Colorimetric Assay of Levodopa

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Abstract □ A new colorimetric assay method for levodopa, based on the reaction with isonicotinic acid hydrazide in alkaline medium, is reported. Data on precision, accuracy, and specificity are also given. The method, owing to its simplicity, is particularly suited for the routine purity control of levodopa.

Keyphrases □ Levodopa—analysis, colorimetric reaction with isonicotinic acid hydrazide □ Colorimetry—analysis, levodopa □ Isonicotinic acid hydrazide—reagent in colorimetric analysis of levodopa

Interest in (−)-3-(3,4-dihydroxyphenyl)-L-alanine (levodopa) shifted from the pure biochemical domain to the therapeutic field after Cotzias *et al.* (1) gave some evidence of its clinical efficacy against Parkinson's disease when administered orally at dosages of 5–8 g./day over an extended period. Due to this massive dosage, analytical methods for assaying the drug should be specific enough to reveal any by-product which, even in a slight amount, could give rise to unsuspected side effects. Existing methods for assaying levodopa are both numerous and sensitive, *e.g.*, fluorometric (2, 3), but do not satisfy the above-mentioned purpose. Some methods, reported as specific, either require previous separation from interfering substances (4) or are based on color reactions given by the phenolic function of the molecule (5–7). These methods present some disadvantages for routine application due to factors governing the color reaction which render the standardization of the analytical procedure rather cumbersome. Hence, the development of simple colorimetric methods specific for the catecholic function was considered.

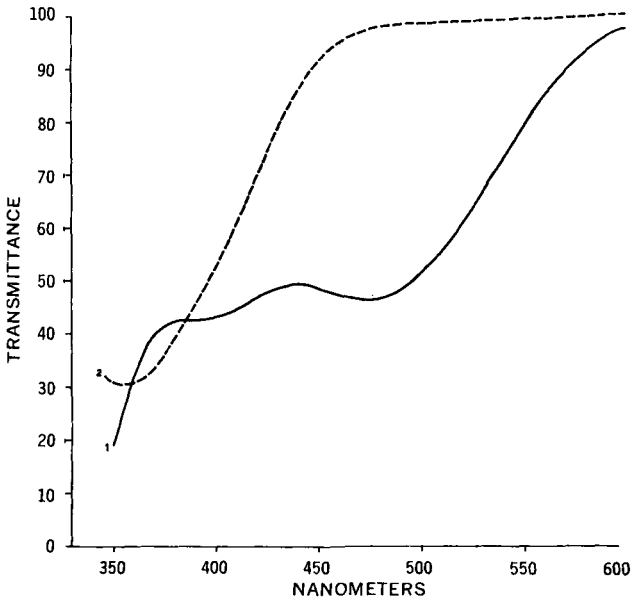

Figure 1—*Absorption curves of levodopa after colorimetric reaction with I as such (1) and after acidification at pH 2.0 (2).*

Hashmi *et al.* (8) recently reported that isonicotinic acid hydrazide (I) is capable of giving a characteristic color when reacted with catechol in an alkaline medium. On the basis of this report, we applied the color reaction to levodopa. The absorptiometric properties of the chromophore as well as the influence of the different parameters (*i.e.*, concentration of reagents, temperature, and most common oxidizing agents) were studied to determine the optimal conditions for the assay procedure. The interference of a number of extraneous substances upon the determination of levodopa with the present method was examined, bearing in mind the possible preparatory route, both from natural (9–11) and synthetic (12) origins. The results also were compared with those obtained using the colorimetric method reported by Heinrich and Schuler (6). This method, considered to be more acceptable than others, is based on the reaction of levodopa with 2-chloro-4-nitrobenzenediazonium naphthalene-1(or 2)-sulfonate (II).

EXPERIMENTAL[1]

All reagents were of analytical grade or comparable purity. The purity of levodopa, obtained from a natural source and repeatedly crystallized from water, was assessed by TLC and by the constancy of significant physicochemical characteristics (UV absorbance, nonaqueous titration, melting point, and rotatory power).

