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DETERMINATION OF THE OPTICAL PURITY OF AMINO ACIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

MODIFICATION OF THE MANNING AND MOORE PROCEDURE*

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SUMMARY

A rapid and accurate method was developed for determining the optical purity of amino acids by the Manning and Moore procedure. Diastereomeric dipeptides, which were derived from the amino acids by the N-carboxy anhydride (NCA) method, were separated and determined by reversed-phase high-performance liquid chromatography (HPLC) instead of with an amino acid analyser. Using the HPLC system, the optical purities of almost all common amino acids, except those of proline and hydroxyproline, were determined within a much shorter period than with the original method, and as little as 0.01% of the contaminating optical antipode was measurable when N-carboxy-L-phenylalanine or -L-leucine anhydride was used as the reagent.

INTRODUCTION

In principle, enantiomers of an amino acid can be separated by various chromatographic methods, either after conversion to a diastereomeric derivative, or using a chiral stationary phase or using a chiral mobile phase. The separation of diastereomeric peptides by gas chromatography was introduced by Weygand *et al.*[2] in 1963 as a sensitive test for the detection of racemization during peptide syntheses. Five years later, the use of an automatic amino acid analyser was recommended by Manning and Moore[3] for the separation and determination of diastereomeric dipeptides. In 1971–72, the possibility of separating an underivatized DL-amino acid by ligand-exchange chromatography on an L-amino acid-bound resin was reported by Davankov and Rogozhin[4] and by Snyder *et al.*[5]. The principle was then widely applied to the separation of various amino acids on various chiral supports, not only using liquid chromatography[6–8] but also gas chromatography[9–11]. In recent years, the use of chiral mobile phases for separating DL-amino acids on ordinary chromatographic supports has been attempted. Dilute solutions of an amino acid–copper com-

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plex[12–14], a peptide-copper complex[15] or an optically active amine–metal chelate[16,17] have been used successfully as eluents.

In order to determine the optical purity of commercial amino acids, we tried to use the Manning and Moore procedure, as it seemed to be the most sensitive and reliable of the newly developed methods. In the original procedure, a neutral or acidic amino acid was converted into a dipeptide by coupling with N-carboxy-L-leucine anhydride (L-Leu-NCA), and an aromatic or basic amino acid with N-carboxy-L-glutamic acid anhydride (L-Glu-NCA). The coupling method was derived from the procedure of Denkewalter and co-workers[18,19] for synthesizing free peptides in an aqueous medium. The NCA procedure is simple and has proved to be free from racemization[18], but difficulties in the synthesis and storage of the reagents, particularly L-Glu-NCA, greatly restricts the wider use of the Manning and Moore method.

Mitchell *et al.*[20] modified the procedure by using *tert.*-butoxycarbonyl-L-leucine N-hydroxysuccinimide ester (Boc-Leu-OSu) instead of unstable NCAs as the reagent for converting test amino acids in to the leucyl-dipeptides. They also improved the chromatographic conditions to eliminate the need to use L-glutamyl-dipeptides to analyse aromatic or basic amino acids.

The separation of peptides on an ordinary amino acid analyser appears convenient, but it is time consuming and the accuracy is not always satisfactory. Therefore, in this study we modified the procedure by using reversed-phase high-performance liquid chromatography (HPLC) for the separation and determination of peptides instead of an amino acid analyser. With HPLC, the analysis of unmodified dipeptides can be performed within a much shorter period than with an amino acid analyser. In addition, eluted peptides can be determined directly by UV absorption with much higher sensitivity than with ninhydrin colorimetry. Optimal separation conditions for the various reaction mixtures using HPLC have been determined for each amino acid after conversion into the L-leucyl or L-phenylalanyl dipeptide; the principle is based mainly on recent work by Kroeff and Pietrzyk[21] and Hancock *et al.*[22].

MATERIALS AND METHODS

Apparatus

A Hitachi Model 638 liquid chromatograph was coupled to a Hitachi Model 635-900 multi-wavelength UV monitor with a sensitivity range of 2.56–0.005 absorbance units full scale (a.u.f.s.) and to a Hitachi Model 056 chart recorder. Stainless-steel columns (150 × 4 mm I.D. for common amino acids, and 300 × 4 mm I.D. for isoleucine and threonine) were slurry-packed with Nucleosil $5C_{18}$ (Macherey, Nagel & Co., Düren, G.F.R.), following the procedure recommended by Hitachi. All runs were performed at ambient temperature.

