Biochemical Pharmacology, Vol. 27, pp. 773–775. Pergamon Press. 1978. Printed in Great Britain.

DIRECT EXTRACTION RADIOASSAY FOR CATECHOL-O-METHYL-TRANSFERASE ACTIVITY

PETER A. GULLIVER* and KEITH F. TIPTON†
Department of Biochemistry, University of Cambridge, Tennis Court Road, Cambridge, CB2 1QW, U.K.

(*Received* 11 *July* 1977; *accepted* 5 *October* 1977)

Abstract—An improved radiochemical assay for catechol-*O*-methyl-transferase is presented that is particularly suitable for use with large numbers of samples. 3,4-Dihydroxyphenylacetic acid is converted to radioactively-labelled homovanillic acid in the presence of s-adenosyl-L-[methyl-^{3}H]methionine. This product is extracted into an organic solvent-scintillant mixture and scintillation counting is performed without further manipulation.

Changes in the activity of catechol-*O*-methyl-transferase (COMT, E.C. 2.1.1.6) have been reported to be associated with a number of disease states [1]. A decrease in the activity of the enzyme in red blood cells has been reported in patients suffering from Parkinsons disease [2], depression [3] and schizophrenia [4]. The availability of a simple and reliable assay method that can be used with large numbers of sample could be of great value in both clinical and enzymological studies on this enzyme. In this paper we describe an assay which is simpler to perform than those used previously.

Of the assays that have been commonly used to assay the activity of COMT, colorimetric assays with non-physiological substrates such as nitrocatechol [5] and bis-catecholphthalein [6] have the disadvantages that they are sensitive to changes in the assay pH and buffer concentration and are difficult to use with crude preparations of the enzyme. The coupled spectrophotometric assay using adenosine deaminase [7] involves measurement of a decrease in absorption at 265 nm and is thus subject to interference by high substrate concentrations and absorbing material present in crude preparation of the enzyme. Fluorimetric methods (e.g. [8]) provide a considerable increase in sensitivity but require extensive extraction of the reaction products. The greatest sensitivity can be provided by radiochemical methods using [^{14}C] of [^{3}H]methyl labelled s-adenosyl-L-methionine [9] which are suitable for use with crude enzyme preparations. Previous methods have, however, involved multiple transfer of volatile organic solvents which is time-consuming and a potential source of error. The method described here avoids such transfers and thus provides a more rapid and accurate assay which can be applied to the batchwise assay of large numbers of samples. After reaction between [^{3}H]methyl labelled s-adenosyl-L-methionine and a 3,4-dihydroxyphenyl acetic acid, the guaiacol product is extracted into an organic solvent and, since the aqueous layer quenches the emission of the unreacted labelled s-adenosyl-L-methionine, it is possible to determine the radioactivity incorporated in the product without further manipulation.

* To whom reprint requests should be sent.
† Present address: Department of Biochemistry, Trinity College, Dublin, 2, Eire.

MATERIALS AND METHODS

s-adenosyl-L-[methyl-^{3}H]methionine was obtained from the Radiochemical Centre, Amersham, U.K. and was diluted with the unlabelled compound in 0.01 M sodium acetate that had been titrated to pH 3.5 with hydrochloric acid and stored deep frozen. 3,4-Dihydroxyphenylacetic acid was obtained from Sigma Ltd., Norbiton Station Yard, Kingston-on-Thames, U.K. and homovanillic acid was obtained from Aldrich Chemical Co. Ltd, 264 Water Road, Wembley, U.K. 2-5-Diphenyloxazole (PPO) and 1,4-di-[2-(5-phenyloxazolyl)-benzene] (POPOP) were obtained from Koch Light Ltd, Colnbrook, U.K. All other chemicals were 'Analar' grade from British Drug Houses Ltd, Poole, U.K.

Catechol-*O*-methyl-transferase was prepared from pig-liver by essentially the method of Gulliver and Wharton [10]. A cruder preparation (a resolubilised, desalted 30–35 per cent saturated ammonium sulphate pellet) was also used. Adenosine deaminase was partially purified from Takadiastase (Sankyo Laboratories, Japan) by using the method of Sharpless and Wolfenden [11] up to and including the dialysis stage.

The scintillation vials and tops used were cleaned by soaking in Decon 90, and then transferred to Pyroneg before being extensively rinsed with tap-water and finally with deionised-distilled water.

