 ng of both 3MT and 4MT substituted for the DA.

Protein determination

Protein concentration was determined by the method of Lowry *et al.* [16], using bovine serum albumin (BSA) as the standard.

Calculations

The concentration of 3MT and 4MT in each sample was determined by comparing each sam-

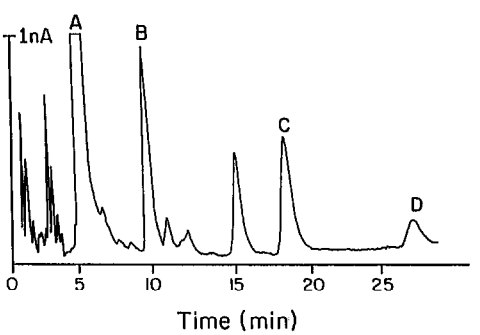

Fig. 2. Typical chromatogram resulting from the described method of measuring mononuclear cell COMT activity. Peaks: A = residual dopamine substrate; B = internal standard, 3-methoxy-4-hydroxybenzylamine; C = 3MT; D = 4MT.

ple to an external and internal standard in the following manner:

$$\frac{\text{peak-area ratio (sample/internal standard)}}{\text{peak-area ratio (external/internal standard)}} \times \text{ng of external standard injected}$$

One unit of COMT activity is defined as 1 pmol 3MT or 4MT produced per mg protein per 10 min, unless otherwise specified.

RESULTS

Mononuclear cell preparation

To ascertain the effect of storage on mononuclear cell COMT activity, whole blood was left in Vacutainer tubes at room temperature and 4°C for 12, 24 and 36 h prior to mononuclear cell isolation. Once the cells were isolated, they were pelleted and frozen at −70°C until used. No significant deterioration in COMT activity occurred in mononuclear cells kept at room temperature for 24 h prior to isolation (255 *versus* 231 U). However, after 36 h of storage at room temperature, COMT activity had decreased to 74% of its original value. The activity in cells maintained at 4°C for 24 h prior to isolation decreased by 14%. All cells used for assay development were isolated from whole blood left at room temperature for <3 h.

The composition of the cell preparations was evaluated microscopically. No erythrocytes were present in the final pellet after 0.2% NaCl treatment. The white blood cell differential count consisted of 84% lymphocytes, 15% monocytes and 1% granulocytes. Cell viability by trypan blue exclusion ranged from 87 to 96%, with a mean of 90%.

Although platelet contamination was minimized, there were still platelets present in the cell pellet as assayed by light microscopy. Therefore, a pure platelet preparation was obtained from platelet-rich plasma and the number of platelets counted with a Coulter counter. Platelet COMT activity was determined by the described procedure and averaged 11.2 pmol 3MT produced per 10^7 platelets per 10 min. Using this average activity, it was determined that COMT activity in platelets accounted for only 6–9% of the total COMT activity measured.

Dependence of COMT activity on pH

The dependence of mononuclear cell COMT activity on pH was determined in a phosphate buffer. Using these conditions, the optimum pH for COMT production of 3MT was 7.3 (Fig. 3). COMT production of 4MT continued to increase with pH until a plateau was reached from pH 7.5 to 7.8, which was the highest pH evaluated. This difference in optimum pH for production of the two methylated products led to a decrease in the *m/p* ratio from 14.6 to 3.7 as the pH was increased from 7.0 to 7.8 (Fig. 3). Decreasing *m/p* ratios have been reported previously with increasing pH for COMT purified from rat liver [17,18]. The reported pH optimum for purified or erythrocyte COMT in phosphate buffer has ranged from 7.8 to 7.9 [1,13].

