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Thermospray liquid chromatography/mass spectrometry study of diastereomeric isoindole derivatives of amino acids and amino acid amides ☆

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Abstract

A thermospray liquid chromatography/mass spectrometry (TSP-LC/MS) method is described for determination of the enantiomeric excess of α-amino acids and α-amino acid amides as their *o*-phthalaldehyde/*N*-acetyl-L-cysteine (OPA/NAC) derivatives.

The source temperature is an important factor in optimizing the sensitivity of the TSP-LC/MS analysis, whereas the repeller voltage is of minor importance. On-column mass spectra were acquired for the OPA/NAC derivatives of several α-amino acids and α-amino acid amides. For the main fragment ions, mass spectra fragmentation pathways are proposed. The applicability of the method is demonstrated by means of the enantiomeric excess determination of valine in a sample from an enzymatic hydrolysis experiment.

Using single ion monitoring, the detection limit of D-valine in the presence of excess L-valine is 10 pmol. The present TSP-LC/MS method is useful for validating the results obtained from LC/UV or LC/fluorescence methods for the enantiomeric excess determination of α-amino acids and α-amino acid amides.

Keywords: TSP-LC/MS; Isoindole derivatives; Amino acids; Amino acid amides; Fragmentation mechanism; Enantiomeric excess determination

1. Introduction

Homochiral forms of α-amino acids are important as chiral auxiliaries in the synthesis of pharmaceuticals, food chemicals and agrochemicals. Chemo-enzymatic processes have been described in which the optical resolution of α-amino acids was achieved through the enantioselective hydrolysis of their acid amides catalyzed by peptidases [1].

In conjunction with this synthesis, analytical methods are required for the control of the enantiomeric purity of both α-amino acids and α-amino acid amides.

Pre-column derivatization procedures using chiral reagents and subsequent separation of the diastereomeric derivatives by high-performance liquid chromatography (HPLC) are frequently used methods for the determination of the enantiomeric excess. Using an optically active thiol compound, i.e. *N*-acetyl-L-cysteine (NAC), in combination with *o*-phthalaldehyde (OPA), it was shown that this reaction was readily applicable to the HPLC analysis of enantiomeric α-amino acids [2,3] and α-amino acid amides [4].

Despite the fluorescent properties of the isoindole derivatives formed, the measurement of these types of derivatives in enzymatic reaction mixtures sometimes lacks specificity. The

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identification of the diastereomeric derivatives of the α-amino acids and α-amino acid amides is typically based upon comparisons of retention times with the standard compounds. In order to confirm identifications made in this way, mass spectrometric data are of unique value. With respect to isoindole derivatives, several mass spectrometric studies have been performed.

In these studies, either probe mass spectrometry [5,6] or gas chromatography/mass spectrometry [7–9] has been used to determine the structures of the products formed. Thermospray mass spectrometry is well suited to the identification of low-molecular-weight polar non-volatile molecules and allows coupling to HPLC systems. Its use for the analysis of phenylthiohydantoin (PTH) amino acids has been reported [10].

The aim of this study was to develop a thermospray liquid chromatography/mass spectrometry (TSP-LC/MS) method to validate the results obtained from a liquid chromatography/fluorometric method for the enantiomeric excess determination of α-amino acids and α-amino acid amides as their OPA/NAC derivatives. For this, TSP-MS conditions were optimized for OPA/NAC derivatives, and mass fragmentation patterns were studied by several derivatized α-amino acids and α-amino amides. From the mass spectra, characteristic ions were chosen for selected ion monitoring. The applicability of the method is demonstrated by means of the enantiomeric excess determination of a sample from an enzymatic hydrolysis experiment.

2. Experimental

2.1. *Materials*

α-Phenylglycine amide (PG-NH_2) was obtained from DSM Research. Other α-amino acid amides and α-amino acids were supplied by Sigma (St. Louis, MO, USA).

For each amino compound, both the racemic form and the L-form of the enantoimer were available. Trifluoracetic acid (TFA) and NAC were supplied by Janssen (Beerse, Belgium). OPA and HPLC-grade methanol were obtained from Merck (Darmstadt, Germany). Water was purified with a Milli-Q system.

All other chemicals were of analytical-reagent grade.

2.2. *Eluent, reagent and derivatization procedure*

The buffer was prepared by dissolving the ammonium acetate salt (50 mM) in water and titrating to pH 6.0 with acetic acid. The percentage of methanol in the mobile phase was 40% (v/v). The OPA/NAC derivatization was carried out as described earlier [4], except that the neutralization of the reaction mixture was carried out with TFA instead of sodium phosphate buffer. To avoid the introduction of sodium borate, i.e. buffer of reaction mixture, into the mass spectrometer, the first 5 ml of column effluent were diverted from the MS after injection of the reaction mixture.

