syrup had very little effect while the higher salt concentrations caused II to "oil out" extensively (Table III). The oily precipitation was a salting-out phenomenon in which the monomeric solubility of II was reduced (11). Demicellization also may have occurred due to electrostatic suppression of the charge on the micelles by the increased ionic environment.

Effect of Salts on Viscosity—Inorganic salts also affect the viscosity of clindamycin palmitate hydrochloride solutions. Salt concentrations of ~400–800 mg/liter in syrup solutions of II caused the viscosity to increase with a fairly sharp peak. When concentrations were converted to anionic strength, as was done for the critical oiling-out point, a very close grouping of the peaks was obtained (Fig. 5). The anionic strengths of the peaks ranged from 0.011 to 0.013, the same range in which haziness or turbidity started in II solutions. At concentrations above this critical range, the viscosity was back to baseline levels well below the oiling-out point.

The chlorides were particularly effective in raising viscosity. Their peaks exceeded 1600 cps whereas the carbonate and sulfates produced more modest maxima. The common ion effect may have been dominant here, suppressing the dissociation of the II hydrochloride salt and thereby reducing the electrostatic repulsion forces between micelles (and also molecules). This can lead to increased intermicellar and intermolecular association forming flow-resistant aggregates or structures.

Following this hypothesis, as the salt concentrations increased, structure formation by II molecules increased until precipitation began, as evidenced by turbidity. The viscosity then peaked out and declined as the drug precipitated out in oily microdroplet form. Well beyond the peak viscosity, the oil droplets coalesced to form visible oil particles, at which point the precipitation was nearly complete.

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Reversed-Phase High-Performance Liquid Chromatographic Investigation of Levodopa Preparations I: Amino Acid Impurities

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Abstract □ A reversed-phase high-performance liquid chromatographic procedure with UV detection at 280 nm is presented for the determination of 6-hydroxydopa, tyrosine, 3-*O*-methyldopa, and other trace amino acid impurities in levodopa preparations. The method is fast and sufficiently sensitive to determine trace impurities at 0.1% of the levodopa concentration with a relative standard deviation of 4–6%. The trace impurities can be estimated at ≤0.01%.

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

Current USP requirements (1) limit the impurities in levodopa [L-3-(3,4-dihydroxyphenyl)alanine] preparations to 0.1% of the levodopa concentration for 6-hydroxydopa [3-(2,4,5-trihydroxyphenyl)alanine] and to 0.5% for 3-*O*-methyldopa [3-methoxytyrosine or 3-(4-hydroxy-3-methoxyphenyl)alanine]. The designated USP method (1) for the estimation of these impurities is a TLC technique with a cellulose stationary phase and an acetic acid–butanol–methanol–water mobile phase. However, this laboratory found that the USP method suffers from a rather lengthy analysis time (>5 hr) and from the fact that the 6-hydroxydopa spot is not easily visualized. In addition, tyrosine, a frequently observed levodopa impurity, yields a spot with nearly the same R_f as 3-*O*-methyldopa. For these reasons, a quicker, simpler, and more universal method would be preferred.

BACKGROUND

A GLC procedure for the determination of levodopa purity was developed (2). However, this technique requires derivatization and was developed for impurities at a level of 1% with a stated detection limit for each component of ~0.1%. Thus, a more sensitive method is needed.

The standard amino acid analyzer procedure of separation on an ion-exchange column followed by detection of the ninhydrin color-forming reaction (3) was attempted but had long elution times (>80 min) and resulted in incomplete resolution of 3-*O*-methyldopa from tyrosine with the pH 3.4 citrate buffer employed[1]. Because of the greater speed and efficiency associated with microparticulate reversed-phase materials as opposed to ion-exchange materials (4) and because of the need to derivatize the amino acids (5) or to deposit a liquid phase on the solid support (6) when normal phase materials are used, a technique involving reversed-phase high-performance liquid chromatography (HPLC) was sought.

