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Characterization of an electrospray ionization ion trap/linear time-of-flight mass spectrometer for phenylthiohydantoin-amino acid analysis

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Abstract

An electrospray ionization ion trap/time-of-flight mass spectrometer was evaluated as a detector for identification of phenylthiohydantoin (PTH)-amino acids, the final products in the Edman sequencing process of peptides and proteins. Each of 22 PTH-amino acids studied can be characterized by well-defined mass spectral patterns. Fragmentation from source excitation followed by a long trapping period (250 ms) gave rise to unique and intense fragmentation products. This allowed for the differentiation of isomers of PTH-leucine/PTH-isoleucine and PTH-α–aminobutyric acid/PTH-α–aminoisobutyric acid. The interpretation of the fragmentation patterns is presented. The detection limits and relative detection sensitivity of 20 standard PTH-amino acids were determined. It is shown that all these compounds can be detected at the 100 fmol level. (Int J Mass Spectrom 176 (1998) 213–225) © 1998 Elsevier Science B.V.

Keywords: Phenylthiohydantoin-amino acid; Edman sequencing; Electrospray ionization; Ion-trap/time-of-flight mass spectrometer

1. Introduction

The use of Edman sequencing chemistry [1,2] for determining the primary structure of a protein is a well-established method in biochemical research. The final products of stepwise chemical degradation of proteins, phenylthiohydantoin (PTH) derivatives of amino acids, are generally analyzed by high-performance liquid chromatography (HPLC) with UV absorbance detection. Despite systematic improvements over the years, the technique still suffers from several limitations [3]. First, the detection method requires a complete separation of PTH-amino acids by HPLC, which is often slow. For sequencers capable of carrying out short degradation cycles, the detection step is the limiting step for speed. Furthermore, the low detectability of PTH-derivatives by UV detection also limits the overall sensitivity of the sequencing process. At present, partial sequences using low picomole amounts of peptides or proteins can be obtained. Finally, a major drawback with UV detection is that it cannot provide structural information for identification of modified or nonstandard amino acids.

Mass spectrometry offers an alternative method for the detection and identification of PTH-amino acids with potentially improved speed, sensitivity, and specificity. The interest in coupling an Edman se-

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quencer with mass spectrometry during the last 25 years has resulted in mass spectrometry characterization of PTH-amino acids by electron ionization [4,5], chemical ionization [6], and thermospray ionization [7]. The use of electrospray ionization (ESI) for studying PTH-amino acids in an ion trap mass spectrometer has recently been reported [8]. There are also several studies on the use of novel sequencing reagents yielding other amino acid derivatives [9–11] that are sensitive for ESI mass spectrometry (ESI-MS) detection. Detection limits for the most sensitive derivatives using ESI range from low femtomole in a quadrupole instrument operated in the selected ion monitoring mode [10] to subfemtomole for tandem mass spectrometry (MS/MS) detection [9]. In addition, these studies illustrate that some structural information on amino acid derivatives can be obtained using ESI-MS. The actual utility of these novel reagents for sequencing synthetic peptides has been reported [12]. The approach of modifying Edman chemistry to yield amino acid derivatives, which are sensitive to ESI-MS detection, may eventually lead to significant advances in Edman sequencing technology.

Our research into combining Edman sequencing with ESI-MS focuses on exploring the capability of an ESI ion trap (IT)/linear time-of-flight (TOF) mass spectrometer for the identification of products generated in a sequencer. These products include both the Edman degradation end products from nonstandard amino acids or modified amino acids and the degraded small peptides with modified sites that are retained in the sequencer. To assess the analytical merits and limitations of ESI IT/TOFMS for this type of application and to use this technique to study structural modifications of proteins, a level of understanding of the detectability of standard PTH-amino acids is essential. In this work, ESI mass spectra of 20 standard PTH-amino acids are reported and the fragmentation patterns of these compounds are discussed. It is shown that ESI IT/TOFMS can be used to differentiate PTH-Leu and PTH-Ile isomers based on their fragmentation patterns. The detection limits and relative detection sensitivity of these PTH-amino acids are also presented. In addition, a discussion of the comparison of our results with those reported on ESI analysis of PTH-amino acids is given.

2. Experimental

2.1. ESI IT/TOFMS instrument

ESI mass spectra of the PTH-amino acids were obtained using an IT/linear TOFMS instrument that has been described previously [13,14]. Briefly, in the two-stage interface, the nozzle (atmospheric) and the sampling (vacuum) plate had openings of 120 and 230 μm, respectively, and were separated by a distance of 4.1 mm. The space between the plates, which is referred to as the collisionally induced dissociation (CID) region, was pumped by two mechanical pumps to maintain a pressure of approximately 200 mTorr. A DC voltage of +4.0 V was applied to the vacuum plate and a variable voltage (20–70 V) was applied to the atmospheric plate. The potential difference or V_{CID} between these plates determined the kinetic energy of the ions. Fragmentation conditions were promoted by increasing the atmospheric plate voltage. Ions exiting the CID region entered a high vacuum region (4×10^{-6} Torr) where a set of lenses focused the ions into the ion trap. The ion trap (pressure 5×10^{-4} Torr) was enclosed by ceramic rings and a buffer gas (helium) was continuously introduced inside the trap. The confinement of ions in the ion trap was achieved by applying a radiofrequency (rf) voltage of 800 $V_{p\text{-}p}$ to the middle (ring) electrode. After a specific trapping time (i.e. 250 ms), the rf voltage was turned off and the ions were ejected from the ion trap by applying a negative pulse (−800 V for 10 μs) to the exit endcap electrode. A voltage of −4100 V was applied to the flight tube to accelerate the ions. The TOF for all ions of different mass-to-charge ratio values was detected by a dual multichannel plate (MCP) detector. The ion signal was amplified using a 500 MHz, 20 dB amplifier (CLC 100, Comlinear Corporation, Fort Collins, CO). A LeCroy (Chestnut Ridge, NY) 9350 M digital oscilloscope, with a time resolution of up to 1 ns per point, was used to capture the signal. The spectra were stored directly from the

oscilloscope to a computer using a data collection system that was developed at the University of Alberta [15].

