High Sensitivity Analysis of Phenylthiohydantoin Amino Acid Derivatives By Electrospray Mass Spectrometry

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A new methodology has been developed for high sensitivity electrospray ionization mass spectrometric analyses of phenylthiohydantoin (PTH) amino acid derivatives. Key components of the methodology are the use of a solvent system consisting of methanol/dichloromethane (1:1 v/v) containing 5-mM lithium triflate, a stainless steel electrode having a relatively large surface area, and a microscale electrospray nozzle that provides for stable electrospray at flow rates in the range of 100–500 nL/min. A linear response for the absolute signal intensity of the protonated molecule was observed for a number of derivatives over the concentration range of 50–1000 fmol/μL. For all except the arginine derivative, there was a decrease in the signal intensity with increasing flow rate with 100–300 nL/min being optimum. Collision induced dissociation (CID) product ion spectra were obtained for 21 derivatives including carboxymethyl cysteine and dehydrothreonine. Leucine and isoleucine can be distinquished on the basis of their CID product ion spectra. A subfemtomole detection limit was demonstrated for the phenylalanine PTH derivative in a selected reaction monitoring (SRM) experiment. Samples from an automated Edman microsequencer run have been analyzed using the new technique and compared to results obtained by conventional high-performance liquid chromatography analysis with UV detection. This work demonstrates the feasibility of using mass spectrometry to identify and quantitate the products generated by automated protein microsequencing using standard Edman degradation chemistry. (J Am Soc Mass Spectrom 1997, 8, 1165–1174) © 1997 American Society for Mass Spectrometry

All commercial protein microsequencing systems use high-performance liquid chromatography (HPLC) with UV detection of the phenylthiohydantoin (PTH) amino acid derivatives formed by Edman degradation chemistry [1, 2]. There have been numerous methodologies developed to use mass spectrometry to analyze PTH derivatives in an effort to improve the speed, sensitivity, and specificity of the sequence analysis. Nearly every ionization method has been investigated, including electron ionization (EI) [3–5], chemical ionization (CI) [6], gas chromatography–mass spectrometry (GC-MS) [7], and thermospray ionization [8]. None of these methods offered a viable alternative to detection by HPLC. More recently, efforts have been directed toward using electrospray (ES) as the ionization method. The electrospray process can be directly coupled to the liquid flow from the protein sequencer, which solves many of the interface problems that plague the other techniques.

PTH amino acid derivatives are inherently insensitive when analyzed by ES mass spectrometry under standard conditions [9, 10]. Consequently, the development of new degradation chemistries that yield more suitable derivatives has been an integral component of recent efforts [9–12]. These derivatives for the most part contain quaternary ammonium groups that are permanently charged or tertiary amine groups that aggressively compete for protons during the ionization process. Aebersold and coworkers have showed detection limits in the low fmole range for 4-(3-pyridinylmethylaminocarboxypropyl) PTH amino acid derivatives (PTH311) when analyzed in the selected ion monitoring mode by using a quadrupole mass spectrometer [12]. Although the adequate sensitivities of these new derivatives have been convincingly demonstrated, the actual use of the new chemistries in an automated microsequencer is problematic. Often there are problems with either the coupling or cleavage kinetics that affect the time and yield for each cycle of chemistry. Additionally, solvents needed to extract the amino acid derivative from the sample support may also result in loss of the remaining peptide or protein. Often there are technical problems with adapting existing instrumentation to the different chemistry. All of these factors conspire to hinder the acceptance of methods based on mass spectrometry by the protein sequencing community.

In the light of recent work by Van Berkel and Zhou concerning the analysis of nonpolar organic molecules

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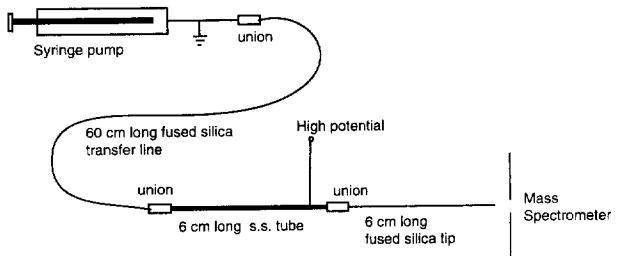


Figure 1. Experimental setup for the PTH amino acid derivative analyses.

by ES [13], we have reexamined the possibility of directly analyzing the standard PTH amino acid derivatives by ES. We report, in this work, the analysis of the standard Edman derivatives in the low femtomole range and compare the inherent sensitivity to dimethylaminopropyl thiohydantoin (DMAP-TH) derivatives previously described [10]. These results demonstrate the very real possibility that mass spectrometry can be coupled to existing protein microsequencers to achieve enhanced performance while still using the standard Edman degradation chemistry.

