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Peptide chiral purity determination: hydrolysis in deuterated acid, derivatization with Marfey's reagent and analysis using high-performance liquid chromatography–electrospray ionization–mass spectrometry

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Abstract

A high-performance liquid chromatography–electrospray ionization–mass spectrometric (LC–ESI–MS) method is presented that allows rapid and accurate determination of amino acid chiral purity in a peptide. Peptides are hydrolyzed in hydrochloric acid-d_1/acetic acid-d_4 and then converted to diastereomers by derivatization with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent). Mixtures of D- and L-amino acid diastereomeric pairs are resolved in one chromatographic separation using conventional reversed-phase high-performance liquid chromatography. Hydrolysis in a deuterated solvent is necessary because the original ratio of D-/L-amino acids present in a peptide changes during acid hydrolysis due to racemization. Peptide hydrolysis in deuterated acids circumvents this problem by labeling each amino acid that racemizes with one deuterium at the α-carbon. An increase in molecular mass of one atomic mass unit allows racemized amino acids to be distinguished from non-racemized amino acids by mass spectrometry. This procedure was used to determine the chiral purity of each amino acid in a purified, hexapeptide by-product (Arg-Lys-Lys-Asp-Val-Tyr) present in a kilogram batch of the synthetic pentapeptide, thymopentin (Arg-Lys-Asp-Val-Tyr).

1. Introduction

The chiral purity of an amino acid in a peptide is usually determined by acid hydrolysis of the peptide and resolution of the amino acid mixture using either direct or indirect chromatographic schemes [1]. Unfortunately, racemization of amino acids occurs concomitant with acid hydrolysis [2] causing a change in the enantiomeric conformation of each amino acid in the peptide. In order to determine the chiral purity of amino acids in a peptide, racemization must be corrected for or circumvented. Correcting for racemization can be carried out by co-hydrolysis

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of a peptide that is nearly identical to the unknown or by combinations of acidic and enzymatic hydrolysis [3]. However, accurate correction factors are difficult to obtain because racemization rates depend on the location of an amino acid in a peptide and differ between individual free amino acids [4].

Methods that label amino acids as racemization occurs are preferred over correction factors because they offer an in situ control. Liardon et al. [5] demonstrated that hydrolysis of peptides in DCl labeled each amino acid that racemized with one deuterium at the α-carbon as shown in Fig. 1A. A subsequent derivatization to increase the volatility of the amino acids was done in the absence of deuterium. This produced a volatile amino acid and removed all excess deuterium except for the deuterium at the α-carbon which

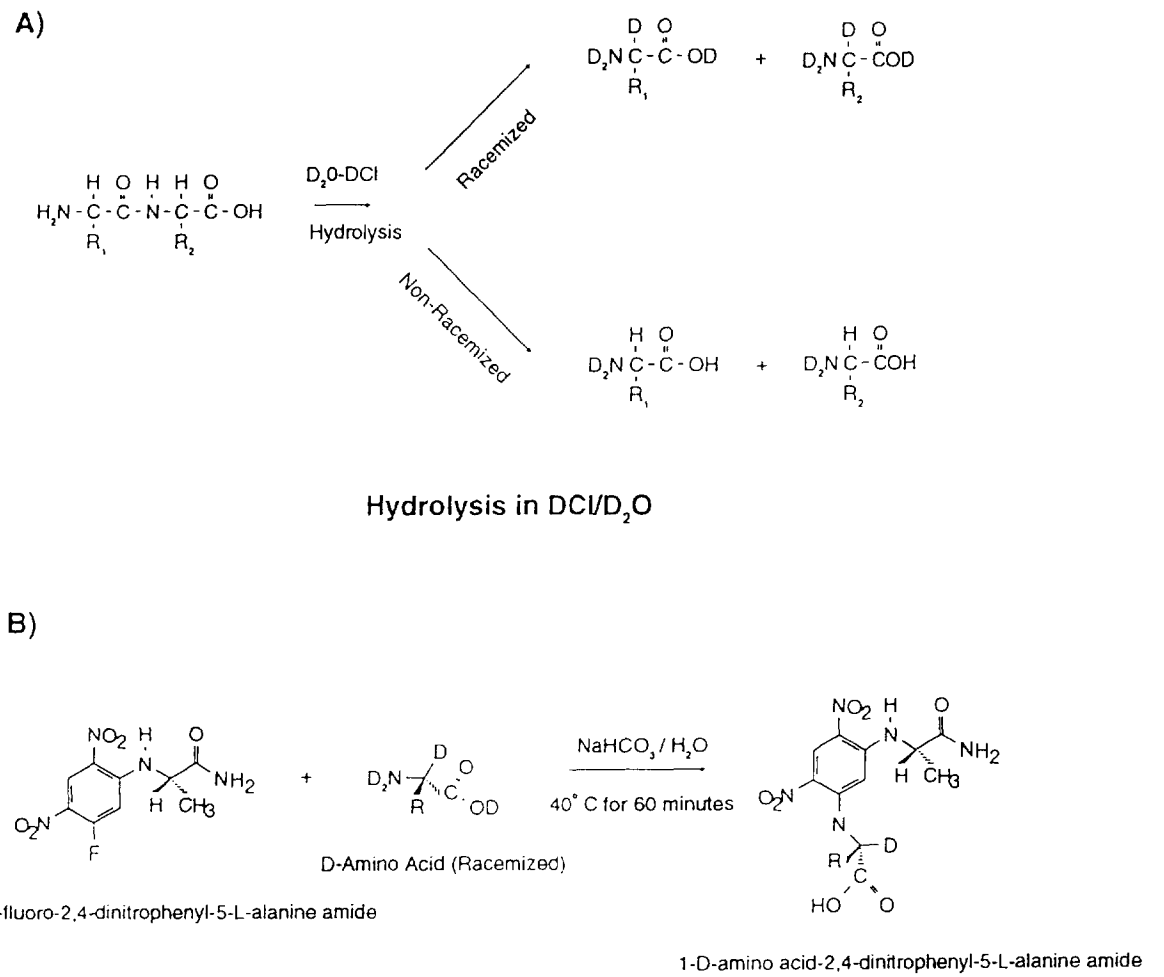

Fig. 1. (A) Amino acid racemization during peptide hydrolysis in a deuterated, acidic solution results in loss of one proton from the α-carbon and is followed by formation of an α-carbon-deuterium bond. (B) Formation of a diastereomer by derivatization of a chiral amino acid with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA).