2.2. Electrospray ionization source

The signal stability for the ESI operation in IT/TOFMS was improved from previous work [13,14] by using a new design for the microspray needle tip. A stainless steel (SS) capillary [inner diameter (i.d.) = 60 μm, outer diameter (o.d.) = 125 μm] was electropolished (as an anode, with copper as the counter electrode in a 1/1/1 solution by volume of orthophosphoric acid/glycerin/water) at one end until the outer diameter was small enough to be inserted into a 100-μm-i.d. fused silica capillary. The most difficult operation was to make a sharp and even cut of the SS capillary piece because the capillary wall was very thin. It was possible to manufacture a satisfactory cut electrolytically (anodization in water/nitric acid solution, 1/1 by volume). The SS capillary cut was formed at the air–liquid interface. The narrow end of the modified SS capillary was inserted inside a fused silica capillary (100 μm i.d., 250 μm o.d.) and protruded 1 mm inside. The tip and fused silica capillary were placed inside a supportive SS capillary (250 μm i.d., 1.59 mm). The mechanical support for this connection was achieved by soldering the middle part of the SS capillary tip to one end of the supportive SS capillary and gluing the fused silica capillary to the other end. Such an assembly was used as the ESI source and it provided a low dead volume connection. For flow injection experiments, a Rheodyne model 7520 injector (0.5 μL volume of injected sample) was used. The electrospray source was connected to the injector by using a 30-cm-long piece of fused silica. A syringe pump (Harvard Apparatus, South Natick, MA) was used to deliver the solvent at a flow rate of 4 μL/min. For continuous infusion experiments, the sample was delivered directly from a syringe to the electrospray source at a flow rate of 4 μL/min. A dc voltage of 2.8 kV was applied to the ESI tip during the ESI process.

2.3. Chemicals and sample preparation

All PTH-amino acids and phenylisothiocarbamoyl (PTC) derivatives were purchased from Sigma Chemical Corporation (St. Louis, MO) and used without purification. HPLC-grade acetonitrile, HPLC-grade water, and ammonium acetate (certified reagent) were purchased from Fisher Scientific Company (Nepean, Ontario, Canada). Stock solutions (1 mM) of PTH-amino acids were prepared by dissolving the required amount of sample in HPLC-grade acetonitrile. Standard solutions and mixtures for ESI-MS analysis were prepared from stock solutions by further dilution. The buffer-solvent composition used for the continuous flow solution and the sample solution was identical (i.e. 80% acetonitrile, 20% 0.25 mM ammonium acetate in water by volume). The continuous flow solution was degassed before use.

3. Results and discussion

3.1. Soft ionization of PTH-amino acids

Fig. 1 shows some representative mass spectra of PTH-amino acids and PTC derivatives obtained by continuous infusion of a 5-μM analyte solution through the electrospray source. They were acquired by using experimental conditions that favor the formation of the molecular ions (i.e. V_{CID} = 25 V). The buffer–solvent composition used provided the optimal signal intensity of the protonated molecular ion with minimal chemical background.

The spectra of all PTH-amino acids, except PTH-(PTC-cysteine), display prominent protonated molecules, MH^+. In the case of PTH-(PTC-cysteine), the ESI spectrum of this compound shows intense fragment ion at *m/z* 205 and no molecular ion peak at *m/z* 374.5 is detected (Fig. 1(D)). Fig. 1 illustrates that ammonium adduct ions, MNH_4^+, are not observed for a low V_{CID} when a relatively low ammonium acetate buffer concentration is used. Alkali metal contamination, probably from the glassware and impurities in the sample, causes the presence of sodium adduct ions (MNa^+) and in some instances potassium adduct ions

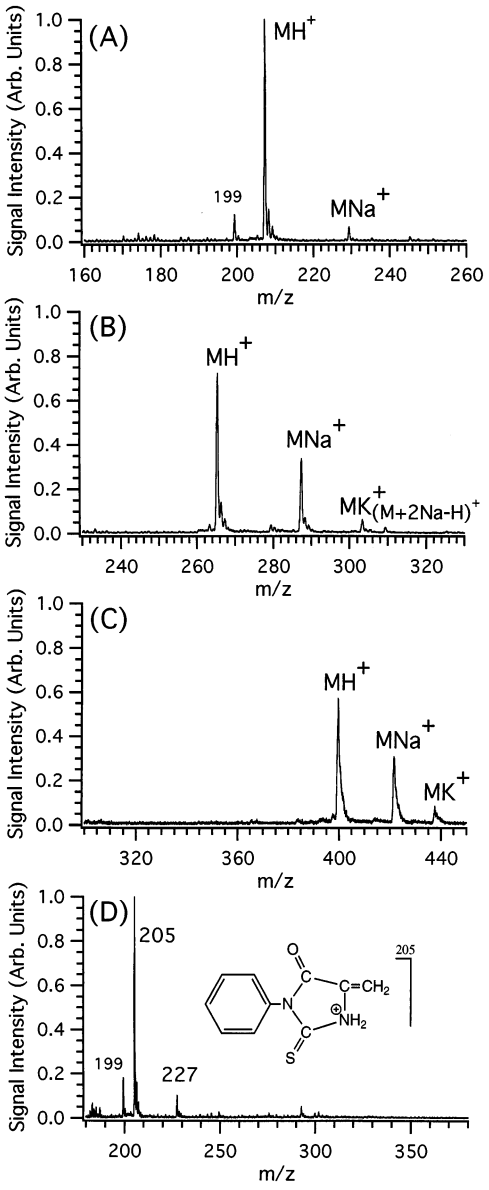

Fig. 1. Molecular ion regions of mass spectra of PTH-amino acids. (A) PTH-alanine, (B) PTH-glutamic acid, (C) PTH-(PTC-lysine), and (D) PTH-(PTC-cysteine); 5-μM sample solutions were analyzed.