The dependence of mononuclear cell COMT activity on pH has been evaluated previously using a Tris buffer and pH 7.7 was shown to be the optimum [12]. Mononuclear cell COMT activity was compared in both sodium–potassium phosphate buffer and 0.3 *M* Tris–HCl buffer using the described method over a pH range of 7.0–8.0. COMT activity was 8% greater in the phosphate

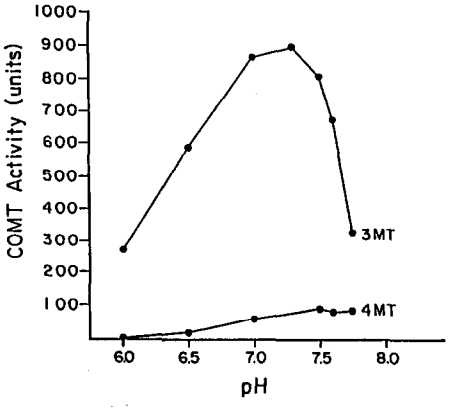

Fig. 3. pH dependence of mononuclear cell COMT activity. Mononuclear cell COMT activity was measured in 0.5 *M* sodium–potassium phosphate buffer at 37°C at various pH values ranging from 6.0 to 7.8. The *m/p* ratio ranged from 29.4 at pH 6.5 to 3.7 at pH 7.8.

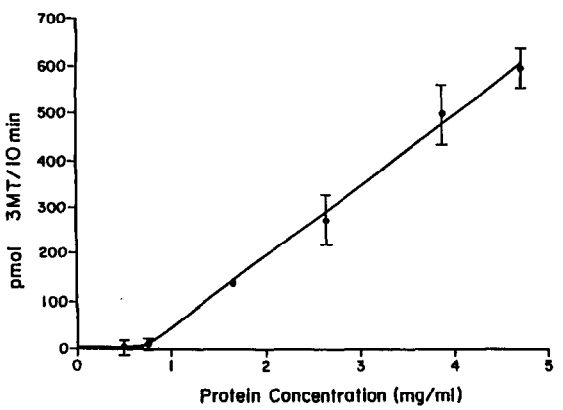

Fig. 4. Effect of increasing mononuclear cell protein on 3MT production. Each point represents the mean ± S.D. of three determinations.

buffer than in Tris–HCl buffer at pH 7.0. At pH 8.0, COMT activity was 88–99% greater in phosphate buffer compared to Tris–HCl buffer.

Linearity of reaction with protein concentration

COMT activity increased linearly with increasing sonicate protein concentration between 0.75 and 4.5 mg/ml (Fig. 4). Below 0.75 mg/ml, the assay lost precision. Augmenting the reaction mixture with an additional 1 mg/ml BSA protein had no effect on COMT activity and did not decrease the variability of the assay at low protein concentrations.

Linearity of reaction with time

COMT production of 3MT in mononuclear cells was linear with incubation time for at least 45 min (Fig. 5). 4MT production by mononuclear cell COMT continued to be linear to an incubation time of 60 min. An incubation time of 10 min was determined to be adequate for routine assay purposes.

Dependence of COMT activity on dopamine and SAM concentration

The dependence of mononuclear cell COMT activity on DA concentration was evaluated and an apparent Michaelis constant (K_M) with respect to 3MT of 0.51 mM was determined (Fig. 6). This appeared to be in good agreement with the value of 0.79 mM reported previously for DA [17]. COMT activity at varying concentrations of the methyl donor, SAM, demonstrated a linearity with SAM concentrations up to approximately 20 μM (Fig. 7). The calculated apparent K_M was 14 μM. The reported K_M value for SAM in lymphocytes using dihydoxybenzoic acid as a substrate was reported to be 2.3 μM [12], but no data using DA were available for comparison.

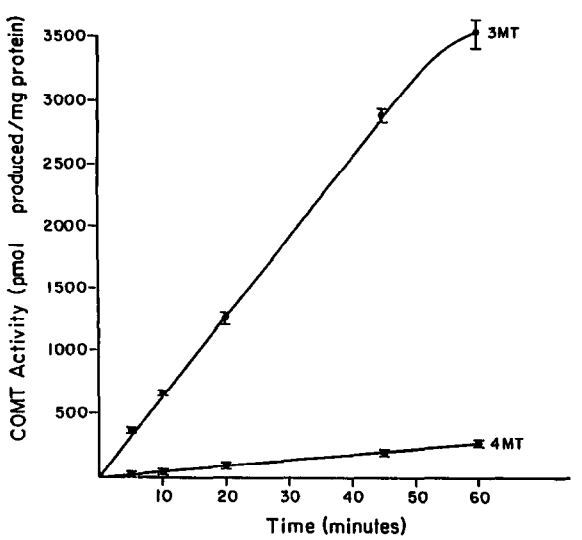

Fig. 5. Effect of increasing incubation time on human mononuclear cell COMT activity. Each point represents the mean ± S.D. of three determinations.

