Journal of Chromatography, 257 (1983) 317–326
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 15,477

DETERMINATION OF THE ENANTIOMERIC PURITY OF LEVODOPA, METHYLDOPA, CARBIDOPA AND TRYPTOPHAN BY USE OF CHIRAL MOBILE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(Received October 28th, 1982)

SUMMARY

Chiral mobile phase high-performance liquid chromatography was used successfully for the determination of the enantiomeric purity of levodopa, methyldopa, carbidopa and tryptophan. The method investigated uses phenylalanine and copper sulfate in the mobile phase and a C_{18} column. Linearity, precision, accuracy, detection limit and interference from expected impurities were assessed. The method is also applicable to the measurement of enantiomeric purity in levodopa tablets and capsules.

INTRODUCTION

The usefulness of chromatographic methods for accurate monitoring of enantiomeric purity has been enhanced by recent developments[1]. High-performance liquid chromatography (HPLC) methods for amino acid resolution include those based on chiral ligand-exchange columns[2–5], chiral columns separating via a hydrogen-bonding mechanism[6], chiral ligand-exchange mobile phases[7–13] and derivatization to diastereomers followed by conventional HPLC separation[14,15]. While several of these approaches are suitable for evaluating the enantiomeric purity of pharmaceutical products, they vary substantially in complexity. A method using only common, commercially available materials and simple sample preparation, avoiding the synthesis of derivatives, would be most easily adopted by potential users. We thus chose to examine the ligand exchange method of Oelrich *et al.*[16] for the enantiomeric purity determination of four chiral compounds currently used in therapy. In this procedure phenylalanine and copper(II) are added to the mobile phase to achieve chiral separation on a C_{18} column.

Due to the apparent toxicity of D-dopa[17,18], the United States Pharmacopeia (USP XX) specifications for levodopa require measurement of specific rotation for

both the bulk drug substance and dosage forms[19]. The Chafetz and Chen method is used to enhance the specific rotation via formation of a cyclic adduct between dopa and formaldehyde, the latter generated *in situ* from methenamine[20]. Since this method for determining enantiomeric purity is non-specific, carbohydrates present in dosage forms, as well as expected chiral impurities, will interfere. HPLC seemed likely to overcome these limitations. We chose to begin our investigation of the applicability of chiral mobile phase HPLC to enantiomeric purity tests with levodopa. Application of the method to similar compounds was also studied, and the method was found suitable for methyldopa, carbidopa and tryptophan.

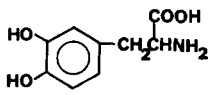
EXPERIMENTAL

Materials

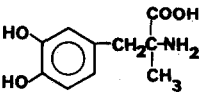
L-Dopa, D-dopa, L-methyldopa, DL-methyldopa, L-tryptophan, D-tryptophan and L-phenylalanine were obtained from Sigma (St. Louis, MO, U.S.A.) and were assayed by the official methods[19]. Reference standard levodopa, methyldopa, carbidopa, 3-(3,4,6-trihydroxy)phenylalanine, 3-methoxytyrosine, 3-O-methylmethyldopa and 3-O-methylcarbidopa were obtained from USP. D-Carbidopa was kindly provided by Merck, Sharp and Dohme Research Labs. (Rahway, NJ, U.S.A.). Structures of analytes are shown in Fig. 1. Copper sulfate was ACS reagent grade (Baker Analyzed). All solvents were HPLC grade.

