Reversed-Phase High-Performance Liquid Chromatographic Investigation of Levodopa Preparations II: Levodopa Determination

GARY W. SCHIEFFER

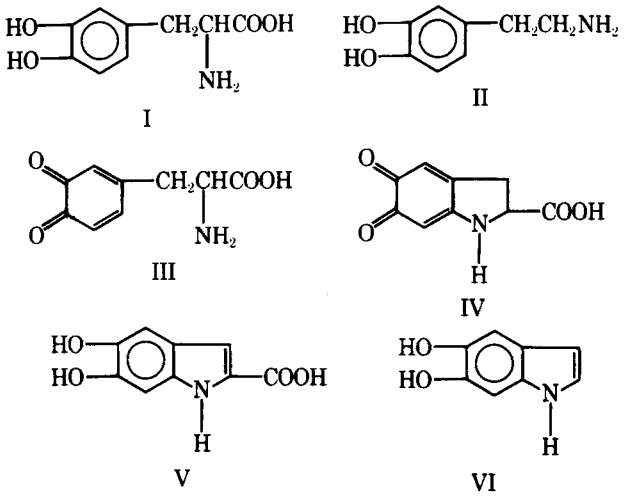
Received March 23, 1979, from the *Pharmaceutical Research Division, Norwich-Eaton Pharmaceuticals, Division of Morton-Norwich Products, Inc., Norwich, NY 13815.* Accepted for publication April 19, 1979.

Abstract □ A reversed-phase high-performance liquid chromatographic procedure with UV detection at 280 nm is presented for levodopa determination in pharmaceutical preparations. The method is fast and specific for levodopa in the presence of its most likely impurities and degradation products.

Keyphrases □ Levodopa—analysis, high-performance liquid chromatography, commercial preparations, impurities, degradation products □ Antiparkinsonian agents—levodopa, high-performance liquid chromatographic analysis, commercial preparations, impurities, degradation products □ High-performance liquid chromatography—analysis, levodopa, commercial preparations, impurities, degradation products

Fluorometric (1), electrochemical (2), GLC (3), and high-performance liquid chromatographic (HPLC) (4, 5) techniques have been presented for levodopa (I). Although some techniques are specific with respect to postulated impurities (3) or levodopa decomposition products formed in alkaline solution (5), a complete study of the specificity of a method with respect to a wide range of possible degradation products has not been reported. Such a specificity study for a proposed levodopa stability-indicating method should include the examination of dopamine (3-hydroxytyramine) (II) [decarboxylated levodopa (6)] and intermediates in the aminochrome formation reaction (7). The latter include the initial oxidation product levodopa-*o*-quinone (III) as well as the cyclization product dopachrome (IV) and the final internal oxidation–reduction products 5,6-dihydroxyindole-2-carboxylic acid (V) and 5,6-dihydroxyindole (VI).

An HPLC method was presented (8) for determining trace amino acid impurities in levodopa preparations. This report discusses extending this technique to include a stability-indicating assay specific for levodopa with respect to the postulated degradation products.



EXPERIMENTAL

Apparatus—The liquid chromatographic apparatus described previously (8) was used. A variable-wavelength absorbance detector[1] adjusted to 485 nm was used for some studies in addition to the 280-nm UV detector used for the assay. Peak areas were determined with a computerized laboratory data acquisition system. The flow rate was 2.0 ml/min.

Reagents and Materials—The levodopa, 6-hydroxydopa, tyrosine, 3-*O*-methyldopa (3-methoxytyrosine), 5-hydroxydopa, and 3-(3-hydroxy-4-methoxyphenyl)alanine described previously (8) were used. 5,6-Dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid were synthesized according to the procedure of Benigni and Minnis (9).

Dopachrome was prepared by adding a few drops of a ferricyanide solution [13 g of potassium ferricyanide and 4.2 g of sodium bicarbonate diluted to 50 ml with water (10)] to 10 ml of a 0.01 *M* phosphoric acid solution containing 2 mg/ml of levodopa, tyrosine, 3-*O*-methyldopa, and hypoxanthine. (The latter three compounds did not react with ferricyanide but were added to demonstrate chromatographic specificity.) The resulting dark-red solution was injected directly.

