Determination of Catechol–*O*-Methyltransferase Activity in Various Tissues by Liquid Chromatography

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Catechol–*O*-methyltransferase activity was determined using liquid chromatography with amperometric detection. With dopamine as a substrate, the method allowed the individual determination of both possible product isomers in the various biological matrices investigated. The selectivity of the electrochemical detector proved advantageous in several aspects, including higher sample throughput, negligible blank values, and simplified chromatograms. The resolution of the reverse phase column permitted the incorporation of an internal standard, 3-methoxy-4-hydroxybenzylamine, to improve the precision to less than 4% RSD for either the 3- or 4-*O*-methyl product isomers. The use of the product isomer preference ratios may be of clinical significance.

Since the first studies by Axelrod and Tomchick (*1*), catechol–*O*-methyltransferase (COMT) has been the subject of continued study from both a clinical as well as a purely biochemical standpoint. As the enzyme responsible for the phenolic methylation of the catecholamines (Figure 1), COMT represents a primary pathway for the termination of neuronal action after neurotransmitter release into the synaptic cleft. COMT is widely distributed in many tissues, with principal activity located in the liver and kidney. It may also be transported throughout the organism via storage in the erythrocytes.

The catecholamine hypothesis of affective disorder (*2*) is based on pharmacological evidence that agents capable of altering the quantity of neurotransmitter released, metabolized, or uptaken can also affect the mood of the individual. Some cases of depression, for example, may be explained by a deficiency in catecholamine levels in critical neuronal zones within the central nervous system. Since COMT is so intimately involved in scavenging the residual norepinephrine or dopamine not taken up into the neuron, it has been stated that COMT activity might be an index of the psychological state of the individual.

The data, however, have not presented a clear picture of COMT's role, if any, in affective disorder. Initial studies using norepinephrine as a substrate demonstrated that women with primary affective disorder had significantly lower COMT activity than either men with primary affective disorder or controls (*3*). The results were later confirmed (*4, 5*). Related follow-up studies using 3,4-dihydroxyphenylacetic acid (DOPAC) as a substrate were inconclusive, showing in one case no significant differences among any clinical groupings (*6*) and in another significantly higher COMT erythrocyte activity in affective disorder patients (*7*). The controversy has been reviewed in the literature (*8*).

One possible explanation for the disparity of views is that the analytical methodology for COMT activity measurements is unsatisfactory. Measurement of COMT has been largely based on either fluorescence, radioenzymatic, or spectrophotometric techniques. The earliest methods used the native fluorescence of the *O*-methylated products at low pH (*1*). To avoid high fluorescence blanks present in crude tissue preparations, radioenzymatic assays based on *S*-adenosyl-L-methionine (with ^{14}C methyl) were later adopted (*9–11*). Although reasonably sensitive, these methods suffer from the lack of multiple product information, isotope expense, and the need for concurrent "blank" runs for background compensation. Spectrophotometric methods have been proposed. Borchardt devised an empirical approach to the assay of liver COMT activity using UV absorption at multiple wavelengths (*12*). The amounts of the predominant 3-*O* methyl and the less studied 4-*O* methyl product could be determined. The assay was not sufficiently sensitive to handle RBC or brain tissue samples. Sophisticated gas chromatographic–mass spectrometric procedures have overcome most of these problems, but these require derivatization reactions (*13*).

Liquid chromatography with electrochemical detection (LCEC) is ideally suited to the determination of COMT activity. LCEC offers the following advantages: (1) low detection limits, on the order of 0.1 pmol of product; (2) the capability of separating all components of interest, useful in this case for quantitating multiple enzymatic products; and (3) high specificity for easily oxidizable catechol and vanillyl compounds. Liquid chromatography with electrochemical detection has been applied to the determination of tyrosine hydroxylase (*14*), phenylethanolamine *N*-methyltransferase (*15*), dopamine-β-hydroxylase (*16*), and dopa decarboxylase (*17*) activity measurements. An early report mentioned the utility of LCEC for liver COMT activity using a low-resolution, pellicular ion-exchange column (*18*). Determination of activity in human erythrocytes was not mentioned and no activity values for other tissues assayed were given.