Knox and Jurand (7) resolved tyrosine from levodopa with reversed-phase HPLC by employing "soap" or ion-pair chromatography; the re-

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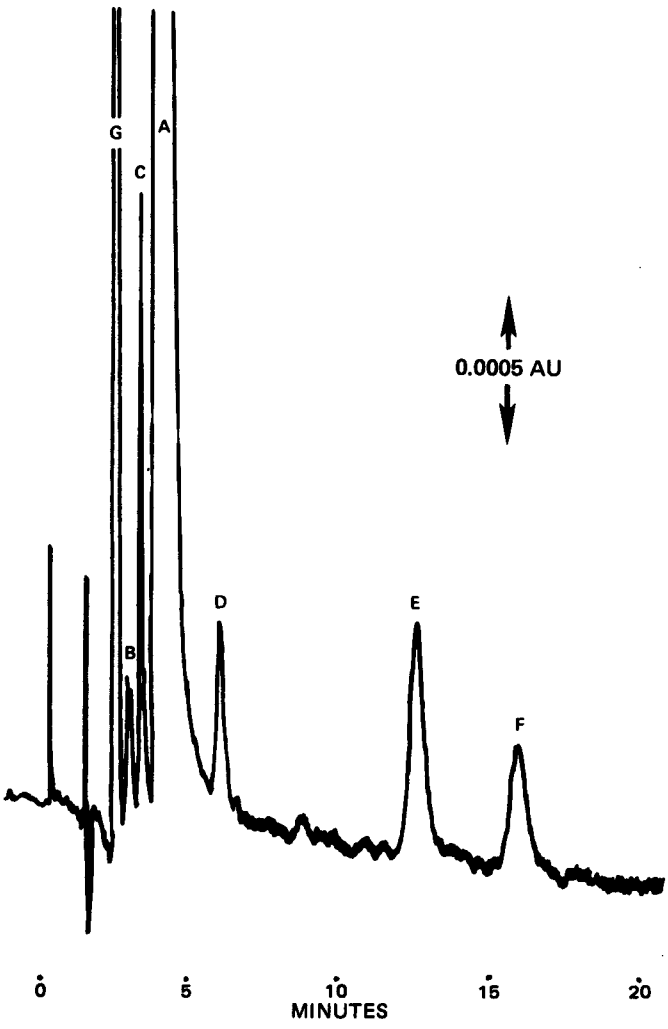

Figure 1—*Chromatographic trace of a standard solution containing 1 mg of levodopa/ml (A), 0.146% 5-hydroxydopa (B), 0.099% 6-hydroxydopa (C), 0.105% tyrosine (D), 0.150% 3-O-methyldopa (E), 0.118% 3-(3-hydroxy-4-methoxyphenyl)alanine (F), and ~0.1 mg of ascorbic acid/ml (G).*

tention arises from either the modification of the hydrophobicity of the hydrocarbon-bonded phase by the adsorption of ion-pairing molecules (8) or the formation of neutral ion-pairs in the eluent (9). However, to obtain reasonably short retention times and sharp peaks, an acid concentration of $>10^{-2}$ *M* (pH ~1.8) was employed, a requirement that can lead to slow hydrolysis of the bonded alkyl groups and eventual column deterioration (7) (pH 2 is the lower limit specified by column manufacturers). Raising the pH to ~2.4 greatly increased the retention times (from ~15 to ~25 min for tyrosine), indicating long analysis times (7).

Recently, Molnar and Horvath (10, 11) reported the reversed-phase HPLC separation of catecholamines and metabolites (including tyrosine and levodopa) utilizing a purely aqueous phosphate buffer mobile phase without ion-pairing reagents. The retention mechanism is apparently the association of the hydrophobic moiety of the molecule with the hydrocarbon-bonded phase, the driving force being the decrease in the nonpolar surface area exposed to the aqueous eluent (12). This paper evaluates a similar technique for determining 6-hydroxydopa, 3-*O*-methyldopa, tyrosine, and other related trace amino acid impurities in levodopa.

EXPERIMENTAL

Apparatus—A high-performance liquid chromatograph[2] with an injection valve and a 280-nm UV detector was used. A commercial[3] 30-cm × 3.9-mm i.d. stainless steel column was obtained prepacked with totally porous 10-μm average diameter silica particles containing an octadecyl monolayer chemically bonded to the silica. The flow rate was 2.0 ml/min.