(MK^+). The abundance and the composition of these adduct ions are compound dependent. The signal intensity for these ions decreases slightly when a higher CID voltage is used. Although these adduct ion peaks appear to complicate the spectra, they can be very useful for confirming the presence of a molecular ion peak. The fragment ions generally are not present as alkali adducts. Only singly charged positive ions are observed in the PTH-amino acid mass spectra.

3.2. Differentiation of PTH-Leu and PTH-Ile

Table 1 lists the molecular ion masses of the PTH-amino acids studied. It is clear that the molecular ion mass is unique for many of the PTH-amino acids and it can be used as the basis of identification. However, the isomers, PTH-Leu and PTH-Ile, cannot be distinguished in this way. In addition, the molecular ion mass information obtained for nonstandard PTH-amino acids does not provide information about the identity of the analyzed compounds. Fragment ion spectra can be readily obtained in ESI-MS. However, the mass spectrometry distinction of leucine and isoleucine derivatives has been reported to be difficult [8–11]. The extent of fragmentation information depends on the analyzed compound and the instrumental configuration used in the experiment. We have examined the possibility of using the ESI IT/TOFMS to distinguish these derivatives based on fragmentation induced in the interface region (i.e. source fragmentation).

In ESI IT/TOFMS, the fragmentation of PTH-amino acids is facilitated by increasing the voltage difference between the atmospheric and vacuum plates (i.e. V_{CID}). Fig. 2 shows the spectra of PTH-Leu and PTH-Ile at increasing V_{CID}. For low V_{CID}, only the protonated molecular peaks (MH^+ = 249) for PTH-Leu and PTH-Ile are observed, as shown in Fig. 2(A) and 2(D), respectively. The increase in the CID voltage promotes fragmentation of the molecular ion and, as a result, the intensity of a fragment ion at *m/z* 86 increases for both species. When the fragmentation voltage was increased further, the fragment ion at *m/z* 69 appeared only in PTH-Ile mass spectrum (Fig. 2(F)). This characteristic fragmentation pattern can be used to distinguish PTH-Ile and PTH-Leu.

It is interesting to compare the fragmentation patterns of PTH-Leu and PTH-Ile observed by using different instrumental configurations under the similar

Table 1
Mass spectral fragmentation patterns of PTH-amino acids

PTH-amino acid	MH^+	Major fragment ions	Minor fragment ions
Proline	233	70 (3.8)[a]	
Leucine	249	86 (1.4)	
Isoleucine	249	86 (1.5), 69 (0.3)	
Valine	235	72 (1.6), 55 (0.4)	
Alanine	207	86 (0.2)	
Glycine	193	165 (0.3), 72 (0.2)	
Aspartic acid	251	191 (0.8), 108 (0.8), 233 (0.3), 174 (0.3), 205 (0.3), 88 (0.3)	
Asparagine	250	233 (1.0), 174 (0.7), 191 (0.3), 87 (0.2)	157, 205
Glutamic acid	265	84 (2.3), 247 (0.3)	102, 205
Glutamine	264	84 (3.2), 247 (1.7)	205
Serine	223	193 (3.1), 60 (0.5), 118 (0.5)	205
Threonine	237	193 (5.5)	219
Tyrosine	299	136 (2.1), 193 (1.9), 119 (0.4)	
Tryptophan	322	193 (12), 130 (8.4), 132 (1.6)	
Histidine	273	180 (2.0), 152 (2.0), 110 (2.0)	83, 93, 170
Phenylalanine	283	120 (1.6)	103, 162
Arginine	292	70 (2.0), 112 (2.0), 275 (1.1), 233 (0.6), 115 (0.3)	60, 129, 140, 156, 157, 174, 205
Methionine	267	61 (0.7), 146 (0.6), 56 (0.5), 104 (0.5), 174 (0.4)	98, 219
PTC-lysine	399.5	306 (6.0), 70 (0.8), 143 (0.7), 116 (0.7), 84 (0.6)	247
PTC-cysteine	374.5[b]	205 (1)	170

[a] 70 (3.8) represents a fragment ion at *m/z* 70, which was 3.8 times more intense than the corresponding protonated molecular ion in the spectrum obtained at V_{CID} = 45 V.
[b] The molecular peak was not observed.

fragmentation conditions. The peaks at *m/z* 86 shown in Fig. 2(B) and 2(E) correspond to the immonium ions, $C_4H_9CHNH_2^+$. The peak at *m/z* 69 observed only in the PTH-Ile spectra (Fig. 2(F)) is probably from the ion formed by NH_3 elimination from the isoleucine immonium ion. The mass spectra (not shown) obtained using a quadrupole mass spectrometer (SCIEX API 100) for both PTH-Leu and PTH-Ile resemble the spectrum of PTH-Leu obtained by IT/TOFMS (Fig. 2(C)). The immonium ion intensities were much smaller and the isoleucine immonium ion fragmentation product at *m/z* 69 was not present in the PTH-Ile spectrum obtained by the single quadrupole system. Thus, it is not possible to differentiate these isomers by source fragmentation in the SCIEX single quadrupole ESI system.

MS/MS studies of PTH-Ile using a commercial ion trap LCQ instrument (Finnigan MAT, San Jose, CA) confirmed the two-step fragmentation process. In the LCQ ion trap mass spectrometer, fragmentation of the isolated MH^+ of PTH-Ile resulted in the intense *m/z* 86 immonium ion without the presence of the *m/z* 69 ion. Further isolation and fragmentation of the *m/z* 86 ion produced the *m/z* 69 ion. In addition, it was possible to obtain distinct spectra for PTH-Leu and PTH-Ile by using only source fragmentation with the LCQ. The key factor for obtaining MS/MS products from source fragmentation is the time duration from excitation to the detection event. A sufficient increase in the internal energy of the ions in the CID source region (collisional excitation) combined with a long duration of the ion's residence in the ion trap (250 ms) produces very unique fragmentation spectra similar to that shown in Fig. 2(F). This is likely to be the property not only for our IT/TOFMS but also for ion trap mass spectrometry systems operated at longer trapping cycles.