Our interest in developing LCEC assays for the catecholamines and their metabolites stems from their clinical application in ascertaining accurate diagnoses and prognoses in neurochemical-related disorders such as pheochromocytoma and neuroblastoma. By merely changing mobile and/or stationary phases, LCEC methods can profile all catecholamines and metabolites to obtain a metabolic picture clearer and more detailed than possible with the assay of only one or two compounds. Our assay for the *O*-methylated metabolites has proved useful in this regard for neuroblastoma (*19, 20*).

As with these earlier studies, the disparity of views concerning the significance of COMT activity in affective psychiatric disorder prompted us to devise a reliable procedure using high resolution reverse phase LC with electrochemical detection. Here we report an assay for COMT activity based on the LCEC approach. A number of different substrates may be used, including 3,4-dihydroxyphenylacetic acid, dopamine, and 3,4-dihydroxybenzylamine. A rapid sample clean-up step is employed prior to the chromatography to remove extraneous peaks and improve the detection limit. The method has been used successfully in rat liver, heart, and brain tissues and

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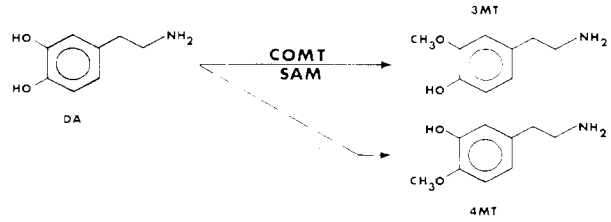

Figure 1. Conversion of dopamine (DA) to 3-methoxytyramine (3MT) and 4-methoxy-3-hydroxyphenethylamine (4MT) via COMT using *S*-adenosylmethionine (SAM) as the methyl donor

human erythrocytes. Both the 3- and 4-*O*-methyl products may be determined from the same injection in less than 8 min.

EXPERIMENTAL

Apparatus. An LC-304 liquid chromatograph equipped with a TL-3 carbon paste electrode (Bioanalytical Systems Inc., West Lafayette, Ind.) was used in this work. The potential of the working electrode was maintained at +850 mV vs. a Ag/AgCl reference electrode.

The separations were performed on a 15 cm × 4.6 mm stainless steel column prepacked with 5-μm reverse phase material (Chromegabond, MC-18; Bioanalytical Systems Inc.).

Mobile Phase. The mobile phase was pH 4.4 phosphate/citrate buffer with 50 mg of sodium octyl sulfate and 90 mL of methanol added to each liter of buffer. The buffer was composed of 560 mL of 0.1 M citric acid and 440 mL of 0.2 M Na_2HPO_4. The flow rate was adjusted to 1.6 mL/min.

Chemicals. *S*-Adenosyl methionine (SAM) and adenosine deaminase were obtained from Sigma Chemical Co. (St. Louis, Mo.). Dopamine-HCl (DA) and 4-methoxy-3-hydroxyphenethylamine (4MT) were purchased from Regis Chemical Co. (Chicago, Ill.) while 3-methoxytyramine (3MT) and 4-hydroxy-3-methoxybenzylamine (MHBA) were supplied by Aldrich Chemical Co. (Milwaukee, Wis.). Sodium octyl sulfate was obtained from Eastman Kodak Co. (Rochester, N.Y.). All other chemicals were reagent grade.

Enzyme Preparation. Adult female Sprague-Dawley rats were decapitated and specific organs were removed, weighed, and homogenized in cold 0.15 M KCl (liver in 20 mL, brain and heart in 5 mL). The tissue homogenates were then centrifuged at 48000 × g for 20 min at 4 °C. Since the enzyme is soluble, the supernatant can be used as a crude source of enzyme.