Assay Procedure (Method I)—*Calibration Curve*—Prepare six standard solutions by dissolving 10, 20, 25, 35, 40, and 50 mg., accurately weighed, of pure levodopa into 1000-ml. volumetric flasks with 1 ml. of 1 *N* HCl and 300 ml. of water; shake vigorously. When dissolved, dilute to volume with water. Pipet a 10.0-ml. aliquot into a 50-ml. flask and add, in order, 10.0 ml. of aqueous I (0.05% w/v), 30.0 ml. of water, and 1.0 ml. of 0.3 *N* NaOH. Shake vigorously and heat for 30 min. at 35 ± 1°. Cool to room temperature and measure the absorbance at 475 nm. in a 1-cm. cell against a reagent blank processed in exactly the same way. Plot the absorbances (mean values of three replicates) against the concentrations (micrograms per milliliter) of the standard solutions.

Sample Preparation and Assay—Add 1.0 ml. of 1 *N* HCl to an accurately weighed sample containing 10–50 mg. of levodopa, shake vigorously, and then add 300 ml. of water. Shake for 20 min. at room temperature, filter into a 1000-ml. volumetric flask, and dilute to volume with water. Proceed as described for the calibration curve. Calculate the titer of the sample from the calibration curve.

Factors Influencing Color Reaction—The absorption spectra (Fig. 1) were recorded after completion of the colorimetric reaction, as described for the assay procedure.

[1] The absorption spectra were recorded on a Beckman DK-2 instrument. The colorimetric readings were taken on a Beckman DU spectrophotometer.

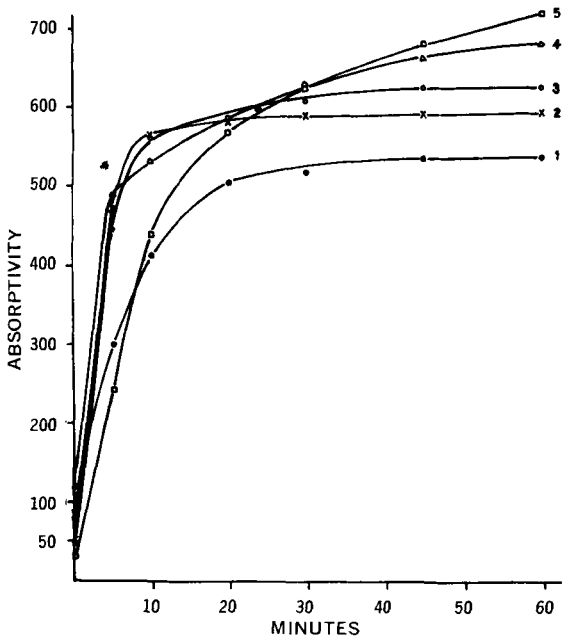

Figure 2—*Influence of time on color formation, at different pH values, at 35°. Key: 1, pH 12.8; 2, pH 12.0; 3, pH 11.7; 4, pH 11.0; and 5, pH 10.5; corresponding in the assay procedure to 1 ml. of NaOH of 5, 1, 0.3, 0.1, and 0.05* N, *respectively.*

All of the data for curves of Figs. 2–5 were obtained following basically the assay procedure, from samples of about 30 mg. of pure levodopa, by varying alkali concentration at 35° (Fig. 2) and 50° (Fig. 3).

In studying the influence of oxygen upon chromophore development (Fig. 4), care was taken to prevent evaporation during the gas-bubbling by presaturating the gas stream by passage through a blank solution. The influence of other oxidizing agents is shown in Fig. 5. Ceric sulfate, potassium ferricyanide, and ammonium persulfate were added to the reaction mixture by dissolving them in the 30 ml. of water, according to the assay procedure. The molar ratios with levodopa were 10:1 for ceric sulfate and potassium ferricyanide

