Biochemical Pharmacology, Vol. 29, pp. 3123–3126.

0006-2952/80/1201-3123 $02.00/0

BENSERAZIDE AND CARBIDOPA AS SUBSTRATES OF CATECHOL-*O*-METHYLTRANSFERASE: NEW MECHANISM OF ACTION IN PARKINSON'S DISEASE

RUSSELL M. HAGAN, MICHAEL J. RAXWORTHY and PETER A. GULLIVER*
Department of Pharmacology, The Worsley Medical and Dental Building, The University, Leeds LS2 9JT, U.K.

(*Received* 30 *April* 1980; *accepted* 4 *July* 1980)

Abstract—The DOPA-decarboxylase (aromatic-L-amino acid decarboxylase) inhibitors benserazide, its active metabolite 2,3,4-trihydroxybenzylhydrazine and carbidopa are substrates of highly purified catechol-*O*-methyltransferase. The affinity and maximal velocity of both benserazide and trihydroxybenzyl hydrazine are extremely favourable implying that these compounds may compete as substrates for catechol-*O*-methyltransferase with L-DOPA. These results are discussed in the light of the ability of benserazide to cause further elevation of plasma L-DOPA than carbidopa and the use of these compounds in Parkinson's disease.

Parkinsonism, a disease predominantly of the elderly characterized by chronic motor dysfunction, is caused by a degeneration of dopaminergic neurons of the nigro-striatal pathway. The degree of dopaminergic deficiency corresponds with the loss of melanin-containing cells of the pars compacta of the substantia nigra.

Replacement therapy utilizes L-DOPA, the normal precursor of dopamine, in doses of up to 8 g/day, which is transported across the blood–brain barrier and subsequently replenishes the depleted dopamine stores. L-DOPA is often combined with an inhibitor of peripheral DOPA-decarboxylase (aromatic-L-amino acid decarboxylase, EC 4.1.1.28) to maintain elevated plasma levels [1]. The two clinically available inhibitors are benserazide [N^1-(DL-seryl)-N^2-(2,3,4-trihydroxybenzyl)hydrazine] (Preparation: MADOPAR 4:1 w/w L-DOPA: benserazide) and carbidopa (α-methyldopahydrazine) (Preparation: SINEMET 10:1 w/w L-DOPA: carbidopa).

Since L-DOPA is effectively catabolized by catechol-*O*-methyltransferase (COMT, EC 2.1.1.6) [2], *O*-methylation becomes the exclusive route of peripheral DOPA metabolism if DOPA-decarboxylase is inhibited. Inhibition of COMT potentiates the effect of L-DOPA in man [3]; and Marx [4] suggested that COMT inhibitors could be used to overcome the decreasing response to chronic L-DOPA therapy and to combat side-effects, which may be due to high concentrations of *O*-methylated DOPA metabolites [3].

Benserazide has been shown to be a potent competitive COMT inhibitor [5–7] and *O*-methylated metabolites have been detected in the urine of rat and man [8] which has led to the present study of benserazide and carbidopa as substrates for COMT. The active benserazide metabolite (2,3,4-trihydroxybenzylhydrazine) and several other COMT substrates were compared to further our understanding of the potential effects of these drugs on peripheral L-DOPA metabolism.

COMT is an enzyme of wide specificity concentrated mainly in the liver [9] from which source it can be purified to homogeneity by affinity chromatography [10]. This highly purified enzyme was used in our investigations.

MATERIALS AND METHODS

All reagents used were of analytical quality (BDH, Poole, U.K.) and dissolved in glass distilled water. All pH measurements are relative to 20°.

Catechol-*O*-methyltransferase was purified from fresh pig liver by the method of Gulliver and Tipton [10]. Briefly, the COMT activity from the supernatant was precipitated by ammonium sulphate, redissolved, subjected to chromatography on Sephadex G75 (Pharmacia, Uppsala, Sweden) and affinity chromatography on 2,6-dimethoxyphenol-azophenyl-methylene-anilino-agarose. The purification scheme is given in Table 1. The magnesium chloride concentration in all buffers was raised to 2.6 mM, as originally used in the affinity chromatography step only.

During purification, COMT activity was assayed by the direct extraction radiochemical method [11] using 3,4-dihydroxyphenylacetic acid as the methyl acceptor and methyl-tritiated *S*-adenosylmethionine as the methyl donor in 0.2 M pH 7.20 triethanolamine hydrochloride buffer, pH 7.20. One unit (U) of activity represents the formation of one micromole of product in one minute.