Experimental Methods

PTH amino acid derivatives were purchased from Sigma (St. Louis, MO). The synthesis of DMAP-TH pheyalanine (Phe) is described in detail elsewhere [14]. Stock solutions of the derivatives were prepared in methanol at a concentration of approximately 1 nmol/μL. Exact concentrations were determined by UV absorption measurements at 269 nm [15]. Lithium trifluoromethane sulfonate (lithium triflate, Aldrich, Milwaukee, WI) was dissolved in methanol/methylene chloride (1:1 v/v) at a concentration of 5 mM, and the solvent was prepared fresh each day. The derivatives from the stock solutions were diluted to concentrations ranging from 50 fmol/μL to 1 pmol/μL using the lithium triflate solution. For the relative response as a function of concentration experiments (see Figure 5 and Table 1), full scan spectra were obtained while infusing the sample solutions at 300 nL/min. The solvent for the conventional electrospray measurements was methanol/water (1:1 v/v) containing 1% acetic acid.

Mass spectral measurements utilized a custom built electrospray needle and electrode assembly (Figure 1). The sample solution was delivered by infusion using a syringe pump (Harvard Apparatus, South Natick, MA). The syringe needle was electrically grounded. A 60-cm-long fused silica transfer line [350 μm outer diameter (o.d.) and 50 μm inner diameter (i.d.)] was used between the syringe and the stainless steel tube electrode (6 cm long, 500 μm o.d., 250 μm i.d.). Alternatively, a 13 cm length of 50-μm stainless steel wire inserted into a 100-μm-i.d. fused silica tube could be used as the electrode. This arrangement had the advantage that the dead volume of the electrospray interface was less, reducing the time needed for cleanup between samples. For certain experiments, a platinum wire was used as the electrode. Prior to use, the stainless steel electrode was cleaned in boiling water containing 5% nitric acid for about 10 min, followed by a 10-min aqueous wash in an ultrasound bath and a final flush with methanol. Both the electrode and heated metal capillary inlet to the mass spectrometer needed to be cleaned every couple of weeks in order to maintain optimal performance. Otherwise, there is a significant loss of sensitivity and an increase in the intensity of background ions.

The electrospray needle was prepared from fused silica tubing (350 μm o.d. and 100 μm i.d.) using a CO_2 laser puller (Sutter Instrument Co., Novato, CA). The final tip dimensions were about 10–20 μm i.d. and 30–40 μm o.d. A 1-cm-long piece of fused silica tubing (92 μm o.d. and 20 μm i.d.) was inserted into the back end of the needle and pushed all the way down to the tip. A piece of hydrophilic polyvinyldifluoride (PVDF) membrane was positioned on top of this piece of fused silica and held in place with a second piece of fused silica tubing. The membrane served as a filter to prevent particulates from blocking the small hole at the tip of the needle.

Conventional electrospray mass spectra, using methanol/water (1:1 v/v containing 1% acetic acid) solvent were collected with the same electrospray setup, except the stainless steel tube electrode was replaced with an assembly described previously [16] in which the transfer line is sheathed with a platinum tube and joined to the electrospray needle with a union having a single vespel ferrule. Thus, contact of the liquid to the metal is limited to the thin gap (<1 mm) between the two pieces of fused silica tubing.

For all experiments, the needle and electrode assembly were mounted on an xyz positioner and the tip of the needle positioned directly in front of the heated metal capillary inlet of the mass spectrometer. Positioning was facilitated by using a CCD camera and video monitor to provide a magnified image of the needle and capillary. No drying gas or sheath liquid was used for any of the experiments.

