Determination of Purity of Levodopa and Levodopa Products by GLC

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Abstract □ A simple GLC procedure for the detection and quantitation of possible impurities in levodopa raw material and commercial dosage forms is presented. The sample is reacted with *N,O*-bis(trimethylsilyl)acetamide in acetonitrile at ambient temperature for 30 min, and the silyl derivatives are separated on a methyl silicone column under isothermal conditions. Peak areas are measured using an electronic digital integrator of wide input signal range capacity. The results obtained by applying the procedure to the analysis of several multicomponent simulated mixtures were in good agreement with theoretical values. Only 3-methoxy-4-hydroxyphenylalanine was detected as a contaminant in each of the five commercial products examined.

Keyphrases □ Levodopa bulk, capsules, and tablets—GLC analysis, 3-methoxy-4-hydroxyphenylalanine detected in commercial products □ 3-Methoxy-4-hydroxyphenylalanine—detected as impurity in commercial levodopa capsules and tablets, GLC □ GLC—analysis, levodopa bulk, capsules, and tablets

Since the institution of levodopa (L-3,4-dihydroxyphenylalanine) as a potent agent for the long-term therapy of Parkinson's disease, several methods have been proposed for its quantitative determination in the presence of its metabolic products in biological media. Urinary and plasma levodopa levels were measured by derivatization of the purified extract of the drug to the *N*-trifluoroacetyl-*n*-butyl ester followed by GLC using electron-capture detection (1). The major metabolite in plasma, 3-methoxy-4-hydroxyphenylalanine, was determined with a modified amino acid analyzer technique (2), while Curzon *et al.* (3) and Sharpless *et al.* (4) quantitated both this compound and the parent levodopa in plasma and in cerebrospinal fluid (5) by fluorometry. A colorimetric assay method for levodopa based on its reaction with isonicotinic acid hydrazide (isoniazid) in alkaline medium was also reported (6). However, none of these methods was really designed for the routine purity screening of the drug and of its pharmaceutical products.

In view of the several routes of synthesis currently being used for the manufacture of levodopa, a number of possible by-products, with unknown biological activity, could be associated with the drug. This, coupled with the shortcomings of other methods, points to a clear requirement for an analytical procedure that would embody the virtues of sensitivity, rapidity, specificity, and amenability to a multiple-analysis setup for the quantitative estimation of levodopa contaminants.

Since several authors reported facile quantitative silylation and successful GLC of amino acids (7–9) and of catecholamines (10, 11), it seemed reasonable to expect levodopa and its intermediates, which, in general, combine the structural features of both amino acids and catechols, to undergo similar transformations to yield silylated products which could be eluted easily.

This paper reports a rapid GLC method suitable for the routine quality control detection and quantitation of tyrosine, 3,4-dimethoxyphenylalanine, 3-methoxy-4-hydroxyphenylalanine, 3-hydroxy-4-methoxyphenylalanine, and 3,4,6-trihydroxyphenylalanine (6-hydroxydopa) in the levodopa raw material and in commercial products. The procedure involves silylation of the drug or drug mixture with *N,O*-bis(trimethylsilyl)acetamide in acetonitrile at room temperature for 30 min prior to isothermal chromatography on a methyl silicone column and measurement of the component peak areas by electronic digital integration.

EXPERIMENTAL

Materials—The following were used: 3,4-dimethoxyphenylalanine[1], 3-hydroxy-4-methoxyphenylalanine[1], L-tyrosine[2], levodopa[3], L-3-methoxy-4-hydroxyphenylalanine (monohydrate)[4], and DL-6-hydroxydopa (monohydrate)[5].

Preparation of Samples—*Response Factor Calibration Mixtures*—For each intermediate, varying quantities ranging from 1 to 4 mg were accurately weighed into four separate 10-ml volumetric flasks, each containing an accurately known amount of levodopa (50–70 mg).