Adenosine deaminase was partially purified from Takadiastase (Koch-Light, Colnbrook, U.K.) using the method of Sharpless and Wolfenden [12] up to and including the dialysis stage.

Kinetic determinations were carried out by the coupled assay method of Coward and Wu [13] as modified by Gulliver and Tipton [11]. The assay contained in a volume of 500 μl : 1.6 mM magnesium

* To whom correspondence should be sent.

Table 1. Purification of catechol-*O*-methyltransferase from pig liver (see Ref. 10)*

Stage	Volume (ml)	Activity (mU/ml)	Total activity (U)	Protein (mg/ml)	Specific activity (mU/mg)	Overall yield (%)	Overall purification (-fold)
Homogenate	2200	90.65	199.43	197	0.46	100	1
Supernatant	1080	130.21	140.63	117	1.11	70.52	2.42
Redissolved 55%-saturating ammonium sulphate precipitate	112	669.55	74.99	209.2	3.20	37.60	6.96
Pooled Sephadex G75 fractions	425	187.1	79.52	8.06	23.21	39.87	50.5
Pooled affinity column fraction	55	766.36	42.15	0.628	1220.38	21.14	2653

* Pig liver (1434 g) was homogenized in 1200 ml 0.01 M triethanolamine hydrochloride buffer pH 8.00 containing 0.13 M potassium chloride, 10 mM mercaptoethanol, 3 mM dithiothreitol, 2 mM phenylmethanesulphonyl fluoride, 2.6 mM $MgCl_2$ and 1 mM EDTA.

The catechol-*O*-methyltransferase assay contained, in a volume of 500 μl : 1.6 mM $MgCl_2$, 3.0 mM 3,4-dihydroxyphenylacetic acid, 0.9 mM Ado–Met (0.374 Ci ^3H per mole), 0.64 U adenosine deaminase, 0.20 M triethanolamine buffer pH 7.20 and 10–100 μl appropriate fraction. 15 minutes incubation at 37°.

chloride, 0.64 U adenosine deaminase, 0.20 M triethanolamine buffer pH 7.20, *S*-adenosylmethionine, a methyl acceptor and 10–50 μl purified catechol-*O*-methyltransferase. The pH and magnesium concentration are both optimal [10, 11]. Initial rates were measured at 37° in a Beckman Model 35 spectrophotometer using a mean of at least eight replicate determinations. A wide range of concentrations of the different varied substrates was used. Apparent kinetic constants were determined by the direct linear plot of Eisenthal and Cornish-Bowden [14].

Bardsley and Tipton (unpublished observations) observed that catecholamines can bind to adenosine deaminase in a radiochemical catecholamine assay. Therefore, control spectrophotometric assays were carried out with up to three times the usual adenosine deaminase concentration in the cuvette with low methyl acceptor concentrations (approximately half the observed Michaelis–Menten constant). This additional adenosine deaminase caused no apparent depression of the observed rates, as would be expected to arise from the binding of the catechols. In addition, radiochemical assays carried out, in the presence and absence of adenosine deaminase, at low concentrations of 3,4-dihydroxyphenylacetic acid again showed no difference in the initial rates observed but did confirm the necessity of adding adenosine deaminase to ensure linear time courses [11].

Protein concentrations above 1 mg/ml were determined by the biuret method [15] and at lower concentrations by the method of Mejbaum-Katzenellenbogen and Dobryszycka [16] using standard curves prepared to bovine serum albumin (Cohn Fraction V, Sigma, Poole, U.K.)

L-DOPA, dopamine and DL-noradrenaline were obtained from Sigma. Isoprenaline was obtained from Ward, Blenkinson & Co, Widnes, U.K. 3,4-Dihydroxyphenylacetic acid was obtained from Calbiochem, Bishops Stortford, U.K.

The following gifts are gratefully acknowledged: benserazide hydrochloride (Roche Products Limited, Welwyn Garden City, U.K.), carbidopa (Merck, Sharpe & Dohme, Hoddesdon, U.K.), trihydroxybenzylhydrazine acetate ethanolate (60%) (Hofman-LaRoche, Basle, Switzerland) and *S*-adenosyl-L-methionine suplhate *p*-toluenesulphonate (AdoMet) (Dr. G. Stramentinoli, BioResearch Milan, Italy).

RESULTS AND DISCUSSION

The calculated apparent kinetic constants are given in Table 2, which also shows the range of concentration of the varied substrates and the concentration of fixed, saturating substrates.