A triple quadruple system is expected to provide PTH-Leu and PTH-Ile differentiation based on MS/MS spectra of isolated immonium ion, judging from the results obtained by low-energy CID in a sector/quadruple instrument [16]. Nevertheless, this

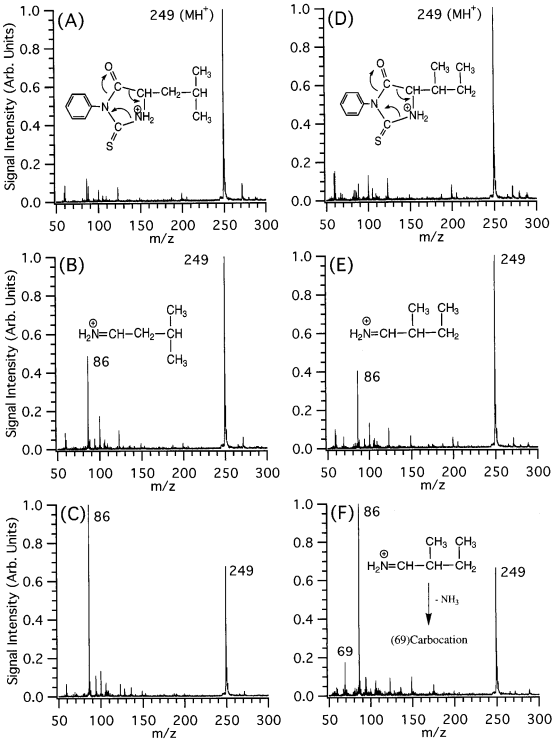

Fig. 2. Mass spectra of PTH-leucine (A–C) and PTH-isoleucine (D–F) at different source voltages: (A) and (D) V_{CID} = 25 V, (B) and (E) V_{CID} = 35 V, (C) and (F) V_{CID} = 45 V.

work demonstrates the ability of differentiating PTH-Leu and PTH-Ile in an IT/TOFMS system.

3.3. Fragmentation patterns of PTH-amino acids

The fragmentation patterns of other PTH-amino acids were investigated. The operating trapping rf voltage suitable for the detection of all PTH-amino acids sets the limit for the lowest mass-to-charge ratio of a trapped ion at 55. This means that all ions with a mass-to-charge ratio smaller than 55 could not be trapped and consequently could not be detected. Table 1 lists all the peaks present in the fragment ion spectra of PTH-amino acids. The intensity of the fragment ion relative to the intensity of the corresponding molecular ion at V_{CID} = 45 V is also given in parentheses for the major fragment ions. Minor fragments are defined as the ion peaks with intensities <10% the intensity of molecular ion peak.

A close examination of the mass spectral data in Table 1 reveals that several groups of PTH-amino acids exhibit similar fragmentation patterns. Fragment ion spectra of aliphatic PTH-amino acids (i.e. PTH-Pro, -Leu, -Ile, and -Val) are dominated by the immonium ions (i.e. $[RCHNH_2]^+$ where *R* is the side chain of the amino acid) and the [MH - PhNCS - CO]$^+$ ions (i.e. [MH - 163]$^+$). Val and Ile immonium ions fragment further by NH_3 elimination to generate the ions at *m/z* 55 and *m/z* 69, respectively. Immonium ions for PTH-Ala and PTH-Gly are not detected. But the spectrum of PTH-Ala displays a peak at *m/z* 86 from [MH - $PhNH_2$ - CO]$^+$. The spectrum of PTH-Gly displays peaks at *m/z* 165 from [MH - CO]$^+$ and *m/z* 72 from [MH - $PhNH_2$ - CO]$^+$.

Acidic and amidic PTH-amino acids (PTH-Asp, -Asn, -Glu, -Gln) show fragmentation products representing H_2O elimination ([MH - H_2O]$^+$ for PTH-Glu at *m/z* 247 and PTH-Asp at *m/z* 233) or NH_3 elimination ([MH - NH_3]$^+$ for PTH-Gln at *m/z* 247 and PTH-Asn at *m/z* 233). PTH-Asp and PTH-Asn spectra display ion products from further fragmentation of the *m/z* 233 ion. The peaks at *m/z* 174 and 205 correspond to the elimination of HNCS and CO, respectively, from the *m/z* 233 ion. The *m/z* 191 fragment ion is [MH - H_2O - CH_2CO]$^+$ for PTH-Asp and [MH - NH_3 - CH_2CO]$^+$ for PTH-Asn. Immonium ions at *m/z* 88 for PTH-Asp and at *m/z* 87 for PTH-Asn are observed. PTH-Glu and PTH-Gln show the dominant fragment ion at *m/z* 84. This peak is probably from 5-pyrrolin-2-one [16], the product of H_2O elimination from the glutamic acid immonium ion at *m/z* 102 and the product of NH_3 elimination from the glutamine immonium ion at *m/z* 101 (the peak at *m/z* 101 is not present in the spectrum). PTH-Glu and PTH-Gln generate less abundant fragment ions at *m/z* 205.

The mass spectra of hydroxilic PTH-amino acids (PTH-Ser, -Thr, -Tyr) and PTH-Trp display a common fragment ion peak at *m/z* 193, corresponding to the protonated PTH ring structure. This characteristic fragment is produced from the molecular ion by the elimination of a neutral species (e.g. acetone in the

case of PTH-Thr, formaldehyde for PTH-Ser). Immonium fragment ions are present in the spectra of PTH-Tyr at *m/z* 136 and PTH-Ser at *m/z* 60. Further elimination of NH_3 from the PTH-Tyr immonium ion results in the ion peak at *m/z* 119. The spectrum of PTH-Ser shows a peak at *m/z* 118 from $[MH - H_2O - CO - HSCN]^+$. Less intense fragments, $[MH - H_2O]^+$, are observed at *m/z* 205 for PTH-Ser and at *m/z* 219 for PTH-Thr. The intense fragment at *m/z* 130 in the mass spectra of PTH-Trp represents the quinolinium ion.