is not exchanged under the experimental conditions. Analysis of the hydrolysate by gas chromatography–mass spectrometry (GC–MS) on a chiral column provided a means for selective detection of non-racemized amino acids. This GC–MS method allowed the original chiral content of a peptide to be determined, but it had two disadvantages which limited broad applicability. Electron ionization (EI) caused fragmentation of amino acids requiring more than one ion be monitored for each amino acid to ensure proper identification. Furthermore, an optically active stationary phase was required for the direct separation of amino acid enantiomers.

Indirect chromatographic schemes for resolution of racemic mixtures allow conventional achiral columns to be used. Indirect methods separate diastereomers of amino acids prepared by derivatizing amino acids with a chiral reagent. Conformational differences between amino acid diastereomers provide chromatographic resolution. Separation is achieved by either high-performance liquid chromatography (HPLC) or gas chromatography (GC). However, the difficulty of preparing volatile amino acid diastereomers by a two-step derivatization procedure for GC separation has made HPLC the preferred method. Of the many methods for preparing amino acid diastereomers for HPLC separation, diastereomers formed with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA, Marfey's reagent) have proven very successful for analyzing complex mixtures of amino acids [6–9].

A liquid chromatographic–electrospray ionization–mass spectrometry (LC–ESI–MS) method for determining the chiral purity of each amino acid in a peptide is presented. Racemization was circumvented by hydrolyzing peptides in ^{2}HCl/[$^{2}H_4$]acetic acid (1:1). Amino acids were then derivatized with FDAA according to the scheme in Fig. 1B and analyzed by LC–ESI–MS on a reversed-phase column. This method allows the original chiral purity of each amino acid in a peptide to be determined using common achiral reversed-phase HPLC columns. Data are presented showing that chromatographic resolution of a mixture of 39 FDAA labeled amino acids can be achieved in a single analysis. Use of ESI instead of EI prevents fragmentation during ionization and allows the protonated molecular ion to be monitored for each amino acid.

The pentapeptide thymopentin (Arg-Lys-Asp-Val-Tyr) is presently being studied in the treatment of asymptomatic human immunodeficiency virus (HIV) infected subjects [10]. The LC–ESI–MS method was applied to a peptide isolated from a kilogram-scale batch of synthetic Arg-Lys-Asp-Val-Tyr. The chiral purity of a series of synthetic standards: Arg-Lys-Lys-Asp-Val-Tyr, Arg-Lys-Lys-Asp-D-*Val*-Tyr, Arg-Lys-Lys-D-*Asp*-Val-Tyr and the isolated peptide were determined using the method described above.

2. Experimental

2.1. Sample preparation

Peptides (0.1 mg) were hydrolyzed in 10-ml Pierce Reacti-vials that had been purged with nitrogen. After 18 h at 130°C in 1:1 hydrochloric acid–acetic acid (HCl–HOAc) or DCl/[$^{2}H_4$]acetic acid (DCl/DOAc), 400 μl of H_2O was added and the sample lyophilized. To this was added 100 μl of 1 *M* $NaHCO_3$ and then 200 μl of 38.7 m*M* 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) in acetone. The solution was vortexed and incubated at 40°C for 60 min. Reactions were quenched by addition of 50 μl of 2 *M* HCl. Samples were diluted 1:10 with mobile phase A (below) and 80 μl of this solution analyzed by high-performance liquid chromatography–mass spectrometry (LC–MS) or HPLC with UV detection at 340 nm.

2.2. Materials, reagents and peptides

HPLC grade acetonitrile and methanol were obtained from Burdick and Jackson (Muskegon, MI, USA). Trifluoroacetic acid (HPLC spectra grade) and 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) were obtained from Pierce Chemical Company (Rockford, IL, USA). ACS grade ammonium formate and formic acid were purchased from Eastman Kodak (Rochester, NY, USA). ACS grade hydrochloric acid was

purchased from Fisher Scientific (Pittsburgh, PA, USA). Water was purified for HPLC using a Millipore (Super 2) water purification system (Milford, MA, USA). Individual D- and L-amino acids were purchased from Sigma (St. Louis, MO, USA). Twenty percent ^{2}HCl (99.96% deuterium) in deuterium oxide (w/w), [$^2H_4$]acetic acid (99.5% deuterium) and deuterium oxide (99.9% deuterium) were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). Except for thymopentin (Cilag AG, Switzerland) and related substances therein, all peptides used in this study were synthesized, purified and characterized at Immunobiology Research Institute. Synthetic peptides and isolated peptides were characterized by amino acid analysis, Edman sequence analysis and mass spectrometric analysis. Isolated peptides were further analyzed by multidimensional NMR. Purity was assessed by reversed-phase high-performance liquid chromatography and thin layer chromatography.

The chiral purity of synthetic peptide standards used in these studies was confirmed by reversed-phase HPLC. Five thymopentin diastereomers containing a single D-amino acid substitution were chromatographically resolved from each other and from thymopentin. Limit of detection for any of the five D-amino acid analogs spiked into thymopentin was 0.1% (w/w).