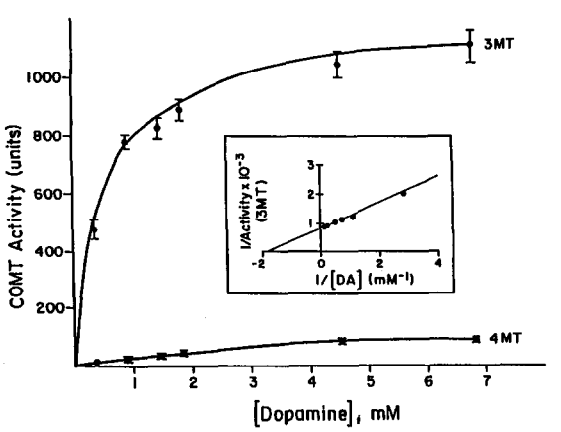

Fig. 6. Effect of increasing dopamine concentration on human mononuclear cell COMT activity. Each point represents the mean ± S.D. of five determinations.

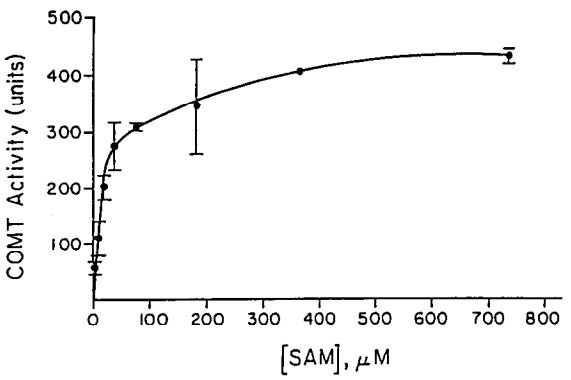

Fig. 7. Effect of increasing SAM concentration on human mononuclear cell COMT activity. Each point represents the mean ± S.D. of three determinations.

Effect of magnesium concentration on COMT activity

Magnesium ion is a necessary cofactor for COMT activity [1]. Other investigators have reported concentrations above 1–2 m*M* to be inhibitory to COMT activity in both phosphate and Tris buffers [9,12,19,20]. However, using the procedure described herein, COMT activity manifested a curvilinear dependence on $MgCl_2$ concentration, with an optimal concentration between 10 and 12 m*M*. COMT activity was not inhibited until a Mg^{2+} concentration ≥ 15 m*M* was reached in the reaction mixture (Fig. 8).

When 0.3 *M* Tris buffer pH 7.3 was substituted in the reaction for the phosphate buffer, an optimal concentration of 1–2 m*M* $MgCl_2$ was found for mononuclear cell COMT activity (data not shown). However, as discussed previously, the relative activity of COMT was higher in the phosphate buffer compared to the Tris buffer, even at a Mg^{2+} concentration of 2 m*M*. Therefore, a Mg^{2+} concentration of 12 mM in a phosphate buffer was considered optimal for use in the procedure.

Mononuclear cell inhibition

Endogenous inhibitors of COMT activity have been reported to be present in mononuclear cells [12]. To determine the extent to which these inhibitors may be affecting the measurement of COMT activity in this assay, the mononuclear cells from four subjects were isolated, resuspended, sonicated and pooled. The pooled sample contained 5.5 mg/ml of protein.

