

Development of Conditions Suitable for Enantiomeric Purity Determination of Peptides using Accurate Mass LC-MS



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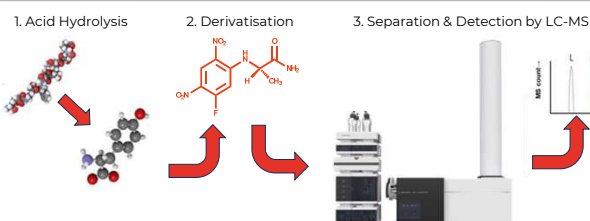
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Abstract

Peptides are becoming an increasingly advanced field of pharmaceutical development, with their therapeutic benefits being marketed globally. The enantiomeric purity of these peptides determines their efficacy and safety for patients. Therefore, it is of great importance to be able to quantify the chiral purity of these molecules. This project aimed to develop conditions for the determination of the enantiomeric purity of a given peptide by reverse phase liquid chromatography mass spectrometry. Various experimental parameters were investigated including acid strength, temperature and duration of hydrolysis and derivatisation. Racemisation is a main side reaction in these peptides, occurring during storage, transport and even during analysis where hydrolysis and racemisation are strongly connected to one another. Samples were hydrolysed in deuterated acid to offset the falsely raised levels of D-amino acids occurring due to racemisation, and to give us a true value for the enantiomeric purity. Marfey's reagent is used to separate the enantiomers as it forms diastereomers which can be separated based on their physical properties. The developed method has furthered understanding into this field, allowing for accurate quantification of enantiomeric purity, essential in ensuring patient safety and the reliability of the pharmaceutical effects of peptide-based medicines.

Introduction

Peptides are an emerging class of therapeutics with applications ranging from cancer treatment to diabetes management. Due to the presence of chiral centres in amino acids, peptides can exist as enantiomers—mirror-image forms that may differ significantly in biological activity. Ensuring the enantiomeric purity of peptide-based drugs is therefore essential for both efficacy and patient safety. This project aimed to develop a reliable method for determining the chiral purity of a synthetic peptide using reverse-phase liquid chromatography coupled with accurate mass spectrometry (LC-MS). A key challenge in this analysis is racemisation, which can occur during peptide synthesis, storage, or hydrolysis, leading to misleading results. To address this, the peptide was hydrolysed using 6N deuterated hydrochloric acid (DCL) to remove the bias caused by racemisation and allow for accurate D-amino acid quantification. The amino acids were derivatised with Marfey's reagent (FDAA), forming diastereomers that could be separated chromatographically.



Results and Discussion

Derivatisation of an amino acid with FDAA reagent produced a diastereomer which allowed for separation of the amino acids due to different physical properties. 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. This work showed that optimal parameters differ between peptides and so development work must be carried out for each to determine the best conditions.

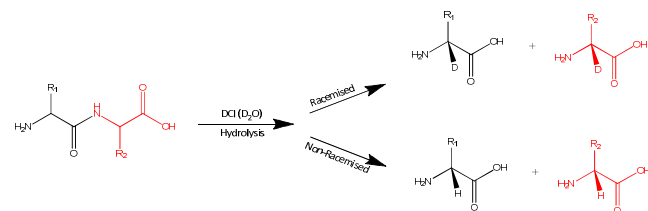


Figure 1: Amino Acid Racemisation during Peptide Hydrolysis using DCL and D₂O

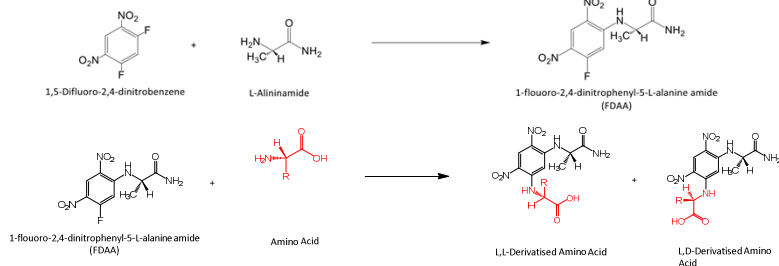


Figure 2: Formation of 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (FDAA) and reaction with amino acid