2.3. Instrumentation

High-performance liquid chromatography

A Perkin-Elmer (Norwalk, CT, USA) system was used for analytical scale method development and consisted of an ISS-100 autosampler, series 410 LC pump and a Waters (Milford, MA, USA) 484 tunable absorbance detector. Separations were carried out on a YMC (Wilmington, NC, USA) Basic B-03-5 250 × 4.6 mm I.D. column. The ammonium formate mobile phase consisted of 1% methanol in both A and B with 5% acetonitrile in A and 60% acetonitrile in B. Mobile phases A and B were each brought to volume with 10 m*M* ammonium formate, pH 5.2. Linear gradients started with 0% B and finished with 100% B in 45 min. The system was allowed to equilibrate for 15 min at 0% B prior to the next analysis. Flow-rate was 1 ml/min with UV detection at an absorbance of 340 nm. For liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) separations a Waters (Milford, MA, USA) system comprised of a 717 autosampler, a 600-MS system controller/pump, a 484 detector, and a YMC Basic MCB-03-5 250 × 2.0 mm I.D. column with post-column split flow was used. Using a linear restrictor and a tee, two-thirds of the flow was split for UV detection at 340 nm and one-third for mass spectrometric detection. Flow-rate at the pump was 0.25 ml/min and in 20 min the linear gradient went from 20% B to 80% B. A trifluoroacetic acid (TFA, 0.05%) mobile phase system was used for most LC–ESI–MS analyses. Methanol and acetonitrile concentrations in the TFA mobile phases were the same as used in the ammonium formate mobile phases.

Mass spectrometry

A FinniganMAT TSQ 700 equipped with a Finnigan electrospray ionization source was used for LC–ESI–MS. Modifications to the standard instrument were limited but included the two following changes. (1) Rough pumping capacity was increased to decrease the time necessary for reaching operating pressure in the manifold after changing the heated capillary. An Alcatel 2012A (310 l/min) that backed up the manifold was replaced with an Edwards EM2-30 (570 l/min) and the ESI roughing pump was changed from an Edwards EM2-30 to a Galileo D045 (35 cfm). (2) A Shimadzu LC-10AD (Columbia, MD, USA) was used to provide co-axial sheath flow. To prevent current from the ESI source from damaging electronics in the sheath pump the methanol coaxial sheath liquid (0.1 ml/min) was grounded using a 1/16 inch metal union attached to the mass spectrometer chassis.

After the post-column split, flow entered the ESI source at 0.083 ml/min. Total liquid flow out of the ESI source was 0.183 ml/min (column + sheath). A typical ESI voltage was + 3.0 kV with the auxiliary and sheath gas nitrogen pressures set at 103.4 and 179.3 MPa,

respectively. Prior to analysis of DNPA-amino acids, a mixture of 1-L-glutamic acid-DNPA and 1-L-glutamine-DNPA was analyzed by LC–ESI–MS to confirm that the mass spectrometer was set to resolve differences of one atomic mass unit.

3. Results and discussion

3.1. HPLC separation of 2,4-dinitrophenyl-5-L-alanine amide amino acids

Derivatization of an amino acid with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) produces a diastereomer with a highly sensitive and specific UV label ($\epsilon = 3 \times 10^4$ at 340 nm) on the α-amino group. Such diastereomers are referred to as 2,4-dinitrophenyl-5-L-alanine amide (DNPA) amino acids or simply DNPA-amino acids. All amino acids including the imino acid proline and the achiral amino acid glycine are easily derivatized with FDAA. In the original report on the use of FDAA, Marfey [6] demonstrated that a select set of five DNPA-amino acids (Ala, Asp, Glu, Met and Phe) could be separated on a conventional reversed-phase column without the addition of chiral modifiers to the mobile phase. Kochhbar and Christen [9] were able to separate a mixture of 19 DNPA-L-amino acids by reversed-phase HPLC. Data in Tables 1 and 2 show that even more complex mixtures consisting of both D- and L-DNPA-amino acids can be resolved by conventional reversed-phase HPLC.

Data in Tables 1 and 2 were acquired by reversed-phase HPLC separation of individual DNPA-amino acid diastereomeric pairs with UV detection at 340 nm. As exhibited by the α-values in Tables 1 and 2, ammonium formate and trifluoroacetic acid mobile phases were capable of separating most diastereomeric pairs. Table 1 shows that each of the 39 DNPA-D- and DNPA-L-amino acids may be resolved using an ammonium formate mobile phase. Excellent resolution was achieved between individual D- and L-amino acid diastereomeric pairs and between all 39 components. Alternatively, the trifluoroacetic acid mobile phase (Table 2) permitted better resolution of the histidine diastereomeric pair, but the individual diastereomeric pairs of glutamine, proline and serine were not resolved. As expected the C_8 YMC-basic column separated hydrophobic diastereomers better than polar DNPA-amino acid diastereomeric pairs. In all cases, DNPA-L-amino acids eluted prior to DNPA-D-amino acids possibly due to greater intramolecular hydrogen bonding in the DNPA-D-amino acids [6].