A 100-μl volume of partially purified COMT was added to either 100 μl of standard phosphate buffer, 100 μl of the buffer containing 1.5 mg/ml BSA or 100 μl of the buffer containing the mono-

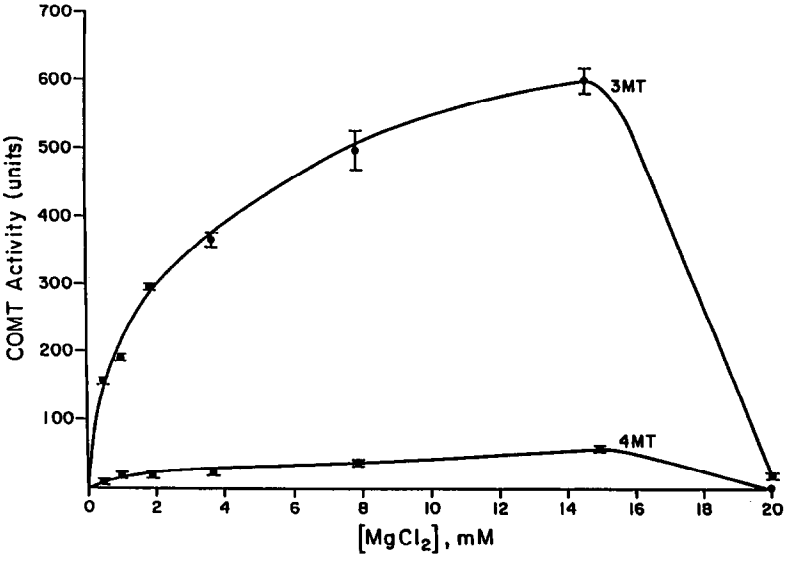

Fig. 8. Effect of increasing $MgCl_2$ concentration on human mononuclear cell COMT activity in 0.5 *M* sodium–postassium phosphate buffer pH 7.3. Each point represents the mean ± S.D. of three determinations.

TABLE I

EVALUATION OF PURIFIED COMT ACTIVITY ALONE, IN THE PRESENCE OF BOVINE SERUM ALBUMIN OR WITH MONONUCLEAR CELL SONICATE

The amount of purified COMT used was equivalent to approximately 15 U where 1 U of activity catalyzes the methylation of 1 nmol of dihydroxybenzoic acid per hour at 37°C, pH 8.0. The protein concentration of the mononuclear cell sonicate was 5.5 mg/ml. Each value represents the mean ± S.D. of three determinations.

Sample	COMT activity (pmol 3MT per 10 min)
Purified COMT	13 ± 1
Purified COMT + 1.5 mg/ml BSA	55 ± 3
Mononuclear cell sonicate	187 + 33
Purified COMT + mononuclear cell sonicate	345 + 38

nuclear cell sonicate. COMT activity in the sonicate was also measured separately.

The combination of mononuclear cell sonicate with purified COMT produced 73% more 3MT than expected by simply adding their activities together (Table I). Some of this enhancement may be due to the increased amount of protein present in the reaction mixture, as the addition of BSA also increased the apparent activity of the purified enzyme (Table I). BSA has previously been shown to enhance COMT activity in dilute preparations [12]. However, the activity of the sonicate–enzyme combination is still 42% above that expected by simply adding the activity of the protein-supplemented enzyme to the sonicate. Therefore, whole mononuclear cell sonicate appears to further augment rather than inhibit the activity of purified COMT.

The mechanism for this enhancement is unknown. However, oxidative inactivation of COMT at its active site has been proposed by other investigators [19]. One possible mechanism of purified COMT activity augmentation by protein sonicate is protection against this oxidative inactivation.

Other investigators have added reducing agents such as dithiothreitol to prevent oxidative inactivation of COMT [19]. However, the addition of 4 m*M* dithiothreitol to the reaction mixture increased 3 MT production by only 5% (1126 ± 145 U *versus* 1075 ± 130 U) and decreased 4MT production by 17% (69 ± 13 U *versus* 83 ± 5 U) compared to controls without dithiothreitol ($n = 5$). Therefore, oxidative inactivation of COMT in crude mononuclear cell sonicate does not appear to occur to an extent that would interfere with accurate measurement of its activity.

Another known inhibitor of COMT activity is S-adenosylhomocysteine [21]. This compound is the by-product formed by the demethylation of SAM [21], as illustrated in Fig. 1. Adenosine deaminase is an enzyme that removes S-adenosylhomocysteine, and thereby has been reported to stimulate COMT activity [13,21]. In order to evaluate the importance of adenosine deaminase to this procedure, the reaction was performed with and without adenosine deaminase ($n = 5$). The addition of adenosine deaminase to the reaction mixture did not increase COMT activity. Nevertheless, we continue to add adenosine deaminase to the reaction mixture as the coefficient of variation in the reaction mixtures containing adenosine deaminase was approximately half that in reaction mixtures without adenosine deaminase (286 ± 15 U *versus* 310 ± 29 U).

