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D-L amino acid analysis using automated precolumn derivatization with 1-fluoro-2,4-dinitrophenyl-5-alanine amide

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Summary. An automated procedure for the precolumn derivatization of enantiomeric amino acid mixtures with 1-fluoro-2,4-dinitrophenyl-5-alanine amide and a liquid chromatographic method for the separation of the derivatives with UV detection are reported. The system described allows to perform routine analyses using microbore columns with a sensitivity at the picomol level. Improvements for the use of this reagent in the protocol of a subtractive Edman degradation procedure of peptides to determine the sequence position of amino acid residues with concomitant identification of their chirality are also described.

Keywords: Amino acid-enantiomeric mixtures – Marfey reagent – Edman degradation – RP-HPLC

Introduction

D-Amino acids have been occasionally found in microorganisms, arthropods, molluscs and plants (Meister, 1965). Recently, a post-translational modification in some gene-encoded peptides leading to the formation of D-amino acids at specific sequence positions has been discovered to take place in microorganisms as well as in vertebrates and invertebrates (Mor et al., 1992). This processing plays a key role in conferring to those molecules either the biological activity or an increased resistance to proteolytic degradation. Moreover, accumulation of D-amino acids, in particular D-aspartic acid, has been related to the aging of proteins (Bada, 1984; Lubec and Lubec, 1993) and elevated levels of D-amino acids were found associated with pathological conditions in humans (Nagata et al., 1987). The need for screening a large number of natural extracts with the aim to isolating peptides containing D-amino acids, as well as the need for checking the stereochemical purity in synthetic products, stimulated the development of procedures for the routine analysis of amino acid enantiomeric mixtures. Enzymatic methods, such as

those based on L- or D-amino acid oxidases have been reported (Meister, 1965). Very recently, another enzymatic procedure employing bacterial D-amino acid transaminase coupled with 2-oxohexanoate has been described (Jones et al., 1994). Moreover, different chromatographic techniques are available for the separation of amino acid racemates. A number of methods that utilize chiral stationary phases (Davankov et al., 1973; Gubitz et al., 1981) or chiral mobile phases (Hare and Gil-Av, 1979; Weinstein et al., 1982) have been reported, but sometimes they are expensive and specific for a single or a few amino acid enantiomeric mixtures. Furthermore, precolumn derivatization with chiral reagents allows to obtain diastereomeric derivatives separable on a standard reversed-phase support (Nimura et al., 1984; Mitchell et al., 1978). Thus, methods utilizing (9-fluorenyl)-ethylchloroformate (Einarsson et al., 1987), Marfey reagent (Marfey, 1984), chiral isothiocyanates (Nimura et al., 1984) or *o*-phthal-dialdeyde and a chiral thiol (Bruckner et al., 1989) have been reported.

We previously described the synthesis and the use of a chiral compound, 1-fluoro-2,4-dinitrophenyl-5-L-alanine (FDNPA), for the determination of the chirality of amino acid residues in the course of sequence analysis of peptides (Scaloni et al., 1991). In order to improve the recovery of the N-terminal DNPA-residue in the course of subtractive Edman degradation, we report in this paper a modification in the hydrolysis procedure of derivatized peptides. Moreover, we describe a fully automated system that permits the analysis of fifteen amino acid pairs plus glycine utilizing the commercially available Marfey reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) (Marfey, 1984) and its enantiomer 1-fluoro-2,4-dinitrophenyl-5-D-alanine amide. The system automatically derivatizes and injects the sample, allowing to perform routine analysis of enantiomeric amino acid mixtures continuously 24 h a day.

Materials and methods

Chemicals

D and L forms of 1-fluoro-2,4-dinitrophenyl-5-alanine amide were purchased from Novabiochem. Standard solutions (0.1 mM) of D and L amino acids (Sigma Chemical Company) were prepared in water and stored at −20 °C. Acetonitrile, 2-propanol and acetone (HPLC grade) were from Farmitalia Carlo Erba. Analytical reagent-grade ortophosphoric acid, triethylamine, ethylacetate and 1,5-difluoro-2,4-dinitrobenzene were from Fluka. Deionized water was first glass-bidistilled and then passed through a SepPak C_{18} cartridge (Millipore).