Chemicals

Amino acids were obtained from Ajinomoto (Tokyo, Japan), Tanabe Seiyaku (Osaka, Japan) and Sigma (St. Louis, MO, U.S.A.) and were used as received. Acetonitrile of HPLC reagent grade, orthophosphoric acid and dipotassium hydrogen orthophosphate of analytical-reagent grade were purchased from Katayama (Osaka, Japan). Water was deionized and then glass distilled.

L-Leu-NCA and L-Phe-NCA were synthesized by the phosgene method[23,24] and used after repeated recrystallization from a dried mixture of ethyl acetate and light petroleum (m.p.: L-Leu-NCA, 77–79°C; L-Phe-NCA, 92–94°C). The NCAs were placed in separate small screw-capped vials (100 mg each), which were then tightly closed and kept over silica gel in a desiccator at −20°C. Boc-L-Leu-OSu was synthesized as described by Mitchell *et al.*[20] (m.p.: 110–112°C). The amount of D-Leu in the starting material, Boc-L-Leu, was determined by the proposed procedure and found to be less than 0.015%.

Preparation of dipeptides

The procedure described by Manning and Moore[3] was followed exactly, except that the amount of amino acid applied to each reaction was increased to 100–200 μmol; thus, an amino acid (100–200 μmol) was placed in a test-tube (16 × 120 mm) and dissolved in 2 ml of borate buffer (0.45 *M*, pH 10.2, at 25°C) and the solution was cooled to 0°C in an ice-bath. Crystals of L-Leu-NCA or L-Phe-NCA (1.2 equiv. for common amino acids and 1 equiv. for lysine) were added to the solution in one step with vigorous agitation on an efficient vibro-mixer. After 2 min, 1.0 *M* hydrochloric acid (0.80 ml) was added to the mixture to stop the reaction. An aliquot of the solution was then subjected to the HPLC analysis.

Chromatography

Each reaction mixture (1–10 μl) was subjected to HPLC after filtration through a Millipore filter (0.22 μm). Potassium phosphate buffers (0.1 *M*) at various pHs were mixed with acetonitrile after filtration through a Millipore filter (0.22 μm), and the mixtures were used as eluents. The concentration of acetonitrile and the pH of the phosphate buffers were adjusted as described in the following section. A flow-rate of 1 ml/min, which was maintained by a pumping pressure of about 140 kg/cm^2, was used in every run. Eluted materials were detected by UV absorption at 210 nm. Detection of minor peaks was ensured by increasing the sensitivity of the UV detector 10- to 500-fold (from 2.56 to 0.005 a.u.f.s.). The amount of each compound separated by HPLC was measured directly by the built-in integrator.

RESULTS AND DISCUSSION

In order to obtain desirable retention times in reversed-phase HPLC, it may be advantageous to convert polar amino acids into their phenylalanyl-dipeptides and aromatic or lipophilic amino acids into their leucyl-dipeptides. To examine the separation conditions for L–L and L–D isomers of leucylphenylalanine, for example, synthetic DL-phenylalanine was subjected to reaction with L-leu-NCA and the reaction mixture was subjected to HPLC. As pointed out by Kroeff and Pietrzyk[21], the capacity factor [$k' = (t_R - t_0)/t_0$, where t_R = retention time and t_0 = void time (*ca.* 1.5 min)] for an L–L isomer is always smaller than that for the L–D isomer of the same peptide.

