Determination of Peptide Chiral Purity using Marfey's Reagent by Accurate Mass LC-MS

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Abstract

Racemisation of D- and L-amino acid chiral residues in a synthetic peptide is one of the most important side reactions in peptide synthesis. The accurate determination of the chirality of amino acids in peptides is crucial for understanding the biological and physiological roles of these stereoisomers. A high-performance liquid chromatography-electrospray ionisation-mass spectrometric (LC-ESI-MS) method is presented that allows rapid and accurate determination of amino acid chiral purity in a peptide. Peptides are hydrolysed in deutrated hydrochloric acid,/deutrated acetic acid, and then converted to diastereomers by derivatisation 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- and L-amino acids present in a peptide changes during acid hydrolysis due to racemisation. Peptide hydrolysis in deuterated acids circumvents this problem by labelling each amino acid that racemises with one deuterium at the α-carbon.

The developed protocol demonstrated excellent selectivity and reproducibility in differentiating D-amino acids from their L-counterparts within peptide sequences. Additionally, the method was successfully applied to the analysis of complex biological samples, showcasing its potential for applications in the fields of proteomics and peptidomics.

Introduction

Due to increased interest in the role of free D-amino acids in biology the development of a robust method for detecting free D-amino acids was required. The chiral purity of an amino acid in a peptide is usually determined by acid hydrolysis followed by amino acid separation using hyphenated chromatographic platforms [1,2]. Acid hydrolysis leads to racemisation, where L-amino acids are converted to D-amino acids and vice versa. In this method if an amino acid undergoes racemisation, the hydrogen on the α-carbon becomes deuterated as shown in Figure 1 reaction below. Consequently, amino acids undergoing racemisation increase by + 1Da in mass and will therefore not be included in the analysis[3]. Analysis of the derivatised amino acids by liquid chromatography-mass spectrometry (LC-MS) on an achiral column provided a means for selective detection of non-racemised amino acids. This method allows the original chiral purity of each amino acid in a peptide to be determined using common achiral reversed-phase HPLC columns.

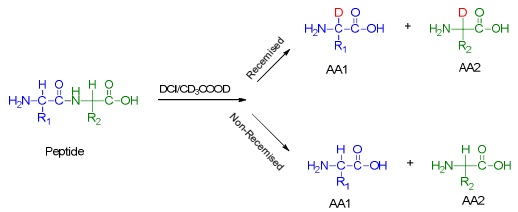


Figure 1: Amino Acid Racemisation during Peptide Hydrolysis using DCl/CD_3COOD

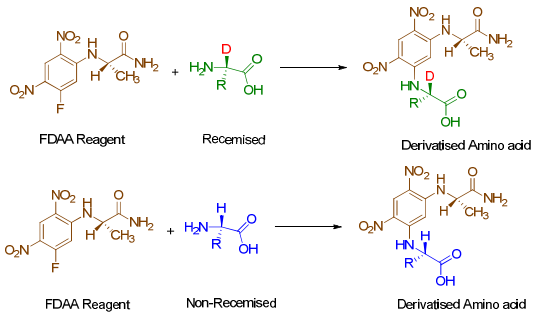


Figure 2: Reaction of FDAA with Amino Acid

Method

Standard Solutions

L- and D- amino acid mixed standards were prepared at a concentration of 50µg/mL in water and derivatised with FDAA at 40°C for 1 hr. Derivatised amino acids were separated by RP-HPLC and analysed by high resolution LC-ESI-MS in negative mode.

Sample Hydrolysis and Derivatisation

Peptide sample was weighed and transferred to a suitable vacuum hydrolysis tube. Hydrolysis of the peptide was carried-out in 6NDCl/ [D4] acetic acid (1:1) for 6 hours at 130°C. The amino acids were then derivatised with FDAA at 40°C for 1 hr and analysed by LC-ESI-MS.

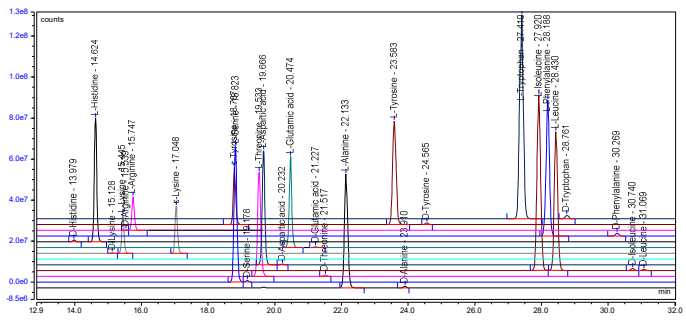
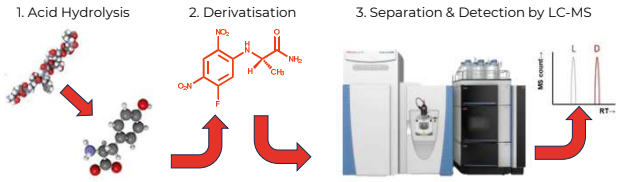