Method

500 μ L of deuterated acid was added to 2 mg of peptide sample and the vial was flushed with Argon and sealed tightly to prevent the ingress of air. The samples were then hydrolysed under with different acid strengths and durations. After hydrolysis, a further 500 μ L of H₂O was added and the samples were freeze dried overnight using liquid nitrogen. After freeze drying the hydrolysed samples were derivatised by adding 100 μ L of 1 M Sodium Bicarbonate, 100 μ L Marfey's reagent (3 mg/mL in acetone) and 200 μ L of UHQ water and heated at varied times and temperatures. The reaction was then quenched with 50 μ L of 2 N formic acid. Finally, the volume of solution was made up to 2 mL to give a sample concentration of 1 mg/mL. Chromatograms of the expected masses of the individual derivatised amino acids (Table 4) were extracted from the total ion chromatograms produced.

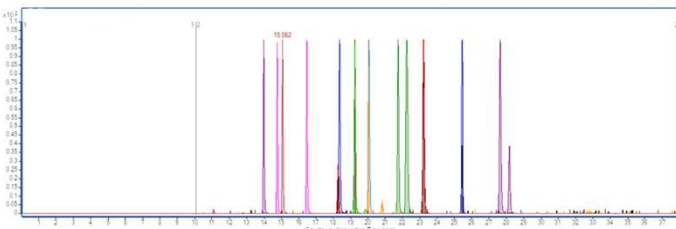


Figure 3: Overlaid Extracted Ion Chromatograms of Derivatised Amino Acids

Optimised Conditions

Hydrolysis

- 6N DCL
- 2 Hours
- 110°C

Derivatisation

- Marfey's Reagent
- 2 Hours
- 40°C

References

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Table 1: Expected Masses of FDAA Derivative Amino Acids

Amino Acid	Marfey's Reagent Derivative [M-H] (Da)
Serine (S)	356.0848
Leucine (L)	382.1368
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
Asparagine (N)	Measure as Aspartic Acid (D)
Aspartic Acid (D)	384.0797 / 386.0923
Tyrosine (Y)	432.1161 / 434.1286
Valine (V)	368.1212
Proline	366.0691

Table 2: Retention Time of Amino Acids

Amino acid	L-AA RT(min.)	D-AA RT(min.)
Alanine	21.7	23.5
Serine	18.3	18.7
Isoleucine	27.5	30.3
Leucine	28.1	30.7
Aspartic acid	19.2	19.8
Lysine	14.9	14.6
Glutamic acid	20.0	20.8
Histidine	14.1	13.4
Arginine	15.2	15.0
Tyrosine	23.2	24.2
Proline	22.2	23.0
Valine	25.4	28.0

Due to hydrogen bonding present in the L,L-diastereomers Marfey predicted that these would elute more quickly than D,L-diastereomers as they are more polar. However, it was found that the D amino acids eluted first for Arginine, Histidine and Lysine, contrary to Marfey's findings this is because they have positive side chains which cause them to have a greater affinity for the polar mobile phase.

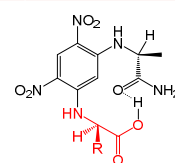


Figure 4: L,L-derivatised Amino Acid showing H-Bonding

Conclusions

This project set out to find an appropriate method to quantify the chiral purity of a peptide containing thirteen different amino acids. The final conditions after considering key parameters of acid strength and duration of hydrolysis and temperature and duration of derivatisation is a two-hour hydrolysis in 6N DCL at 110°C followed by a two-hour derivatisation at 40°C with Marfey's reagent. These conditions minimised racemisation and maximised peak area for the amino acids present in this peptide. While these parameters were optimised individually, they were not tested together in one method and so future analysis would involve combining these conditions to confirm correlation between them. The selected conditions are different from those used for previous chiral purity analysis on different peptides, this emphasises that time must be built into customer project quotes for method development when developing peptide specific methods such as for highly bridged, synthetic or cyclic peptides. This work has contributed to further understanding of chiral purity testing in Almac and has helped build a solid foundation for future amino acid analysis.