The conditions for HPLC were adjusted according to the following principles: (i) the peak of an L–L isomer should have a retention time of about 5 min and that of an L–D isomer should be about 10 min and (ii) the resolution [$R = 2(t_{R2} - t_{R1})/(w_1 + w_2)$, where w = band width at the baseline] between any two adjoining peaks should

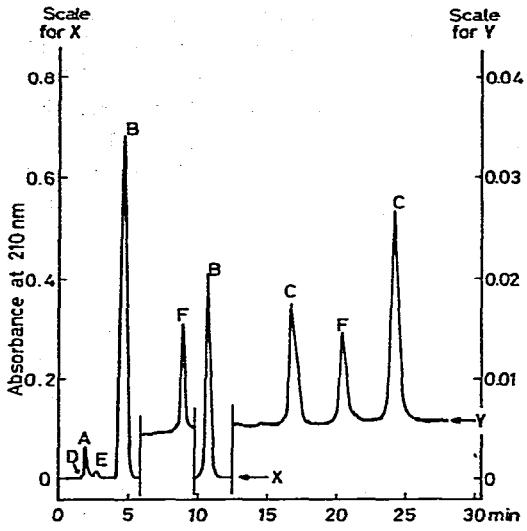

Fig. 1. Separation of a reaction mixture of DL-Phenylalanine with L-Leu-NCA (1.2 equiv.) by HPLC. Eluent: 0.1 *M* H_3PO_4–K_2HPO_4 (pH 4.5)/CH_3CN (84:16). See text for assignment of each peak. X, Recorded at normal sensitivity; Y, recorded at higher sensitivity (×20).

be greater than 1.0. These two conditions were controlled by adjusting the pH and the acetonitrile concentration of the eluents.

Fig. 1. shows a chromatogram of the above reaction mixture which was obtained under optimal resolution conditions. Assignment of each peak was made by comparing the retention times with those of authentic samples. As can be seen the formaion of the following side-products in the reaction mixture was observed: tripeptide (C), Leu–Leu–Phe, formed by an additional coupling of the NCA with the principal product (B), Leu–Phe; a free amino acid (D), Leu, by decomposition of the NCA; a dipeptide (E), Leu–Leu, by dimerization of the NCA; and hydantoic acids (F), formed by reverse coupling of the NCA with the test amino acids. The formation of these side-products was rationalized by Hirschmann *et al.*[19] in 1967. A was a peak for

TABLE I

AREA OF PEAKS SHOWN IN FIG. 1

Peak	*Peak area (%)*	*Recovery ratio of antipodes*
Unreacted Phe	4.7	
L-Leu–L-Phe	46.9	1.18
L-Leu–D-Phe	40.0	
L-Leu–L-Leu–L-Phe	1.7	0.39
L-Leu–L-Leu–D-Phe	4.4	
L–L-Hydantoic Acid	0.9	0.64
L–D-Hydantoic Acid	1.4	
Total	100.0	

the unreacted amino acid, phenylalanine. The separation of these peaks from those of the principal products was satisfactory, as can be seen in Fig. 1. The recoveries of the separated compounds are given in Table I. The deviation of the recovery ratio for L-Leu-L-Phe to L-Leu-D-Phe (1.18) from unity could be attributed to the difference in the reactivity of the corresponding optical antipodes with an optically active NCA and/or in the UV absorption coefficient of the paired compounds.

To examine the constancy of the recovery ratio at various concentrations of the optical isomers, mixtures of L- and D-phenylalanine were prepared in several known ratios in the range from 0.001:1 to 1:1. Each mixture was subjected to reaction with Leu-NCA, and each reaction was repeated for several times to ensure reproducibility of the analysis. The recovery ratio of L-Leu–L-Phe to L-Leu–D-Phe was then meassured by HPLC and plotted on the same graph (curves I and II in Fig. 2.).

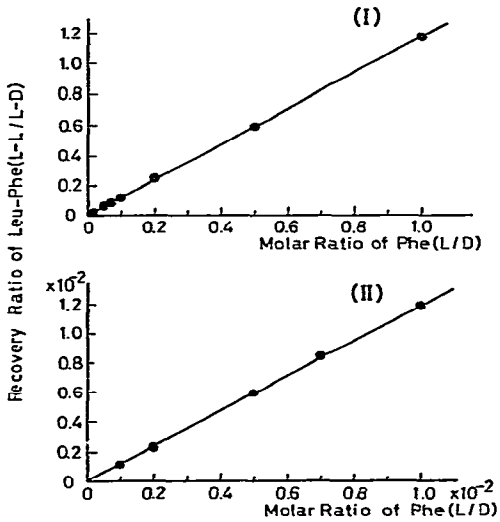

Fig. 2. Correlation between molar ratio of Phe(L/D) and recovery ratio of Leu–Phe(L–L/L–D) determined by the proposed method in the reactions of Phe(L/D) with L-Leu-NCA. I, Higher region of molar ratios of Phe(L/D); II, lower region of molar ratios of Phe(L/D).