Levodopa-*o*-quinone was generated electrochemically (11, 12) with a specially designed precolumn tubular electrode cell (13). Hypoxanthine[2] and dopamine hydrochloride[3] were obtained commercially. All other reagents and chemicals were ACS reagent grade or the equivalent.

Mobile Phase—A 0.01 *M* NaH_2PO_4 solution was adjusted to pH 3.5 with phosphoric acid. This solution was filtered daily through a 0.5-μm membrane filter[4] and degassed prior to use.

Internal Standard Solution—About 150 mg of hypoxanthine was weighed into a 100-ml volumetric flask, dissolved on a steam bath with 40 ml of 0.1 *M* phosphoric acid, and diluted to volume with water.

Levodopa Standard Solution—About 50 mg of reference standard levodopa was accurately weighed into a 50-ml volumetric flask, dissolved with 5 ml of 0.1 *M* phosphoric acid, and diluted to volume with water. Exactly 10.0 ml of this solution was added to a 100-ml volumetric flask containing 20.0 ml of the internal standard solution, and the contents were diluted to volume with water.

Sample Solution—Twenty levodopa capsules or tablets were ground and mixed well, and a quantity sufficient to yield 250 mg of levodopa was accurately weighed into a 250-ml volumetric flask. Fifty milliliters of 0.1 *M* phosphoric acid was added, and the flask was shaken mechanically for 30 min. The contents were diluted to volume with water, and a portion of the solution was centrifuged. Exactly 10.0 ml of the centrifugate and 20.0 ml of the internal standard solution were added to a 100-ml volumetric flask, the contents of which were diluted to volume with water and further clarified by filtration through the 0.5-μm filter.

Table I—Linearity

Parameter	Area Ratios		Height Ratios	
	Levodopa	Hypoxanthine	Levodopa	Hypoxanthine
Correlation coefficient	0.9999	0.9999	0.9995	0.9999
Standard error of estimate ($S_{y/x}$)	0.004	0.004	0.02	0.007
Intercept[a], %	0.2	3.0	−0.5	3.8
Variation[b], %	0.6	0.3	2.0	0.4

[a] The (y intercept/$\bar{y}$) × 100, where $\bar{y}$ is the average y. [b] ($S_{y/x}/\bar{y}$) × 100.

[1] Model 770, Schoeffel Instrument Corp., Westwood, N.J.
[2] Heterocyclic Chemical Corp., Harrisonville, Mo.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Millipore.

0022-3549/79/1000-1299$01.00/0

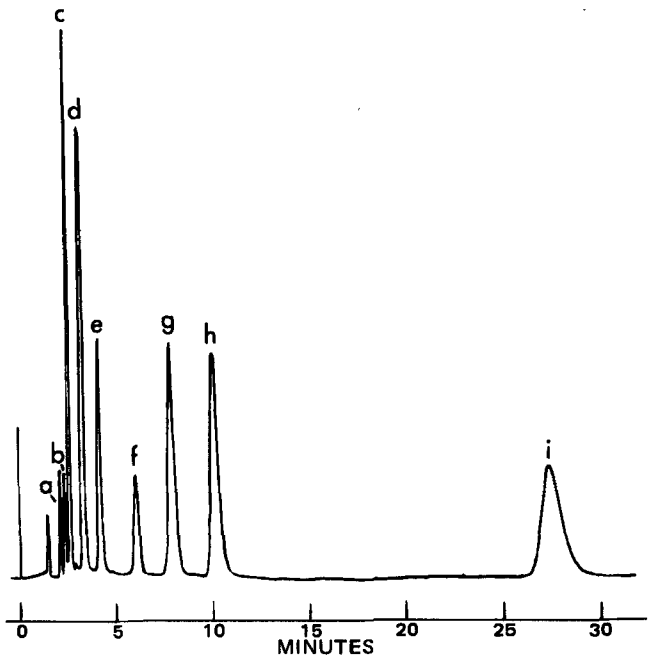


Figure 1—*Chromatogram of levodopa, hypoxanthine internal standard, and possible interferences: (a) 5-hydroxydopa, (b) 6-hydroxydopa, (c) levodopa, (d) dopamine and tyrosine, (e) hypoxanthine, (f) 3-O-methyldopa, (g) 3-(3-hydroxy-4-methoxyphenyl)alanine, (h) 5,6-dihydroxyindole, and (i) 5,6-dihydroxyindole-2-carboxylic acid.*