Acid hydrolysis

Samples of peptides to be hydrolysed (100–400 pmol) were dried in Gilson polypropylene microtubes (cat. n. 450831) and enclosed in a Pyrex container with 6N HCl (1 ml). The hydrolysis of FDAA-derivatized peptides was performed in the presence of a large excess of nitrobenzene derivatives placed in a separate tube. The container was evacuated, flushed with nitrogen, evacuated again and sealed. Hydrolysis was performed in the vapor phase at 110 °C for 20–24 h (amino acid composition analysis) or 4 h (N-terminal analysis). After the hydrolysis, the tubes were dried in a SpeedVac concentrator (Savant) to remove drops of condensed HCl.

Preparation of derivatized amino acids

Automatic derivatization of amino acid mixtures was achieved using a Gilson autosampling injector consisting of two modules, a model 231 sample injector equipped with a code 9 modified thermostated rack, and a model 401 diluter, both controlled by the sample controller keypad. The rack modification realized by INMECA, Aprilia, Rome, allows to perform the sample derivatization at 50 °C, whereas the reagent solutions are kept at 5 °C. The rack position 18/2 was modified to accomodate glass vials (Pierce, 32 × 12 mm I.D.) filled with the loading solvent (H_2O/acetonitrile/ ethylacetate 91:8:1, v/v/v). The dried samples, placed in the portion of the rack thermostated at 50 °C, were automatically dissolved in 7 µl of buffer (triethylamine/H_2O/acetonitrile 1:2:3, v/v/v) and derivatized by adding 10 µl of FDAA reagent (0.5 mg/ml in acetonitrile/acetone 4:1, v/v). After 120 min of incubation, the dried reaction mixture was dissolved in 23 µl of loading buffer and injected into the column. Stock solutions of the reagents were kept in glass vials sealed with a crimper and stored at −20 °C over a period of weeks without appreciable degradation.

HPLC analysis

The liquid chromatographic system (Kontron Instruments AG, Zurich Switzerland) consisted of two model 420 pumps, a model 430 UV-visible detector equipped with a 3 µl flow cell, a Gilson model 811C dynamic mixer (65 µl mixing chamber), and a Rheodyne injection valve (model 7125) equipped with a 20 µl sample loop. Gradient formation, quantitation of the chromatographic peaks, and data treatment were performed by a Kontron Instruments model 450 MT data system. The separation of derivatized amino acids was obtained using a PhaseSep ODS2 column (250 × 2.0 mm, 3 µm) protected by a 2 mm stainless-steel filter (Rheodyne). For the chromatographic separation, a gradient of acetonitrile/2-propanol (4:1, v/v) in 40 mM triethylamine-phosphate buffer containing 40 mM Titriplex, pH 2.3, at a flow rate of 200 µl/min, was used. The total running time, including column recycling, is about 120 min. The absorbance of the effluent was monitored at 338 nm.

Results and discussion

Amino acids derivatized with Marfey reagent, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA), can be separated into their D and L-forms, with elution volumes often differing by several minutes, using conventional RP-HPLC conditions. In the original version of the method (Marfey, 1984), FDAA was used to derivatize five amino acid enantiomeric mixtures: aspartic and glutamic acids, alanine, methionine and phenylalanine. Sodium bicarbonate was used to provide the basic conditions necessary for the coupling of the reagent to the amino group. More recent papers report i) a separation of the FDAA-diastereomers by a combined 2D-TLC/RP-HPLC procedure (Nagata et al., 1992), ii) a RP-HPLC analysis limited to the L-amino acids present in protein hydrolysates (Kochhar and Christen, 1989) or iii) a RP-HPLC analysis of racemic mixtures using different chromatographic buffers depending on the amino acid nature (Szokan et al., 1988). The conditions described in some cases give overlapping peaks or require a HPLC analysis of the sample recovered from TLC.