2.3. *Instrumentation*

HPLC was performed using a Gilson (Villiers-le-Bel, France) Model 302 pump and a Gilson Model 231-401 autosampling injector for derivatization and injection. The injection loop had a 20 μl capacity. The column used was a Nucleosil 120-C18 (250 × 4.0 mm i.d., 5 μm) from Macherey, Nagel & Co (Düren, Germany). The flow rate was 1.0 ml min^{-1} and the separations were carried out at ambient temperature.

The MS system used was a Finnigan MAT TSQ 70 triple quadrupole mass spectrometer equipped with a thermospray interface (Finnigan MAT, San José, CA, USA). The third quadrupole was operated both in the full scanning mode and mass-selective mode, while the first and second quadrupoles were in the rf-only mode of operation. All experiments were carried out in the filament-off mode. To facilitate the ionization of the amino acid derivatives studied, post-column addition of 1% (v/v) aqueous TFA solution at a flow-rate of 0.25 ml min^{-1} was effected with a Gilson Model 302 pump. The solution was added to the column effluent using a Lee (Frankfurt, Germany) visco-jet micromixer. After optimization, source temperature and repeller voltage were set at 200°C and 120 V, respectively. The vaporizer temperature was kept at 90°C. TFA was used for calibration of the mass spectrometer up to 1000 Daltons. Scanning was performed from 115 to 515 Daltons with a scan time of 1.5 s.

3. Results and discussion

3.1. Optimization of MS conditions

Using L-Val as test compound, on-column mass spectral data were acquired for the OPA/NAC derivative of this amino acid. Characteristic mass peaks of the compound studied were then selected in order to investigate the influence of these mass peaks. In Fig. 1 the intensities of five ions with respect to the intensity of the most abundant ion in the spectrum, i.e. m/z 234, are shown as a function of the source temperature.

The absolute intensity of the m/z 234 ion and the intensity of m/z 251 showed a maximum at source temperatures between 175 and 200°C. The intensity of m/z 266 was hardly influenced by the source temperature.

The m/z 250 ion showed a maximum intensity at 225°C, while the protonated molecular ion (m/z 379) and the m/z 248 ion reached their maxima at 275°C.

Variation of the repeller voltage in the range 20–170 V did not result in changes of the intensities (with respect to the intensity of m/z 234) of the mass peaks studied. However, the

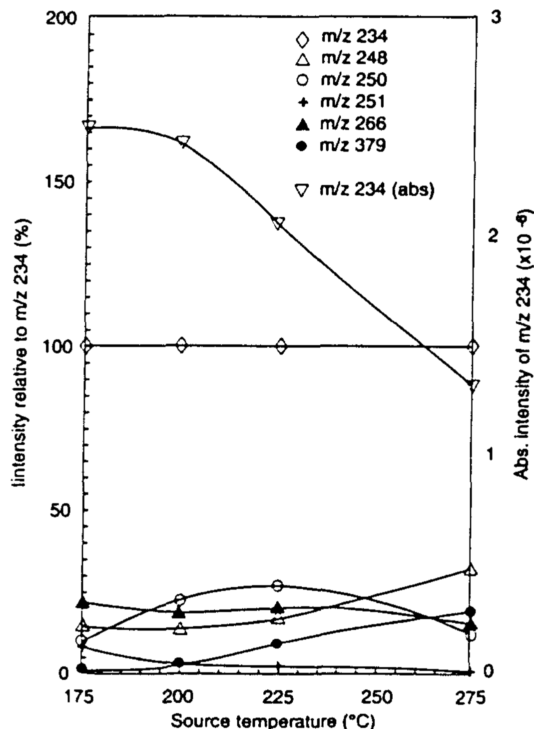

Fig. 1. Intensities of characteristic ions of the OPA/NAC derivative of L-Val as a function of source temperature (abs = absolute). Repeller voltage: 120 V. For other conditions, see Experimental section.

absolute intensity of the m/z 234 ion did correlate with the repeller voltage. A source temperature of 200°C and a repeller voltage of 120 V were found to be the best compromise for obtaining good intensities for all mass peaks studied.

3.2. Mass spectral data of OPA/NAC derivatives

On-column mass spectra were acquired for the OPA/NAC derivatives of the following amino acids and amino acid amides: Ala, Val, Phe, Leu, PG, Ala-NH_2, Val-NH_2, Leu-NH_2, Leu-NH_2 and PG-NH_2. For all derivatives studied, the spectra showed the protonated molecular ion $(MH)^+$.