COMT from red blood cells (RBC) was isolated in a similar fashion. Blood was drawn from healthy volunteers by venipuncture into heparinized collection tubes. The tubes were centrifuged at 2000 × g to separate the plasma from the cells. The plasma and the top layer of the packed cells were carefully removed and discarded. Two mL of packed cells were removed and diluted with 5 mL of cold water. This solution was allowed to stand on ice for 15 min to ensure the lysing of the cells. The tubes were then centrifuged at 15000 × g to remove the membrane fraction. The supernatant was used as the source of the enzyme.

Procedure. The standard incubation mixture was composed of the following reagents in a final volume of 600 μL. Supernatant (50 μL from liver, 100 μL from brain, heart, or RBC) was added to a 1.5-mL conical polypropylene centrifuge tube which contained 200 μL of 0.5 M pH 7.8 phosphate buffer. Then 100 μL of 10 mM SAM, 50 μL of 10 mM $MgCl_2$, 50 μL of water (liver assay only), and 50 μL of adenosine deaminase (3U) were added and thoroughly mixed using a vortex mixer. The tubes were placed in a constant temperature bath (37 °C) and preincubated for 5 min. The enzyme reaction was initiated by the addition of 50 μL of 50 mM DA. A 5-min incubation was sufficient for the liver samples whereas heart and brain supernatants were incubated for 25 min. RBC supernatant was incubated for 1 h. The reaction was stopped by the addition of 50 μL of 4 M $HClO_4$ solution which also contained the internal standard, MHBA. The amount of MHBA added to each tube was 4.66 nmol. The tubes were vortexed and then centrifuged for 5 min at 15000 × g in an Eppendorf Model 5412 Centrifuge (Brinkmann Instruments, Westbury, N.Y.).

Four hundred μL of the supernatant was transferred to a glass conical screw cap vial. The pH of this solution was adjusted to 6.5 by the addition of 2.5 mL of a pH 8.1 4% borate/1% EDTA solution. Following pH adjustment, the solution was poured onto a small isolation column which contained a cation-exchange resin, Bio-Rex 70, 100–200 mesh (BioRad Laboratories, Richmond, Calif.). The effluent was discarded. The preparation and regeneration of this resin and the columns for the isolation of *O*-methylated catecholamine metabolites has been described elsewhere (*19*). The conical vials were washed with water and the washings were also poured onto the columns. The columns were subsequently washed with 3 mL of water, discarding the effluent. One mL of 0.7 M H_2SO_4 was applied to the column, still discarding the effluent. Finally 3 mL of the same 0.7 M H_2SO_4 solution were added to the column and this eluate was collected. One hundred μL was injected onto the LC.

A blank was prepared in similar fashion for each of the supernatants described earlier. The blank consisted of deproteinizing the sample with the perchloric acid/internal standard solution prior to the addition of substrate. This "zero time" blank was compared to blanks which used COMT inhibitors (e.g., metal ions) and was found to be equivalent.

A standard solution containing 3-methoxytyramine (2.6 nmol/mL), 4-hydroxy-3-methoxyphenethylamine (2.5 nmol/mL), and the internal standard 3-methoxy-4-hydroxybenzylamine (MHBA) in 0.1 M $HClO_4$ was injected with every set of samples. The concentration of internal standard in this solution (1.03 nmol/mL) was made equal to the concentration expected in the prepared tissue extracts if 100% recovery occurred. The recovery of MHBA accurately follows those of both the 3- and 4-*O* methylated products and thus normalizes the data.