It was found that all the observed values were distributed on the same straight line, and the detection limit of the measurement of a minor component was 0.01% with a relative standard deviation of 10%. Thus, it can be concluded that the recovery ratio of 1.18 is common to the reaction of L-Leu-NCA with any mixtures of phenylalanine at different ratios of the D- and L-isomers. Similarly, recovery ratios for enantiomeric mixtures of various amino acids were determined using synthetic DL-amino acids as test samples, as these were considered to be exact 1:1 mixtures of the D- and L-isomers. The choice of NCA for each amino acid, conditions for the separation of the diastereomers formed, separation parameters and recovery ratios are listed in Table II.

As lysine and cysteine are bifunctional, the side-chain functional groups should be protected before applying the NCA method. With lysine, however, if all of the reaction products with an NCA are separable by HPLC, it might be possible to

TABLE II

HPLC SEPARATION CONDITIONS AND RECOVERY RATIO FOR DIASTEREOMERS

Dipeptide	*Eluent*		*k'*		*R*	*Recovery ratio* (L–L/L–D)
	pH	*CH_3CN (%)*	L–L	L–D		
Leu–Ala	3.0	3	1.5	5.7	7.0	0.86
Leu–Val	3.0	12	1.2	7.3	13.2	0.87
Leu–Phe	4.5	16	1.8	6.3	13.1	1.18
Leu–Trp	1.6	19	2.8	5.5	4.3	1.21
Leu–Met	1.6	12	1.5	5.3	4.9	1.12
Leu–Gln	3.0	–	2.1	4.5	4.6	0.91
Leu–Asp	3.0	–	2.0	3.7	4.4	0.94
Leu–Glu	3.5	1	1.4	4.4	6.4	0.94
Leu–Arg	4.5	–	1.2	5.1	9.8	1.03
Phe–Lys	3.5	–	3.4	6.9	6.9	1.42
Leu–Cys (MB)*	4.5	20	3.7	9.2	13.1	1.08
Phe–Leu	4.5	15	1.8	6.4	11.5	1.16
Phe–Tyr	3.5	12	1.9	5.2	6.8	1.06
Phe–Ser	6.25	–	2.6	6.5	7.4	1.15
Phe–Asn	5.0	–	2.5	5.4	6.7	1.26
Phe–His	4.5	–	3.1	9.0	9.0	1.13

* MB: *p*-methoxybenzyl.

determine the optical purity by the present procedure. When an equimolar amount of L-Phe-NCA was reacted with DL-Lys under normal reaction conditions, the formation of following six principal products is expected: L-Phe–L-Lys, L-Phe–D-Lys, L-Lys(L-Phe), D-Lys(L-Phe), L-Phe–L-Lys(L-Phe) and L-Phe–D-Lys(L-Phe). The conditions for the separation of all of these compounds by HPLC were examined, and the maximal separation was obtained when the mixture was analysed as shown in Fig. 3; nevertheless, the separation of L-Lys(Phe)/D-Lys(Phe) and Phe–L-Lys(Phe)/Phe–D-Lys(Phe) was unsatisfactory. Therefore, it was considered that the optical purity of

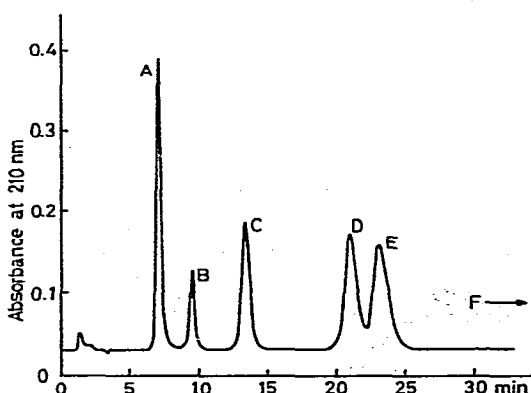

Fig. 3. Separation of a reaction mixture of DL-lysine with L-Phe-NCA (1 equiv.) on HPLC. Eluent: 0.1 *M* H_3PO_4–K_2HPO_4 (pH 3.5). Peaks: A = L-Phe–L-Lys; B = Phe; C = L-Phe–D-Lys; D = L-Lys(L-Phe); E = D-Lys(L-Phe); F = L-Phe–Lys(L-Phe).