Simulated Mixtures—Appropriate amounts of each desired component (0–4 mg) were accurately weighed into a 10-ml volumetric flask along with a precisely known quantity of levodopa (50–70 mg).

Commercial Products—The formulations were sampled as follows: 10 capsules were emptied and their contents were weighed and thoroughly mixed. In the case of the tablets, 10 were selected at random, weighed, and finely powdered. An amount of powder equivalent to about 60–70 mg of levodopa was accurately weighed into a 10-ml volumetric flask.

Derivatization Procedure—Each sample of drug or drug mixture was treated with *N,O*-bis(trimethylsilyl)acetamide reagent[6] (2.5 ml), diluted to volume with "silylation grade" acetonitrile[6], and allowed to stand for 30 min in the dark at room temperature with occasional shaking. At the end of this period, the oily globules dissolved to give a clear homogeneous solution or, in the case of the commercial products, a clear supernate.

Gas Chromatography—Five microliters of solution was injected into a gas chromatograph[7] (flame-ionization detector), fitted with a 5% OV-101[6] on Chromosorb W/HP[8] (100–120 mesh) U-shaped glass column [1.82 m (6 ft) × 6 mm o.d.] preconditioned at 265° for 18 hr. The support was coated using a Hi-Eff[9] fluidizer maintained at 150° for 2 hr with a suitable nitrogen flow to ensure gentle yet thorough drying of the packing material. Temperatures were: column, 170° (isothermal); injection port, 255°; and detector, 260°. Gas flows were: nitrogen, 60 ml/min; hydrogen, 40 ml/min; and air, 380 ml/min.

The detector signal was fed to a continuous balance dual pen,

[1] Courtesy of Norwich Pharmacal Co.
[2] Wyeth Inc.
[3] Monsanto Chemical Co. This material was found by GLC to contain about 0.27% of 3-methoxy-4-hydroxyphenylalanine, but due allowance was made for this in the calculation of the results.
[4] Monsanto Chemical Co.
[5] Chemical Dynamics Corp.
[6] Pierce Chemical Co.
[7] Bendix series 2500.
[8] Chromatographic Specialties Ltd.
[9] Applied Science Laboratories.