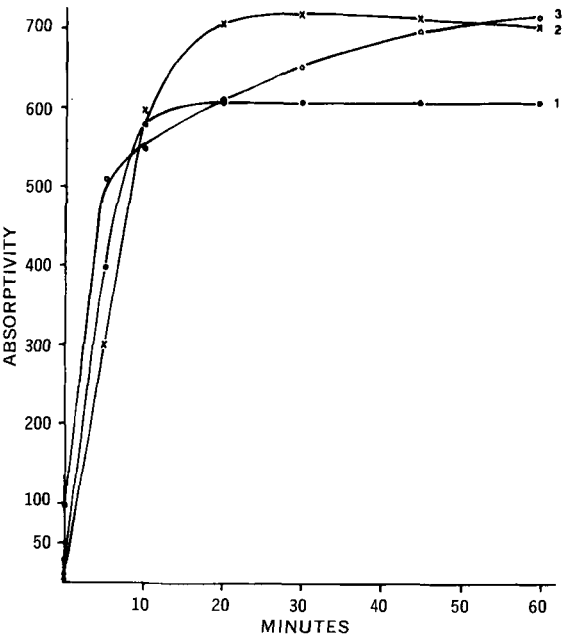

Figure 3—*Influence of time on color formation, at different pH values, at 50°. Key: 1, pH 12.0; 2, pH 11.7; and 3, pH 11.0; corresponding in the assay procedure to 1 ml. of NaOH of 1, 0.3, and 0.1* N, *respectively.*

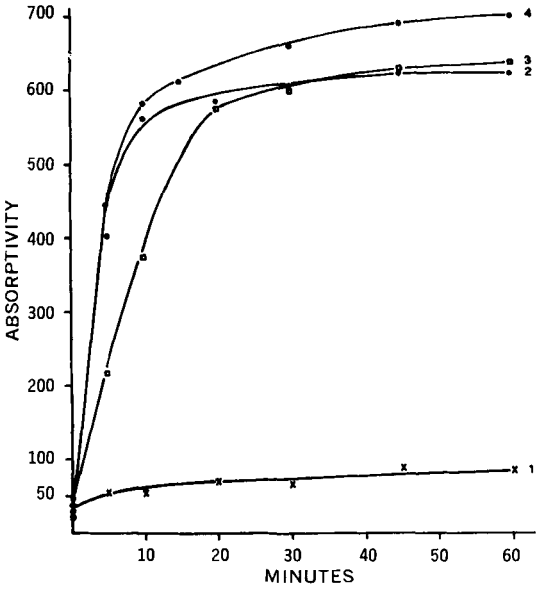

Figure 4—*Influence of oxygen on color formation. Key: 1, nitrogen bubbling; 2, standard conditions (atmospheric air); 3, air bubbling; and 4, oxygen bubbling.*

and 5:1 for ammonium persulfate. Manganese dioxide was reacted as follows: 25 mg. of manganese dioxide was shaken in 500 ml. of water, the suspension was filtered, and 30 ml. of the filtrate was utilized for the reaction. The trials with ceric sulfate were carried out using 1 *N* NaOH instead of 0.3 *N* NaOH because of the acidity of ceric sulfate. The precipitate formed under this condition was filtered off before taking colorimetric readings.

Linearity Studies—The curves (Fig. 6) were drawn by plotting the concentrations of the standard solutions (abscissa) *versus* the absorbance values at 35 and 50°, following the assay procedure.

Precision—The precision of the assay procedure at an intermediate point of the calibration curve was assessed with 40-mg. samples of pure levodopa, treated according to the assay procedure.

Accuracy—The recovery trials were performed by adding known quantities (by weight) of pure levodopa to three different samples of crude levodopa from natural (A) and synthetic (B) sources. The analyses were carried out before and after addition according to the assay procedure.

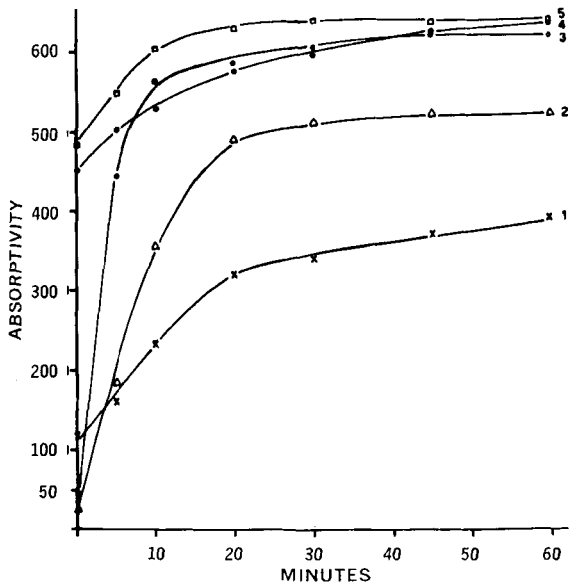

Figure 5—*Influence of oxidizing agents on color formation. Key: 1, ceric sulfate; 2, ammonium persulfate; 3, assay procedure conditions; 4, potassium ferricyanide; and 5, manganese dioxide.*