Substrate Solution Cleanup. Two mL of a 50 mM DA solution were added to a glass conical screw cap vial along with 200 μL of 5% metabisulfite and 200 μL of 10% EDTA. One mL of 3 M pH 8.7 Tris buffer was then added to the vial followed by rapid addition of 300 mg of acid washed alumina. The vials were agitated for 5 min on a reciprocal shaker. Upon removal the alumina was allowed to settle. The liquid layer was removed by aspiration and discarded. The alumina was washed three times with water, discarding each washing. After the last water wash, the alumina was aspirated to near dryness. One mL of 0.2 M $HClO_4$ was added to elute the dopamine from the alumina. The vial was swirled briefly and allowed to stand for 20 min. Prior to the solution being used as the source of substrate, it was filtered with a syringe filter assembly (Millipore, Bedford, Mass.) to remove alumina fines. Based on previous alumina recovery, studies, the DA concentration was 50 mM.

Calculations. For each sample as well as the standard solution, the peak heights of MHBA, 3MT, and the 4-methoxy product were measured and the peak height ratios of each product to the internal standard calculated. For either product, the number of nanomoles produced in any sample may then be calculated using the respective peak height ratios and the relationship

$$\frac{\text{peak ht ratio (sample)}}{\text{peak ht ratio (std solution)}} \times \text{nmol of product injected (std soln)}$$

The nanomoles produced may then be related to enzyme activity units such as nmol/min-mg protein or nmol/min-g tissue.

RESULTS AND DISCUSSION

The enzyme assay was tested according to the usual parameters of linearity with time and enzyme concentration, as well as replicate precision. Both the 3- and 4-*O* methyl metabolites were quantitated in the evaluation of these assay parameters. Either isomer gave equivalent results with these tests.

The linearity of the assay under the pseudo first-order kinetic conditions is illustrated in Figure 2 using crude rat liver homogenate. Because of the high activity of the liver preparation, the data were linear up to a maximum incubation period of 15 min. After that time, a loss in reaction rate was noted, evidence of product inhibition or a falloff from saturating Michaelis–Menten conditions. The addition of adenosine deaminase, which removes the inhibitory by-product *S*-adenosylhomocysteine (*21*), improved the linearity slightly

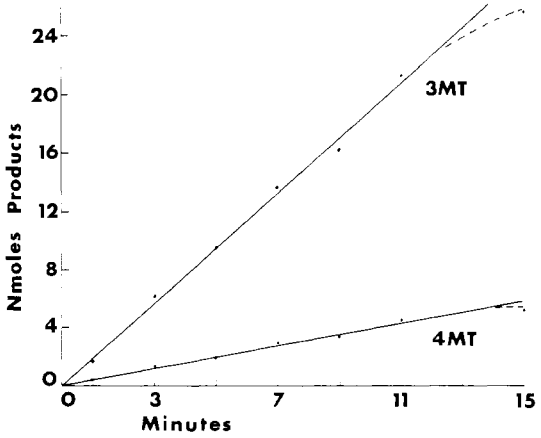

Figure 2. Product formation vs. time for each isomer

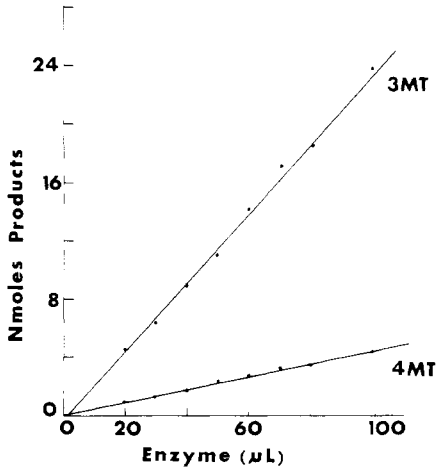

Figure 3. Product formation vs. increasing enzyme concentration. Conditions were as in text, using crude liver homogenate

at longer incubation times. Both the 3- and 4-*O* methylated metabolites showed the same trends. For liver, a 5-min incubation was sufficient. Incubations of 20 min to 1 h were used for low activity tissues and erythrocytes.