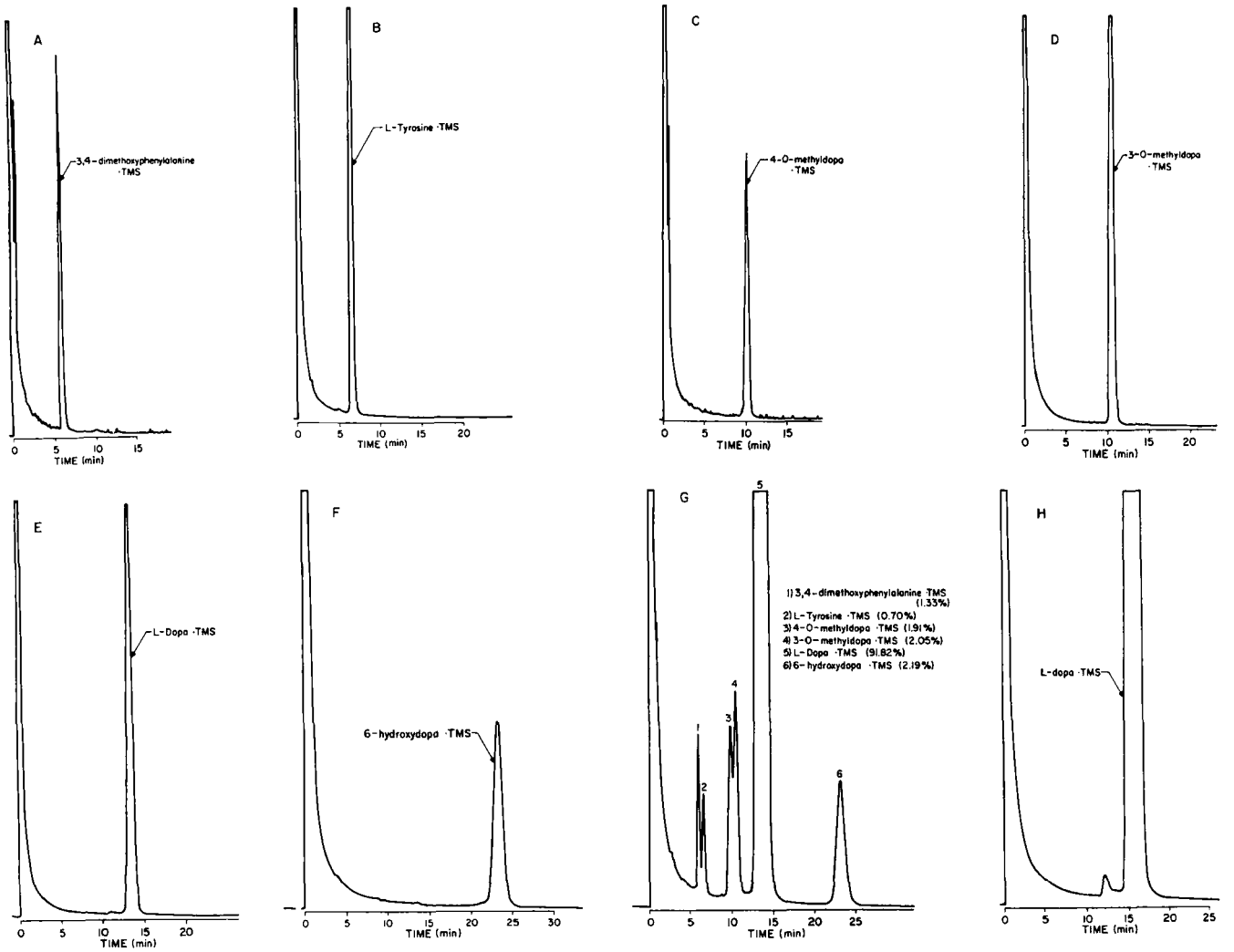

Figure 1—*Gas chromatograms of the trimethylsilyl (TMS) derivatives of levodopa and its possible contaminants. Key: A, pure 3,4-dimethoxyphenylalanine; B, pure* L-*tyrosine; C, pure 3-hydroxy-4-methoxyphenylalanine (4-O-methyldopa); D, pure 3-methoxy-4-hydroxyphenylalanine (3-O-methyldopa); E, pure levodopa* (L-*dopa); F, pure 6-hydroxydopa; G, simulated mixture of components A–F; and H, commercial levodopa product (500-mg capsule) contaminated with 3-O-methyldopa (about 0.25%).*

1-mv recorder[10] with variable chart speed set at 0.508 cm (0.20 in.)/min and connected to an automatic printout electronic digital integrator[11] with a selected input signal range of 0–100 mv. The attenuation was kept constant at 20×10^{-11} afs.

Calculations—The response factor (relative to levodopa) for an impurity I is given by Eq. 1:

$$f_I = \frac{\text{area ratio } (I_s/\text{levodopa}_s)}{\text{weight ratio } (I/\text{levodopa})} = \frac{Y_{I_S}}{X_I} \qquad \text{(Eq. 1)}$$

where the subscript s denotes the silyl derivative. For the computation of the impurity levels in simulated mixtures and commercial dosage forms, the weight ratio X_I is determined by Eq. 2:

$$X_I = \frac{Y_{I_S}}{f_I} \qquad \text{(Eq. 2)}$$

and is expressed as a weight unit fraction of the actual levodopa content. For a mixture consisting of a number of impurity components, $I, J \ldots Z$, the total recovered relative weight units, taking levodopa as unity, is expressed in Eq. 3:

$$1 + X_I + X_J + \ldots + X_Z = T \qquad \text{(Eq. 3)}$$

The level of impurity I can be calculated as a percentage of the total weight of drug found:

$$I = \frac{100X_I}{T} \qquad \text{(Eq. 4)}$$

Similarly:

$$\%J = \frac{100X_J}{T} \qquad \text{(Eq. 5a)}$$

$$\%Z = \frac{100X_Z}{T} \qquad \text{(Eq. 5b)}$$

and:

$$\%\,\text{levodopa} = \frac{100}{T} \qquad \text{(Eq. 5c)}$$

RESULTS AND DISCUSSION

In this study, the most efficacious procedure for the silylation of levodopa and its by-products involved the use of *N,O*-bis(trimethylsilyl)acetamide in acetonitrile. In this system, complete derivatization of levodopa was achieved in about 15 min at room temperature, although it was preferable to allow about 30 min since L-tyrosine and 3,4-dimethoxyphenylalanine, which might be present as contaminants, appeared to require a somewhat longer reaction period. Other solvents such as pyridine or dimethylformamide could be used but, since more time was needed (about 2 hr), they were considered to be less feasible as media for a routine

[10] Minneapolis-Honeywell, Elektronik 19 strip-chart recorder.
[11] Kent, model Chromalog 2.

Table I—GLC Data on Levodopa and Possible Contaminants

Compound	Molecular Weight (Fully Silylated Derivative)	Retention Time of Derivative, min	Relative[a] Retention Time of Derivative	Relative[a] Response Factor of Derivative
3,4-Dimethoxyphenylalanine	369.58	6.15	0.447	0.604 (±5.3%)
L-Tyrosine	397.70	6.80	0.495	0.886 (±1.8%)
3-Hydroxy-4-methoxyphenylalanine	427.73	10.25	0.745	0.754 (±3.4%)
3-Methoxy-4-hydroxyphenylalanine	427.73	10.90	0.793	0.726 (±3.0%)[b]
Levodopa	485.88	13.75	1.000	1.000
6-Hydroxydopa	574.05	23.40	1.702	0.919 (±2.4%)[b]

[a] Relative to levodopa. [b] Calculated for the anhydrous form.

Table II—Detector Linearity Data for Impurity Concentrations[a] in the Range of About 0.1–1%

Compound	Concentration, %	Area Counts	Concentration, %	Area Counts	Concentration, %	Area Counts	Concentration, %	Area Counts	Concentration, %	Area Counts
3,4-Dimethoxyphenylalanine	1.45	13,781	0.73	6911	0.29	2703	0.15	1287	0.07	593
L-Tyrosine	1.07	11,139	0.54	5612	0.21	2208	0.11	1058	0.06	496
3-Hydroxy-4-methoxyphenylalanine	1.62	14,364	0.81	7093	0.32	2796	0.16	1427	0.08	657
3-Methoxy-4-hydroxyphenylalanine	1.96	16,084	0.98	8211	0.39	3370	0.20	1581	0.10	743
6-Hydroxydopa	1.63	16,499	0.82	8147	0.33	3255	0.16	1501	0.08	732

[a] Calculated as a percentage of total weight of drug found.

Table III—Analysis of Simulated Mixtures

Compound	1		2		3		4		5	
	% Calc.	% Found	% Calc.	% Found	% Calc.	% Found	% Calc.	% Found	% Calc.	% Found
3,4-Dimethoxyphenylalanine	1.55	1.45	2.42	2.27	3.18	2.93	—	—	1.33	1.12
L-Tyrosine	0.66	0.57	2.36	2.33	—	—	1.23	1.07	0.70	0.51
3-Hydroxy-4-methoxyphenylalanine	—	—	2.38	2.13	—	—	2.37	2.14	1.91	1.62
3-Methoxy-4-hydroxyphenylalanine	3.83	4.09	—	—	2.38	2.12	—	—	2.05	1.96
Levodopa	93.96	93.89	92.84	93.27	89.33	89.82	94.56	95.16	91.82	92.81
6-Hydroxydopa	—	—	—	—	5.11	5.13	1.84	1.63	2.19	1.98