Methods

The radiochemical assay of COMT was performed in standard screw-topped glass scintillation vials. Each assay contained in a total volume of 500 μl: 1.6 mM $MgCl_2$, 3.0 mM 3,4-dihydroxyphenylacetic acid, 0.9 mM ^{3}H-labelled s-adenosyl-L-methionine (0.418 Ci/mole), 0.64 units adenosine deaminase, 0.08 M pH 8.00 triethanolamine hydrochloride buffer and catechol-*O*-methyl-transferase. The scintillation vials were allowed to equilibrate at 37° in a shaking water-bath before the reaction was initiated by the addition of the s-adenosyl-L-methionine. Blanks were prepared in the same way but replacing the enzyme solution with an equal volume of buffer. After suit-

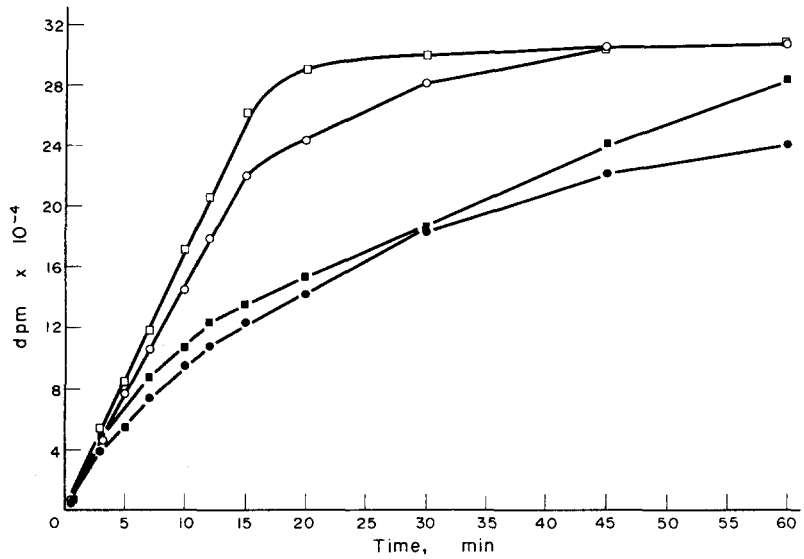

Fig. 1. Time-courses of the radiochemical assay of catechol-*O*-methyl-transferase (COMT). Assays were performed as described in the text and in the presence or absence of 0.64 units adenosine deaminase (ADA) —●— 0.0148 units purified COMT. —○— 0.0148 units purified COMT + ADA. —■— 0.0177 units crude COMT. —□— 0177 units crude COMT + ADA.

able fixed time intervals the reaction was stopped by the addition of 1.00 ml 3 M HCl and 10 ml of a toluene to isoamyl alcohol mixture (3:2; v/v) [8] containing 0.4 per cent PPO and 0.01 per cent POPOP (w/v in both cases) was added to each reaction mixture. The mixture was subjected to vortex mixing for 15 sec to extract the ^{3}H-labelled homovanillic acid that had been formed in the reaction. The mixture rapidly separated into two phases on standing and was counted in a Nuclear Enterprises NE 8310 scintillation counter without further treatment. Counting efficiency was calculated by reference to an external standard, values of about 20 per cent were typically obtained.

For comparative purposes the coupled assay method of Coward and Wu [7] was used. The assays were carried out in 2 mm pathlength cuvettes using the same assay mixture as in the radiochemical assay except that the concentrations of 3,4-dihydroxyphenylacetic acid and s-adenosyl-L-methionine were reduced to 1.56 mM and 0.456 mM respectively to avoid excessive absorption at 265 nm and resulting possibilities of instrumental deviation from Beers Law. The reaction was incubated at 37° and followed

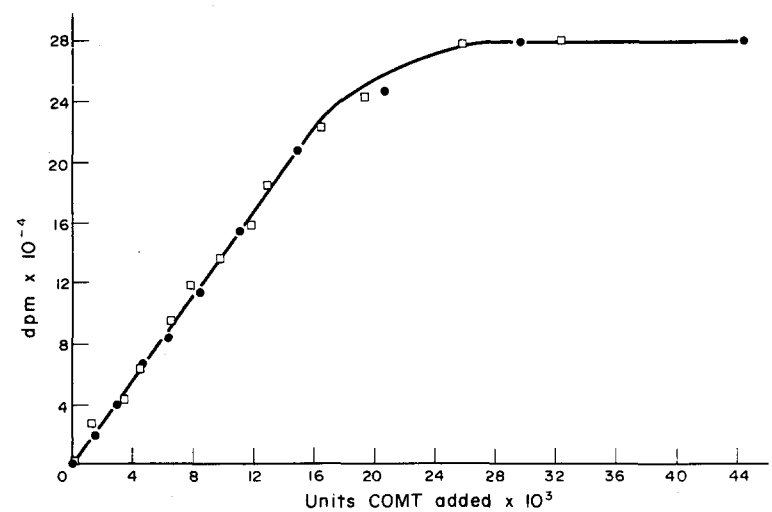

Fig. 2. The effect of enzyme concentration on the extent of the reaction catalysed by catechol-*O*-methyl-transferase. The radioactive assay was used as described in the text. The results are expressed as dpm extracted after 15 min incubation time. 925 dpm corresponds to the formation of 1 nm labelled product. Above 0.015 units enzyme the curve became non-linear because true initial rates are no longer being measured at this incubation time. Results are shown with the purified enzyme (—●—) and the enzyme activity in the crude extract (—□—).

at 265 nm in a Beckman DU Spectrophotometer fitted with a linear densitometer and scale expansion unit.