Positive ion electrospray mass spectra were collected on a MAT 900 (Finnigan MAT, San Jose, CA) magnetic sector instrument over the mass range m/z 100–400 with a 1-s scan time. The spray potential was typically 1–1.2 kV above the 4.7-kV source potential. During the electrospray operation, the measured current from the electrode power supply was 0.7 μA. With only the 4.7-kV acceleration potential applied, observed current back to the grounded syringe needle was 0.4 μA. Collision induced dissociation (CID) product ion spectra were obtained using an LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA). Spectra were collected over the mass range from m/z 50 to m/z 450. A relative collision energy setting of 10%–14% was used. The automatic gain control (AGC) feature of the LCQ was used to determine how many ions were loaded into the trap. An AGC MS^n target value of $2e+007$ (measure of the number of ions in the cell) was

used for all CID product ion spectra. A constant ion injection time of 300 ms was used for the selected reaction monitoring (SRM) experiment with PTH-Phe (see Figure 10). The electrode potential was typically 800–1000 V.

Samples from the sequencer run (see Figure 11) were generated by a prototype multiple sample sequencer currently under development at the City of Hope in Duarte, CA. Samples were analyzed by both HPLC with UV detection at 269 nm and by mass spectrometry. Identical 5-μL aliquots from each of the first three cycles were analyzed in each instance. To remove amine salt contamination from the sequencer, the samples for mass spectral analysis were loaded onto a short capillary column (4 cm × 0.2 mm diameter) packed with Vydac C_{18} reverse phase support and mounted on a HPLC switching valve. The column was flushed with 30 μL of water, then switched online and eluted with methanol/water (1/1, v/v) containing 5-mM lithium triflate flowing at 2 μL/min to the stainless steel tube electrode and needle assembly described above. When the lithium triflate solution arrived at the electrode, as indicated by an increase in the electrospray current, the flow was decreased to 0.3 μL/min and mass spectra were collected over the mass range of m/z 100–400 using the MAT 900 magnetic sector mass spectrometer.

Results and Discussion

Until recently, the electrospray ionization method has only been applied to compounds with easily ionizable functionality. With the realization that an electrospray source functions as an electrochemical cell [17], Van Berkel and Zhou reasoned that it should be possible to achieve a one electron oxidation of nonpolar organics [13]. Using a metal electrospray needle and a solvent system consisting of dichloromethane and acetonitrile with lithium triflate as the electrolyte, they demonstrated the analysis of a number of aromatic hydrocarbons with excellent sensitivity. Although the PTH ring is certainly more polar than aromatic hydrocarbons and contains two nitrogen atoms that can be protonated, the proton affinity is reduced considerably by adjacent carbonyl and thiocarbonyl moieties. As a consequence, only a weak signal is observed in the positive ion electrospray spectrum for the protonated molecule when analyzed using a typical electrospray solvent system (methanol:water, 1:1, 1% acetic acid). The analysis of PTH-Phe (Figure 2A) is typical of the results obtained for derivatives other than histidine (His) and arginine (Arg), which have easily protonated side chain functionality. Even at a level of 1 pmol/μL, the signal for the protonated molecule was only about a factor of 4 greater that the chemical background noise in the spectrum. Using Van Berkel's solvent system (acetonitrile: dichloromethane, 1:1, 5-mM lithium triflate), there was an improvement in the absolute intensity of the signal for the protonated molecule and a very significant decrease in the level of the chemical noise (Figure 2B). There was no evidence in the spectrum for a one electron oxidation to yield the odd electron molecular ion (m/z 282). Even better sensitivity was obtained by substituting methanol for the acetonitrile (Figure 2C); the explanation for this observation requires further study. The choice of lithium triflate as the conducting electrolyte was key to the success of Van Berkel's methodology and is a dominant factor in these experiments as well. Simply substituting lithium triflate for acetic acid in the aqueous methanol solvent system greatly improved sensitivity (Figure 2D). Some degree of lithium ion attachment was observed in spectra obtained from the solvent system containing water, whereas none was observed if only organic solvents were used.

