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DETERMINATION OF THE ENANTIOMERS OF α-H-α-AMINO ACIDS, α-ALKYL-α-AMINO ACIDS AND THE CORRESPONDING ACID AMIDES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY[a]

A. DUCHATEAU*, M. CROMBACH, J. KAMPHUIS, W. H. J. BOESTEN, H. E. SCHOEMAKER and E. M. MEIJER
DSM Research, P.O. Box 18, 6160 MD Geleen (The Netherlands)

SUMMARY

o-Phthalaldehyde in combination with N-acetyl-L-cysteine is a useful derivatization reagent for the optical resolution of enantiomeric α-H-α-amino acids, α-alkyl-α-amino acids and the corresponding acid amides. By using reversed-phase high-performance liquid chromatography with a mobile phase containing copper(II) acetate and L-proline, the diastereomeric derivatives of the α-amino compounds can be separated under isocratic conditions. The rate of reaction of α-alkyl-α-amino compounds with *o*-phthalaldehyde–N-acetyl-L-cysteine can be increased by selectively increasing the amount of *o*-phthalaldehyde in the reaction mixture. When the derivatization parameters were controlled automatically, the derivatization process showed good reproducibility and the method was found to be suitable for quantitative measurements. The method was applied to monitor the enantiomeric purity of α-H-α-amino acids and α-alkyl-α-amino acids obtained by enantioselective hydrolysis of the corresponding acid amides using an aminopeptidase.

INTRODUCTION

Optically pure α-amino acids (α-AA) are of great interest to the biochemist and pharmaceutical chemist. One of the routes to obtain these compounds is through organic synthesis of racemic α-amino acid amides (α-AA-NH_2) followed by the use of a broad-specificity aminopeptidase to achieve resolution on a large scale[1,2]. In conjunction with this synthesis, analytical methods are required to monitor the enantiomeric purity of both α-AA and α-AA-NH_2. Several high-performance liquid chromatographic (HPLC) methods have been developed for the optical resolution of α-H-αAA[3–8]. Resolution of α-alkyl-α-AA enantiomers has been achieved by ligand-exchange chromatography (LEC) on both reversed-phase columns[9] and chiral columns[10].

The aim of this work was to develop an HPLC method for the enantiomeric resolution of α-H-α-AA and α-alkyl-α-AA together with the corresponding acid amides. With the aid of LEC, enantiomeric mixtures of α-H-α-AA and α-H-α-AA-NH_2

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could be separated[11]. However, the same approach was unsuccessful with the α-alkyl substituted analogues.

In this paper, we report on the use of *o*-phthalaldehyde (OPA) in combination with N-acetyl-L-cysteine (NAC) for the derivatization of α-H-α-AA, α-alkyl-α-AA and the corresponding acid amides. For the amino compounds studied, we optimized the derivatization reaction. By using a copper complex of L-proline (L-Pro) as an additive to the mobile phase, separation of the amino acid and amide enantiomers could be achieved in an isocratic HPLC run. When the derivatization process was automated, the method was found to be suitable for quantitative measurements.

EXPERIMENTAL

Materials

α-H-α-AA-NH_2, α-alkyl-α-AA and α-alkyl-α-AA-NH_2 were synthesized in our laboratory[1,2]. α-H-α-AA were procured from Sigma (St. Louis, MO, U.S.A.). For each compound, both the racemic form and at least one optically pure enantiomer were available. NAC was obtained from Janssen (Beerse, Belgium). OPA, HPLC-grade methanol (CH_3OH) and acetonitrile (CH_3CN) were supplied by Merck (Darmstadt, F.R.G.). Water was purified with a Milli-Q system. All other chemicals were of analytical-reagent grade.

Instrumentation

The chromatographic system consisted of a Hewlett-Packard (Palo Alto, CA, U.S.A.) Model 1081B liquid chromatograph and a Gilson Model 231-401 auto-sampling injector for derivatization and injection. The injection loop had a 20-μl capacity. The columns used were Nucleosil-120-C_{18} (250 × 4.0 mm I.D., 5 μm, and 125 × 4.0 mm I.D., 3 μm) from Marcherey, Nagel & Co. (Düren, F.R.G.). The flow-rate was 1 ml/min and the column temperature was kept at 40°C. The derivatives were monitored with a Waters Assoc. (Milford, MA, U.S.A.) Model 420 fluorescence detector. For excitation a 338-nm band-pass filter was used, and for emission a 415-nm long-pass filter was chosen. Quantitation was performed with a Hewlett-Packard 3350 Laboratory Automation System.