While very good resolution of all DNPA-amino acid diastereomeric pairs can be achieved, differences in absorption between diastereomeric pairs have been observed. If absorbance at 340 nm is the sole method of detection, then integrated areas must be corrected for quantitative

Table 1
HPLC separation using the ammonium formate mobile phase of all 19 common D- and L-amino acid pairs and glycine derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide

Amino acid	Ammonium formate			AA-DNPA R_F ratio (D/L)
	Retention time (min)			
	L	D	α	
Ala	10.93	13.66	1.356	1.25
Arg	10.29	11.17	1.124	1.27
Asn	7.88	9.63	1.375	1.13
Asp	7.98	9.85	1.394	1.16
Cys	10.89	12.56	1.218	1.22
Glu	9.42	11.35	1.313	1.40
Gln	8.97	10.03	1.186	1.31
Gly	11.03		N.A.	N.A.
His	9.08	9.23	1.026	N.D.
Ile	17.01	20.94	1.286	1.24
Leu	17.58	21.28	1.257	1.19
Lys	9.67	10.57	1.140	1.24
Met	14.54	17.98	1.304	1.22
Phe	18.33	21.23	1.192	1.32
Pro	11.57	13.82	1.270	1.02
Ser	8.48	9.93	1.277	1.19
Thr	9.01	12.28	1.566	1.32
Trp	18.37	20.52	1.142	1.24
Tyr	13.34	15.67	1.230	1.28
Val	14.29	18.22	1.355	1.27

N.A. = not applicable and RF ratio = UV response factor ratio at 340 nm.
N.D. = not determined.

Table 2
HPLC separation using the trifluoroacetic acid mobile phase of all 19 common D- and L-amino acid pairs and glycine derivatized with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide

Amino acid	Trifluoroacetic acid		
	Retention time (min)		
	L	D	α
Ala	18.34	19.98	1.108
Arg	14.45	15.38	1.083
Asn	13.93	14.21	1.026
Asp	15.88	16.33	1.035
Cys	11.66	13.08	1.167
Glu	17.11	17.77	1.047
Gln	14.99	14.99	1.00
Gly	17.21		N.A.
His	11.85	13.37	1.175
Ile	25.36	28.03	1.121
Leu	25.64	28.10	1.109
Lys	13.98	15.03	1.098
Met	22.38	24.62	1.117
Phe	25.95	27.85	1.083
Pro	19.12	19.12	1.00
Ser	14.80	14.80	1.00
Thr	15.54	17.57	1.164
Trp	25.20	26.52	1.060
Tyr	18.05	20.73	1.180
Val	22.88	25.49	1.133

N.A. = not applicable.

analysis. Prior to beginning work with ESI–MS detection, the UV response factors for all DNPA–amino acid diastereomeric pairs at 340 nm were determined (Table 1). The ammonium formate mobile phase was used because of the better resolution achieved for all DNPA-amino acids. Each diastereomeric pair was analyzed as a mixture of 0% D, 5% D, 15% D and 25% D. Graphical analysis of the observed DNPA-D-amino acid area percent versus the actual DNPA-D-amino acid percent yielded a line the slope of which represents the DNPA-D-amino acid to DNPA-L-amino acid response factor ratio. Laboratories with access to LC–ESI–MS will not require such corrections because all 19 response factors were found to have a value of one by mass spectrometry. However, as described in detail below, mass spectrometry can also be used to unequivocally distinguish racemized from non-racemized amino acids. Racemization of amino acids during peptide hydrolysis has been a more perplexing problem to solve than separation of amino acids.

3.2. Racemization during peptide hydrolysis

Fig. 2 illustrates one of the challenges presented in attempting to use single amino acids as

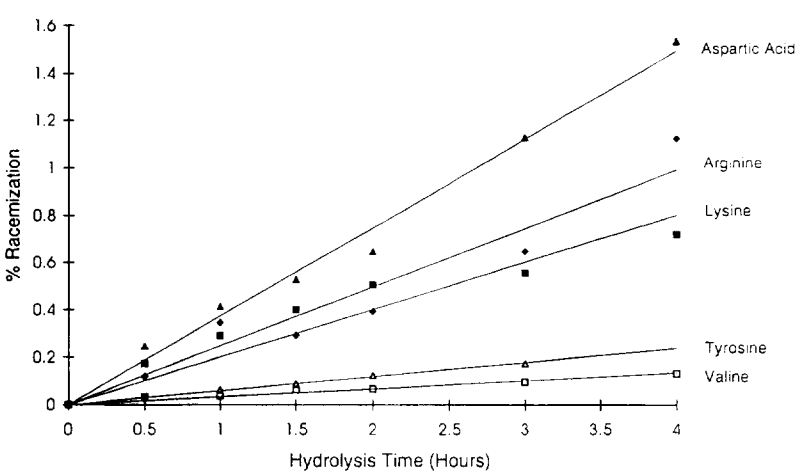

Fig. 2. Example of the variability in racemization between free amino acids during conditions of acid hydrolysis. Individual amino acids were incubated for various times at 130°C in HCl–HOAc (1:1).

controls for racemization. The five amino acids found in thymopentin were incubated in HCl–HOAc at 130°C for varying lengths of time (Fig. 2). Degree of racemization differed between amino acids and increased over time for all amino acids tested. The observed difference in racemization between aspartic acid and valine at each time point is expected based on the mechanism of racemization [11]. The carbanion intermediate formed during amino acid racemization is stabilized by the carboxyl side chain of aspartic acid and destabilized by the alkyl side chain of valine. A further complication during the hydrolysis of thymopentin and other peptides arises from the location of a given amino acid residue within the primary sequence [4]. A given amino acid will exhibit rates of racemization that are specific to the amino acid sequence in which it is located. Differences in racemization between amino acids and the dependence of amino acid location within a peptide preclude the use of free amino acids or related peptides as controls for racemization.

Fig. 3 shows HPLC separations for a mixture of five DNPA-amino acid standards for the amino acids found in thymopentin (Fig. 3A) and a mixture of the same DNPA-amino acids produced by hydrolysis of thymopentin (Fig. 3B) in HCl–HOAc. Fig. 3A illustrates the excellent resolution that can be achieved with DNPA-amino acids. More than the expected ten responses are observed in Fig. 3A,B, because FDAA reacts with water (DNPA-OH), at the ϵ-amino group of lysine (*R*-Lys) and at the ρ-hydroxyl group of tyrosine (*R*-Tyr) [12]. Formation of bis-DNPA-amino acids was minimal under the derivatization conditions used. At higher concentrations of FDAA bis-DNPA-amino acid products may be significant [12].