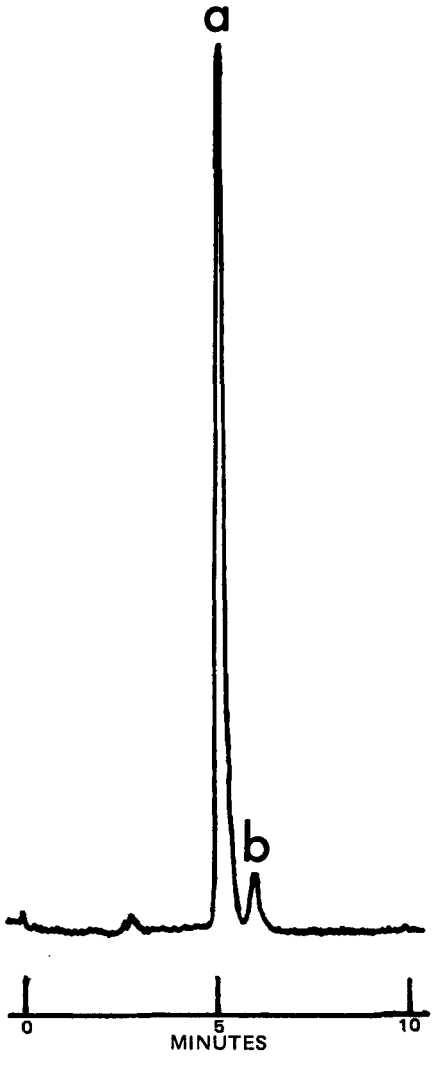


Figure 2—*Chromatogram of levodopa (a) and electrochemically generated levodopa-o-quinone (b). Electrode generation potential was 0.8* versus *Ag–AgCl reference electrode. Flow rate was 1.0 ml/min.*

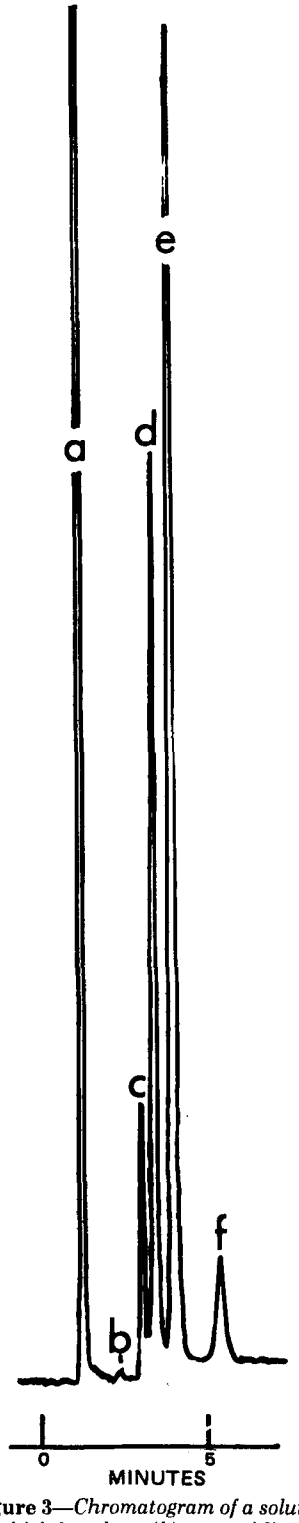


Figure 3—*Chromatogram of a solution in which levodopa (b) was oxidized to dopachrome (d) with ferricyanide (a). Tyrosine (c), hypoxanthine (e), and 3-O-methyldopa (f) remained unoxidized.*