The effect of increasing the enzyme concentration at a fixed incubation time was also examined and is shown in Figure 3 for both products as they were formed in the liver homogenate. Fifty μL of liver and corresponding larger volumes of the lower activity heart, lung, and erythrocyte preparations were chosen.

The proposed LCEC method demonstrated excellent precision. A series of eight samples was prepared separately from the same crude liver homogenate and incubated for 5 min. Mean peak height ratios (± standard deviation) for the 3- and 4-*O* methylated isomers were 1.28 ± 0.04 and 0.213 ± 0.0076, respectively. The relative standard deviations for these samples were only 3.3 and 3.6%. Since both isomers may be measured in the same run, it is also convenient to calculate the ratio of meta/para product preference. When the meta/para product ratios were calculated for this same series of replicate samples, the precision tightened to 1.9% relative standard deviation. In addition to discerning measurable product preference, the use of the meta/para ratio improved assay precision considerably by eliminating small errors due to pipetting enzyme or internal standard or post-incubation sample preparation.

Figure 4 demonstrates the ability of the LCEC technique to determine COMT activity. Samples from homogenized liver tissue of three different rats are presented followed by a blank using denatured enzyme. The initial major peak is the substrate dopamine, followed by the internal standard (IS), 3-methoxytyramine, and 3-hydroxy-4-methoxyphenethylamine

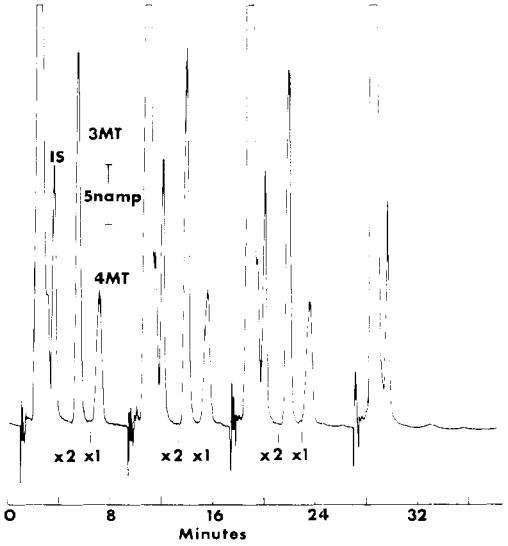

Figure 4. Typical chromatograms obtained from the described procedure. The first three samples were from different liver samples while the fourth injection was a zero-time blank of the third liver sample

Table I. COMT Activity in Various Tissues

tissue	activity
rat liver (n = 6)	120 ± 22[a]
rat brain (n = 6)	0.87 ± 0.16[a]
rat heart (n = 6)	0.88 ± 0.31[a]
human RBC (n = 6)	0.14 ± 0.067[b]

[a] Activity expressed as nmol 3MT per min per gram of tissue. [b] Activity expressed as nmol 3MT per min per mL packed red blood cells.

(4MT). A small peak unresolved from the trailing edge of the substrate peak was evident during normal incubations but was not identified. The sensitivities employed (10–200 nA full scale) did not tax the high sensitivity of the amperometric detector. All peaks of interest were resolved in under 8 min.

Several types of "blanks" were evaluated, including those lacking substrate, enzyme, or cofactor; those created by adding metal ions complexing the active site; and those created by stopping the reaction at zero time. The most stringent blank was the latter one and it was adopted for this assay. The last of the four samples shown in Figure 4 was a "zero time" blank containing all essential components, incubated for the usual time. Less than a 1% contribution for either peak was evident. The blank shown here was representative of those seen in all other tissue samples and therefore not run for each sample. Alternately, 50 μL of a 1.0×10^{-2} M $CuSO_4$ solution added prior to the substrate quenched the reaction completely.

Samples of heart, liver, and brain from the rat as well as the soluble fraction of human erythrocytes were assayed for COMT activity. The results of the populations studied are presented in Table I. These values compare favorably to those reported earlier in the literature (22). Since several authors have not used dopamine as substrate, direct comparison cannot be made in these cases. The trends, however, are consistent.