The fragment ion mass spectrum of PTH-His shows $[MH - PhNH_2]^+$ at *m/z* 180, $[MH - PhNH_2 - CO]^+$ at *m/z* 152, and the immonium ion at *m/z* 110. The minor peaks can be attributed to the product of NH_3 elimination from the immonium ion (*m/z* = 93) and the protonated methylimidazole (*m/z* = 83). A similar pattern is observed for PTH-Phe. The intense immonium ion at *m/z* 120 and a less abundant $[MH - PhNH_2 - CO]^+$ ion at *m/z* 162 are observed. The product of NH_3 elimination from the immonium ion is also present at *m/z* 103.

PTH-Arg and PTH-Met spectra show multiple fragment ion peaks because of the breakage of the side chain *(R)* of the amino acid. The major fragment ions for PTH-Arg include *m/z* 275 from $[MH - NH_3]^+$, *m/z* 233 from $[MH - guanidine]^+$, *m/z* 112 from $[RCH_2NH - NH_3]^+$, and *m/z* 70 from $[RCHNH_2 - guanidine]^+$. The major fragment ions for PTH-Met are *m/z* 174 from $[MH - PhNH_2]^+$, *m/z* 146 from $[MH - PhNH_2 - CO]^+$, *m/z* 104 from $[RCHNH_2]^+$, *m/z* 61 from $[CH_3SCH_2]^+$, and *m/z* 56 from $[RCHNH_2 - CH_3SH]^+$.

The mass spectrum of PTH-(PTC-Lys) shows the most intense fragment ion at *m/z* 306 from $[MH - PhNH_2]^+$, which fragments further to the immonium analog, $[MH - PhNH_2 - PhNCS - CO]^+$, at *m/z* 143. The elimination of HCN from the ion at *m/z* 143 results in the product at *m/z* 116. An alternative fragmentation pathway generates $[MH - PhNH_2 - HNCS]^+$ at *m/z* 247. This product ion fragments further to $[247 - PhNCS - CO]^+$ at *m/z* 84 and $[247 - PhNCS - CH_2CO]^+$ at *m/z* 70. The molecular ion of PTH-(PTC-Cys) is readily fragmented, and the spectrum shows a dominant peak at *m/z* 205 from $[MH - PhNH_2 - CS_2]^+$ and a less intense fragment ion peak at *m/z* 170.

In all fragment ion spectra of PTH-amino acids, several peaks at *m/z* 136, 106, and 94 from the product ions of PTH ring cleavage are observed. It should also be noted that the choice of V_{CID} can strongly affect the intensity and identity of the fragments ions in the spectra of PTH-amino acids. If necessary, additional fragmentation information may be obtained by a further increase of the CID voltage. For example, it was possible to observe the hydroxytropylium ion at *m/z* 107 and the tropylium ion at *m/z* 91 in the PTH-tyrosine spectrum at V_{CID} = 55 V.

The ESI mass spectral results discussed above indicate that the fragment ion spectra, in combination with the molecular ion information, can provide unambiguous identification of standard PTH-amino acids. Consequently, no chromatographic retention information is required from the chemical identification point of view.

3.4. Detection of nonstandard PTH-amino acids

Understanding the fragmentation patterns should also assist in detecting nonstandard PTH-amino acids. Fig. 3 shows the mass spectra obtained for PTH-α–aminobutyric acid and PTH-α–aminoisobutyric acid. These two isomers cannot be distinguished on the basis of detection of the molecular ion. Fig. 3 shows that the fragmentation pattern for both PTH-nonstandard amino acids resembles those of aliphatic PTH-amino acids (e.g. very intense immonium ion at *m/z* 58 from $[MH - PhNCS - CO]^+$ and less intense ion at *m/z* 100 from $[MH - PHNH_2 - CO]^+$). More importantly, different fragment ion mass spectra were obtained for these two isomers. The spectrum of PTH-α-aminoisobutyric acid (Fig. 3(A)) displays a prominent peak at *m/z* 134 from $[MH - HNCS - CO]^+$, which does not appear in the spectrum of PTH-α–aminobutyric acid. Thus, these two isomers can be readily identified.

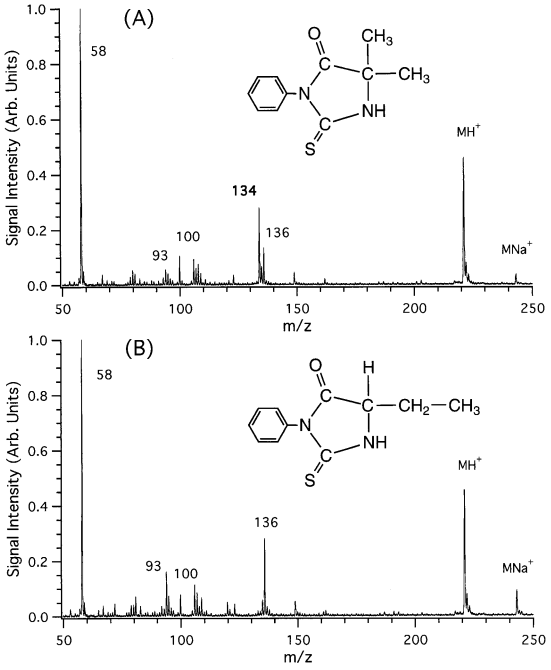

Fig. 3. Mass spectra of two isomeric nonstandard PTH-amino acids obtained with V_{CID} = 45 V. (A) PTH-α-aminoisobutyric acid and (B) PTH-α-aminobutyric acid.