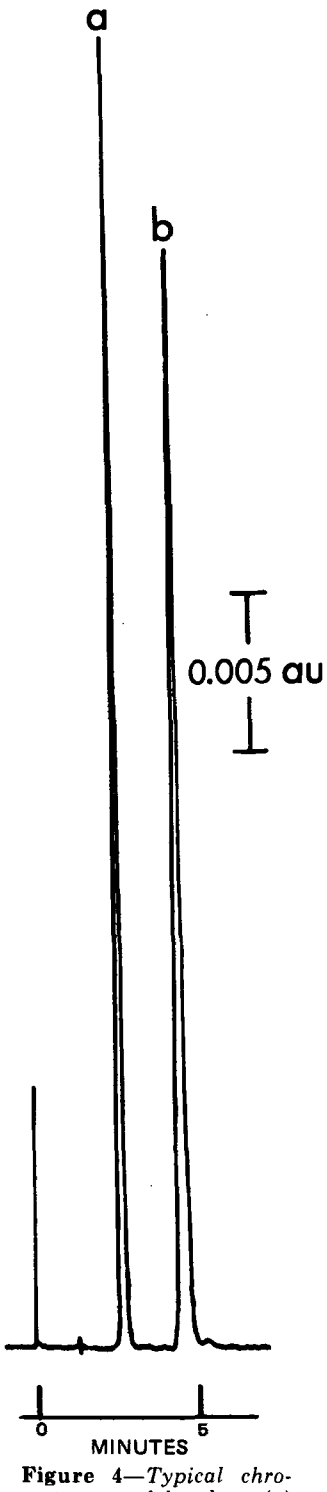


Figure 4—*Typical chromatogram of levodopa (a) and hypoxanthine (b) (1 μg of levodopa and 3 μg of hypoxanthine were injected).*

Synthetic samples were made by mixing known quantities of reference standard levodopa and excipients in the dry state and treating the mixture according to the procedure for the actual samples.

Sample Analysis and Linearity—Ten microliters each of the standard and sample solutions were injected. The amount of levodopa present in the sample, expressed as milligrams of levodopa per gram of sample weight, was calculated from:

$$\text{levodopa} = 5(R_x/R_s)(W_s/W_x) \qquad \text{(Eq. 1)}$$

Table II—Assay of Levodopa in Actual Commercial Samples by HPLC, USP Spectrophotometric, and Amino Acid Analyzer (AAA) Techniques

Sample	Levodopa per Dosage Unit, mg	n	Amount Found[a] HPLC[b]	USP[c]	AAA[c]
1	100	14	100.4 (0.7)	—	100.7
2	100	5	105.9 (0.7)	—	—
3	100	5	104.4 (0.8)	—	—
4	250	4	99.4 (0.8)	99.9	99.7
5	250	5	99.3 (1.1)	—	—
6	500	4	91.3 (2.0)	91.6	92.3

[a] Expressed as percent label for the ungrouped data (ranging from 50 to 150% of the nominal sample size). [b] Values in parentheses represent relative standard deviations. [c] Average of two or three assay values.

where R_x and R_s are the peak height or area ratios (levodopa to hypoxanthine) of the sample and standard solution, respectively; W_s is the weight of the levodopa standard in milligrams, and W_x is the weight of the sample in grams.

Linearity studies were performed by (*a*) varying the levodopa concentration over 0.02–0.2 mg/ml and holding the internal standard concentration constant, and (*b*) varying the internal standard concentration over 0.075–0.6 mg/ml and holding the levodopa concentration constant.

RESULTS AND DISCUSSION

The initial attempt was to combine the HPLC assay of levodopa impurities described previously (8) with a stability-indicating levodopa assay. However, the pH 2.5 phosphate buffer eluent did not resolve the decarboxylated degradation product, dopamine, from levodopa, although the four other postulated degradation products were resolved from levodopa and the impurities (8). The pH had to be increased to 3.5 to achieve a separation.

With the pH 3.5 phosphate buffer eluent, adequate resolution was obtained among levodopa, the hypoxanthine internal standard, and the impurities (8). (The pH 3.5 buffer could not be used for the impurities assay because the resolution of 6-hydroxydopa and tyrosine from levodopa was not sufficient to determine these impurities accurately at the 0.1% level.) In addition, dopamine as well as 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid, the final products in the levodopa aminochrome reaction (7), were all resolved. A chromatogram containing all of these postulated interferences is shown in Fig. 1.