To accurately quantitate enzyme activity, the analytical method should be capable of measuring all changes that occur during the incubation period within the constraints of linearity, reproducibility, and accuracy. Most spectrophotometric and radioenzymatic techniques for COMT activity detect only gross changes in activity, such as an increase in disintegrations per minute or a change in absorbance at a selected wavelength. Unless chromatography is employed prior to detection, multiple product information is unavailable. With radioen-

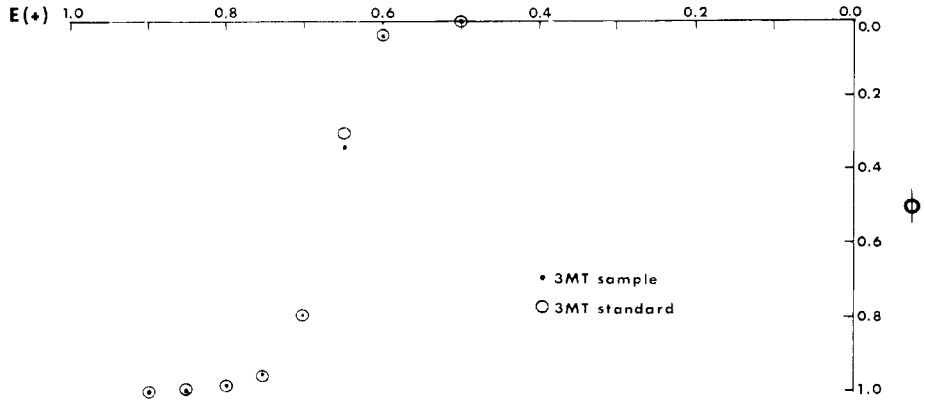

Figure 5. Hydrodynamic voltammograms of the sample extract peak for 3MT (black dots) and of 3MT standard (open circles)

zymatic techniques, for example, the chromatography must be done off-line so as to allow sufficient counting time.

The use of microparticulate reverse phase over pellicular cation-exchange media has many advantages. The small RP particle diameter allows for a separation of higher resolution in approximately $^1/_5$ the time per sample, and all peaks are normalized by an internal standard. Furthermore, since the mechanism of solute retention in reverse phase liquid chromatography is based on hydrophobicity, the reverse phase column may be used with a number of different substrates, including acidic and neutral compounds not separable on a cation-exchange column. As an example, DOPAC was used as substrate in our initial studies and the production of homovanillic acid (HVA) was followed on the same column using a different mobile phase (0.1 M phosphate buffer, pH 2.8, with 10% methanol (v:v)). Sample cleanup for the acidic compounds consisted of simply deproteinizing with 4 M $HClO_4$, filtering, and injecting the incubation mixture directly. The reproducibility of the procedure using the DOPAC/HVA reaction was 3.4% relative standard deviation with 8 identical liver samples incubated for 15 min. It should be mentioned that no internal standard was necessary to achieve this precision. With these organic acids, the chromatography was highly dependent on both the pH and the methanol content of the mobile phase. Other substrates such as 3,4-dihydroxyacetophenone could be employed on the reverse phase column but were not tested here. In the present approach, there is no need for these artificial substrates which were originally adopted for their chromogenic properties.

Dopamine was preferred as the substrate for several reasons. Our initial attempts with DOPAC in heart and brain were less than satisfactory owing to impurities present in the standard. Under saturating Michaelis–Menten conditions, where the substrate concentration can be as high as 30 mM for this enzyme, it is not unusual to find several impurities present at concentrations sufficient to interfere with the product peaks. These compounds are thought to arise during the synthesis of the standard, since the catechol function is protected by methyl groups which are later cleaved with strong acid. Therefore, the presence of *O*-methyl compounds in "pure" substrate solution was not unexpected. Fresh solution from three different manufacturers showed similar composition. With DOPAC, approximately 6 such peaks were detected. With dopamine, three peaks were evident, one co-eluting with authentic 3-methoxytyramine. Dopamine was readily purified by using a very effective, small-scale alumina clean-up step. All extraneous peaks were eliminated. The purification of DOPAC was less successful. The commercial availability of both the 3- and 4-*O*-methyl products of dopamine was also a consideration.