Eluent, reagent and derivatization procedure

The mobile phase consisted of 2.5 m*M* copper(II) acetate buffer, titrated to pH 6.0 with ammonium acetate, and 5 m*M* L-Pro. The concentrations of CH_3OH and CH_3CN varied, depending on the compounds studied, and are indicated in the tables. The OPA reagent was prepared by dissolving 3.3 mg of OPA per ml of water–CH_3OH (1:1, v/v). For the NAC reagent, 4 mg of NAC were dissolved per ml of water–CH_3OH (1:1, v/v). Amino acids and amino acid amides were dissolved in water.

Derivatization was performed automatically with a Gilson Model 231-401 system. Into an empty vial the following were successively dispensed: 35 μl of sample solution, 35 μl of OPA reagent, 35 μl of NAC reagent and 175 μl of 0.4 *M* potassium borate buffer (pH 9.4). The largest volume, i.e., the borate solution, was added at a high speed in order to ensure good mixing of the reaction medium. The vial was allowed to stand for at least 2 min at room temperature, after which 140 μl of 1 *M* sodium phosphate buffer (pH 3.5) were added to neutralize the mixture. An

aliquot of the reaction mixture was then injected directly into the chromatographic system.

RESULTS AND DISCUSSION

Choice of the mobile phase

For the optical separation of OPA–NAC derivatives of α-H-α-AA, reversed-phase systems have been described[5,6,12], in which the usual buffer salts (sodium acetate or sodium phosphate) in combination with an organic solvent were used as mobile phases. A different approach was taken by Lam[13], who used mixed chelation to resolve the OPA–NAC derivatives of α-H-α-AA. We compared both approaches for the optical resolution of α-CH_3-Val, Val and the corresponding α-AA-NH_2. The results are given in Table I. The separation factors (α) of the enantiomers of the α-AA and α-AA-NH_2 studied are comparable in both chromatographic systems. In contrast to Lam and Malikin[14], we found that the use of reversed-phase ligand-exchange chromatography (RP-LEC) did not improve the enantioselectivity as compared with RP-HPLC. However, mixed chelation changes the selectivity of the system with respect to the α-AA and the corresponding α-AA-NH_2. The α-value of the last-eluting α-AA enantiomer and the first-eluting α-AA-NH_2 enantiomer (Table I) is much smaller in the RP-LEC system than in the RP-HPLC system. The decrease in the α-value in the RP-LEC system results from a decrease of the k' value of the α-AA-NH_2.

The difference in retention characteristics of α-AA-NH_2 in an RP-LEC system and an RP-HPLC system may be explained as follows. The polar amide function of α-AA-NH_2 may interact with free silanol sites on the RP column and this interaction will result in an additional increase in retention time. In the presence of Cu^{II} and L-Pro mixed-ligand complexes of α-AA-NH_2 with Cu^{II}–L-Pro will be formed. In the resulting complex the polar acid amide group will be coordinated with Cu^{II} and will thus be unavailable to interact with the free silanol sites in the matrix. The absence of the latter interaction may explain the observed decrease in retention time when the RP-LEC system is used. The relative retention time of α-AA and α-AA-NH_2 was also dependent

TABLE I

SELECTIVITY (α) OF THE OPA–NAC DERIVATIVES OF Val, Val-NH_2, α-CH_3-Val AND α-CH_3-Val-NH_2 ENANTIOMERS IN RP-HPLC AND RP-LEC SYSTEMS

Mobile phases: RP-LEC, 5 m*M* L-Pro–2.5 m*M* copper(II) acetate adjusted to pH 6.0 with ammonium acetate–40%CH_3OH; RP-HPLC, 2.5 m*M* sodium acetate adjusted to pH 6.0 with acetic acid–15% CH_3OH.

Enantiomer	α^a		*Enantiomer*	α^a	
	RP-HPLC	*RP-LEC*		*RP-HPLC*	*RP-LEC*
L-Val I	1.35	1.33	D-α-CH_3-Val I	1.22	1.23
D-Val II			L-α-CH_3-Val II		
L-Val-NH_2 I	1.22	1.17	D-α-CH_3-Val-NH_2 I	1.22	1.27
D-Val-NH_2 II			L-α-CH_3-Val-NH_2 II		
D-Val I	10.03	1.96	L-α-CH_3-Val I	8.67	1.16
L-Val-NH_2 II			D-α-CH_3-Val-NH_2 II		

[a] $\alpha = t_{RII}/t_{RI}$.

on the organic modifier used in the RP-LEC system. By using CH_3CN instead of CH_3OH, the α-value of L-α-H-α-AA and D-α-H-α-AA-NH_2 increased by a factor of 2 on average.