Instrumentation

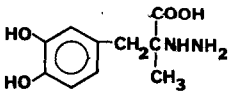
The chromatograph consisted of a single-piston pump (Milton Roy Mi-



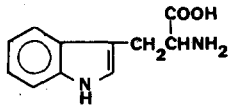
Dopa



Methyldopa



Carbidopa



Tryptophan

Fig. 1. Structures of the drugs for which HPLC enantiomeric purity tests were investigated.

nipump, Laboratory Data Control (LDC), Riviera Beach, FL, U.S.A.) with pulse damper and pressure gauge, a sample injection valve (Model 7125, Rheodyne, Cotati, CA, U.S.A.) equipped with a 20-μl or a 50-μl loop, a Supelcosil LC-18 column (250 mm × 4.6 mm I.D., Supelco Inc., Bellefonte, PA, U.S.A.), a fixed-wavelength detector (LDC UV III Monitor) with a 280-nm filter and an integrating recorder (Model 282, Linear Instruments, Irvine, CA, U.S.A.). Detector attenuation and/or integration rate were changed between D and L peaks as necessary. A recording integrator (Model 3390A, Hewlett-Packard, Palo Alto, CA, U.S.A.) was used for a few of the determinations. Specific rotations were measured with an automatic polarimeter (Model 241, Perkin-Elmer, Norwalk, CT, U.S.A.) equipped with a jacketed cell and a water bath. Mobile phases were filtered through a 0.45-μm cellulosic membrane filter (Metricel Alpha-450, Gelman Instrument, Ann Arbor, MI, U.S.A.).

Chromatographic conditions

Mobile phase compositions and typical chromatographic parameters are given in Table I. Flow-rates were *ca.* 1 ml/min. Parameters were calculated as in ref. 19. The void volume was taken as the first baseline disturbance, usually sinusoidal, caused by an injection of water observed at sensitive attenuation.

TABLE I

TYPICAL CHROMATOGRAPHIC PARAMETERS, SEPARATION CHARACTERISTICS OF DRUG ENANTIOMERS AND CHROMATOGRAPHIC CONDITIONS

α = Separation factor.

Compound	k'_D	k'_L	α	R_s	*Mobile-phase composition*			*Sample preparation solvent*
					L-Phe	*Copper(II)*	*% Methanol*	
Dopa	1.7	2.4	1.4	2.9	0.006 *M*	0.003 *M*	0	Mobile phase
Methyldopa	1.9	2.6	1.2	1.9	0.012 *M*	0.006 *M*	0	Mobile phase–water (50:50)
Carbidopa	7.4	9.5	1.2	3.4	0.006 *M*	0.003 *M*	0	1% Hydrochloric acid
Tryptophan	5.8	7.0	1.2	2.6	0.008 *M*	0.004 *M*	10	Mobile phase

A higher concentration of metal and ligand was required for methyldopa than the other analytes in order to achieve adequate resolution. In the dopa system mobile phase D- and L-methyldopa exhibited capacity factors (k') of 4.2 and 4.8, respectively, with a resolution of 1.6, which did not give baseline separation. Higher mobile-phase concentration was found to lead to less retention and more resolution. The ability of the detector to cope with high background absorbance was the limiting factor to the amount of metal and ligand that could be added and thus to the resolution achievable.

Since equilibration was found to require several hours, it was most convenient to recycle mobile phase through the system continuously. Mobile phase was not permitted to remain on the column with no flow. When system shutdown was necessary, at least 50 ml of degassed water were pumped through the column. At least 200

ml of methanol–water (50:50) were required to remove completely the phenylalanine–copper complex from the column, as indicated by cessation of the elution of visibly blue solvent from the column and stabilization of the baseline monitored with detector and recorder. Column stability was satisfactory. All work was done with one column, which was used for more than six months.

Sample preparation

Sample solvents are given in Table I. Concentrations were 0.5 to 1.0 mg/ml. Known enantiomer mixtures for validation studies were prepared by dilution of stock solutions, except for those used for comparison with polarimetry, which were thoroughly mixed in a mortar and pestle. Dosage-form samples (capsule contents or powdered tablets) were shaken mechanically with solvent for 30 min before diluting to volume and filtering through a 0.45-μm cellulosic membrane filter (HAWP, Millipore, Bedford, MA, U.S.A.). Components of spiked samples and authentics were weighed directly into the volumetric flasks used for sample preparation.