In low activity samples such as erythrocytes the sample clean-up procedure using the Bio-Rex 70 ion-exchange resin was mandatory. The technique is fast, simple, and allows for 15–20 parallel sample preparations. The elution system on the isolation columns was designed to optimize product recovery. Borate buffer was added to the quenched incubation mixture to bring the pH up to 6.5 and, more importantly, complex the unused dopamine at its catechol function to yield a species unretained by the ion exchanger. Under saturating conditions, some dopamine was still present in the final chromatogram but its overall magnitude was within acceptable limits and much less than if a noncomplexing buffer had been employed. Absolute recoveries of the *O*-methylated metabolites ranged from 85 to 100% and were normalized by the internal standard, which behaved in identical fashion. If only liver COMT activity were to be determined, the cleanup and internal standard could be avoided entirely.

Table II. Normalized Current Values vs. Applied Potential for Liver Extract

potential, mV[a]	ϕ, 3MT	ϕ, 4MT
+850 (sample)	1.00	1.00
(standard)	1.00	1.00
+750 (sample)	0.95	0.83
(standard)	0.96	0.85
+700 (sample)	0.79	0.54
(standard)	0.79	0.54
+650 (sample)	0.34	0.15
(standard)	0.30	0.12

[a] Potentials are vs. a Ag/AgCl reference electrode.

When using LC for trace organic analysis, positive identification of an eluted compound can be difficult. Retention time information is helpful but certainly not unambiguous. Since most LCEC determinations involve nanogram amounts of injected materials, it is impossible to obtain spectral information from a collected fraction.

Plotting hydrodynamic voltammograms for specific chromatographic zones provides additional evidence for the identification of an eluting compound. The hydrodynamic voltammograms are produced by making repeated injections of a sample at different potentials and plotting the peak height (current response) vs. the applied potential. To facilitate comparison of the sample voltammogram to that of a standard, it is useful to divide each peak height by that measured at the most positive potential. The resulting normalized current function, ϕ, is the value plotted against the applied potential. If the suspected compound in a particular chromatographic zone and a standard are truly the same, then the curves will be identical. Figure 5 illustrates this technique for the 3MT produced from a liver incubation. The hydrodynamic voltammogram generated for the 3MT standard and the enzymatic 3MT match perfectly. The enzymatic 4MT was tested as well and yielded equivalent results. It is not often necessary