Employment of the RP-HPLC system for enantioselective analysis will necessitate the use of gradient elution to obtain the enantiomers of an α-AA and α-AA-NH_2 within a reasonable time. However, this will be at the expense of the resolution of the α-AA-NH_2 enantiomers. Use of the RP-LEC system gives the possibility of analysing the enantiomers of both α-AA and α-AA-NH_2 under isocratic conditions. In our further study, we therefore chose to use the RP-LEC system, employing CH_3OH as organic modifier.

Enantioselective analysis

The enantioselectivity of the RP-LEC system was tested with several α-H-α-AA, α-alkyl-α-AA and the corresponding acid amides. By varying the concentration of CH_3OH in the mobile phase, each tested α-AA and the corresponding α-AA-NH_2 could be baseline separated into their enantiomers in one analysis. Table II shows the chromatographic data for various α-H-α-AA and the corresponding α-AA-NH_2. The data for the α-alkyl-α-AA and α-alkyl-α-AA-NH_2 are given in Table III. Representative chromatograms are given of an α-H-α-AA and the α-H-α-AA-NH_2 (Fig. 1) and of an α-alkyl-α-AA and the α-alkyl-α-AA-NH_2 (Fig. 2). As far as the order of elution of the enantiomers is concerned, all the tested α-AA-NH_2 show the same order as the corresponding α-AA. The order of elution of the tested α-H-α-AA was L- before D-. For the aliphatic α-H-αAA, the same order of elution was reported by Lam and Malikin[14]. The order of elution of the α-alkyl-α-AA studied was the reverse of that of

TABLE II

CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s) OF OPA–NAC DERIVATIVES OF α-H-α-AA AND THE CORRESPONDING α-H-α-AA-NH_2

For chromatographic conditions, see Experimental.

Methanol concentration (%)	*α-H-α-AA α-H-α-AA-NH₂*	k'_L	k'_D	α	R_s[b]
20	Ala	5.3	5.9	1.11	2.4
	Ala-NH_2	10.7	13.4	1.25	5.9
25	But	7.3	8.9	1.22	5.0
	But-NH_2	15.1	19.3	1.28	7.0
40	Val	1.8	2.4	1.33	4.7
	Val-NH_2	4.7	5.5	1.17	3.3
	Leu	2.8	3.2	1.14	1.4
	Leu-NH_2	9.5	11	1.16	3.3
35	α-Ph-Gly	6.7	7	1.05	1.2
	α-Ph-Gly-NH_2	14.4	16.1	1.12	2.4
	Phe	9.0	9.7	1.08	1.8
	Phe-NH_2	25.4	26	1.02	-
	β-Bz-Ala	27.7	30.5	1.10	2.2
	β-Bz-Ala-NH_2	50	58	1.16	2.7

[a] $\alpha = k'_D/k'_L$.

[b] $R_s = 1.177\ (t_{R_D} - t_{R_L}/W_{\frac{1}{2}L} + W_{\frac{1}{2}D})$.

TABLE III

CAPACITY FACTOR (k'), SELECTIVITY (α) AND RESOLUTION (R_s) OF OPA–NAC DERIVATIVES OF α-ALKYL-α-AA AND THE CORRESPONDING α-ALKYL-α-AA-NH_2

For chromatographic conditions, see Experimental.

Methanol concentration (%)	*α-Alkyl-α-AA* *α-Alkyl-α-AA-NH_2*	k'_D	k'_L	α^a	R_s^b
30	α-CH_3-But	5.1	5.7	1.12	2.2
	α-CH_3-But-NH_2	8.6	9.8	1.14	2.9
40	α-CH_3-Val	3.1	3.8	1.23	3.7
	α-CH_3-Val-NH_2	4.4	5.6	1.27	4.8
37.5	α-CH_3-Leu	9.1	10.1	1.11	2.4
	α-CH_3-Leu-NH_2	14	15.5	1.11	2.8
35	α-CH_3-α-Ph-Gly	7.9	8.7	1.10	2
	α-CH_3-α-Ph-Gly-NH_2	13.5	14.6	1.08	1.8
37.5	α-CH_3-Phe	8.2	9.8	1.20	2.7
	α-CH_3-Phe-NH_2	14.0	16.7	1.19	4.3
	α-CH_3-β-Bz-Ala	18.9	20.3	1.07	1.5
	α-CH_3-β-Bz-Ala-NH_2	28	30.8	1.10	2.2
	α-C_2H_5-Phe	40	44	1.10	1.9
	α-C_2H_5-Phe-NH_2	67.8	83.9	1.24	4.3

[a] $\alpha = k'_L/k'_D$.