RESULTS AND DISCUSSION

Method validation

Due to the possibility that the actual species being eluted, the diastereomeric complexes, might possess different molar absorptivities[5], it was necessary to determine whether enantiomeric purity could be measured directly from area ratios or whether calibration curves were required. The experiment consisted of preparing various dilutions of stock D- and L-isomer solutions, chromatographing each, calculating the ratio of the area of the D peak to the sum of the areas of the D and L peaks (expressed as % D) and comparing the result with the % D calculated from weights used to prepare stock solutions, dilutions made and assay values for the enantiomers used. Four to seven injections of each dilution were averaged. The results are presented in Table II. The difference between recovered and added % D was less than 0.5 % for sixteen of the seventeen samples and less than 1.0 % for all samples. Standard deviation was less than 0.5 % for thirteen samples and less than 1 % for all. Correlation coefficients were greater than 0.986. The data indicate that estimation of enantiomeric purity by area ratios for the substances tested has the accuracy, precision and linearity needed for drug analysis. While a sample containing some of the undesired isomer is necessary for demonstration of system suitability, neither standards nor calibration curves are required for routine analysis. Typical chromatograms are shown in Figs. 2 and 3.

HPLC resolution of carbidopa enantiomers has not been previously reported. Carbidopa is a hydrazino acid, rather than an amino acid, and thus would be expected to form a more stable complex with copper. This is reflected in the greater retention and increased tailing seen for carbidopa, as compared with methyldopa. The slight deviation from linearity observed at higher levels of the D enantiomer appears to be connected with this tailing, but is not so great as to necessitate construction of a calibration curve.

The accuracy of the method is further demonstrated by Table III, in which HPLC is compared with the Chafetz and Chen specific rotation method for dopa. The difference between HPLC and specific rotation results was less than 0.30 % for all four

TABLE II

VALIDATION OF ENANTIOMERIC PURITY TESTS FOR LEVODOPA, METHYLDOPA, CARBIDOPA AND TRYPTOPHAN, DETERMINATION OF LINEARITY, PRECISION AND ACCURACY

Column headings: Added = % D in D + L added to samples; Average recovered = average % D in D + L recovered from samples; S.D. = standard deviation of % D recovered; $r - a$ = difference between % D recovered and % D added.

	Added	*Average recovered*	*S.D.*	$r - a$
Levodopa	11.68	11.59	0.51	−0.09
	6.31	6.41	0.12	+0.10
	3.37	3.26	0.07	−0.11
	2.44	2.27	0.08	−0.17
	Linear regression of r on a: slope = 1.01; y intercept = −0.14; correlation = 0.998.			
Methyldopa	10.63	10.96	0.70	+0.33
	5.33	5.22	0.04	−0.11
	2.13	2.21	0.28	+0.08
	1.12	1.23	0.26	+0.09
	Linear regression of r on a: slope = 1.02; y intercept = 0.0116; correlation = 0.996.			
Carbidopa	11.49	10.53	1.02	−0.96
	5.45	5.06	0.59	−0.39
	2.12	2.18	0.49	+0.06
	1.05	1.08	0.12	+0.03
	Linear regression of r on a: slope = 0.896; y intercept = 0.22; correlation = 0.986.			
Tryptophan	11.40	11.89	0.35	+0.47
	5.41	5.60	0.19	+0.19
	2.10	2.06	0.10	−0.04
	1.04	1.15	0.29	+0.11
	0.52	0.58	0.05	+0.06
	Linear regression of r on a: slope = 1.04; y intercept = 0.00; correlation = 0.999			

samples, with a correlation coefficient greater than 0.999. Such agreement would be expected only if the samples were free of impurities which might interfere with either method. The materials used in these experiments were determined to be free of likely impurities, as indicated by the absence of peaks at the k' values of the substances investigated for method specificity, discussed below. Under these conditions, results from rotation measurements and HPLC are equivalent. This would not be the case if chiral impurities were present in the sample. It should be noted in passing that samples for measurement of optical rotation were found to begin precipitating within a few minutes after opening the flask to withdraw an aliquot at the end of the reaction time, even if maintained in the bath in the dark. This precipitate interfered with multiple readings of some samples.