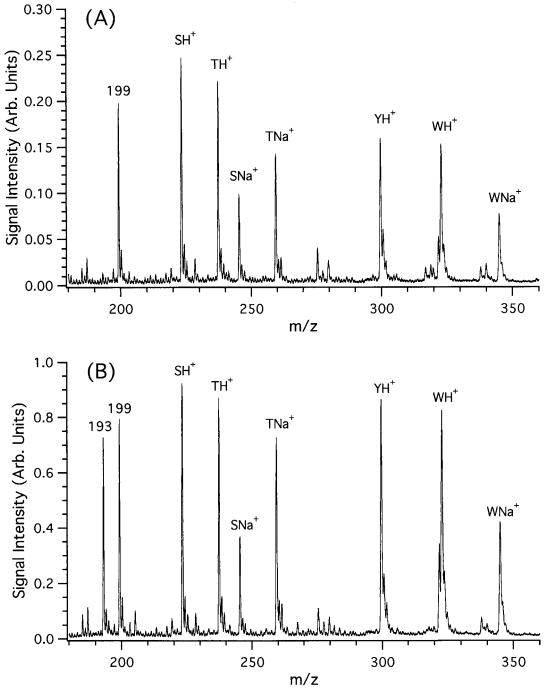

Fig. 4. Mass spectra of a mixture of PTH-S(serine), PTH-T(threonine), PTH-Y(tyrosine) and PTH-W(tryptophan) obtained at different source voltages: (A) V_{CID} = 25 V and (B) V_{CID} = 28 V. MH^+ (where M is the single letter code of an amino acid) represents the protonated molecule and MNa^+ represents the sodium adduct.

3.5. Isobaric interferences in mixture analysis

The data shown in Table 1 may suggest possible isobaric interferences for the fragment ions, molecular ions, and adducts of different PTH-amino acids. However, this isobaric interference can be controlled for simple mixtures. For example, the peak overlap of MH^+ from PTH-His and MNa^+ from PTH-Asp is not significant, as either derivative can be identified in the presence of the other. A serious peak overlap may exist for MH^+ of PTH-Gly at *m/z* 193 and the intense fragment ions at *m/z* 193 from PTH-Ser, -Thr, -Tyr, and -Trp. Fig. 4 shows the spectrum of the mixture of the PTH-amino acids that are found to easily fragment to generate the *m/z* 193 ion. When a low CID potential (V_{CID} = 25 V) was used (Fig. 4(A)) the intensity of the *m/z* 193 peak was at the background level. Using a larger CID potential (V_{CID} = 28 V), as shown in Fig. 4(B), the intensity of this peak makes the identification of PTH-Gly impossible.

Fig. 5 shows the mass spectra of a simple mixture of PTH-Ile and PTH-His at different source voltages. At a low CID voltage, the MH^+ peaks are very intense. At a higher fragmentation voltage (V_{CID} = 45 V), even the isomer differentiation information (i.e. *m/z* 69 for PTH-Ile) can be obtained as shown in Fig. 5(B). This shows that the identification of PTH-amino acids for a simple mixture is still possible. However, for the analysis of complex mixtures of PTH-amino acids, separation is still required for unambiguous identification. This is particularly true in cases in which the relative amounts of PTH-amino acids in a mixture vary greatly.

3.6. Chemical background

Impurities in samples can further complicate the mass spectral interpretation. More importantly, the presence of impurities can cause adverse effects on

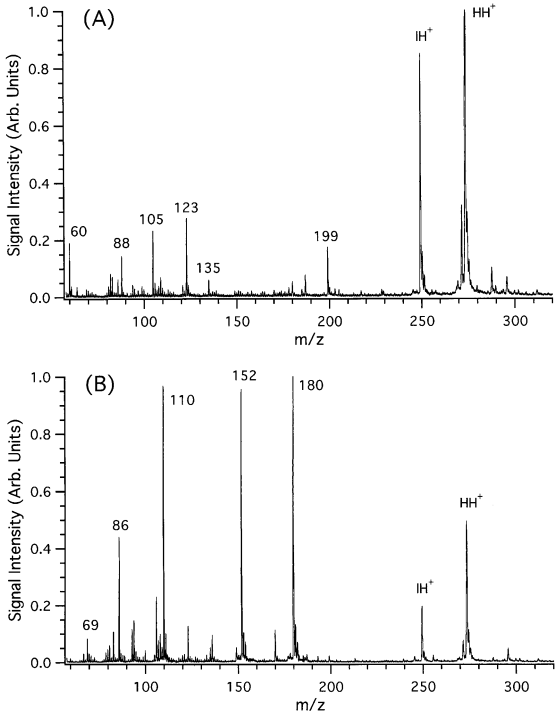

Fig. 5. Mass spectra of a mixture of PTH-I(isoleucine) and PTH-H(histidine) obtained at (A) V_{CID} = 25 V and (B) V_{CID} = 45 V.

the detection of PTH-amino acids. For samples from a protein sequencer, the impurities can be from the reagents and various byproducts of the sequencing process.

An attempt was made to detect PTH-amino acids directly from a sequencer. Aliphatic amines used in the sequencing process totally suppressed the PTH-amino acid signals. This failed attempt suggests that although PTH-amino acid identification using ESI IT/TOFMS can be carried out based on the mass spectral patterns, some type of chromatographic separation is still required for the analysis of PTH-amino acids in a complicated matrix. We note that the purposes of this type of separation are to eliminate any impurities that may interfere with PTH-amino acid detection and to reduce the possible isobaric interference, rather than to achieve total separation of the PTH-amino acids for retention information. We are in the process of examining different strategies, including the use of alternative reagents in a protein sequencer and the use of a fast liquid chromatography (LC) separation method, to reduce the interference from chemical background.

3.7. Detectability of PTH-amino acids

The detection sensitivities of PTH-amino acid were examined using an ESI IT/TOFMS. This sets the limit of detection, a very important parameter used to judge the applicability of this method. Knowing that PTH-amino acid identification relies mainly on the molecular ion mass, the detectability based on monitoring these prominent ions was evaluated. Using the ESI setup in the flow injection configuration, a series of injections for various amounts of each PTH-amino acid were performed.

The relative signal intensities for 20 PTH-amino acids were determined and the results are shown in Fig. 6. An amount of 500 fmol of each compound was injected (5 repetitions). The signal response of the molecular ion peak MH^+ (*m/z* 205 fragment ion in case of PTH-(PTC-cysteine)) for each PTH-amino acid was monitored using a spectral recording rate of 4 Hz. The resulting histogram (detector response as a function of time) represents the signal for an injected species, calculated as an integrated peak area above the average level of the baseline.