screening program. Other silylation reagents such as hexamethyldisilazane or trimethylsilylimidazole in toluene were also generally satisfactory, but these systems required a number of hours at reflux temperature before complete derivatization was effected. Although the structures of the derivatives[12] obtained with *N,O*-bis(trimethylsilyl)acetamide were not elucidated, the preponderance of evidence in the literature (7–11) suggests that, with this reagent and its fluorinated analog, silylation at all vulnerable sites to give the silylamine–silyl ester normally occurs with amino acids. However, it is known that *N*-silylated derivatives, like other silylamines, hydrolyze very readily and must be carefully protected from moisture. Therefore, it is conceivable that, while the fully *N,O*-silylated compound might be formed *in situ* under the present conditions, it suffers hydrolysis in the syringe or at the injection port to afford the fully *O*-silylated product (with free amino group). In any case, with the proposed method, one sharp GLC peak was obtained for the silyl derivative of each intermediate and of levodopa itself (Figs. 1A–1F). An unambiguous separation of the components of a synthetic mixture of six compounds is shown in Fig. 1G.

As well as conferring increased volatility and thermal stability, the replacement of the active hydrogen atoms by trimethylsilyl groups has the additional effect of eliminating, or at least substantially reducing, the polarity of the molecule. Intermolecular adsorptive interactions between relatively nonpolar solutes and nonpolar stationary phases, such as the methyl silicone used in this study, are rather weak so that one can usually safely assume a strictly molecular weight-dependent elution order. That this is the case with silylated hydroxyphenylalanines on OV-101 is clearly illustrated in Table I, which lists the retention time and relative (to levodopa) retention time of each compound in ascending order of molecular weight calculated as the fully *N,O*-silylated derivative. For the chromatography of these compounds, it was necessary to maintain the injection port temperature above 250°. At lower temperatures, incomplete elution of the less volatile derivatives occurred, resulting in reduced responses and reappearance of these peaks as ghosts or breakdown products which could have invalidated subsequent analyses.

Each response factor (relative to levodopa) in Table I represents the average of four determinations at varying weight ratios of compound to levodopa. The quantities weighed out were selected to represent an impurity range of about 1–10%, even though levels above 1% would not normally be encountered in the analysis of the raw material and of commercial dosage forms. To establish the detector linearity below 1%, appropriate dilutions were made with acetonitrile to give impurity concentrations down to about 0.1%. The area counts of the peaks were found to be proportional to those obtained with the higher levels (Table II). It can be reasonably assumed that the limit of detectability for each component is at least 0.1%. The precision of each measured value is expressed in terms of its coefficient of variation, which is given

[12] Characterization of these compounds using mass spectrometry and other selected techniques is presently in progress.

Table IV—Determination of the Impurity Levels in Commercial Dosage Forms

Sample Number and Dosage Form	Manufacturer	Dosage Level, mg/unit	Percent Impurity[a]
I, capsules	A	250	0.99
	A	250	0.97
II, capsules	A	500	0.35
	A	500	0.35
III, capsules	B	500	0.25
	B	500	0.25
IV, tablets	B	500	0.23
	B	500	0.26
V, capsules	C	250	0.20
	C	250	0.21

[a] Only one impurity, 3-methoxy-4-hydroxyphenylalanine, was detected and is expressed as a percentage of total drug found.

beside its respective response factor value. Theoretically, it is evident that the greater the increase in molecular weight from the underivatized compound to the trimethylsilyl product, relative to the analogous change effected with levodopa, the greater is the detector response. For example, 3,4-dimethoxyphenylalanine with only two available sites for trimethylsilyl attack (carboxylic and amino functions) would give less derivatized product per unit weight and, therefore, a lower response factor than 3-methoxy-4-hydroxyphenylalanine, which has an additional phenolic site, or than levodopa, which can incorporate four trimethylsilyl groups. In this study, the value obtained for 6-hydroxydopa was less than that expected on the basis of five susceptible sites for trimethylsilyl attack. The reason for this anomaly was not investigated but is probably related to some interplay between instrumental parameters and elution characteristics of the compound.

