Spectrofluorometric Determination of Carbidopa [L-(−)-α-Hydrazino-3,4-dihydroxy-α-methylhydrocinnamic Acid] in Plasma

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Abstract □ A spectrofluorometric assay is described for carbidopa utilizing *p*-dimethylaminobenzaldehyde. The fluorophore is dimethylaminobenzalazine which fluoresces intensely in chloroform saturated with trichloroacetic acid. The assay was applied to the analysis of carbidopa in monkey plasma.

Keyphrases □ Carbidopa—spectrofluorometric determination, monkey plasma □ L-(−)-α-Hydrazino-3,4-dihydroxy-α-methylhydrocinnamic acid—spectrofluorometric determination, monkey plasma □ Spectrophotofluorometry—analysis of carbidopa in monkey plasma

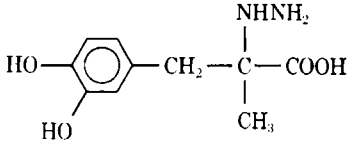
Carbidopa [L-(−)-α-hydrazino-3,4-dihydroxy-α-methylhydrocinnamic acid] is an inhibitor of aromatic amino acid decarboxylase, and it is able to potentiate levodopa in the treatment of Parkinson's disease (1–3). Carbidopa decreases the peripheral decarboxylation of dopa but not the conversion of dopa to dopamine in the brain, since carbidopa does not penetrate the blood–brain barrier (4).

To measure levels of carbidopa in plasma after oral administration of the drug in therapeutic doses, a sensitive assay was required. A fluorometric method was developed which depends upon the formation of dimethylaminobenzalazine, chloroform solutions of which fluoresce in the presence of trichloroacetic acid.

EXPERIMENTAL

Analytical Method—Trichloroacetic acid[1], chloroform[2], *p*-dimethylaminobenzaldehyde[3], and anhydrous magnesium sulfate[1] were used in these studies.

Plasma (2 ml.) was mixed with aqueous 10% trichloroacetic acid (3 ml.) and centrifuged (15 min.). The supernate was removed (as much as possible) and shaken with chloroform (2 ml.) for 15 min.; the aqueous layer was removed (as much as possible) and mixed with 0.4% ethanolic *p*-dimethylaminobenzaldehyde (2 ml.). The solution was heated at 70° for 45 min., cooled, and shaken with chloroform (3 ml.) for 10 min. The chloroform layer was removed following centrifugation, and anhydrous magnesium sulfate (10 mg.) was added. After centrifugation, the solution was analyzed spectrophotometrically in a spectrophotofluorometer[4] using quartz cells (10-mm. lightpath). Carbidopa analyzed in this manner exhibited a maximum value of fluorescence at an excitation wavelength of 466 nm. and an emission wavelength of 546 nm.



I: carbidopa

[1] Baker Analyzed.
[2] Fisher Analytical.
[3] Fisher Certified.
[4] Aminco-Bowman.

Table I—Reproducibility of the Fluorescence Assay for Carbidopa[a]

Carbidopa, mcg./2 ml.	Arbitrary Fluorescence Units ± *SD*
2	10.5 ± 0.7
1	4.9 ± 0.4
0.5	2.3 ± 0.3
0.1	0.4 ± 0.05

[a] Four experiments.

Identification of Fluorophore—Carbidopa (0.55 g.) was dissolved in the least amount of 1 *N* HCl possible, and ethanol (10 ml.) was added. This solution was added to an ethanolic solution (10 ml.) of *p*-dimethylaminobenzaldehyde (0.74 g.). After 30 min. at 70°, solid sodium acetate was added until precipitation occurred. The precipitate was filtered and recrystallized from dimethylformamide to yield 346 mg. material, m.p. 274–275°.

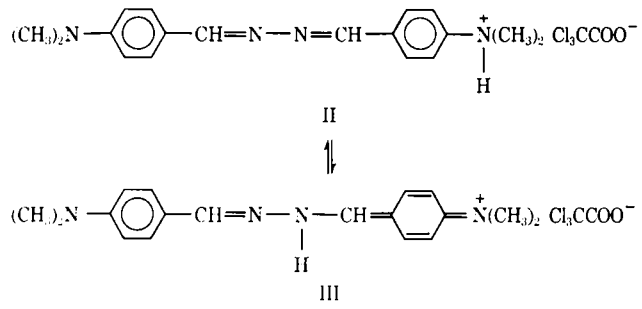
Animal Experiments—Five female rhesus monkeys (3–4 kg.) were dosed orally with an aqueous solution of carbidopa. Three received a dose of 20 mg./kg. and two received a dose of 2 mg./kg. Plasma was drawn at 1, 2, and 4 hr., and levels of carbidopa were measured with a calibration curve constructed by using normal plasma spiked with known amounts of carbidopa and plotting the fluorescence of the chloroform solution (after correcting for the blank value) *versus* the concentration of carbidopa in plasma.