Several substrates caused reproducible deviations from apparent Michaelis–Menten kinetics [17] at high concentrations as indicated in Table 2. However, linear double reciprocal plots were obtained throughout the major portion of the concentration range used [18]. The reason for these anomalous rate increases are unknown but they may represent a general response to a hydrophobic environment since the enzyme preparation is homogeneous [10].

The apparent K_m values for adenosylmethionine observed in the presence of saturating benserazide and 3,4-dihydroxyphenylacetic acid (0.91 and 0.035 mM, respectively) agree well with those observed previously [10] using a similar enzyme preparation and assay procedure. The apparent K_m for 3,4-dihydroxyphenylacetic acid is higher, however, than that previously obtained (1.46 mM compared to 0.64 mM) [10] which may imply a variation in the form of pig liver COMT since the V_{max} is also higher (1320 mU/mg protein as opposed to 705.6 mU/mg). The initial specific activity of the homogenate from which the enzyme was prepared is also higher (0.46 mU/mg as opposed to 0.16 mU/mg).

These discrepancies may be due to the inherent biological variation in agricultural animals or perhaps due to the purification of a different predominant isoenzyme [19].

The apparent Michaelis constants observed for benserazide and its metabolite, 2,3,4-trihydroxybenzylhydrazine, are remarkably low, indicating a high

Table 2. Apparent kinetic constants of pig liver COMT for various substrates*

Varied substrate	Concentration range of varied substrate (mM)	Apparent K_m (mM) ± St. Er. of mean	Apparent V_{max} (mU/mg protein) ± St. Er. of mean	Number of determinations	Fixed saturating substrate	Concentration of fixed saturating substrate (mM)
Benserazide	0.09–1.20	0.38 ± 0.015	1698.3 ± 31.8	6	Adenosylmethionine	0.46
Adenosylmethionine	0.03–0.12	0.091 ± 0.004	1862.5 ± 34.8	3	Benserazide	3.00
Trihydroxybenzylhydrazine	0.02–2.00	0.090 ± 0.004	1080.0 ± 20.2	3	Adenosylmethionine	0.46
Carbidopa†	0.75–3.00	2.48 ± 0.10	220.0 ± 4.1	3	Adenosylmethionine	0.46
L-DOPA	1.00–3.00	1.83 ± 0.07	330.0 ± 6.2	3	Adenosylmethionine	0.46
(±)α-Methyldopa	0.50–8.00	1.60 ± 0.06	105.1 ± 2.0	3	Adenosylmethionine	0.46
Dopamine†	0.45–1.80	0.75 ± 0.03	835.8 ± 15.6	3	Adenosylmethionine	0.46
3,4-Dihydroxyphenylacetic acid	0.30–3.00	1.46 ± 0.06	1320.0 ± 24.7	3	Adenosylmethionine	0.46
Adenosylmethionine	0.03–0.15	0.035 ± 0.001	1140.0 ± 21.3	3	3,4-dihydroxy phenylacetic acid	3.00
(±)Noradrenaline†	0.18–1.20	0.86 ± 0.034	1182.0 ± 22.1	3	Adenosylmethionine	0.46
(±)Isoprenaline†	0.11–0.80	0.36 ± 0.014	574.9 ± 10.8	4	Adenosylmethionine	0.46

* Determined by the assay of Coward and Wu [13] as modified by Gulliver and Tipton [11] in 1.6 mM magnesium chloride and 0.20 M triethanolamine hydrochloride buffer, pH 7.20.
† denotes deviations from Michaelis–Menten kinetics observed at high varied substrate concentration.

affinity for the enzyme. The K_m for trihydroxybenzylhydrazine is as low as that observed for adenosylmethionine with benserazide as the methyl acceptor and, since it was an impure sample of an unstable compound, the actual K_m may be much lower. The maximal velocities observed for these substrates are very high and both benserazide and 2,3,4-trihydroxybenzylhydrazine are better substrates for COMT than carbidopa (K_m = 2.48 mM; V_{max} = 220 mU/mg) or L-DOPA itself (K_m = 1.83 mM; V_{max} = 330 mU/mg).