Figure 3: SIM Channels of 1% D-Amino Acids in L-Amino Acids



Results and Discussion

Derivatisation of an amino acid with FDAA reagent produced a diastereomer with high sensitivity and specificity. All amino acids were robustly derivatised with FDAA resulting in 2,4-dinitrophenyl-5-L-alanineamide (DNPA) derivative amino acids. Chromatographic peaks were extracted according to the different m/z values listed in Table 1. Figure 3 shows typical separation of all D- and L- derivatised amino acid derivatives by RP-HPLC-MS. Accurate mass determination provided extra selectivity that allowed additional confidence in results.

Table 1: SIM Masses of FDAA Derivative Amino Acids

Amino Acid	Marfey's Reagent Derivative $[M-H]^-$ (Da)
Serine (S)	356.0848
Leucine (L)	382.1368
Glutamine (Q)	Measure as Glutamic Acid (E)
Alanine (A)	340.0899
Glutamic Acid (E)	398.0954 / 400.1079
Lysine (K)	397.1477
Arginine (R)	425.1539
Isoleucine (I)	382.1368
Histidine (H)	406.1117
Threonine (T)	370.1004
Asparagine (N)	Measure as Aspartic Acid (D)
Aspartic Acid (D)	384.0797 / 386.0923
Tyrosine (Y)	432.1161 / 434.1286
Tryptophan (W)	455.1321 / 460.1635
Phenylalanine (F)	416.1212
Valine	368.1212
Proline	366.0691
Methionine	400.0932
Cysteine	372.0619

Table 2: Retention Time of Amino Acids

Amino acid	L-AA RT(min.)	D-AA RT(min.)
Alanine	22.1	23.9
Serine	18.8	19.2
Threonine	195.	21.5
Isoleucine	27.9	30.8
Leucine	28.4	31.1
Aspartic acid	19.6	20.2
Lysine	15.4	15.1
Glutamic acid	20.5	21.2
Histidine	14.6	14.0
Phenylalanine	28.2	30.
Arginine	15.8	15.6
Tyrosine	23.6	24.5
Tryptophan	27.3	28.7
Proline	22.9	23.7
Valine	26.002	28.6
Methionine	25.6	27.9
Cysteine	15.7	17.0

In this MS-platform multiple deprotonated molecular ions $[M-H]^-$ were monitored for each expected m/z value corresponding to a non-racemised DNPA-amino acid present in the peptide prior to hydrolysis. Glutamine and asparagine were deaminated during hydrolysis to form glutamic acid and aspartic acid. Therefore, glutamine and asparagine were identified as the corresponding acid derivative.

Conclusions

In conclusion the combination of Accurate Mass LC-MS and FDAA provides a powerful tool for the determination of D-amino acid levels in synthetic peptides, facilitating the characterisation of peptide stereochemistry and supporting the development of bioactive peptides for therapeutic applications.

References

1. Visser, W.F., Verhoeven-Duif, N.M, Opho, R., Bakker, S.; Klomp, L.W.; Berger, R.; de Koning, T.J. A sensitive and simple ultra-high-performance-liquid chromatography–tandem mass spectrometry-based method for the quantification of d-amino acids in body fluids. J. Chromatogr. A 2011, 1218, 7130–7136.
2. Goodlett, D.R.; Abuaf, P.A.; Savage, P.A.; Kowalski, K.A.; Mukherjee, T.K.; Tolan, J.W.; Corkum, N.;Goldstein, G.; Crowther, J.B. Peptide chiral purity determination: Hydrolysis in deuterated acid, derivatization with Marfey's reagent and analysis using high-performance liquid chromatography-electrospray ionization-mass spectrometry. J. Chromatogr. A 1995, 707, 233–244.
3. Danielsen, M., Nebel, C., Dalsgard, T.K., Simultaneous Determination of L- and D-Amino Acids in Proteins: A Sensitive Method Using Hydrolysis in Deuterated Acid and Liquid Chromatography–Tandem Mass Spectrometry Analysis, Foods. 2020 Mar; 9(3): 309
4. Marfey, P.: Determination of D-amino acids. II. Use of a bifunctional reagent, 1,5-difluoro-2,4-dinitrobenzene. Carlsb. Res. Commun. 49, 591–596 (1984)