Prior to the work with the DNPA-amino acids, the chiral purity of each residue in thymopentin was determined to be greater than 99.9% (see Experimental). The observation of DNPA-D-amino acids in Fig. 3B indicates that racemization occurred during hydrolysis. This conclusion is based on separate experiments, where very little racemization ($<0.06\%$) of the five amino acids in thymopentin occurred as a result of FDAA derivatization. One way to circumvent the racemization observed in Fig. 3B is to carry out hydrolysis in deuterated acids being careful to exclude any source of protons. Any amino acid that racemizes will be labeled with one deuterium at the α-carbon. This allows racemized amino acids to be distinguished from non-racemized amino acids by an increase of one atomic mass unit using mass spectrometry [5].

In the original GC–MS method [5] peptides were hydrolyzed in ^{2}HCl, derivatized for increased volatility and ionized by EI. Fragmentation of the molecular ion during EI forced multiple ions to be monitored for each derivatized amino acid. Recently, thermospray ionization has also been shown to cause fragmentation of DNPA-amino acids [13]. Using ESI–MS prevents fragmentation of DNPA-amino acids during ionization and allows only the protonated molecular ion to be monitored for identification of each DNPA-amino acid. This is an advantage over the GC–MS method because more amino acids can be monitored per separation. While chiral analysis of free amino acids not subjected to hydrolysis can be carried out as in Fig. 3, analysis of the chirality of amino acids originating in a peptide require hydrolysis in deuterated acids followed by selected-ion monitoring (SIM) mass spectrometric analysis to distinguish non-racemized amino acids.

3.3. Detection of non-racemized amino acids by LC–ESI–MS

Amino acids undergoing racemization during hydrolysis in a deuterated medium increase in molecular mass by one atomic mass unit. At unit resolution a quadrupole mass spectrometer can distinguish between two ions that differ by only one m/z value. This allows non-racemized amino acids to be distinguished from amino acids that racemize in an environment of deuterium ($>99.99\%$). In a typical LC–ESI–MS experiment multiple protonated molecular ions ($[M+H]^+$) are monitored for each expected m/z value corresponding to a non-racemized amino acid. The total-ion chromatogram for such an experiment will indicate the D- and L-amino acids