Among the different derivatives studied, the mass spectra showed a high degree of commonality. All derivatives showed the $(MH-18)^+$, $(MH-113)^+$, $(MH-129)^+$, $(MH-131)^+$, $(MH-145)^+$ and $(MH-163)^+$ ions. The presence of all ions was validated by reconstruction of the mass chromatograms and these ions were all significantly present (signal-to-noise ratio better than 3). Except for Val-NH_2, the $(MH-145)^+$ ion was the base peak in all spectra. Further, the protonated molecular ion of the native amino acid (or amino acid amide) was present in each mass spectrum. Some typical differences were noticed between the mass spectra of derivatized amino acids and those of the amino acid amides. The $(MH-18)^+$ peak was more prominent in the case of the amino acid amides, the $(MH-44)^+$, $(MH-95)^+$ and $(MH-128)^+$ ions were absent in the case of the amino acid amides, while $(MH-161)^+$ did not occur in the mass spectra of OPA/NAC amino acids. Obviously, the type of side-chain of the amino acid (or amino acid amide) does not play an important role with respect to the mass fragments obtained, as no differences between aromatic and aliphatic side-chains could be observed. Table 1 summarizes the major mass peaks found and their intensities.

The D-forms of the amino compounds studied were equivalent to the L-forms with respect to the mass peaks and their intensities. The difference in ionization efficiency between L- and D-forms was found to be less than 5%.

Possible mass fragmentation pathways for the OPA/NAC amino acids and amino acid amides are shown in Fig. 2. In this figure, the $(MH-18)^+$ and $(MH-44)^+$ peaks indicate the loss of a molecule of water and carbon dioxide,

Table 1
Mass losses of OPA/NAC derivatives of L-α-amino acids and L-α-amino acid amides [a]

Δ	OPA/NAC derivatives								
	Ala	Ala-NH_2	Val	Val-NH_2	Phe	Leu	Leu-NH_2	PG	PG-NH_2
0	+	+	+	+ +	+	+	+ +	+	+
−18	+	+ +	+	B	+	+	+ +	+	+ +
−44	+	−	+	−	+	+	−	+ +	−
−95	+	−	+	−	+	+	−	+	−
−113	+ +	+ +	+	+	+ +	+ +	+	+ +	+
−128	+	−	+	−	+	+	−	+	−
−129	+	+ +	+ +	+ +	+	+ +	+ +	+	+ +
−131	+ +	+	+ +	+ +	+	+	+	+	+
−145	B	B	B	+ +	B	B	B	B	B
−161	−	+	−	+ +	−	−	+	−	+ +
−163	+ +	+ +	+	+ +	+	+	+ +	+ +	+ +

[a] Δ, mass loss from the protonated molecular ion; −, not detected; +, intensity < 10%; + +, intensity > 10%; B, base peak.

respectively. This loss may represent either a fragmentation or a thermally induced intramolecular rearrangement. In the case of the OPA/NAC amino acids, the substituent at either the 1-position or the 2-position of the isoindole ring can give rise to these losses. The $(MH\text{-}129)^+$, $(MH\text{-}131)^+$, $(MH\text{-}161)^+$, $(MH\text{-}163)^+$ peaks result from the loss of a moiety of the thio substituent. Two mechanisms seem to be involved here. One appears to involve elimination of unsaturated forms of the thio moiety from the protonated molecular ion and gener-