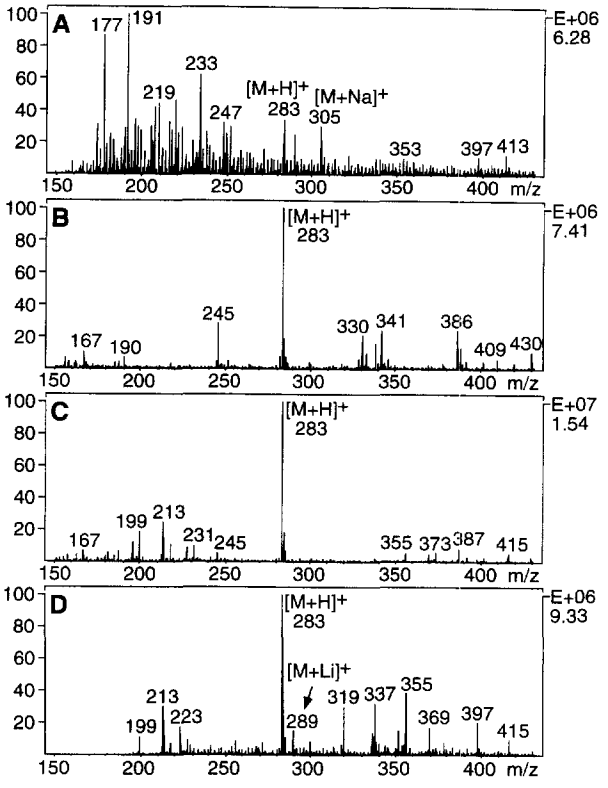


Figure 2. Positive ion electrospray spectra for a 1-pmol/μL solution of PTH-Phe in (**A**) methanol:water 1:1, 1% acetic acid, (**B**) acetonitrile:dichloromethane 1:1, 5-mM lithium triflate, (**C**) methanol:dichloromethane 1:1, 5-mM lithium triflate, (**D**) methanol: water 1:1, 5-mM lithium triflate.

The exact nature of the electrochemical reactions that lead to the increased protonation of the PTH derivatives are not known, but a number of experiments have been done to determine the role of various parameters. For all derivatives other than arginine, the flow rate had a dramatic effect on the intensity of both the sample and background ions. Results for the analysis of the PTH-Phe derivative are typical (Figure 3). At 0.3 μL/min (Figure 3A), the protonated molecule ($[M+H]^+$) was the most intense ion in the spectrum. As the flow rate was increased to 2.0 μL/min (Figure 3B), the intensity of

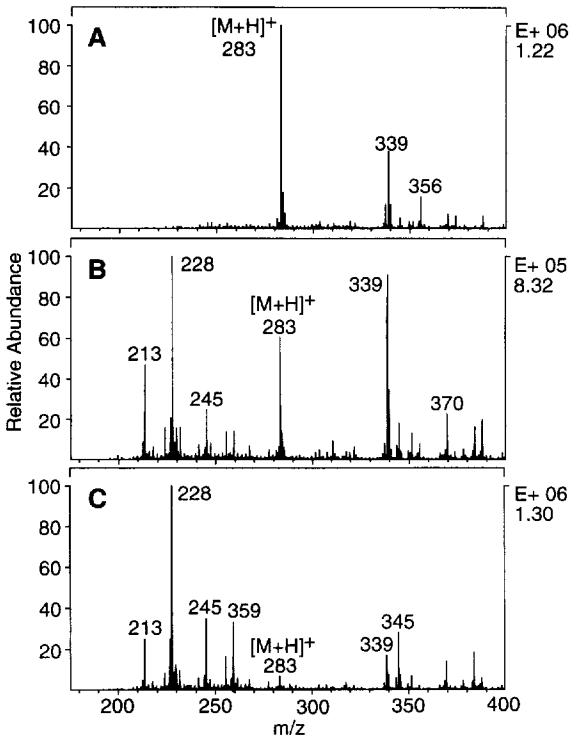


Figure 3. Positive ion electrospray mass spectra of a 250-fmol/μL solution of PTH-Phe in 5-mM lithium triflate MeOH/methylene chloride (1:1 v/v) solvent at flow rates of (**A**) 0.3 μL/min, (**B**) 2 μL/min, and (**C**) 4 μL/min.