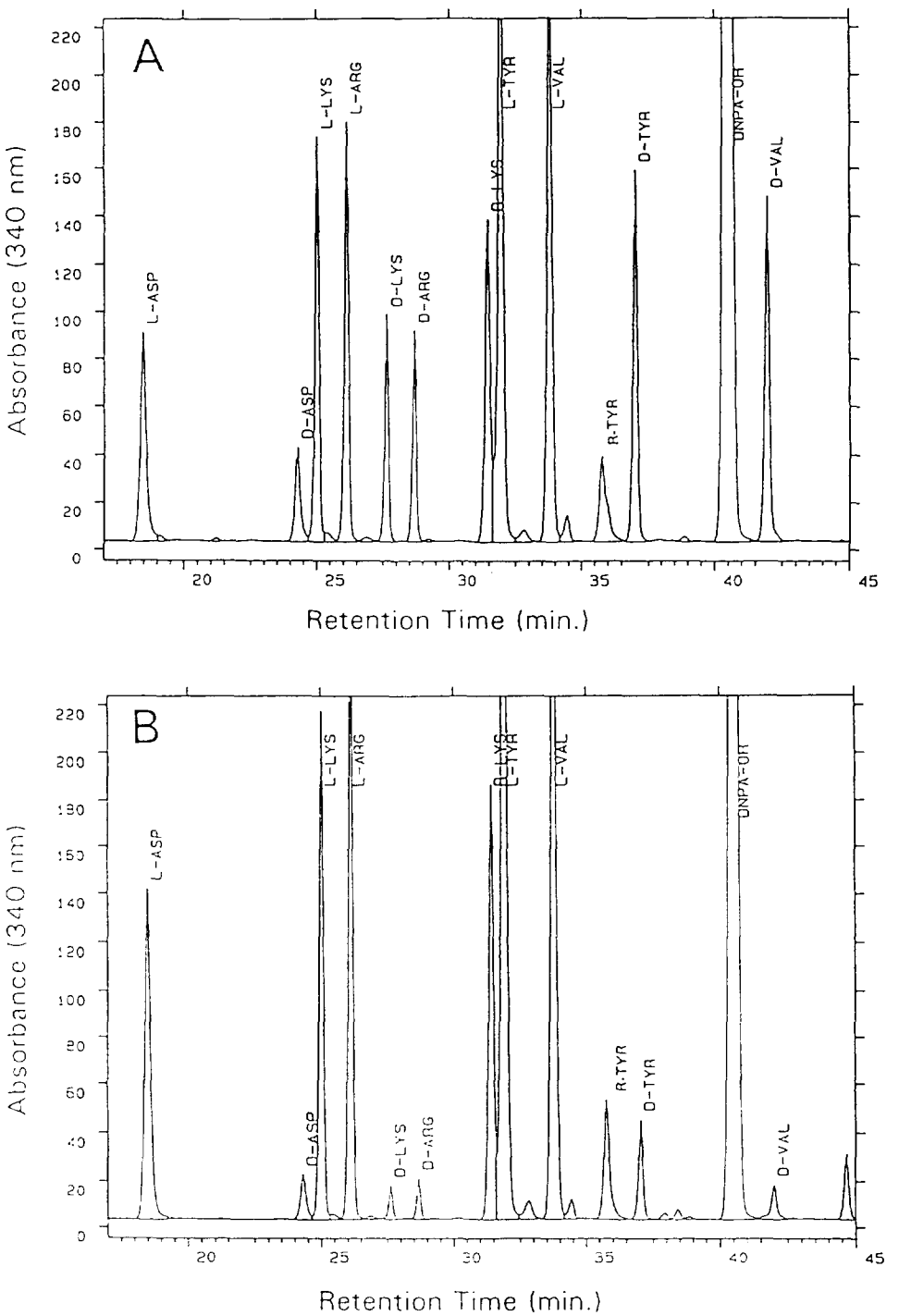

Fig. 3. Reversed-phase separations of 2,4-dinitrophenyl-5-L-alanine amide (DNPA) amino acids: (A) from a mixture of the D- and L-DNPA-diastereomers of arginine, lysine, aspartic acid, valine, and tyrosine, and (B) after hydrolysis of thymopentin in HCl–HOA (1:1). Separation was carried out using the ammonium formate mobile phase and detection was at 340 nm. DNPA-OH is hydrolyzed FDAA, *R*-Lys is ε-DNPA-lysine and *R*-TYR is ρ-DNPA-tyrosine.

present in the peptide prior to hydrolysis. Any racemized amino acids are excluded from consideration because of an increase in molecular mass of one atomic mass unit. For a peptide with five different amino acids, all five DNPA-amino acids can be examined in a single LC–ESI–MS experiment by monitoring only five ions. Alternately, if only one amino acid in a peptide is suspected of being a D-isomer, then the mass spectrometer can be scanned over a narrow range to detect that isomer. This concept is presented in Figs. 4 and 5 by comparing the results of peptide hydrolysis in HCl–HOAc against hydrolysis in DCl–DOAc.

Thymopentin was hydrolyzed overnight in HCl–HOAc (Fig. 4A,B). Hydrolysates were subjected to derivatization with FDAA and analyzed by LC–ESI–MS. In Figs. 4 and 5 lysine is used as an example of how one amino acid in a peptide can be checked for enantiomeric configuration. Data in Fig. 4 was acquired by scanning over a narrow range around the protonated molecular ion for DNPA-lysine (m/z 399). Extracted ion chromatograms displayed for $[\text{DNPA-lysine} + \text{H}]^+$ (m/z 399) (Fig. 4A) and $[\text{DNPA-lysine-d}_1 + \text{H}]^+$ (m/z 400) (Fig. 4B) each show three peaks. The three species were identified as: $[\alpha\text{-DNPA-L-lysine} + \text{H}]^+$ (1), $[\alpha\text{-DNPA-D-lysine} + \text{H}]^+$ (2) and $[\epsilon\text{-DNPA-L-lysine} + \text{H}]^+$ (3). Peak 2 was due to racemization of L-lysine to D-lysine during hydrolysis. This assignment was based on findings that none of the five amino acids in thymopentin underwent racemization during FDAA derivatization alone. The three responses in Fig. 4B represent the same species as in Fig. 4A, but are due to the natural abundance of deuterium.

When thymopentin was hydrolyzed in DCl–DOAc under conditions similar to those used with HCl–HOAc, then only two responses were observed for the extracted ion chromatogram of $[\text{DPNA-lysine} + \text{H}]^+$ (m/z 399) (Fig. 5A). Responses 1 and 3 are the same as those identified in Fig. 4A. In Fig. 5B the extracted ion chro-

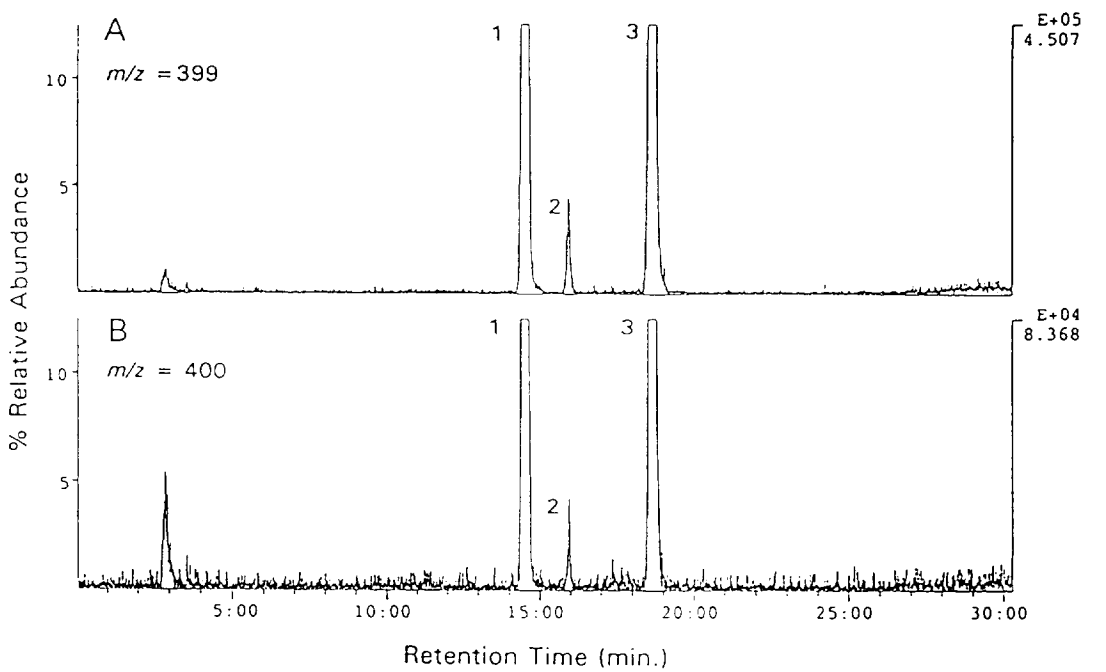

Fig. 4. Scanning LC–ESI–MS analysis (m/z 394–404 in 1.0 s) of DNPA-lysine from thymopentin hydrolyzed in HCl–HOAc. Extracted single ion chromatograms are shown for (A) $[\text{DNPA-lysine} + \text{H}]^+$ (m/z 399.1) and for (B) $[\text{DNPA-lysine-d}_1 + \text{H}]^+$ (m/z 400.1). The three annotated peaks were identified as: $[\alpha\text{-DNPA-L-lysine} + \text{H}]^+$ (1), $[\alpha\text{-DNPA-D-lysine} + \text{H}]^+$ (2) and $[\epsilon\text{-DNPA-L-lysine} + \text{H}]^+$ (3).