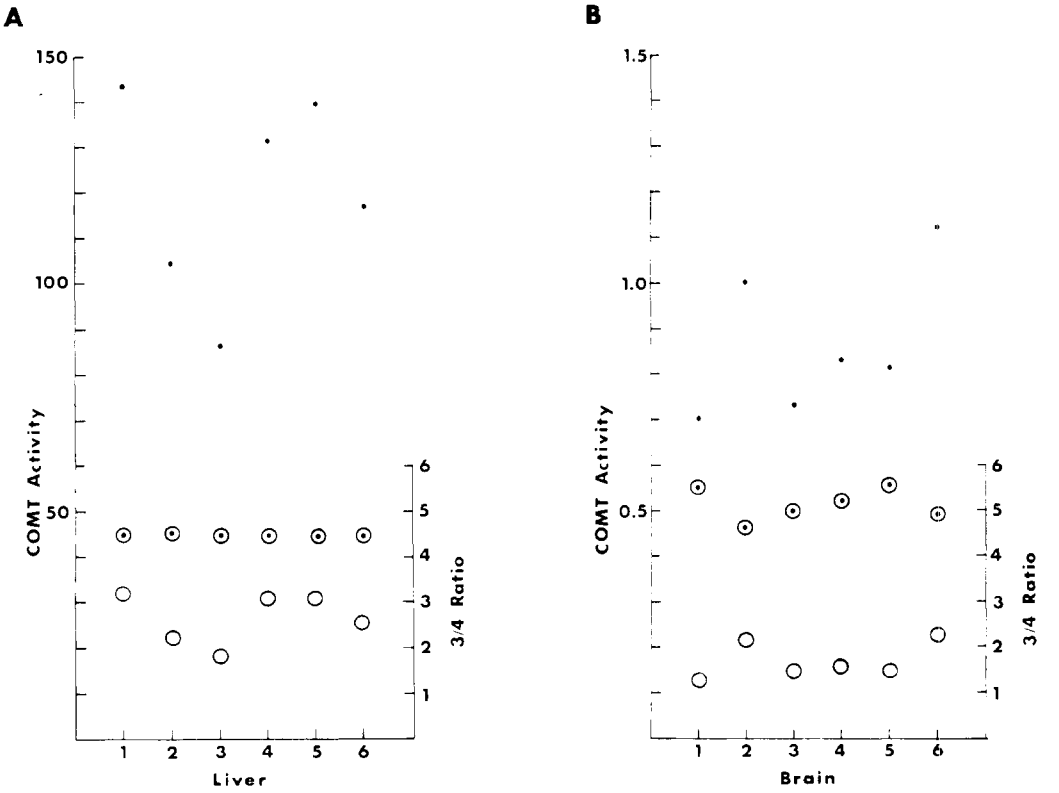

Figure 6. Both the 3-*O* methyl (solid dots) and the 4-*O* methyl (open circles) product velocities for liver (A) and brain (B) were plotted. Product isomer ratios are shown as dots inside circles. Activity units for both plots are in nmol-min^{-1}-g^{-1}

to scan a large potential range. Usually 3–4 points spaced over 300 mV are sufficient to confirm the presence of a compound. Table II compares the data for 3MT and 4MT from liver incubations.

Since both the para and meta *O*-methyl metabolites may be quantitated, the assay permits the calculation of product preference ratios. As discussed earlier, these ratios may be determined with excellent precision over a series of replicate samples and eliminate small errors due to factors such as pipetting enzyme or internal standard. More importantly, the ratios offer useful biochemical information about the enzyme itself and its product preference.

To demonstrate the value of multiple product information, meta/para ratios were calculated for the six individual rat liver and brain samples described in Table I. In Figure 6A, both the 3-*O*-methyl and 4-*O*-methyl product velocities are plotted for liver homogenate. Note, however, the wide range in the 3-*O*-methyl production rate, from 86 to 144 nmol/min-g of tissue. When indexed against the 4-*O*-methyl product as a ratio, however, the values become nearly identical (3/4 ratio = 4.56 ± 0.21, RSD = 4.6%). The constancy of the meta/para ratio, in contrast to the wide fluctuations in the absolute activities, suggests that this factor is more directly related to the chemistry of the enzyme per se—perhaps through the enzyme structure; its isoenzymes and their individual meta/para preferences; the tertiary arrangement, etc. Similar results were seen in the brain and plotted in Figure 6B. The 3/4 ratio for brain was 5.15 ± 0.36, a value significantly different from the liver samples ($p < 0.01$).

In the case of affective disorders, where clinical reports relying on gross erythrocyte COMT activity have been contradictory, the underlying differences if any between controls and patients may be in the enzyme itself. Several studies have demonstrated that COMT activity is largely under genetic control (*7*, *23–25*), and the contradictory findings so far might be explained by different molecular forms of the enzyme with varying substrate specificities, meta/para preferences, etc. With respect to the present assay, the ability to measure meta/para preference as well as the absolute activity with either isomer should prove advantageous.

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