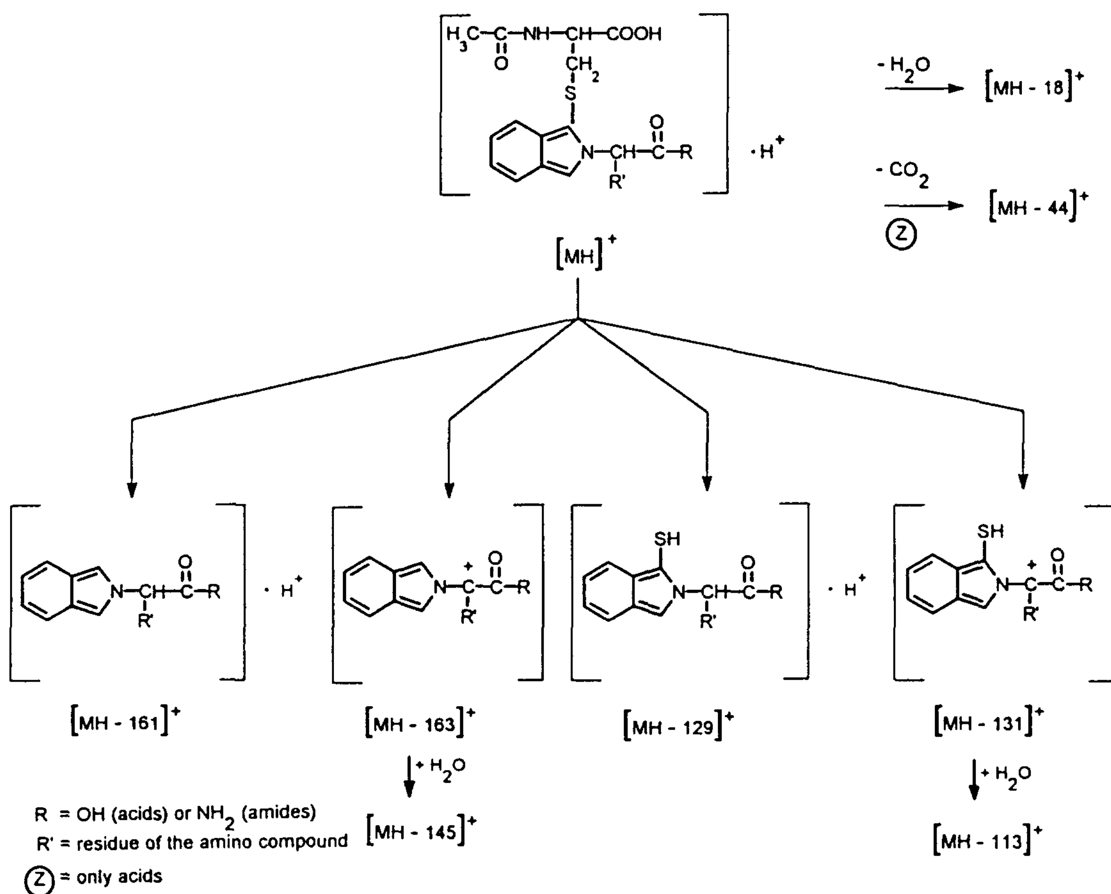

Fig. 2. Proposed mass spectral fragmentation pathways for OPA/NAC derivatives of α-amino acids and α-amino acid amides.

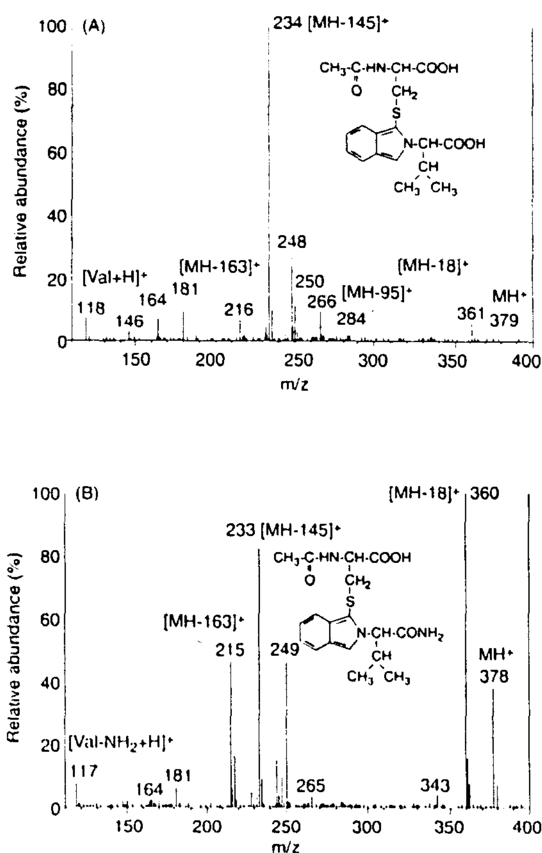

Fig. 3. Mass spectra of the OPA/NAC derivatives of L-Val (A) and L-Val-NH_2 (B).

ates the $(MH\text{-}129)^+$ and $(MH\text{-}161)^+$ ions, whereas the $(MH\text{-}131)^+$ and $(MH\text{-}163)^+$ ions may result from elimination of the protonated thio moiety. The occurence of $(MH\text{-}113)^+$ and $(MH\text{-}145)^+$ ions may be explained by the addition of a molecule of water to $(MH\text{-}131)^+$ and $(MH\text{-}163)^+$ ions, respectively. The assigned fragment ions in Fig. 2 indicate that mass losses mainly occur at the thio substituent on position 1 of the isoindole ring. Apart from the fragmentation pathways given in Fig. 2, some of the fragment ions mentioned in Table 1 may be formed in another way. For instance, hydrolysis of the isoindole derivative in the vaporizer region will give rise to NAC (m/z 164) and a neutral part. Protonation of the latter generates the $(MH\text{-}145)^+$ fragment ion. Besides m/z 164, all spectra also show m/z 181, which may represent the cluster ion of NAC with ammonia. The $(MH\text{-}113)^+$ ion may be the result of the clustering of $(MH\text{-}145)^+$ with methanol.