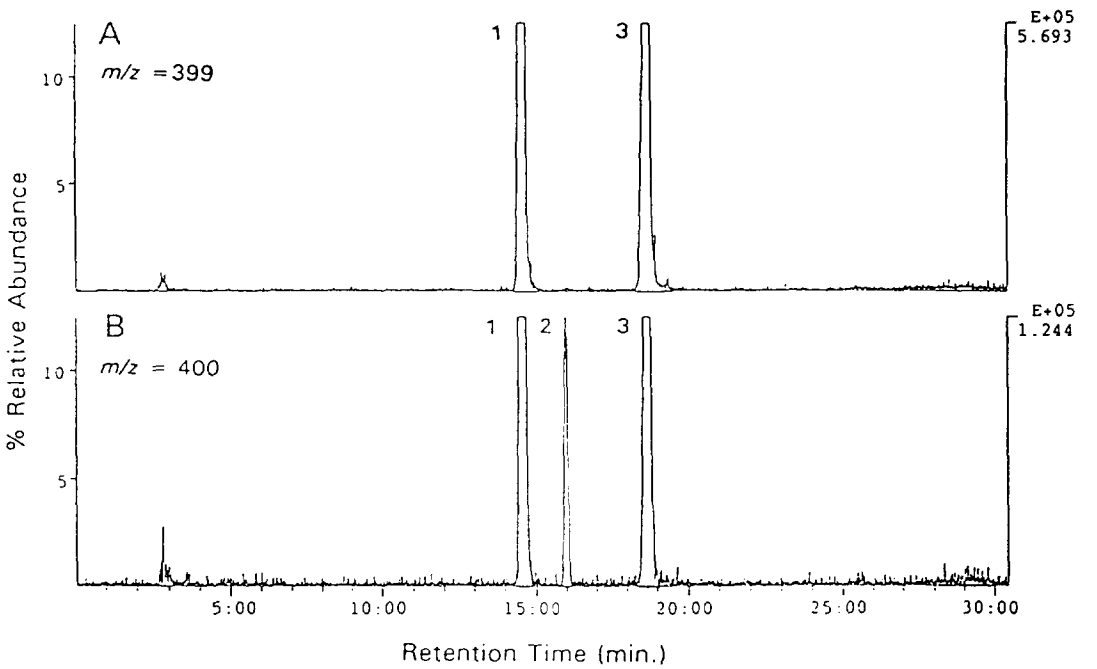

Fig. 5. Scanning LC–ESI–MS analysis (m/z 394–404 in 1.0 s) of DNPA-lysine from thymopentin hydrolyzed in DCl–DOAc. Extracted single ion chromatograms are shown for (A) $[\text{DNPA-lysine} + H]^-$ (m/z 399.1) and for (B) $[\text{DNPA-lysine-d}_1 + H]^+$ (m/z 400.1). The three annotated peaks were identified as: $[\alpha\text{-DNPA-L-lysine} + H]^+$ (1), $[\alpha\text{-DNPA-D-lysine} + H]^+$ (2) and $[\epsilon\text{-DNPA-L-lysine} + H]^+$ (3).

matogram for the protonated molecular ion of DNPA-lysine-d_1 (m/z 400) is shown. In Fig. 5A response 2, identified in Fig. 4A as $[\alpha$-DNPA-D-lysine-$d_1 + H]^+$,is absent. Any D-lysine present in thymopentin prior to hydrolysis would elute at 16.0 min in Fig. 5A. Figs. 4 and 5 illustrate the power of this LC–ESI–MS method to determine the enantiomeric configuration of amino acids present in a peptide prior to hydrolysis. Any amino acids that racemize during hydrolysis can be effectively circumvented so that only non-racemized amino acids are observed.

3.4. Application of the LC–ESI–MS method

This LC–ESI–MS method was used to establish the chiral purity of a thymopentin related substance. An unknown peptide, originally present at less than 0.1% relative to thymopentin, was isolated by reversed-phase HPLC and characterized. Tandem mass spectrometry and Edman chemistry identified the sequence as Arg-Lys-Lys-Asp-Val-Tyr. From heteronuclear NMR data the peptide was suspected of containing either all L-amino acids, a D-aspartic acid substitution for L-aspartic acid or a D-valine substitution for L-valine. Three standards were synthesized for comparison to the unknown and to validate the LC–ESI–MS method: Arg-Lys-Lys-Asp-Val-Tyr, Arg-Lys-Lys-*D-Asp*-Val-Tyr and Arg-Lys-Lys-Asp-*D-Val*-Tyr. The unknown and standards were separately hydrolyzed in DCl–DOAc, derivatized with FDAA and analyzed by LC–ESI–MS as shown in Fig. 6.