Figure 6—*Calibration curves. Key: 1, 35°; and 2, 50°. Abscissa values refer to the levodopa concentrations of the standard solutions.*

Interferences—The influence of possible interfering substances was determined by adding increasing amounts (by weight) of the selected compounds to pure levodopa (35 mg.) and performing the analysis according to the assay procedure. A second set of parallel trials was carried out following Heinrich and Schuler's (6) method (Method II) by reading absorbances at 415 nm.

RESULTS AND DISCUSSION

A characteristic orange color develops when levodopa is reacted with isonicotinic acid hydrazide (I) in alkaline aqueous medium, with an absorption maximum at 475 nm. The course of the color reaction was studied as a function of the temperature, the alkali concentration, and the presence of oxidizing agents. Based on preliminary trials, the concentration of I was established at a level exceeding the minimum amount and was kept constant. The absorption spectra of the chromophore under the assay procedure conditions and adjustment of pH (pH 2.0) after color development are given in Fig. 1. Orientative tests on the influence of temperature on chromophore formation led to the conclusion that temperatures below 35° lowered the reaction rate significantly and temperatures above 50° decreased the stability of the chromophore. From examination of the curves in Figs. 2 and 3, it appears that, both at 35 and 50°, a sodium hydroxide concentration of 0.3 *N* is a good compromise for the intensity and steadiness of the color. Under such conditions, a reaction time of between 20 and 30 min. seems most appropriate. The use of 1 *N* NaOH appears, from these curves, competitive with 0.3 *N* NaOH. It gives a steady color, although less intense, in a shorter period. However, the 1 *N* NaOH was not employed due to the bad reproducibility of the data and to a marked deviation from the linearity for concentrations of levodopa above 35 mcg./ml.

In Fig. 4, comparative graphs show that chromophore development is depressed if atmospheric oxygen is excluded. No appreciable improvement is obtained by bubbling air, but some increase of the color intensity is observed when atmospheric air is replaced by oxygen. Under the assay procedure conditions, no appreciable influence on the color development was found by varying the atmospheric oxygen available in the reaction flask or the agitation speed and time.

The data of the exploratory trials with oxidizing agents are shown in Fig. 5. Ceric sulfate, ammonium persulfate, and potassium ferricyanide do not provide results comparable with those obtained by the simple atmospheric oxygen, probably because of degradation of levodopa or of the chromophore which is formed. On the contrary, manganese dioxide seems slightly more effective than atmospheric oxygen. It is probably worthwhile studying the possibility of utilizing manganese dioxide in this reaction, in spite of some complications associated mainly with the difficulty of standardizing it as a reagent.

Table I—Accuracy Data

	Sample			Pure Levodopa Added, mg.	Total Levodopa Calculated, mg.	Total Levodopa Found, mg.	$\frac{\text{Found}}{\text{Calc.}} \times 100$
	Source	Percent Titer[a]	Weight, mg.				
A_1	Natural	91.8	30.30	10.96	38.77	38.50	99.30
			20.64	21.16	40.11	39.67	98.90
			10.30	32.03	41.49	41.08	99.01
A_2	Natural	63.8	30.60	15.55	35.07	35.09	100.05
			20.90	20.26	33.59	33.42	99.49
			20.50	31.23	44.31	43.70	98.62
B	Synthetic	36.0	20.45	10.52	17.88	18.16	101.56
			20.80	26.78	34.27	34.16	99.68
			10.25	31.25	34.94	33.91	97.05

[a] Assessed by Method I.