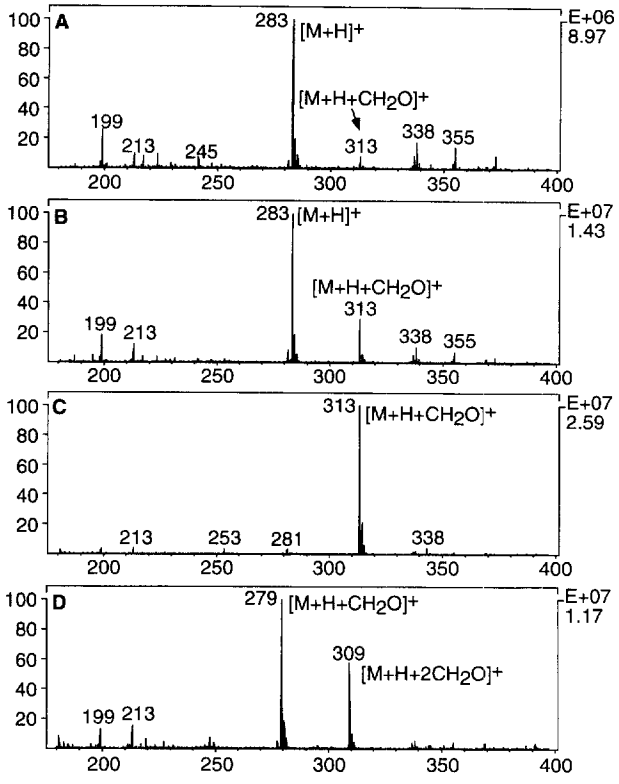
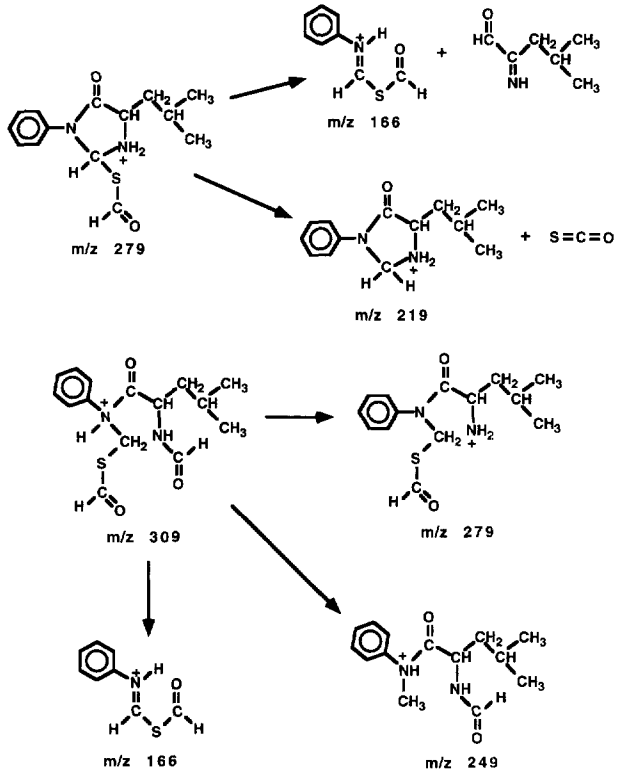


Figure 4. Positive ion electrospray spectra for (**A**) PTH-Phe using a 4-cm-long platinum wire electrode, (**B**) PTH-Phe using a 5-cm-long platinum wire electrode, (**C**) PTH-Phe using a 9-cm-long platinum wire electrode, and (**D**) PHT-Leu using a 9-cm-long platinum wire electrode.

MH^+ decreased by a little more than a factor of 2. At the same time, however, a number of strong background ions appeared. The structures and sources for most of these background ions have not been determined, but they appear to be related to impurities in the solvents or contaminants on the surfaces of hardware components. Ion series whose members differ by the mass of lithium triflate are observed primarily in the mass range beyond that needed for the analysis of the PTH derivatives (data not shown). As the flow rate was increased to 4 μL/min (Figure 3C), the intensity of MH^+ has decreased to less than 10% of its original value and is dwarfed by the strong background ions.