The occurrence of the free amino acid (or amino acid amide) in the mass spectra may result from a pyrolytic cleavage process that affects the isoindole ring.

3.3. Determination of enantiomeric excess

For the routine analysis of the enantiomeric excess of amino acids obtained by enantioselective hydrolysis of the corresponding amino acid amides, an HPLC method combined with fluorometry is used in our laboratory [4]. Using

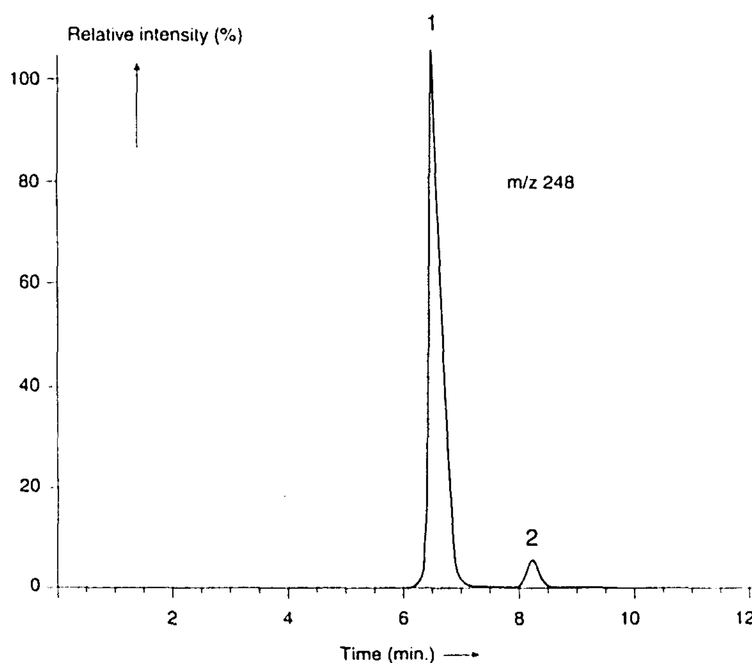

Fig. 4. Mass chromatogram of a sample taken during the course of an L-Val synthesis: (1) L-Val, (2) D-Val.

this detection technique, matrix components sometimes interfere in the chromatographic separation of the diastereometric OPA/NAC derivatives. In order to validate the results obtained by this method, the current TSP-LC/MS method was applied. For the determination of the enantiomeric excess, the technique of selected ion monitoring was used. At first, the OPA/NAC derivative studied was measured in the full scanning mode. The most specific mass peaks for the derivative were then chosen for selected ion monitoring. As an example, typical mass spectra of the OPA/NAC derivatives of L-Val and L-Val-NH_2 are shown in Fig. 3. With respect to valine, m/z 216, m/z 234 *and* m/z 248 were chosen for selected ion monitoring. All three ions were characteristic fragment ions of the protonated molecular ion (m/z 379) i.e. $(MH-163)^+$, $(MH-145)^+$ and $(MH-131)^+$, respectively.

For the OPA/NAC derivative of D-Val, the detection limits, based on a signal-to-noise ratio of three, were: 450 pmol (full scanning mode), 20 pmol (selected ion monitoring of m/z 216, m/z 234, and m/z 248) and 10 pmol (selected ion monitoring of m/z 248). As an application, the enantiomeric excess of a sample from L-Val synthesis, taken in the course of a synthesis on the laboratory scale, was determined. The mass spectra were similar to the spectra of the standard components at the corresponding retention times. A typical mass chromatogram of a sample from L-Val synthesis is shown in Fig. 4.

The calculated enantiomeric excess of the sample shown in Fig. 4 was 90%, with a relative standard deviation of 1% ($n = 3$).

4. Conclusions

Selection of specific ions from isoindole derivatives of amino acids and amino acid amides can be made on the basis of the fragmentation mechanisms described in this paper.

By means of the ions chosen, enantiomeric excess determinations of laboratory scale samples can be carried out without interferences from other matrix compounds.

The TSP-LC/MS method is useful for the validation of liquid chromatography/fluorometric methods.

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