TABLE II

ELUTION TIMES OF CATECHOLAMINES AND THEIR METHYLATED PRODUCTS AS DETERMINED USING THE DESCRIBED CHROMATOGRAPHIC CONDITIONS

Catecholamine	Elution time (min)
MHPG	1.30
Epinephrine	2.15
Norepinephrine	2.22
NM	3.38
Dopamine	5.00
Met	5.92
MOPET	6.07
HVA	7.34
MHBA	8.52
3MT	16.29
4MT	27.00

TABLE III

MONONUCLEAR CELL COMT ACTIVITY IN EIGHT RANDOMLY SELECTED CRITICALLY ILL INFANTS AND CHILDREN

Patient	Age	Diagnosis	Catecholamine infusion rate (μg/kg/min)	COMT activity (pmol 3MT per mg protein per 10 min)	3MT/4MT ratio
1	2 years	Meningococcal meningitis	Dopamine; 3	417	2.7
2	4 years	Cervical vertebral dislocation with spinal shock	None	508	8.8
3	4 months	Meningitis	Dopamine; 3	571	9.0
4	3 weeks	Single ventricle; pulmonary stenosos; S/P Blalock–Taussig shunt	None	585	8.7
5	8 years	S/P posterior spinal fusion; bacterial pneumonia	None	493	8.5
6	6 years	Meningococcal meningitis	None	674	7.5
7	17 months	Hypertrophic cardiomyopathy; 25% surface area full thickness burn	Dobutamine; 10 Dopamine; 3	407	11.8
8	19 months	Tetralogy of Fallot repair	None Dopamine; 8	600 869	N.D.[a]

[a] Not determined.

Recovery

An average of 48 ± 5% of a known quantity (0.05 ml) of 3MT stock solution (255 ng/ml) was recovered from the described procedure (n = 22). 4MT demonstrated a similar mean recovery fraction of 45 ± 5% from a 0.05-ml aliquot of the 255 ng/ml stock solution carried through the procedure (n = 22). The internal standard, MHBA, extracted in a similar fashion, has a mean recovery of 39 ± 4% from a 0.05-ml aliquot of the 955 ng/ml stock solution (n = 22).

Reproducibility

Nine samples prepared simultaneously were used to determine a within-day coefficient of variation of 6.9% for 3MT and 5.2% for 4MT. In order to determine the between-day coefficient of variation, mononuclear cells were isolated and frozen in six aliquots at −70°C. One aliquot was assayed every other day over a two-week period. The between-day coefficient of variation was 11%.

Clinically significant compounds that might co-chromatograph with the internal standard, 3MT or 4MT were sought. These included catecholamines, as well as several of their methylated metabolites. The methylated catecholamines such as normetanephrine (NM), metanephrine (Met), 4-hydroxy-3-methoxyphenylethanol (MOPET), 4-hydroxy-3-methoxy phenylglycol (MHPG) and homovanillic acid (HVA) were dissolved in 0.1 M perchloric acid and injected onto the column. The elution time for each of these compounds was separated by more than 1 min from either 3MT, 4MT or the internal standard (Table II). Epinephrine and norepinephrine both eluted earlier than DA (Table II).

Mononuclear cell COMT activity in critically ill patients

Mononuclear cell COMT activity was measured in eight randomly selected critically ill pediatric patients using the described method (Table III). The average activity was 532 ± 93 3MT units and 75 ± 39 4MT units. This COMT activity reflects a four-fold increase from previously reported mononuclear cell COMT activity [12]. The m/p ratio for five of the eight patients were similar, with a mean of 8.5 ± 0.6. However, one patient demonstrated a low m/p ratio of 2.7, and the m/p ratio in another patient was 11.8.