Van Berkel and Zhou observed a similar dependence of sample and background ion signal intensity as a function of flow rate [17]. The flow rates for our experiments are much lower than those of Van Berkel and Zhou, and protonated molecules are formed rather than radical cations. Nevertheless, the flow rate dependence argues for an intimate association of the sample molecules with the surface of the electrode in order to form the observed species. A second experiment serves to reinforce this view. When the 6-cm-long stainless steel tube was replaced with a short metal union with less than 1 mm of metal exposed to the liquid, no ions were observed for the PTH-Phe sample even with a sample concentration of 1 pmol/μL and a lithium triflate concentration of 5 mM. Under these conditions, the measured current value is 0.2 μA compared to 0.6 μA for the 6-cm-long stainless steel tube. Interestingly, the same background ions were observed, indicating that formation of these species is not dependent on the surface area of the electrode.

Spectra were also acquired using a Pt wire electrode placed within the fused silica transfer line. For a short length of wire (4 cm), results for the analysis of PTH-Phe were similar to those obtained using the stainless steel tube electrode (Figure 4A). In addition to the ion for the protonated molecule ($[M+H]^+$, m/z 283), an ion 30 mass units higher (m/z 313) was observed. As the length of the Pt wire electrode was increased (Figure 4B), the intensity of the m/z 313 ion increased relative to the protonated molecule. With a 9-cm electrode, the MH^+ ion was totally absent and only the m/z 313 ion remained (Figure 4C). The 30 mass unit addition was observed for a number of different PTH derivatives, and in some instances, a second addition of 30 mass units was observed, as in the case of PTH-Leu analyzed under the same conditions (Figure 4D). We have attributed the 30 mass unit additions to an electrochemical oxidation of the methanol solvent molecules to form formaldehyde, which subsequently reacts with the PTH derivatives. CID product ion spectra were obtained for both the +30 (m/z 279) and the +60 (m/z 309)



Scheme I

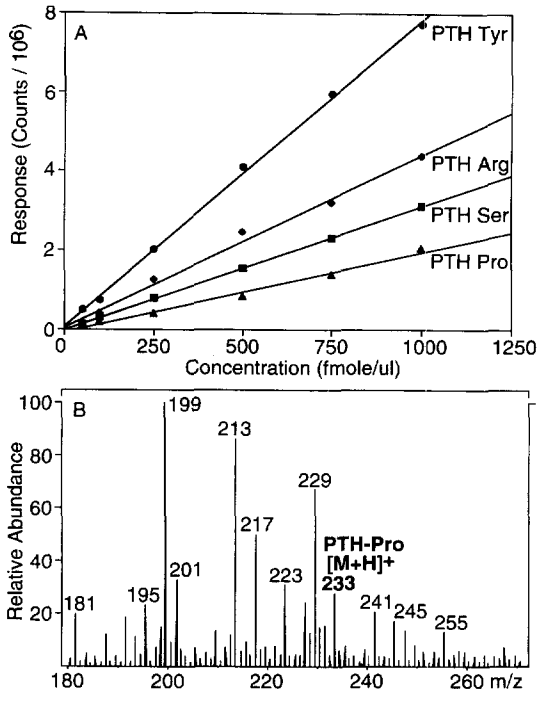


Figure 5. (**A**) Absolute signal intensity of the protonated ion for PTH-Tyr, PTH-Arg, PTH-Ser, and PTH-Pro as a function of the sample concentration. Each data point is the average of 100 individual spectra. (**B**) Positive ion electrospray mass spectrum of a 50-fmol/μL solution of PTH-Pro (5-scan average).

adducts for the PTH-Leu derivative using an ion trap mass spectrometer. Tentative structures have been assigned (Scheme I) for the major observed CID product ions. The creation of the solvent adducts does not offer any advantages in terms of increased sensitivity or quality of the tandem mass spectra, and has the complication of creating multiple species for some of the derivatives. Consequently, the use of platinum electrodes was not explored further.

Relative responses as a function of concentration were obtained for nine of the PTH derivatives (PTH-Phe, Tyr, Arg, Asp, Leu, Val, Pro, Ser, and Gly). For each, the response was linear over the concentration range of 50–1000 fmol/μL (in Figure 5A, for clarity, only curves for PTH-Pro, Ser, Arg, and Tyr are shown). There is an approximately fourfold difference between the most sensitive (PTH-Tyr) and least sensitive (PTH-Pro) derivatives (Table 1). The data used to construct Figure 5A and Table 1 were obtained using a 100-scan average for each analysis and corresponding to a total sample consumption of 38 fmol. A five-scan average is sufficient to provide an easily detected signal for even the least sensitive derivative (PTH-Pro, Figure 5B), corresponding to 1.9 fmol of the sample consumed. Thus, there is sufficient inherent sensitivity in the analysis for microsequencing at subpicomole levels.