Reagents and Materials—Levodopa, 6-hydroxydopa, tyrosine, and 3-*O*-methyldopa equivalent to USP specifications were used. In addition, an azalactone amino acid synthesis was used to prepare 5-hydroxydopa [3-(3,4,5-trihydroxyphenyl)alanine] (13) and 3-(3-hydroxy-4-methoxyphenyl)alanine (14). All other reagents and chemicals were ACS reagent grade or equivalent.

Mobile Phase—A 0.01 *M* NaH_2PO_4 solution was adjusted to pH 2.5 with phosphoric acid. This solution was filtered daily through a 0.5-μm membrane filter[4] and degassed prior to use.

Levodopa Standard Solution—A standard trace amino acid solution was prepared by accurately weighing (using an electrobalance[5]) 1–5-mg quantities of the amino acids to be determined, transferring them to a 100-ml volumetric flask, dissolving them with 10 ml of 0.1 *M* phosphoric acid, and diluting the solutions to volume with distilled water. About 50 mg of reference standard levodopa was accurately weighed and transferred to a 50-ml volumetric flask and dissolved with 5 ml of 0.1 *M* phosphoric acid. Exactly 5.0 ml of the standard trace amino acid solution and ~5 mg of ascorbic acid were added to the flask, and the contents were diluted to volume with distilled water. (The ascorbic acid was added to inhibit 6-hydroxydopa oxidation.)

Sample Solution—A quantity of the typical commercial levodopa preparation sufficient to yield 50 mg of levodopa was weighed and transferred to a 50-ml volumetric flask. Five milliliters of 0.1 *M* phosphoric acid and 5 mg of ascorbic acid were added. The flask was shaken mechanically for 15 min to effect levodopa dissolution, and the solution was diluted to volume with distilled water. A portion of the sample was centrifuged and filtered through the 0.5-μm filter.

Synthetic samples containing known quantities of trace amino acid impurities were prepared by adding 1-mg quantities of the accurately weighed trace amino acids to a 1-liter volumetric flask containing a quantity of the levodopa preparation sufficient to yield 1 g of levodopa. About 100 mg of ascorbic acid was added, and the dry mixture was shaken mechanically for 15 min. Prior to analysis, 100 ml of 0.1 *M* phosphoric acid was added to the flask, and the flask was shaken mechanically for 15 min to effect dissolution. The contents were diluted to volume, and a portion was centrifuged and filtered as before.

Sample Analysis and Linearity—Fifty microliters of the standard and sample solutions were injected. The amount of each trace amino acid present in the sample, expressed as percent of levodopa concentration, was calculated from:

$$\%\ \text{levodopa} = 0.1 W_s (h_u/h_s) \qquad \text{(Eq. 1)}$$

where W_s is the weight, in milligrams, of the trace amino acid used to prepare the standard trace amino acid solution, and h_u and h_s are the peak heights observed for the sample and standard solutions, respectively.

A linearity study was performed by analyzing actual sample solutions containing known quantities of amino acid impurities.

Table I—Linearity

Parameter	5-Hydroxydopa	6-Hydroxydopa	Tyrosine	3-*O*-Methyldopa	3-Hydroxy-4-methoxyphenylalanine
Correlation coefficient	0.9968	0.9997	0.9997	0.9997	0.9997
Standard error of estimate ($S_{y/x}$)	1.29	1.95	0.453	1.35	0.565
Intercept[a], %	−3.7	−3.6	13.4[b]	13.3[b]	0.72
Variation[c], %	6.9	2.4	1.8	1.7	2.0

[a] The (*y* intercept/$\bar{y}$) × 100, where $\bar{y}$ is the average *y*. [b] The significant positive intercepts observed for these compounds are due to the presence of these amino acids as trace impurities in the levodopa. [c] ($S_{y/x}/\bar{y}$) × 100.

RESULTS AND DISCUSSION

A sample chromatogram obtained with a new column is shown in Fig.