The HPLC profile of 15 pairs of enantiomeric amino acids plus glycine, after precolumn derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide is shown in Fig. 1. The conditions for the chromatographic analysis are

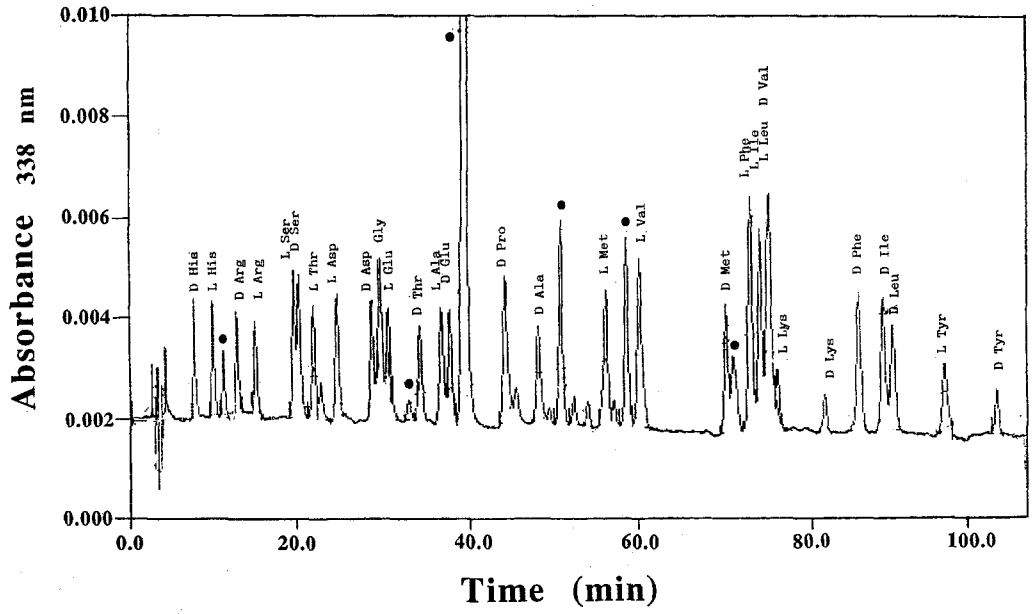

Fig. 1. RP-HPLC elution profile of a standard mixture of D and L- amino acids plus Gly (150 pmol each) derivatized with L-FDAA. The peaks denoted with • are reagent-related. For chromatographic conditions see the experimental section

Table 1. Gradient parameters for the separation of FDAA-derivatives

Time (min)	Composition (%)[a]		Curve (type)
	A	B	
0.00	80	20	
3.00	80	20	0
30.00	76	24	1
100.00	55	45	1
100.20	10	90	1
105.00	80	20	1

[a] Solvents: A, 40 mM triethylammonium phosphate buffer, 40 mM Titriplex, pH 2.3; B, acetonitrile/2-propanol (4:1 v/v). Gradient curves: *0*, isocratic; *1*, linear.

given in Table 1. Each D-amino acid derivative is well separated from the corresponding L-stereoisomer; only the coelution of L-Leu with D-Val occurs. D-diastereomers were reported to be eluted from the column after the L-diastereomers as a result of a hypothesized intramolecular hydrogen bonding (Marfey, 1984). Similar results were obtained in our case, except for histidine and arginine derivatives. If the derivatization is performed with the D-form of the reagent, the elution order of the amino acid diastereomers is reversed. The use of the D-reagent allows an unequivocal determination also in the case of the coelution previously mentioned. With both reagents, each amino acid derivative can be detected and quantitated in the presence of a large excess of the corresponding diastereomer.

Traditionally, precolumn derivatizations are performed off-line from the liquid chromatographer. They are therefore time-consuming and errors inevitably occur when using manual procedures. Moreover, decomposition of sample derivatives takes place when analysis is delayed. The system here described is based on the complete automation of both derivatization and injection. During the analysis of a sample the next one is derivatized. Different thermostating of the reagents (5 °C) and samples (50 °C) allows the storage of the reagents without degradation. The substitution of triethylamine for sodium bicarbonate originally used in the derivatization mixture allows the