Results for the three control peptides Arg-Lys-Lys-Asp-Val-Tyr, Arg-Lys-Lys-*D-Asp*-Val-Tyr and Arg-Lys-Lys-Asp-*D-Val*-Tyr are shown in Fig. 6. Data was acquired in three experiments by continuously monitoring the same five ions during each LC–ESI–MS experiment. Chromatographic resolution of all DNPA-amino acids allowed the original chiral purity to be determined quickly from the total ion chromatogram for each peptide. The method accurately pre-

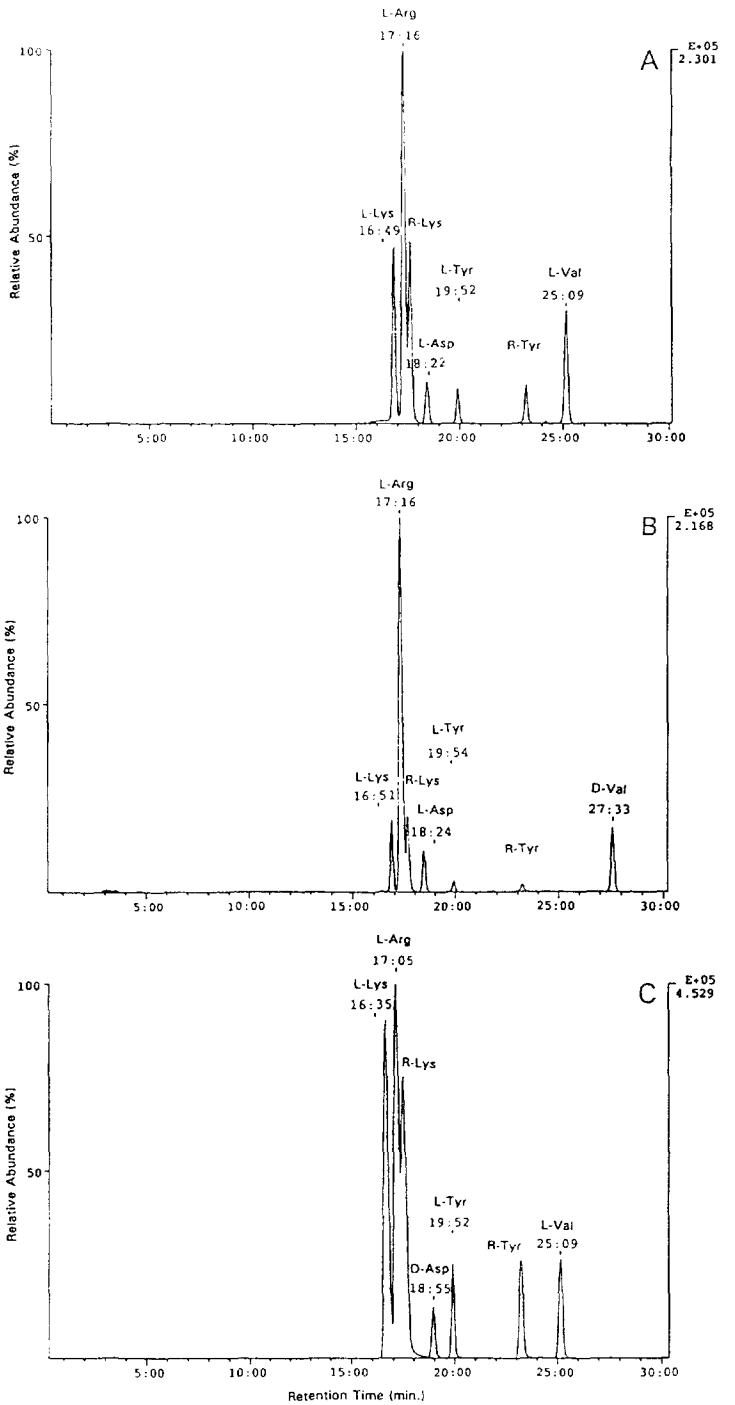

Fig. 6. Analysis by LC–ESI–MS of (A) Arg-Lys-Lys-Asp-Val-Tyr, (B) Arg-Lys-Lys-Asp-D-*Val*-Tyr and (C) Arg-Lys-Lys-D-*Asp*-Val-Tyr hydrolyzed in DCl–DOAc for 12 h at 130°C. One ion was monitored for each of the five anticipated DNPA-amino acids (e.g. for [DNPA-L-lysine + H]$^+$ m/z 399.1 ± 0.1 amu in 0.3 s) and the summed ion chromatograms displayed. *R*-Lys is [ε-DNPA-lysine + H]$^+$ and *R*-Tyr is [ρ-DNPA-tyrosine + H]$^+$.

dicted the chiral purity of the three standards. Results for the unknown identified it as being identical to the all L-isomer Arg-Lys-Lys-Asp-Val-Tyr in Fig. 6A. The LC–ESI–MS results were corroborated in separate HPLC experiments where the unknown peptide co-eluted with Arg-Lys-Lys-Asp-Val-Tyr.

4. Conclusions

We have extended the advances in reversed-phase separation of DNPA-amino acid diastereomers to resolution of 39 DNPA-amino acids. By combining this advance with peptide hydrolysis in deuterated acids to circumvent racemization, ESI–MS detection was used to unambiguously detect non-racemized amino acids in peptide hydrolysates. Simple, volatile mobile phases allowed DNPA-amino acids to be resolved and detected by ESI–MS analysis. By varying the gradient conditions complex mixtures were readily resolved. Isomers such as DNPA-isoleucine and DNPA-leucine were distinguishable because of the high chromatographic resolution that was achieved. Use of ESI–MS to analyze DNPA-amino acids prevented the fragmentation of amino acid derivatives common with electron ionization [4] and thermospray ionization [13]. This advantage over the GC–EI–MS method can be used to monitor a greater number of ions per LC–ESI–MS analysis or to monitor fewer ions at a lower limit of detection. A second advantage of ESI over EI is that amino acids need not be derivatized to increase volatility and this decreases sample preparation time. The LC–ESI–MS method as described provides a rapid and accurate determination of the enantiomeric purity of each amino acid in a given peptide. We are now applying the method to larger peptides and proteins.

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