Lys may be determined by the present procedure by measuring the amount of Phe–L-Lys or Phe–D-Lys as the index. Actually, the recovery was always constant, as was expected, and the ratio was determined to be 1.42 when synthetic DL-lysine was used as the test compound.

With proline, neither L-Phe-NCA nor L-Leu-NCA gave products with satisfactory separations using HPLC under the present conditions; this may be due to the presence of an equilibrium between their *cis*- and *trans*-conformers. In this instance, the reaction mixture was analysed more satisfactorily by the original Manning and Moore method using an amino acid analyser.

Both isoleucine and threonine have four optical isomers; thus, four diastereomers should be formed after coupling with Phe-NCA. Even under the optimal separation conditions so far examined using a longer column (see Figs. 4 and 5), however, the resolution between *threo*- and *allo*-type isomers with the same optical configuration is not satisfactory, except for the separation between L-Phe–D-Thr and L-Phe–D-*allo*-Thr. Therefore, the determination of a small amount of an *allo*-isomer with the same optical configulation in a *threo*-type amino acid, or *vice versa*, may be difficult under the present conditions. However, when detecting racemization of isoleucine or threonine, the product should be the *allo*-type isomer with the opposite optical configuration, and the measurement should be possible even under the present conditions. As completely racemized *allo*- or *threo*-amino acids were not available with for isoleucine and threonine, we could not determine the exact recovery ratio for each pair of isomers and, as a result, measurement of contamination was not as accurate as for other amino acids.

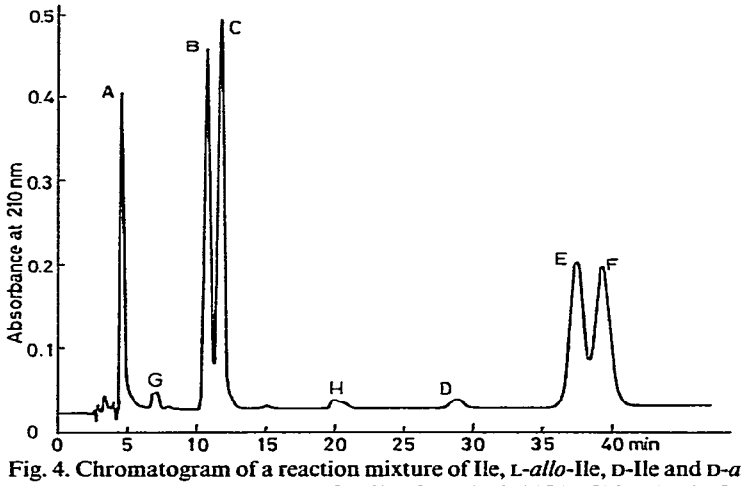

Fig. 4. Chromatogram of a reaction mixture of Ile, L-*allo*-Ile, D-Ile and D-*allo*-Ile (1:1:1:1) with L-Phe-NCA (1.2 equiv.). Eluent: 0.1 *M* H_3PO_4–K_2PO_4 (pH 6.1)/CH_3CN (87:13). Column: Nucleosil $5C_{18}$ (300 × 4 mm I.D.). Flow-rate: 1 ml/min. Peaks: A = Phe; B = L-Phe–L-Ile; C = L-Phe–L-*allo*-Ile; D = L-Phe–L-Phe; E = L-Phe–D-Ile; F = L-Phe–D-*allo*-Ile; G = (L–L)-hydantoic acids (estimated); H = (L–D)-hydantoic acids (estimated).