Table 2. Program for the automated analysis of D and L amino acids

1	RACK CODE 9	41	PRINT C9/54	81	HEIGHT
2	AUXIL 6/1	42	TUBE 19/3	82	ASPIR 0/50/0
3	INPUT C/1	43	HEIGHT	83	HEIGHT 7
4	INPUT C0/43	44	ASPIR 0/50/0	84	ASPIR 0/C6/0
5	INPUT A0/6	45	HEIGHT 5	85	WAIT 2
6	INPUT C8/36	46	ASPIR 0/C9/0	86	TUBE C2/C3
7	INPUT C9/54	47	WAIT 2	87	HEIGHT 0
8	INPUT C6/24	48	HEIGHT	88	DIS 0/C6/0
9	C1=0	49	ASPIR 0/3/0	89	FOR C7=1/5
10	FOR C2=1/6	50	TUBE C2/C3	90	ASPIR 0/10/0
11	FOR C3=1/6	51	HEIGHT	91	DIS 0/10/0
12	C1=C1+1	52	DIS 0/3/0	92	PRINT C7/28
13	PRINT /6	53	DIS 0/C9/0	93	WAIT 2
14	WAIT A0	54	FOR C4=1/3	94	NEXT C7
15	PRINT C8/36	55	ASPIR 0/3/0	95	ASPIR 0/C6/0
16	TUBE 19/5	56	DIS 0/3/0	96	WAIT 2
17	HEIGHT	57	PRINT C4/28	97	ASPIR 0/2/0
18	ASPIR 0/50/0	58	WAIT 2	98	WAIT 2
19	HEIGHT 5	59	NEXT C4	99	PRINT C6/31
20	ASPIR 0/C8/0	60	DIS 0/2/0	100	TUBE 0/0
21	WAIT 2	61	HEIGHT 4	101	WAIT 3
22	HEIGHT	62	WAIT 2	102	DIS 0/C6/0
23	ASPIR 0/3/0	63	DIS 0/4/0	103	WAIT 2
24	HEIGHT	64	RINSE	104	DIS 0/3/0
25	TUBE C2/C3	65	DIS 0/500/6	105	WAIT 5
26	HEIGHT	66	C5=0	106	INJECT 1
27	DIS 0/3/0	67	FOR C5=1/C0	107	AUXIL 7/2
28	DIS 0/C8/0	68	PRINT C5/43	108	WAIT 100
29	FOR C4=1/3	69	WAIT 2	109	INJECT 0
30	ASPIR 0/3/0	70	PRINT C1/1	110	DIS 0/500/5
31	DIS 0/3/0	71	WAIT 2	111	RINSE
32	PRINT C4/28	72	PRINT /74	112	DIS 0/500/6
33	WAIT 2	73	WAIT 2	113	IF C1−=C
34	NEXT C4	74	PRINT C1+1/1	114	GO TO 119
35	DIS 0/2/0	75	WAIT 2	115	NEXT C3
36	HEIGHT 4	76	PRINT /80	116	NEXT C2
37	WAIT 2	77	WAIT 2	117	WAIT 8000
38	DIS 0/4/0	78	NEXT C5	118	AUXIL 6/0
39	RINSE	79	PRINT C6/24	119	HOME
40	DIS 0/500/6	80	TUBE 20/2		

drying of the sample directly in the reaction rack, avoiding the formation of non volatile salts during the acid neutralization step. The program for the derivatization of amino acid racemates and for sample injection is reported in Table 2.

The linearity of the response of all the amino acids analyzed was verified in the range 50–1000 pmol. Table 3 reports the color factors obtained by automatic integration in the HPLC system used. The use of L-norvaline (eluting 0.8 min later than L-Val) as internal standard allows to calculate the recovery for each sample. The amino acid compositions of two natural peptides containing a D-amino acid, analyzed using this system, is reported in Table 4.