DISCUSSION

Inter-individual differences in COMT activity have been shown to be important in the clinical response to some catechol-containing drugs [5]. Catecholamine infusions are frequently utilized in critically ill patients as pharmacologic agents, leading to serum concentrations several hundred-fold above endogenous levels [22]. Further studies are required to determine whether COMT could become the rate-limiting enzyme in the metabolism of these exogenous catecholamines. If that occurs, then inter-individual variation in COMT activity should impact on serum catecholamine concentrations, and perhaps also influence the clinical response to these widely used drugs. The purpose of this study was to develop a method for measuring COMT activity which could be employed easily in patient populations receiving catecholamine infusions. These patients are almost always critically ill, and frequently include children, as well as patients, who have undergone open heart surgery.

A method for measuring COMT activity in clinical samples should ideally possess the characteristics listed in Table IV. The first characteristic is that the substrate should be a catecholamine to which patients are actually exposed. The m/p methylation ratios for physiologic and non-physiologic catecholamines have been shown to differ [17]. Furthermore, it is unclear if membrane-bound and soluble COMT represent two distinct isoenzymes [2,37]. Under these circumstances, it is crucial to use a clinically employed catecholamine as a substrate in order to simulate the clinical situation most closely.

The method should be capable of separately quantifying both methylated products. It has been suggested that the abnormally high levels of 4MT produced from endogenous dopamine are responsible for some psychiatric disorders, as well as Parkinson's disease [13,34,38,39]. This ap-

TABLE IV

OPTIMAL CHARACTERISTICS OF A METHOD USED TO MEASURE COMT ACTIVITY IN CLINICAL SAMPLES

Each reference is marked with an "×" to indicate the presence of a particular characteristic. N.R. = not reported.

Factor	Present method	1	12	13	18	19	20	23	24	25	26	27	28	29	30	31	32	33	34	35	36
Use of a clinically employed catecholamine as substrate	×	×		×	×						×	×			×	×				×	
No radioisotope necessary	×			×	×							×	×	×	×	×	×	×	×	×	×
Capable of metabolite separation, detection and quantification	×			×	×										×	×	×	×	×	×	×
Clinically accessible tissue	×			×		×	×	×	×		×				×			×			
Tissue not transfused clinically	×		×																		
Small sample size (≤5 ml blood)	×			×		×		×	×		N.R.				N.R.			N.R.			
Limit of detection ≤5 pmol	×		N.R.	N.R.												×	×	×	×	×	×
Single step product separation	×	×	×	×		×		×	×	×		×	×	×		×	×	×	×	×	×

parent inter-individual variation in *m/p* metabolism by COMT may also play a role in determining the efficacy and potency of exogenous catecholamines.

Furthermore, an *m/p* ratio of 11.8 is reported here in a patient exposed to dobutamine, a synthetic catecholamine, compared to a ratio of 8.5 in the other patients. This suggests that certain drugs may actually alter the *m/p* ratio of metabolites produced by COMT. Additionally, it is unknown whether the *m/p* ratio changes over time in patients exposed to high doses of catecholamines for a prolonged period of time.

It is preferable not to employ radioisotopes in the assay. Radiometric methods fail to separately detect and quantitate the two methylated metabolites [1,12,20,23–26]. They can be hampered by large blanks and are costly [12].

The optimal tissue to use in clinical studies of COMT should meet several criteria. It should be easily obtained and stored in order to simplify the collection of multiple samples over time. It should not be a tissue that is transfused into patients, thereby avoiding exogenous sources of the enzyme. Additionally, the activity in the tissue should reflect enzyme activity in other metabolically important organs or tissues. Mononuclear cells possess all of these characteristics. They are easily obtained and remain intact during storage with simple freezing. Many critically ill patients receive transfusions of erythrocytes, but do not receive mononuclear cell transfusions. Additionally, relative COMT activity in mononuclear cells has been shown to reflect the activity in erythrocytes, lung and kidney [11,12]. These characteristics simplify the collection of multiple clinical samples over time. Most critically ill patients receive transfusions of erythrocytes, but do not receive mononuclear cell transfusions. Therefore, exogenous sources of COMT are avoided by using mononuclear cells. Additionally, the COMT activity in mononuclear cells has been shown to reflect the activity in other more metabolically important tissues [11,12].