[b] $R_s = 1.177\ (t_{R_L} - t_{R_D}/W_{\frac{1}{2}L} + W_{\frac{1}{2}D})$.

the α-H-α-AA analogues in all instances. This phenomenon may be explained by the fact that replacement of the hydrogen on the α-carbon by a methyl or ethyl group leads to a change in intramolecular binding in the diastereomers, through which the order of elution reverses.

For Ala, using the chromatographic conditions listed in Table I, we found in both the RP-HPLC and RP-LEC system that the order of elution of the enantiomers was L- before D-, which is the reverse of that reported previously[8,12].

Depending on the nature of the substituent on the α-carbon of α-AA and α-AA-NH_2, different concentrations of CH_3OH were used to regulate the retention times. Comparison of the retention behaviours of the α-H-α-AA studied showed that the retention times of the aliphatic compounds increased with increasing carbon content of the substituent (Leu > Val > But > Ala). With the aromatic compounds,

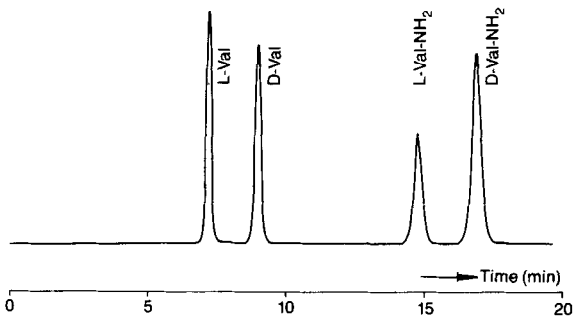

Fig. 1. Chromatogram of OPA–NAC derivatives of L,D-Val and L,D-Val-NH_2. For chromatographic conditions, see Experimental.

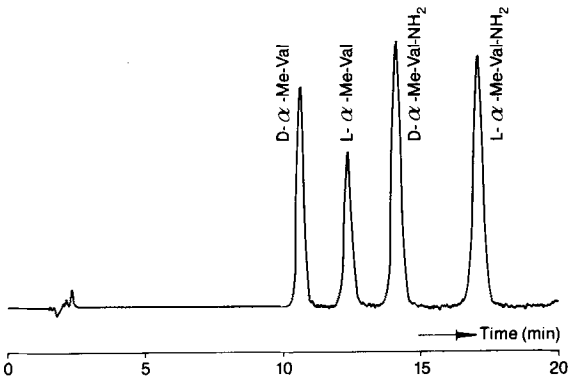

Fig. 2. Chromatogram of OPA–NAC derivatives of L,D-α-CH_3-Val and L,D-α-CH_3-Val-NH_2. For chromatographic conditions, see Experimental. Me = methyl.

the k' values increased with increasing number of methylene units between the phenyl group and the α-carbon (β-Bz-Ala > Phe > α-Ph-Gly). Because of the higher carbon content, α-alkylated compounds were retained more than the corresponding α-H analogues.

For all the aliphatic and aromatic enantiometric pairs studied, the α-values ranged from 1.1 to 1.3, except for Phe-NH_2 (1.02). Val and α-CH_3-Val showed the highest enantioselectivity. Among the α-H-α-AA studied by Lam and Malikin[14], the Val enantiomers also showed the highest α-value.

Rate of the OPA–NAC derivatization reaction

In accordance with others[9,15,16], we found that α-alkyl-α-AA react more slowly that the α-H analogues with the OPA reagent, under the same reaction conditions. The rates of derivative formation of some α-AA and α-AA-NH_2 are shown in Fig. 3 as a function of the reaction time. For Val and α-Ph-Gly, both α-H-α-AA, the maximum fluorescence occurs after about 2 min, whereas for α-CH_3-Val and α-CH_3-Val-NH_2, maximum fluorescence has still not been reached after 25 min. In order to improve the rate of reaction of α-alkylated compounds with the OPA–NAC reagent, we studied the influence of both OPA and NAC concentrations in the reaction mixture. Fig. 4 shows the fluorescence response of the OPA–NAC derivative of α-CH_3-Val as a function of the reaction time at different reagent concentrations. Compared with an equimolar excess of OPA–NAC, a 10-fold molar increase in OPA leads to an increase in the reaction rate, whereas a 10-fold molar increase in NAC decreases the reaction rate. These results indicate that the thiol competes with the amine for the OPA. By using an excess of OPA with respect to NAC, nucleophilic attack of the sterically hindered α-alkyl-α-AA on the carbonyl function of OPA is thus facilitated. By choosing a 100-fold molar excess of OPA and a 10-fold molar excess of NAC with respect to the α-alkyl-AA, maximum fluorescence is obtained within 10 min.