Various problems were encountered in attempting to measure the rotation of methyldopa by the compendial method[19]. The aluminum chloride solution used as

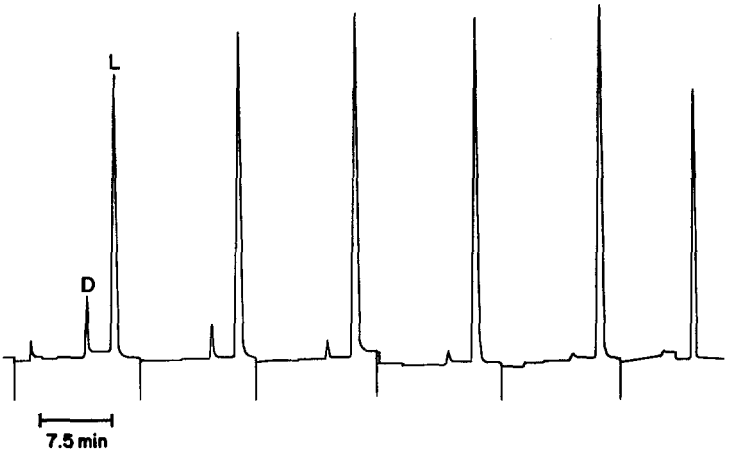


Fig. 2. Chromatograms of L-dopa containing various amounts of D-dopa. From left to right: 11.5% D (in D + L); 6.5% D; 3.5% D; 2.5% D; 1.5% D. Chromatographic conditions given in Table I.

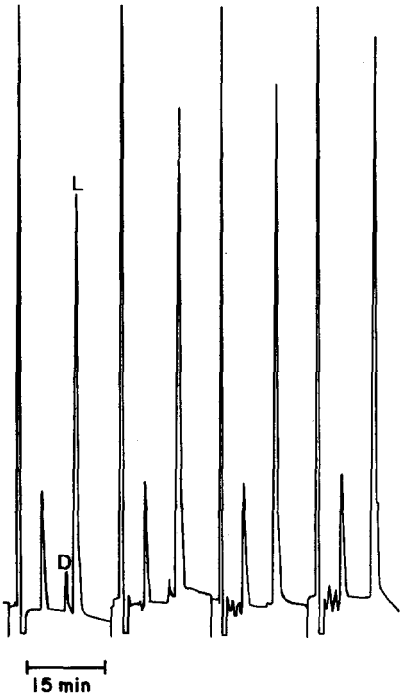


Fig. 3. Chromatograms of L-carbidopa containing various amounts of D-carbidopa. From left to right: 9.4% D (in D + L); 4.4% D, 1.7% D, 0.8% D. Chromatographic conditions given in Table I.

TABLE III

COMPARISON OF METHODS FOR DETERMINATION OF ENANTIOMERIC PURITY OF LEVODOPA

All results expressed as % D in D + L, standard deviation. $[\alpha]_D^{20}$ −165.2 from ref. 20 used for specific rotation.

Mixture No.	*HPLC*	*Specific rotation*	*HPLC − Specific rotation*
1	7.36 ± 0.12	7.10 ± 0.30	+0.26
2	5.99 ± 0.13	5.79 ± 0.06	+0.20
3	3.34 ± 0.08	3.50 ± 0.12	−0.16
L-Dopa from Sigma	1.39 ± 0.15	1.59 ± 0.06	−0.17

Linear regression of HPLC on specific rotation: slope = 0.914; y = intercept = 0.365; correlation = 0.999.

sample solvent[19] is extremely viscous, causing difficulty in cell filling and lowering energy transmission to 20%. The DL-methyldopa sample purchased from Sigma contained some impurity which caused the solution to turn yellow, giving an energy transmission of 5% and a reading which drifted severely. The actual angle measured for L-methyldopa was *ca.* 0.9° with substantial drift. For these reasons a meaningful comparison of HPLC and specific rotation for methyldopa could not be conducted. Since the amount of carbidopa available was limited and since its rotation is also measured in aluminum chloride solution, rotations for carbidopa were not determined. All of the difficulties encountered in the measurement of rotation can be avoided by using HPLC to evaluate enantiomeric purity.