Despite these advantages, mononuclear cells contain relatively little COMT activity [12]. Small sample sizes are a requirement for making human studies possible, especially when studying pediatric patients or analyzing serial samples from a single patient. Recently, HPLC–ED has been utilized successfully to measure COMT activity in microsamples of human erythrocytes and small intestine [13,33,37]. However, these HPLC methods failed to achieve the sensitivity (5 pmol) to measure COMT activity from small quantities of mononuclear cells when using dopamine as a substrate.

One of the methods utilized ion-exchange columns to selectively separate the methylated products from substrate [13]. These columns required large amounts of strong acids for elution of the methylated products. This resulted in product dilution that precluded detection of COMT activity from clinically obtainable samples of mononuclear cells. Several methods eliminate any separation of substrate from the methylated products, and merely deproteinate their samples with acidification and centrifugation [31–35]. This eliminates product loss during a clean-up procedure. However, these methods primarily use rat liver, an organ with a high specific activity, as the source of COMT [31,32,34,35]. This allowed for injection of quantities of supernatant as low as 5 μl [31], as well as the use of amperometric detection ranges of 10–20 nA [32–34]. These methods failed to adequately separate substrate from methylated products at the amperometric detection range of 0.5-1 nA which was necessary to measure COMT in small quantities of mononuclear cells.

A similar method for measuring COMT activity in human erythrocytes without a step to separate product from substrate has been published recently [33]. It appeared to provide the necessary sensitivity to measure COMT activity in small quantities of mononuclear cells. However, it employed dihydroxybenzoic acid as the substrate. We were unable to reproduce this sensitivity using dopamine as the substrate.

Therefore, a procedure to separate product from substrate was necessary to permit the use of extremely sensitive amperometric detection ranges. The ideal separation procedure to use in a method analyzing clinical samples should be

both efficient and simple enough for multiple sample analysis. The described organic solvent extraction procedure permits the use of amperometric detection ranges of 0.5–1. Additionally, since the procedure involves a single step, it is simple and rapid enough to easily handle up to 25–30 samples per day.

The method reported in this paper possesses all of the characteristics necessary for measuring COMT activity in clinical samples. The use of dopamine as a substrate mimics the clinical situation in critically ill patients where dopamine is an endogenous catecholamine, as well as one that is used frequently in pharmacologic doses as a pressor/inotrope. The organic extraction procedure described removes a sufficient amount of the substrate to allow the use of extremely sensitive amperometric detection, and allows the products to be concentrated in a small volume of perchloric acid. This leads to a limit of detection of 3 pmol of either 3MT or 4MT, which allows the measurement of COMT activity from as little as 0.1 mg of protein. This amount of mononuclear cell protein can often be obtained from as little as 2–3 ml of whole blood.

This procedure permitted the determination of COMT activity in mononuclear cells in eight randomly chosen critically ill patients. The values measured were four-fold higher compared to previously reported values in human mononuclear cells [12]. Different substrates were used in the two studies, making direct comparisons difficult. However, dopamine is generally considered the less active substrate, so that the COMT activity reported here should be lower rather than higher. Additionally, the two methods examine COMT activity in two different populations: healthy adult volunteers *versus* critically ill children. The effect of physiologic stress on COMT activity is unknown.

The present procedure utilizes whole mononuclear cell sonicate in contrast with the previously described method which measured COMT from the soluble portion of the cell only. Since 50% of the mononuclear cell COMT activity is membrane-bound [12], using this fraction of the cell can account for a two-fold higher activity compared to the previously reported activity.

Finally, the previously reported procedure was a radiometric procedure that was complicated by relatively large blanks which may have inadvertently lowered the reported activity. Additionally, it was hampered by the presence of endogenous inhibitors which may have interfered with the accurate measurement of COMT activity. Neither of these problems were encountered during the procedure described herein.

In summary, we report here a simple, relatively rapid method for measuring COMT activity in small amounts of tissue. This method should facilitate future clinical studies of the role of this enzyme in the metabolism of exogenous catecholamines in critically ill patients.

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