RESULTS AND DISCUSSION

The time course of the reaction measured by the radiochemical assay was shown to be linear with time for at least 15 min with both the purified and crude enzyme preparations at enzyme concentrations up to 0.015 units, (1 unit is defined as the amount of enzyme catalysing the production of 1 μm product per min). At higher concentrations, shorter incubation times would be necessary to observe true initial rates. The presence of adenosine deaminase was shown to be essential to obtain linear time-courses, as shown by Fig. 1, presumably because it catalysed the conversion of the highly inhibitory S-adenosyl-L-homocysteine to the relatively innocuous inosyl derivative [7].

Using an assay time of 15 min, the rate of reaction was shown to be proportional to enzyme concentration up to activities of 0.015 units as shown in Fig. 2. The blank value was less than 3 per cent of the assay value when 0.015 units of enzyme activity were used.

Storage of assay samples after extraction at room temperature for periods of up to 14 days caused no significant change in the radioactivity in either the assay samples or the blank, indicating that S-adenosyl-L-methionine does not break down to produce extractable radioactive material under these conditions and that the activity of COMT has been completely inhibited. Removal of the organic layer for separate counting by freezing the aqueous layer and decanting (cf 12) resulted in considerably more variable counting efficiency due to loss of scintillant and offered no significant increase in sensitivity. Separation of the organic phase would, however, be necessary if ^{14}C-labelled S-adenosyl-L-methionine were used because of less efficient quenching of the emission by aqueous solutions in this case.

The extracted radioactive material was identified as homovanillic acid by thin layer chromatography on silica gel plates developed with chloroform–methanol–acetic acid (6:1:1; v/v/v). In this system authentic samples of dihydroxyphenylacetic acid and homovanillic acid exhibited R_f values of 0.52 and 0.69 respectively. Under the conditions of the assay, no detectable amounts of dimethoxyphenylacetic acid were found when purified catechol-*O*-methyl-transferase was used. The stoichiometry of the reaction was investigated by comparing the formation of homovanillic acid with the formation of the other product, S-adenosyl-L-homocysteine, as determined by the coupled assay [7]. The results indicate a 1:1 stoichiometry with a maximum error of 2 per cent if an extraction efficiency of 100 per cent was assumed. This was confirmed by the observation that no further radioactivity appeared in the organic phase in subsequent extractions.

The assay procedure described here is simple accurate and convenient for use with large numbers of samples. It can be used at other pH values and with other buffers than that used here. If 0.08 M pH 8.00 potassium phosphate buffer was used in the assay mixture, instead of triethanolamine hydrochloride, identical results were obtained if the $MgCl_2$ concentration was increased 6 mM. This assay method would also be suitable for use with plant catechol-*O*-methyl-transferases if caffeic acid were used as methyl acceptor [13]. In comparison with this assay, a system using either dopamine or noradrenaline as the substrate in which the reaction was terminated by addition of borate buffer prior to extraction was found to give blank rates that increased with time, unless the organic phase was removed immediately after extraction, because of alkaline hydrolysis of S-adenosyl-L-methionine. In addition, this method may suffer from the disadvantage that the addition of borate does not completely inhibit catechol-*O*-methyl-transferase [14].

Acknowledgement—We thank the Medical Research Council for a grant in support of this work.

REFERENCES

1. H. C. Guldberg and C. A. Marsden, *Pharm. Rev.* **27**, 135 (1975).
2. J. M. Frère and A. Barbeau, *Lancet* **ii**, 269 (1971).
3. D. L. Dunner, C. K. Cohn, E. S. Gershon and F. K. Goodwin, *Archs Gen. Psychiat.* **25**, 348 (1971).
4. P. Poitou, M. Assicot and C. Bohuon, *Biomedicine* **21**, 91 (1974).
5. W. F. Herblin, *Analyt. Biochem.* **51**, 19 (1973).
6. P. J. Anderson and A. D'Iorio, *Biochem. Pharmac.* **17**, 1943 (1966).
7. J. K. Coward and F. Y.-H. Wu, *Analyt. Biochem.* **55**, 406 (1973).
8. J. Axelrod, *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 5, pp. 748–51. Academic Press, New York (1962).
9. R. E. McCaman, *Life Sci.* **4**, 2353 (1965).
10. P. A. Gulliver and C. W. Wharton, *Biochem. Pharmac.* **25**, 2033 (1976).
11. T. K. Sharpless and R. Wolfenden, *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 12, pp. 126–31. Academic Press, New York (1967).
12. S. Otsuka and Y. Kobayashi, *Biochem. Pharmac.* **13**, 995 (1964).
13. J. Poulton, H. Grisebach, J. Ebel, B. Schaller-Hekeler and K. Halbrock, *Archs Biochem. Biophys.* **173**, 301 (1976).
14. J. K. Coward, E. P. Slisz and F. Y.-H. Wu, *Biochemistry* **12**, 2291 (1973).