Two parameters (basicity and hydrophobicity of a compound) appear to influence the ESI signal response for PTH-amino acids. Most basic PTH-amino acids (arginine, histidine) displayed the highest sensitivity. Among all the rest of PTH-amino acids with comparable basicity, ESI signal responses ranged from larger for more hydrophobic species (PTH-Pro, -Val, -Phe) to smaller for less hydrophobic ones (PTH-Glu, -Asp). Data from Fig. 6 indicate that the response factor differs by 20 for extreme PTH-amino acids. Thus, for nonseparation mass spectrometry detection, the signal intensity of a major product from a particular Edman degradation sequence step could be smaller than the signal intensity for the highly sensitive product carried over from the previous step.

Detection limits for 20 PTH-amino acids were determined from the flow injection experiment using

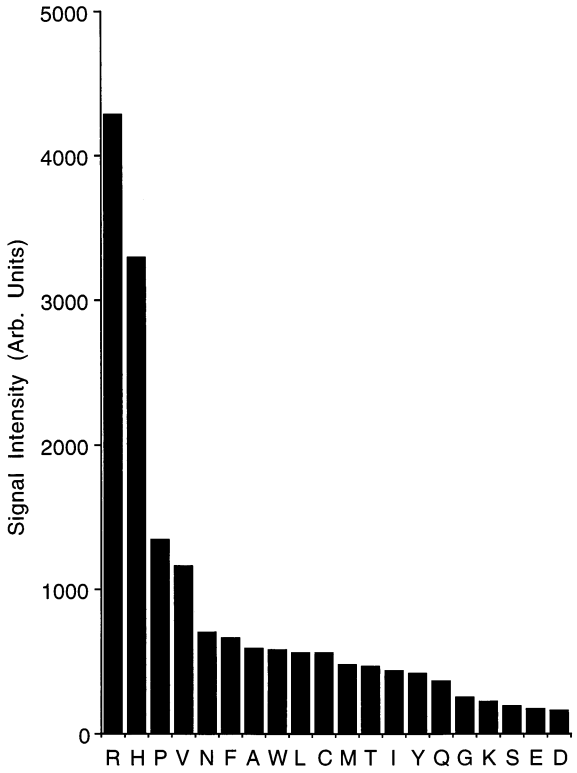

Fig. 6. Signal intensities of PTH-amino acids obtained from an injection of 500 fmol of each sample in a flow injection experiment. One letter abbreviation represents the corresponding PTH-amino acid.

ESI IT/TOFMS as the detector. The protonated molecular ion peak of each PTH-amino acid [*m/z* 205 fragment ion in the case of PTH-(PTC-cysteine)] was monitored to create repetitive histograms corresponding to 100, 250, and 500 fmol of injected sample. The signal was calculated as the peak height above the average level of the baseline and the noise was represented by the standard deviation of the baseline. Table 2 lists the values for the signal-to-noise (S/N) ratios obtained in the flow injection experiment. For compounds characterized as not very sensitive, a subpicomole level of detection for all PTH-amino acids was achieved. In fact, it was possible to identify all PTH-amino acids at 100 fmol with an S/N ratio of not worse than 5. PTH-Arg, the most sensitive compound, displayed lower than expected S/N ratio values at 500-fmol and 100-fmol injections. This was the only compound that exhibited adsorption in the injection system. Severe band broadening of an injected sample and a long lasting, variable background contributed to the low S/N value, especially for large amounts of sample.

3.8. Comparison with other studies

Recently, Zhou et al. [8] reported a study of PTH-amino acid analysis by using an ion trap mass spectrometer. Aside from the difference in the type of mass spectrometry instrument used in our studies and the work of Zhou et al. [8], different approaches were used to increase the detectability for PTH-amino acids. The method of Zhou et al. relies on the use of a buffer–solvent system containing 5 mM lithium triflate in 1:1 methanol/dichloromethane. PTH-amino acids in such a medium flow through the SS capillary to the nanospray tip at 100–300 nL/min. The increased sensitivity of PTHs in ESI is achieved by the electrochemical modification of these compounds at the surface of the SS capillary electrode. The detection sensitivity and limits of detection in the work of Zhou et al. [8] were determined from spectral responses with continuous sample introduction and reported as concentration limits.

In our approach, the solvent system consists of an acetonitrile, water, and ammonium acetate buffer commonly used for separation of PTH-amino acids by HPLC. The flow rate used for ESI is generally 4 μL/min, compatible to microcolumn separation. The detection sensitivity and detection limits were determined from injection of a fixed volume of sample (0.5 μL) and reported as the absolute amounts detected. As discussed earlier, for PTH-amino acid analysis from a sequencer, some form of separation is required before the introduction of the samples to the mass spectrometer. Thus, we believe that a study involving analyte injection gives a closer approximation to a real world application, although better concentration limits are always observed with continuous sample introduction in our experimental setup.

The detectability of the ESI IT TOF MS method by using continuous sample introduction is illustrated in Fig. 7, which shows a representative ESI spectrum

Table 2
Detectability of PTH-amino acids for ESI IT/TOFMS

PTH-amino acid	500 fmol	250 fmol	100 fmol	50 fmol	25 fmol
Arginine	29	—	45	19	10
Histidine	—	—	—	83	52
Proline	287	109	77		
Asparagine	125	97	43		
Valine	130	64	24		
Phenylalanine	112	57	24		
Alanine	143	49	23		
Leucine	143	69	22		
Tyrosine	69	38	20		
Isoleucine	79	41	18		
PTC-lysine	75	35	18		
Tryptophan	67	37	18		
Methionine	61	33	18		
Threonine	82	40	16		
PTC-cysteine	67	44	15		
Glutamine	55	26	11		
Glycine	46	26	10		
Serine	39	18	7		
Glutamic acid	32	18	7		
Aspartic acid	30	16	5		

obtained by the continuous infusion of a mixture of PTH-Pro, PTH-Leu, and PTH-Phe at a concentration of 50 fmol/μL per component. The solvent system used for ESI was composed of 80% acetonitrile and 20% 0.25 mM ammonium acetate in water (by volume). The sample was delivered at 1 μL/min. The spectrum shown in Fig. 7 was acquired by summing