Depending on the structure of the α-AA and α-AA-NH_2 studied, different fluorescence intensities were obtained. Some examples are given in Fig. 3. The maximum fluorescence intensities for the α-AA-NH_2 were about one order of magnitude lower than those for the corresponding α-AA, while the α-alkylated

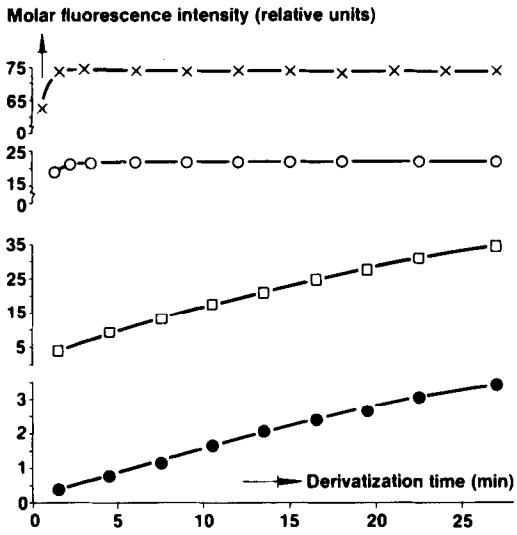

Fig. 3. Molar fluorescence response of the OPA–NAC derivatization as a function of reaction time for (×) Val, (○) α-Ph-Gly, (□) α-CH_3-Val and (●) α-CH_3-Val-NH_2. The molar excess of both OPA and NAC was 10-fold with respect to the compounds studied.

compounds showed lower intensities than the α-H-analogues. Differences in fluorescence intensity were also seen for the enantiomeric forms of the compounds studied. For the α-AA and α-alkyl-α-AA-NH_2 enantiomers, differences in specific fluorescence between 0 and 30% were observed. In some instances the D-isomer showed a higher fluorescence than the L-isomer, whereas in other instances the reverse was seen; however, no relationship could be established with structural aspects of the compounds. For the α-H-α-AA-NH_2 consistency was found, *viz.*, all D-isomers studied

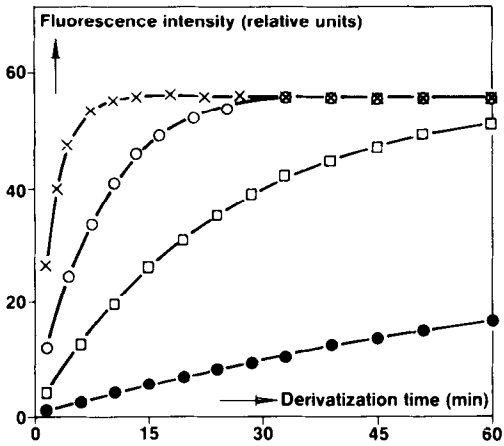

Fig. 4. Fluorescence response of the OPA–NAC derivatization as a function of reaction time for α-CH_3-Val. Molar excess of reagent: (×) OPA 100, NAC 10; (○) OPA 100, NAC 100; (□) OPA 10, NAC 10; and (●) OPA 10, NAC 100.

had a higher fluorescence intensity (by a maximum factor of about 2) than the L-isomers.

The difference in the fluorescence intensities of the OPA–NAC derivatives could be attributed to differences in intramolecular bonding in the isoindole reaction product. According to Lindner[17], intramolecular binding can occur between the carboxyl function of NAC and the isoindole nitrogen. These interactions, which will be dipolar or hydrogen bonding, may change by replacing an α-H by an α-alkyl group, by replacing a carboxyl function by an acid amide function or by replacing the entire L-enantiomer by the D-configuration.

Quantitative determinations were carried out by comparing the peak areas of samples with those of standard solutions, employing the external standard method. As an example of the linearity, precision and detection limit of the method, data are given for α-CH_3-Val. The linearity of the amount–response relationship was established over the range 2–20 nmol for each of the enantiomers. Linear regression analysis from calibration graphs indicated that the correlation coefficient for both enantiomers was 0.9999. The within-run precision of the assay gave a coefficient of variation of $<2\%$ ($n = 5$) over the range 2–20 nmol. The detection limit for L-α-CH_3-Val, based on a signal-to-noise ratio of 2, was 10 pmol.

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