Method specificity

Another advantage of HPLC over rotation measurements is specificity. This can be seen from the behavior of the impurities for which tests are specified in USP XX monographs[19]. The data tabulated in Table IV indicate that 3-methoxytyrosine elutes much later than dopa, while trihydroxyphenylalanine appeared just before L-dopa. Resolution between the two was 0.9. Low levels of the trihydroxy compound would produce some interference, while the 3-methoxy compound would not. In an attempt to improve this situation, a mobile phase containing only copper sulfate was pumped over a column which had been extensively equilibrated with phenylalanine. This type of system has been reported to give increased retention and resolution compared with one with phenylalanine in the mobile phase[21]. However, we found the performance of such a system to be unacceptable. The stability and reproducibility of the chromatographic system were very poor without phenylalanine in the mobile phase. Retention times were observed to decrease with each injection. In addition the following k' values were noted: D-dopa, 3.5; L-dopa, 7.7; trihydroxyphenylalanine, 8.8; methoxytyrosine, 31.6. Since a small peak after a large peak is much more difficult to distinguish than the opposite order, this situation is clearly less favorable. For both these reasons this avenue was not pursued further.

Table IV demonstrates that the 3-O-methyl precursors of methyldopa and carbidopa do not interfere with this test. When carbidopa was allowed to stand in

TABLE IV

SPECIFICITY OF HPLC ENANTIOMERIC PURITY TESTS; CHROMATOGRAPHIC BEHAVIOR OF LIKELY IMPURITIES

	k'
Mobile phase 0.006 *M* L-Phe, 0.003 *M* copper(II)	
D-Dopa	1.7
Trihydroxyphenylalanine	2.4
L-Dopa	2.6
D-Methyldopa	4.2
L-Methyldopa	4.8
D-Carbidopa	7.4
3-Methoxytyrosine	8.9
L-Carbidopa	9.5
3-O-methylcarbidopa	20.6
	27.8
Mobile phase 0.012 *M* L-Phe 0.006 *M* copper(II)	
D-Methyldopa	1.9
L-Methyldopa	2.4
3-O-methyldopa	8.7
Mobile phase 0.008 *M* L-Phe, 0.004 *M* copper(II), 10% methanol	
D-Tryptophan	5.8
L-Tryptophan	7.0
D- and L-Tyrosine	47.0

aqueous solution a peak appeared at a k' of 20.6, which is the same k' as that of the first of the two peaks observed with 3-O-methylcarbidopa. The height of this peak increased as the carbidopa solution was allowed to stand for increased periods of time. This peak was not observed when 1% hydrochloric acid was used as the sample solvent. While carbidopa is not stable in mobile phase, methyldopa and dopa are more stable in mobile phase than in water. Mobile phase containing carbidopa exhibits a blue to green color change. In addition, the rapid appearance of early eluting peaks (one with a retention time corresponding to that of methyldopa) and a decrease in the intensity of the carbidopa peak were observed. When 1% hydrochloric acid is used as sample solvent a large vacancy peak is observed at a retention time *ca.* four times that of carbidopa. Dopa, methyldopa and carbidopa do not interfere with each other. The data thus indicates that all but one of the likely contaminants of these substances do not interfere with HPLC, while all of them could interfere with the measurement of specific rotation.