RESULTS

Eighty nanograms of carbidopa/2 ml. of plasma could be detected by this method. The sensitivity of the method could be increased by using larger volumes of plasma since this caused no concomitant increase in background fluorescence. To test the reproducibility of the method, plasma samples (2 ml.) containing carbidopa (1, 0.5, 0.25, and 0.05 mcg./ml.) were analyzed (Table I). The intensities of the fluorescence obtained in these experiments were compared to those of standard solutions of the azine in chloroform saturated with trichloroacetic acid. It was determined that the reaction and the extraction of the product were essentially complete (93.5% ± 4.4 *SD*) over the 0.1–2-mcg. range of carbidopa/ml. of plasma. A water bath temperature of 70° and a 45-min. incubation were optimum conditions for maximum intensity of fluorescence. The latter was reduced when ethyl acetate or butanol was substituted for chloroform. Magnesium sulfate, unlike sodium chloride or hydrochloric acid, did not decrease the sensitivity of the assay. Trichloroacetic acid served a dual purpose: precipitating

Table II—Identification of Dimethylaminobenzalazine by Comparative TLC

Solvent System	R_f of Fluorescent Product	R_f of Authentic Dimethylaminobenzalazine
Benzene–acetic acid (3:2)	0.05	0.05
Benzene–acetic acid (2:3)	0.1	0.1
Benzene–acetic acid (4:1)	Origin	Origin
Benzene–acetone–water–acetic acid–butanol (equal parts)	0.92	0.92
Ethanol–acetic acid (1:1)	0.81	0.78



Scheme I—Ion-pair formation between dimethylaminobenzalazine and trichloroacetic acid

protein and increasing the fluorescence intensity of the product. There was no advantage in using trichloroacetic acid concentrations greater than 10%.

Carbidopa was shown to form dimethylaminobenzalazine (mixed melting point 274°) when reacted with dimethylaminobenzaldehyde. The structure of the product was confirmed by the use of mass spectroscopy and comparative TLC (Table II), which also did not indicate the presence of another product.

Anal.—Calc. for $C_{18}H_{22}N_4$: C, 73.45; H, 7.48; N, 19.05. Found: C, 73.52; H, 7.85; N, 18.98.

The recovery of the product and the reproducibility of the assay at levels anticipated after clinical doses[5] of carbidopa in man were found to be satisfactory.

Interfering Substances—No interference was produced by dopa, dopamine, phenylhydrazine, tryptamine, or tyrosine at levels of 3, 1, 1, 10, and 10 mcg./ml. of plasma, respectively. The 3-*O*-methyl derivative of carbidopa will interfere, but this was not detected as a metabolite of carbidopa. Hydrazine will also interfere, and the method forms the basis for a very sensitive assay for hydrazine. However, hydrazine has not been identified as a metabolite of carbidopa[6].

Plasma Levels of Carbidopa in Monkeys—Levels of carbidopa were measured in the plasma of monkeys dosed with carbidopa (see *Experimental*). Maximum levels were usually achieved within 2 hr. (Table III). These doses gave plasma levels which were in the range described by the calibration curve, *i.e.*, 0.1–2 mcg./2 ml. plasma (Table I).

[5] Plasma levels of radioactivity in man (nine subjects) after administration of a single 50-mg. oral dose of carbidopa-^{14}C when expressed as equivalents of carbidopa ranged from 0.05 to 0.9 mcg./ml. (unpublished results of the authors).

[6] Unpublished observations of the authors.

Table III—Levels of Carbidopa[a] in Monkey Plasma after an Oral Dose

Monkey	Dose, mg./kg.	Carbidopa in Monkey Plasma—Hours 1	2	4
1	20	0.7	2.0	0.3
2	20	1.6	0.5	0.2
3	20	2.6	1.8	0.8
4	2	0.15	0.05	<0.03
5	2	<0.03	0.03	0.18

[a] Expressed as micrograms of carbidopa per milliliter of plasma.

DISCUSSION

In the present investigation, carbidopa formed the corresponding azine with dimethylaminobenzaldehyde without preliminary hydrolysis; it was not possible to detect the hydrazone. Structure III (5) may be the isomeric form of II that is responsible for the fluorescence properties of dimethylaminobenzalazine (Scheme I). This follows a similar suggestion made by Glazko *et al.* (6) to explain the observation that solutions of reserpine in ethylene dichloride become strongly fluorescent in the presence of trichloroacetic acid.

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