The results of the GLC analyses of five simulated mixtures are given in Table III. Each quoted experimental value represents the average of duplicate injections and is expressed as a percentage of total amount of drug recovered. The theoretical percentages were calculated on a similar basis. Even with mixtures having closely spaced component peaks, such as 3,4-dimethoxyphenylalanine and tyrosine or 3-methoxy-4-hydroxyphenylalanine and 3-hydroxy-4-methoxyphenylalanine, good accuracy was achieved, although in some cases slight underestimations can be noted. While each intermediate examined is indeed a possible contaminant in levodopa samples, more than one impurity was never encountered in any given sample of raw material or finished dosage form. Thus, the inclusion of simulated Mixture 5 (Fig. 1G) was not intended to illustrate a practical situation but simply to demonstrate the feasibility of the procedure as a reliable qualitative and quantitative monitor of purity.

The results of the analyses of five commercial products in both capsule and tablet forms from three manufacturers at two dosage levels are presented in Table IV. A typical chromatogram of such a sample, after silylation of an aliquot of the powder with *N,O*-bis(trimethylsilyl)acetamide reagent in acetonitrile, is shown in Fig. 1H. As readily observed, 3-methoxy-4-hydroxyphenylalanine, but no other impurity, was detected in each product. Of these, Product I had the highest level (about 1%). The three- to fivefold difference existing between the 3-methoxy-4-hydroxyphenylalanine levels in Product I and the four remaining preparations again raises the seemingly ubiquitous question of the importance of sound pharmaceutical workmanship. While the presence of the latter contaminant will not result in a significant loss of therapeutic activity, it may represent a potential hazard (3), especially since levodopa is administered in high doses and chronically.

CONCLUSIONS

There appears to be no entirely adequate method for the detection and quantitation of the impurities in levodopa raw material and commercial dosage forms. To our knowledge, previously proposed GLC methods utilized conversion of the drug to its trifluoroacetylbutyl derivative but, in our experience, this process is not only time consuming but gives rise to a number of reaction by-products which tend to vitiate the area measurements of the relevant peaks. Therefore, there seemed to be a need for a more specific and accurate method suitable for routine screening of the drug, simpler in its manipulative aspects, and, perhaps, adaptable to automation. The present GLC procedure should represent a step forward in this direction.

REFERENCES

(1) K. Imai, N. Arizumi, M. Wang, S. Yoshiue, and Z. Tamura, *Chem. Pharm. Bull.*, **20**, 2436(1972).

(2) J. H. Fellman, J. R. Joyce, and J. J. Strandholm, *Clin. Chim. Acta*, **32**, 313(1971).

(3) G. Curzon, B. Kantamaneni, and J. Trigwell, *ibid.*, **37**, 335(1972).

(4) N. S. Sharpless, M. D. Muenter, G. M. Tyce, and C. A. Owen, Jr., *ibid.*, **37**, 359(1972).

(5) N. S. Sharpless and D. S. McCann, *ibid.*, **31**, 155(1971).

(6) N. Maggi and A. Cometti, *J. Pharm. Sci.*, **61**, 924(1972).

(7) C. W. Gehrke, H. Nakamoto, and R. W. Zumwalt, *J. Chromatogr.*, **45**, 24(1969).

(8) C. W. Gehrke and K. Leimer, *ibid.*, **53**, 201(1970).

(9) A. E. Pierce, "Silylation of Organic Compounds," Pierce Chemical Co., Rockford, Ill., 1968, pp. 218–243.

(10) P. Capella and E. C. Horning, *Anal. Chem.*, **38**, 316(1966).

(11) P. F. G. Boon and A. W. Mace, *J. Pharm. Pharmacol. Suppl.*, **21**, 49S(1969).

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