Table II—Influence of Addition of Different Compounds on Colorimetric Readings of Levodopa

Compound	Percent by Weight Addition to Levodopa	Method I—Absorbance Values (475 nm.) Before Addition	After Addition	Percent Deviation	Method II (6)—Absorbance Values (415 nm.) Before Addition	After Addition	Percent Deviation
L-Tyrosine	5.0	0.440	0.435	−1.14	0.345	0.346	+0.29
	20.0		0.441	+0.23		0.349	+1.16
	50.0		0.449	+2.05		0.354	+2.61
3-Amino-L-tyrosine	5.0	0.440	0.443	+0.68	0.345	0.370	+7.25
	20.0		0.442	+0.45		0.393	+13.91
	50.0		0.441	+0.23		0.436	+26.38
L-Tryptophan	5.0	0.440	0.443	+0.68	0.345	0.347	+0.58
	20.0		0.441	+0.23		0.342	−0.87
	50.0		0.439	−0.23		0.367	+6.38
Resorcinol	5.0	0.440	0.449	+2.05	0.345	0.354	+2.61
	20.0		0.432	−1.82		0.430	+24.64
	50.0		0.439	−0.23		0.620	+79.71
Hydroquinone	5.0	0.440	0.469	+6.59	0.345	0.341	−1.16
	20.0		0.497	+13.00		0.350	+1.45
	50.0		0.644	+46.36		0.363	+5.22

The application of the color reaction to the levodopa assay was considered, taking into account the linearity, precision, and accuracy of the method. The calibration graphs in Fig. 6 show that for pure levodopa concentrations ranging from 10 to 50 mcg./ml., the Lambert–Beer law is substantially obeyed, especially at 35°. The precision of the standard procedure was ascertained on six replicate analyses. The relative standard deviation was 0.59 (mean value of absorbance 0.503, range 0.006), and it was calculated according to the recommended guidelines (13). In practice, it was found that there is no need to check the calibration curve of the standard procedure unless the batch of I is changed. The accuracy trials were carried out on different samples of levodopa, at various purity levels, from natural (A) and synthetic (B) sources. Table I shows that the recovery data are satisfactory for both type of samples.

The influence of possible interfering substances in Assay Method I is summarized in Table II. Substances other than aminoacids, such as hydroquinone and resorcinol, were selected on the basis of their functional similarity to some by-products of natural levodopa (9–11). The potential interfering substances with levodopa of synthetic origin were those directly involved in the synthetic process, namely tyrosine and 3-aminotyrosine. For comparison purposes, Table II also reports the data, obtained on the same samples, using Method II (6). Method II was chosen due to its simplicity and accuracy. The examination of the data demonstrates that Method I is more specific. In fact, the only appreciable interference using Method I is for 1,4-hydroquinone; with Method II, interferences are more numerous and are particularly relevant for 3-aminotyrosine and resorcinol.

CONCLUSIONS

The chromophore produced in the reaction between levodopa and I in alkaline medium varies as a function of pH. Under acidic conditions the absorption maximum shifts from 475 to 355 nm. (Fig. 1). The color variation can be reverted after realkalinization of the medium. Investigations on the presence of a reversible equilibrium of the different forms of the chromophore and on the consequent presence of ionizable functions associated to it are the subject of further studies to acquire information for structure elucidation. Preliminary oxidation by atmospheric oxygen seems to be the necessary step for color formation (Fig. 4).

The application of the color reaction, as reported in the present paper, seems to be particularly suitable for assaying levodopa in routine analysis due to its precision and accuracy. The simplicity of the analytical procedure, moreover, allows this method to be used in automated analysis during the production process of levodopa for clinical uses (14).

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Acetaminophen Colorimetry as 2-Nitro-4-acetamidophenol

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Abstract □ A method previously proposed for assay of acetaminophen and its tablet formulations was found to give comparable results with three commercial elixir formulations. The procedure was adapted to an automated assay apparatus for analysis of the drug in tablets containing acetaminophen alone and formulated with other drugs. Relative standard deviations of the automated method were about 1.4%.

Keyphrases □ Acetaminophen tablets and elixir—colorimetric analysis, as 2-nitro-4-acetamidophenol □ 2-Nitro-4-acetamidophenol—colorimetric derivative of acetaminophen, analysis in tablets and elixir □ Colorimetry—analysis, acetaminophen in tablets and elixir, as 2-nitro-4-acetamidophenol

Chafetz *et al.* (1) described a simple and selective colorimetric assay for acetaminophen and its tablet preparations in which the drug is measured as 2-nitro-4-acetamidophenol after reaction with nitrous acid. Since the procedure requires only the successive addition of reagents, it was predicted that it would be easily adaptable to an automated assay apparatus. The realization of this prediction is described, along with data obtained by extending the colorimetric method to the assay of acetaminophen elixir.