The most likely impurities in tryptophan are other amino acids, as indicated by recent proposed modifications of its USP Monograph[22]. The wavelength used in this method results in only phenolic and indole amino acids detectable. Chromatography further improves selectivity; using a mobile phase containing 15% methanol, tyrosine gave a k' of 47, so that it clearly will not interfere. Hydroxytryptophan has been reported to elute well before tryptophan[16]. The results indicate that HPLC has greatly enhanced selectivity for determining tryptophan enantiomeric purity, as compared with polarimetry.

Method sensitivity

Limits of quantitation and detection, taken as signal-to-noise ratio of three, were as follows (expressed as % D in D + L): dopa and tryptophan, 0.03%; methyldopa, 0.42; carbidopa, 0.09%. The higher limits for methyldopa are connected with the higher concentration of mobile phase. This necessitated the use of water in the reference side of the detector to keep the signal within the detector range and led to an increase in both short-term noise and drift. Attempts to use a mobile phase without phenylalanine, analogous to that discussed above, resulted in increased retention but decreased resolution (D-methyldopa $k' = 5.6$, L-methyldopa $k' = 6.7$; resolution, $R_s = 1.3$). This resolution is not adequate for estimation of low levels of D in L.

Dosage-form analysis

Table V presents analytical results for three levodopa dosage-form samples from two different manufacturers. Differences between recovered and added % D were less than 0.10% for all eight samples. Equivalent results were obtained whether samples were shaken or 30 or 60 min, indicating adequate extraction of the drug and the small amount of contaminating enantiomer found in 30 min. In addition to the spiked samples reported in the table, two authentic samples consisting of lactose, talc and L-dopa were analyzed. The L-dopa used in these samples was that obtained from Sigma, the analysis of which indicated that it contained *ca.* 1.5% D (see Table III). Recoveries of 1.38% and 1.54% D were obtained. A placebo sample, without dopa, gave no peaks.

The results indicate that HPLC is an appropriate method for determining the enantiomeric purity of levodopa dosage forms and that expected excipients, including carbohydrates, do not interfere. By comparison with starting material, it should thus be possible to determine by HPLC whether any racemization occurs during dosage-form preparation or storage.

No D enantiomer was observed in levodopa, methyldopa or carbidopa USP reference standards. This verifies not only the enantiomeric purity of these standards, but also the purity of the L-phenylalanine used as a mobile phase component.

TABLE V

ENANTIOMERIC PURITY OF LEVODOPA DOSAGE FORMS AND RESULTS OF SPIKE RECOVERY STUDIES

Headings as in Table II; % D found in product standard deviation.

Manufacturer	*Product*	*% D found in product*	*Added*	*Recovered*	*r − a*
1	100-mg capsule	0.085 ± 0.023	1.41	1.48	+0.07
			1.14	1.10	−0.04
			0.66	0.70	+0.04
			0.77	0.73	−0.04
2	100-mg capsule	0.136 ± 0.055	0.87	0.87	0.00
			0.97	0.90	−0.07
	250-mg tablet	0.136 ± 0.033	0.96	0.95	−0.01
			0.99	1.02	+0.03

CONCLUSION

HPLC, using a chiral mobile phase containing L-phenylalanine and copper(II), appears to be a practical method for determining the enantiomeric purity of dopa, methyldopa, carbidopa, tryptophan and levodopa dosage forms. It is particularly suitable for a semiquantitative, limit test of the compendial type, in which the desired result is a determination of whether or not an impurity is present in a sample in amounts exceeding a limiting value. The results demonstrate that HPLC can provide this determination in a reliable manner.

ACKNOWLEDGEMENTS

This work was supported in part by the Science Advisor Research Associate Program of the U.S. Food and Drug Administration, a fellowship from the United States Pharmacopeia awarded to L.G. and NIH BRSG grant RR-05830 for aid in purchase of the polarimeter. We thank Ms. Avery Schroeder for assistance with experimental work and Dr. Barry L. Karger and Dr. Michael F. Delaney for helpful advice and counsel.

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