The optical purities of some commercially avaialable amino acids and some protected amino acid derivatives prepared in our laboratory were determined by the proposed procedures and the results are given in Table III. Lower optical purities

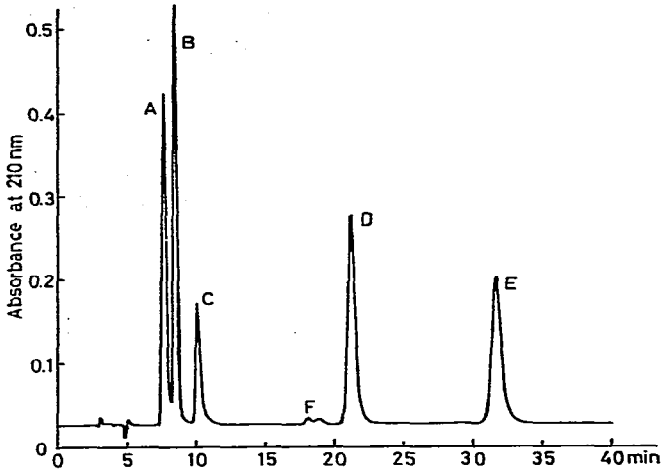

Fig. 5. Chromatogram of a reaction mixture of L-Thr, L-*allo*-Thr, D-Thr and D-*allo*-Thr (1:1:1:1) with L-Phe-NCA (1.2 equiv.). Eluent: 0.1 *M* H_3PO_4–K_2HPO_4 (pH 4.5)/CH_3CN (97:3). Column: Nucleosil $5C_{18}$ (300 × 4 mm I.D.). Flow-rate: 1 ml/min. Peaks: A = L-Phe–L-Thr; B = L-Phe–L-*allo*-Thr; C = Phe; D = L-Phe–D-*allo*-Thr; E = L-Phe–D-Thr; F = (L–L)-hydantoic acid (?).

TABLE III

OPTICAL PURITIES OF COMMERCIALLY AVAILABLE AMINO ACIDS AND THEIR DERIVATIVES DETERMINED BY THE HPLC METHOD

The values given are means of triplicate, measurements with a relative standard deviation of 10%.

Compound	*Content of the optical antipode (%)*	*Compound*	*Content of the optical antipode (%)*
L-Ala	0.03	L-Phe	0.01
D-Ala	0.07	D-Phe	0.01
L-Asp	0.02	L-Ser (A)	1.93
D-Asp	0.20	L-Ser (B)	1.24
L-Asn	0.04	D-Ser	1.31
D-Asn	0.04	L-Ser (Bz)*	0.09
L-Arg	0.04	L-Ser**	0.06
D-Arg	0.10	L-Trp	0.01
L-Glu	0.03	D-Trp	0.01
D-Glu	1.08	L-Tyr	0.02
L-Gln	0.09	D-Tyr	0.02
D-Gln	0.11	L-Val	0.03
L-His	0.01	D-Val	0.03
D-His	0.04	Boc-D-Val	0.01
L-Leu	0.02	Boc-D-Ala	0.03
D-Leu	0.97	Boc-D-Gln	0.02
L-Lys	0.07	Boc-D-Leu	0.11
D-Lys	0.06	Boc-D-Met	0.03
L-Met	0.02	Boc-D-Arg (Tos)***	0.04
D-Met	0.18		

* Bz = benzyl.

** Recovered from L-Ser(Bz) by catalytic hydrogenolysis.

*** Tos = tosyl (*p*-toluenesulphonyl).

were observed with L-serine, D-glutamic acid and D-leucine. However, when these amino acids were converted into their Boc derivatives or side-chain derivatives, a significant improvement was observed in their optical purities.

Finally, we used the proposed HPLC technique to compare the optical purity of D-phenylalanine through the leucyl dipeptide derived by Mitchell *et al.*'s procedure[20] with that derived by the NCA procedure[3]. The former procedure showed 0.08 % of phenylalanine in the D-phenylalanine, whereas the latter procedure showed the presence of at most 0.01 %. This result may be explained by our previous finding[25] that an appreciable amount of racemization occurs at the C-terminal amino acid residue when a free amino acid or peptide is acylated by the active ester method. Details of this phenomenon will be published separately. Although the N-hydroxysuccinimide ester procedure caused the least racemization at the C-terminal of the common active esters tested so far, we found that the NCA method is superior to the active ester method in accuracy for determining the optical purity of amino acids.

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