The apparent kinetic constants for dopamine, noradrenaline and isoprenaline are included for comparison since COMT is known to methylate these compounds *in vivo* and, in fact, represents the major route of isoprenaline metabolism [20]. Other workers have obtained conflicting kinetic data due to variations in the source of COMT, its degree of purification, the assay system used and especially the pH of the assay medium, since Flohé and Schwabe [21] reported that the K_m for adrenaline decreased sharply with increasing pH. The work of Fiebig and Trendelenburg [22] on the perfused rat heart and other tissue studies has yielded apparent Michaelis–Menten constants 20–25 times lower than those reported here, implying the enzyme may be more active *in vivo*. The Michaelis–Menten constants obtained here compare well with those of Ball *et al.* [23], Tong and D'Iorio [24] and White and Wu [25]. The maximal velocities cannot be compared because of differences in degree of purification. However, some workers (see e.g. [7]) may have been misled into reporting very low K_m's and maximal velocities because of inhibition by the product, *S*-adenosylhomocysteine which was not removed by adenosine deaminase in these experiments.

The general conclusion than can be drawn from this work is that benserazide will cause a profound inhibition of L-DOPA metabolism by two mechanisms: (a) competition for the major catabolic enzyme, COMT, and (b) causing a large demand on the restricted methyl donor pool [26]. This may be reflected in the ability of benserazide to cause higher plasma L-DOPA levels when administered in conjunction with L-DOPA than carbidopa [1] which is a poor COMT substrate. The decrease in the methyl donor pool would also decrease the production of toxic *O*-methyl derivatives. The methylated derivatives of benserazide produced by COMT may not inhibit DOPA-decarboxylase since, although benserazide causes higher plasma L-DOPA concentrations than carbidopa initially, its effect declines more rapidly.

Acknowledgements—We thank the MRC for financial support, Mr. John Savage of Leeds Abbattoir for his cooperation, and Dr. G. Stramentinoli of BioResearch, Milan, for the gift of adenosylmethionine.

REFERENCES

1. A. Lieberman, E. Estey, G. Gopinathan, T. Ohashi, A. Sauter and M. Goldstein, *Neurology* **28**, 964 (1978).
2. R. J. Wurtman, C. Chou and C. Rose, *J. Pharm. exp. Ther.* **174**, 351 (1970).
3. A. D. Ericsson, *J. Neurological Sci.* **14**, 193 (1971).
4. J. L. Marx, *Science* **203**, 737 (1979).

5. R. J. Baldessarini, *J. Pharm. Pharmac.* **24**, 78 (1972).
6. R. J. Baldessarini and E. Greiner, *Biochem. Pharmac.* **22**, 247 (1973)
7. N. T. Buu, O. Kuchel and M. T. Parent, *Can. J. Biochem.* **55**, 771 (1977).
8. D. E. Schwartz and R. Brandt, *Arzn. Forsch. (Drug Res.)* **28**, 302 (1978).
9. J. Axelrod and R. Tomchick, *J. biol. Chem.* **233**, 702 (1958).
10. P. A. Gulliver and K. F. Tipton, *Eur. J. Biochem.* **88**, 439 (1978).
11. P. A. Gulliver and K. F. Tipton, *Biochem. Pharmac.* **27**, 773 (1978).
12. T. K. Sharpless and R. Wolfenden, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), Vol. 12, pp.126–131. (1967).
13. J. K. Coward and F. Y-H. Wu, *Analyt. Biochem.* **55**, 406 (1973).
14. R. Eisenthal and A. Cornish-Bowden, *Biochem. J.* **139**, 715 (1974).
15. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
16. W. Mejbaum-Katzenellenbogen and W. M. Dobryszycka, *Clinica Chim. Acta* **4**, 515 (1979).
17. L. Michaelis and M. L. Menten, *Biochem. Z.* **49**, 333 (1913).
18. H. Lineweaver and D. Burk, *J. Am. Chem. Soc.* **56**, 658 (1934).
19. G. D. Rock, J. H. Tong and A. D'Iorio, *Can. J. Biochem.* **48**, 1326 (1970).
20. M. E. Conolly, D. S. Davies, C. T. Dollery, C. D. Morgan, J. W. Paterson and M. Sandler, *Br. J. Pharmac.* **46**, 458 (1972).
21. L. Flohé and K-P. Schwabe, *Hoppe-Seyler's Z. Physiol. Chem.* **353**, 463 (1972).
22. E. R. Fiebig and U. Trendelenburg, *Naunyn-Schmiedeberg's Archs. Pharmac.* **303**, 21 (1978).
23. P. Ball, R. Knuppen, M. Haupt and H. Breuer, *J. Clin. Endocr. Metab.* **34**, 736 (1972).
24. J. H. Tong and A. D'Iorio, *Can. J. Biochem.* **55**, 1108 (1979).
25. H. L. White and J. C. Wu, *Biochem. J.* **145**, 135 (1975).
26. S. H. Mudd and J. R. Poole, *Metabolism* **24**, 721 (1975).