[2] ALC/GPC 202 equipped with an M6000 pump and a U6K injector, Waters Associates, Milford, Mass.
[3] μBondapak C_{18}, Waters Associates, Milford, Mass.
[4] Millipore.
[5] Cahn Instrument Co., Paramount, Calif.

Table II—Analysis of Spiked Samples

Compound	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6		Average Recovered[a], %
	Added, %	Found, %	Added, %	Found, %	Added, %	Found, %	Added, %	Found, %	Added, %	Found, %	Added, %	Found, %	
6-Hydroxydopa	0.099	0.098	0.112	0.110	0.142	0.145	0.104	0.095	0.096	0.095	0.060	0.059	98 (4)
Tyrosine	0.106	0.088	0.100	0.085	0.115	0.108	0.161	0.134	0.141	0.130	0.080	0.069	87 (5)
3-*O*-Methyldopa	0.110	0.104	0.100	0.105	0.150	0.157	0.128	0.123	0.135	0.123	0.178	0.166	97 (6)
5-Hydroxydopa											0.139	0.160	115
3-Hydroxy-4-methoxyphenylalanine											0.084	0.092	110

[a] Values in parentheses are relative standard deviations (1σ) for the data.

Table III—Analysis of Commercial Samples for Trace Amino Acid Impurities

Sample	Levodopa Dosage, mg	6-Hydroxydopa, %	Tyrosine, %	3-*O*-Methyldopa, %
1	250	0	0.013 (0.003)[a]	0.02 (0.01)[a]
2	100	0.005	0.010	0.202
3	100	0	0.012	0.010
4	100	0.007	0.022	—
5	250	0.003	0.017	0
6	500	0	0.012	0.010

[a] Values in parentheses are standard deviations (1σ); $n = 4$. Data were obtained on 4 separate days with two different columns.

1. The two USP-monitored compounds, 6-hydroxydopa and 3-*O*-methyldopa, as well as the frequently observed impurity tyrosine and the related compounds 5-hydroxydopa and 3-(3-hydroxy-4-methoxyphenyl)alanine, all were resolved. A much older column, which had shown loss of resolution through long usage with other reversed-phase separations, still yielded acceptable resolution of all amino acids in the present study, although the retention times of the slower eluting components were ~30% less with this column. Increasing the eluent pH from 2.5 to ≥3 further reduced the retention times observed with both columns and resulted in a significant loss of resolution between the 6-hydroxydopa and levodopa peaks.

Linearity data for the trace amino acids are presented in Table I. Linearity was observed over the concentration ranges studied: 0.02–0.2% for 6-hydroxydopa and tyrosine, 0.04–0.2% for 5-hydroxydopa and 3-(3-hydroxy-4-methoxyphenyl)alanine, and 0.06–0.6% for 3-*O*-methyldopa (0.1% represents 50 ng of injected sample).

Samples spiked with known quantities of trace amino acids yielded the recovery values listed in Table II after correction for any trace quantities of amino acids initially present (usually <0.02%). The results indicate sufficient precision and accuracy for trace analysis at the 0.1% level.

The results of an analysis of several commercial levodopa preparations from four different manufacturers are listed in Table III. Except for Sample 2, the trace impurities were ≤0.02%. [Sample 4 contained 10 mg of carbidopa as an active ingredient, which interfered with both the HPLC and the TLC determinations of 3-*O*-methyldopa, although the two compounds could be resolved by increasing the HPLC eluent pH to 3.5 (15).]

The method employed is relatively fast. Prior derivatization, the addition of a liquid stationary phase, or the addition of an organic solvent or ion-pairing reagent to the mobile phase is not required. Less than 1.5 hr is required for the preparation of the eluent, standard, and sample solutions, and <15 min is required for elution of the major amino acids.

The method also is sufficiently precise without an internal standard and sufficiently sensitive to determine accurately trace contaminants at the 0.1% level and to estimate them at levels of <0.01%. The resolution problems associated with the amino acid analyzer and TLC approaches are avoided, and the chromatogram is a documented record that yields an objective evaluation of the impurity content as opposed to subjective evaluation of the intensities of TLC spots.

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