We previously described the use of the Marfey reagent for the determination of the chirality of the N-terminal amino acid residue in the course of subtractive Edman degradation of peptides (Scaloni et al., 1991). However, the conditions used for the hydrolysis of the peptide bond affected the recovery of the derivatized amino acids. In order to improve this procedure, we have studied the efficacy of various scavengers in preventing the destruction of diastereomers during acid hydrolysis. Hence, different nitro-benzene derivatives in a separate tube were placed in the vapor phase hydrolysis apparatus. The relative recovery of three different amino acid derivatives is reported in Fig. 2. 1,5-Difluoro-2,4-dinitro-benzene (DFDNB) gave in all cases the best recovery. The use of scavenger wetted with few microliter of acetone facilitates this scope. The recovery of L-amino acid derivatives was always higher than that of the corresponding isomer, probably as a result of an improved stability to the acid conditions. The application of this modification to the original method was recently utilized for the determination of the chemical structure of antibacterial and haemolytic peptides from the skin of *Bombina*

Table 3. Peak area per picomole of amino acids after derivatization with FDAA and RP-HPLC separation

Amino acid	Peak area/pmol (counts $\times 10^3$)
Histidine	9.55 ± 0.17
Arginine	17.82 ± 0.42
Serine	24.10 ± 0.13
Aspartic acid	16.74 ± 0.27
Glycine	33.02 ± 0.75
Threonine	17.56 ± 0.13
Glutamic acid	19.54 ± 0.24
Proline	19.07 ± 0.11
Alanine	25.52 ± 0.23
Methionine	24.74 ± 0.26
Valine	29.89 ± 0.27
Lysine	21.51 ± 0.92
Phenylalanine	26.48 ± 0.34
Isoleucine	25.34 ± 0.26
Leucine	29.32 ± 0.32
Tyrosine	16.71 ± 0.34

Table 4. Amino acid composition of bioactive peptides determined by automatic derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide

	Ala-deltorphin II[a]	Leu-deltorphin[b]
L-His		1.0 (1)
L-Ser		1.5 (2)
L-Asp		1.6 (2)
Gly	0.9 (1)	1.1 (1)
L-Thr		1.0 (1)
L-Glu	0.9 (1)	
L-Ala		1.7 (2)
D-Ala	0.6 (1)	
L-Val	2.0 (2)	0.8 (1)
L-Phe	1.2 (1)	2.6 (3)
L-Ile		2.1 (2)
D-Leu		0.8 (1)
L-Tyr	0.6 (1)	0.7 (1)

Opioid peptides from [a] *Phyllomedusa bicolor* and [b] *Phyllomedusa burmeisteri*. The composition from sequence analysis of each peptide is indicated by the numbers in parentheses.

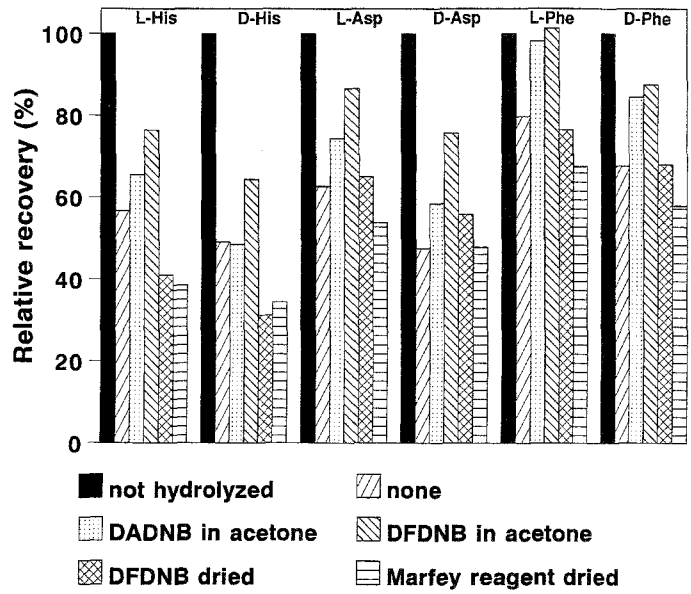

Fig. 2. Recovery of derivatized amino acids in the presence of nitro-benzene derivatives (dried or wetted with acetone) after vapor phase hydrolysis with 6N HCl, 4 h at 110 °C. The coefficient of variation was in the range of 5% (n = 3). DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DADNB, 1,5-diamino-2,4-dinitrobenzene

variegata (Mignogna et al., 1993) and from cultures of *Pseudomonas syringae* pv. *syringae* (Scaloni et al., 1994).

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