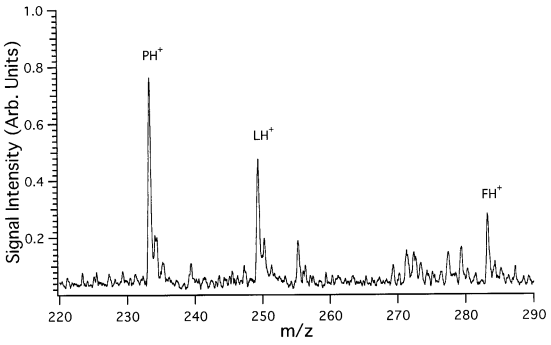

Fig. 7. Mass spectrum of a mixture of PTH-P(proline), PTH-L(leucine), and PTH-F(phenylalanine) at 50 fmol/μL obtained by using continuous sample introduction. The sample was delivered at 1 μL/min in the buffer–solvent (80% acetonitrile and 20% 0.25 mM ammonium acetate in water by volume). The spectrum was acquired by summing 100 scans for a period of 25 s and smoothed by using Savitzky-Golay algorithm of 15 points.

the same number of scans (i.e. 100) as that used in the work of Zhou et al. [8]. Because the scan time used in our work was 250 ms per spectrum versus 1.5 s in Zhou et al.'s work and the flow rate used was 1 μL/min versus 0.3 μL/min, the total sample consumed to obtain the spectrum shown in Fig. 7 was about half of that used in Zhou et al.'s work (i.e. 21 fmol vs. 38 fmol). The spectrum was smoothed by using Savitzky-Golay algorithm of 15 points. The S/N ratios for the molecular ion peaks of PTH-Pro, PTH-Leu, and PTH-Phe are 47, 28, and 15, respectively, compared with 4, 8, and 12 reported by Zhou et al. It is clear that different levels of detection are obtained from the two studies, which reflect the differences in the type of instrument and experimental conditions used. At this stage, it is difficult to ascertain the extent of contribution to the differences observed by the individual experimental parameter. Nevertheless, some general observations on the experimental results obtained in our instrument can be discussed below.

First, a spectrum similar to that shown in Fig. 7 can be obtained by the summing of only 10 scans, instead of 100 scans. The detection limit is governed by the chemical background noise. Thus, spectral summing

of >10 scans does not reduce the noise level further. Note that the amount of sample consumed to produce a spectrum like the one shown in Fig. 7 by summing 10 scans is only 2 fmol. When such an amount was introduced to the system by injection, no signal was observed. In fact, even when 0.5 μL of a 50 fmol/μL solution of PTH-Pro, PTH-Leu, or PTH-Phe was introduced by injection, only PTH-Pro could be detected. The injection experiment accounts for the analyte dispersion or dilution effect during injection to a continuous stream of carrier solvent as well as adsorption losses during the transport of the sample plug. It is clear that one cannot infer the absolute amount of detection from a continuous injection experiment.

We also studied the flow rate effect on the detection sensitivity of our method. It was found that for continuous injection the signal intensity was independent of flow rate used in the range 0.5–6 μL/min. A flow rate <0.5 μL/min causes the spray to become unstable. For a flow rate >6 μL/min, the less effective desolvation process resulted in the presence of cluster peaks in the spectrum, hence a decrease in sensitivity. We have also attempted to use 5 mM lithium triflate in 1:1 methanol/dichloromethane as the ESI solvent in our instrument. Despite extensive efforts we could not obtain any signals of PTH-Pro, PTH-Leu, or PTH-Phe by using continuous injection of a 50-fmol/μL solution. It appears that the optimal solvent system used for PTH-amino acid detection, particularly in cases involving electrochemical modifications of analytes, is dependent on the instrument used (e.g. the design of the ESI needle tip).

It should be noted that the method of generating CID spectra in our work is different from that of Zhou et al. We rely on the use of source fragmentation and they used CID in the ion trap. Different fragmentation products were observed in some cases. Because we do not have the CID capability in our current IT/TOF system, a direct comparison of source fragmentation and CID under the same ESI conditions cannot be made. Nevertheless, the ability to generate unique fragmentation patterns for PTH-amino acid identification by source fragmentation should facilitate the PTH-amino acid analysis, because no precursor ion selection is required in this method. This is particularly true for on-line HPLC/MS analysis. By contrast, this method lacks control over the selection (isolation) of the parent ion for fragmentation. However, with an efficient separation attained by HPLC, possible sources of interferences for identification can be reduced.

4. Conclusions

An ESI IT/TOFMS system was evaluated as a detector for identification of PTH-amino acids. Each of the PTH-amino acids studied can be characterized by well-defined ion peaks in the mass spectra of these compounds. With the exception of PTH-(PTC-cysteine), a protonated molecular peak can be used for identification of these derivatives. Fragmentation patterns obtained by source excitation followed by a long trapping period (250 ms) gave rise to unique fragmentation products. This allows for the differentiation of isomers (PTH-Leu, PTH-Ile, and PTH-α–aminobutyric acid, PTH-α–aminoisobutyric acid). The detectability of PTH-amino acids for the ESI IT/TOFMS was examined. Results for the relative signal responses of the 20 standard PTH-amino acids indicate that the response factor between the most sensitive and the least sensitive compound can be as high as 20. Sensitivity disproportionation must be considered in the identification procedure. Subpicomole detection of PTH-amino acids was demonstrated. However, samples from a sequencer require a purification procedure for sensitive detection. The purpose of such a step is not the total separation of PTH-amino acid but the elimination of interfering species. We are in the process of developing fast microcolumn-LC separation methods, compatible with the ESI IT/TOF mass spectrometer optimized at a spectral recording speed of 4 Hz, for direct analysis of PTH-amino acids from a sequencer.

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