The initial oxidation product in the aminochrome reaction, levodopa-*o*-quinone, eluted immediately following the levodopa peak and did not interfere (Fig. 2). Dopachrome eluted just before the hypoxanthine internal standard peak and did not interfere (Fig. 3). [The dopachrome peak in Fig. 3 also was observed when the absorbance detector was set at 485 nm, a characteristic absorption maximum for aminochromes (7).]

Linearity data of levodopa and hypoxanthine, obtained by plotting peak area or peak height ratios *versus* weight ratios, are presented in Table I. Linearity was observed over the ranges studied: 0.2–2.0 μg for levodopa and 0.8–6.0 μg for the hypoxanthine internal standard. A typical chromatogram is shown in Fig. 4.

Fifteen synthetic samples prepared with levodopa weights ranging from 50 to 150% of theory (100% representing 100 mg of levodopa/dosage unit or 515 mg/g) yielded an average recovery and relative standard deviation of 99.0 ± 0.9% for the ungrouped data. A linear regression analysis of milligrams of levodopa found *versus* milligrams added yielded a straight line with a slope of 0.98 ± 0.02 and an intercept of 0.5 ± 1.4 (appended values are 99% confidence limits), indicating a slope and intercept not significantly different from 1 and 0, respectively, and no bias in the method.

The results of an analysis of actual commercial samples obtained from four different manufacturers are presented in Table II. Some of the HPLC assays were checked with the USP UV spectrophotometric technique (14) and with an amino acid analyzer technique utilizing an ion-exchange column and a pH 4.05 citrate buffer eluent[5]. Excellent agreement was found among the techniques. Furthermore, a six-point standard addition of a portion of Sample 1 using the HPLC technique yielded an assay value of 100.8% of label, in excellent agreement with the sample assay. [Sample 3 contained 10 mg of carbidopa/dosage unit as an active ingredient, which eluted between the hypoxanthine and 3-*O*-methyldopa peaks (Fig. 1) and did not interfere.]

In summary, an HPLC stability-indicating assay was developed that is specific for levodopa in pharmaceutical preparations with respect to at least five postulated degradation products and five impurities. The method is fast, allowing injections every 5 min and the assay of ~25 samples/8-hr day. The stability-indicating assay may be applied in conjunction with the impurities assay (8) merely by adjusting the eluent pH from 2.5 to 3.5.

REFERENCES

(1) J. C. Johnson, G. J. Gold, and D. H. Clouet, *Anal. Biochem.*, **54**, 129 (1973).

(2) W. D. Mason, *J. Pharm. Sci.*, **62**, 999 (1973).

(3) J. R. Watson, *ibid.*, **63**, 96 (1974).

(4) R. M. Riggin, R. L. Alcorn, and P. T. Kissinger, *Clin. Chem.*, **22**, 782 (1976).

(5) A. G. Ghanekar and V. D. Gupta, *J. Pharm. Sci.*, **67**, 1247 (1978).

(6) W. H. Vogel, *Naturwissenschaften*, **56**, 462 (1969).

(7) R. A. Heacock, *Chem. Rev.*, **59**, 181 (1959).

(8) G. W. Schieffer, *J. Pharm. Sci.*, **68**, 1296 (1979).

(9) J. D. Benigni and R. L. Minnis, *J. Heterocycl. Chem.*, **2**, 387 (1965).

(10) G. L. Mattok and R. A. Heacock, *Can. J. Chem.*, **42**, 481 (1964).

(11) M. D. Hawley, S. V. Tatawawadi, S. Piekarski, and R. N. Adams, *J. Am. Chem. Soc.*, **89**, 447 (1967).

(12) A. W. Sternson, R. McCreery, B. Feinberg, and R. N. Adams, *J. Electroanal. Chem. Interfacial Electrochem.*, **46**, 313 (1973).

(13) G. W. Schieffer, *Anal. Chem.*, in press.

(14) "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975, p. 281.

ACKNOWLEDGMENTS

The author is grateful to B. Stevenson and C. Finch for the synthesis of 5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid, to S. Casamento for the sample assay, and to P. J. Palermo for helpful discussions.

[5] G. S. Denning, Jr., Norwich-Eaton Pharmaceuticals, Norwich, N.Y., unpublished results, 1975.