Analyses were also performed on an equimolar mixture of 17 PTH amino acid derivatives (Gly, Ser, Pro, Val, Thr, Leu, Asn, Asp, Gln, Glu, His, Phe, Arg, Tyr, Try, and Lys) using the methanol/methylene chloride/lithium triflate solvent system at a concentration of 50 fmol/μL (Figure 6). Protonated ion peaks were observed for each of the derivatives. The protonated molecules corresponding to dehydroserine (m/z 205) and dehydrothreonine (m/z 219) are also observed. PTH-Arg and His were unusual in that each was accompanied by an ion two mass units lower. These ions were also observed when the compounds were run individually. The amount of the MH^+-2 ion relative to the protonated molecule is quite variable, and thus far we have not been able to correlate this behavior to any particular experimental parameter. The signal for PTH-Arg is somewhat suppressed when analyzed with the other derivatives, particularly at the lower flow rates. Background ions derived from the solvent are generally restricted to the low and high mass ends of the mass range of interest, and none of the prominent background ions have the same mass as any of the PTH derivatives.

Previously, we described the characteristics of DMAP-TH amino acid derivatives [10]. Using the standard electrospray solvent system (water:methanol 50:50, 1% acetic acid), the analysis of DMAP-TH-Phe was 50 times more sensitive than the analysis of PTH-Phe. Using the methanol–dichloromethane–lithium triflate solution, under optimal conditions, the signal intensity for a 50-fmol/μL DMAP-TH-Phe is 3.6 times greater than that obtained under optimal conditions using the standard solvent system (data not shown). However,

Table 1. Relative sensitivities over the concentration range of 50 fmol/μL–1.0 pmol/μL and signal-to-noise ratios at the 50-fmol level for the protonated molecular ion of the Asp, Phe, Gly, Leu, Pro, Arg, Ser, Val, Tyr PTH derivatives

Amino acid	Asp	Phe	Gly	Leu	Pro	Arg	Ser	Val	Tyr
Relative sensitivity (counts/fmol/μL)	3300	4000	2370	3190	2020	4340	3130	3030	7760
Signal-to-noise ratio (50 fmol/μL analysis)	8	12	4	8	4	10	4	7	25

the signal for DMAP-TH-Phe is only 4.2 times more intense than for the PTH-Phe derivative (Figure 7) when both are analyzed using the methanol–dichloromethane–lithium triflate solution. Adapting the DMAP-TH chemistry to an automated microsequencer proved to be very problematic, particularly in terms of extracting the cleaved amino acid residue from the sample membrane. Conditions sufficient to extract sufficient amounts of the DMAP-TH derivatives also resulted in washout of the sample. With the new electrospray procedure for analyzing the PTH derivatives, standard Edman chemistry can be used with only a marginal difference in sensitivity compared to that of the DMAP derivatives.

CID product ion spectra for all 20 PTH derivatives have been determined on an ion trap mass spectrometer (Table 2). The Ala and Gly derivatives yield a number of ions, some of which are not readily assigned. Assorted cleavages of the PTH ring account for most of the major ions (Figure 8). For both derivatives, the most intense ion corresponds to protonated phenylisothiocyanate (m/z 136). Spectra for the other derivatives are characterized by one or two product ions that are much more intense than the others. For Asp, Glu, and the carboxymethyl derivative of Cys, the dominant ion is formed by loss of water from the sidechain carboxylic acid moiety. Possible structures for the observed ions are given in Schemes II, III, and IV. CID spectra for the Asn, Gln, and Arg derivatives are similar in that the most prominent ion results in loss of ammonia from the sidechain. The Asn and Gln spectra are nearly devoid of other product ions.

For the Phe, Ile, Leu, Pro, and Val derivatives, the dominant product is the imminium ion incorporating the sidechain (Figure 8, cleavage a). Other ions are of

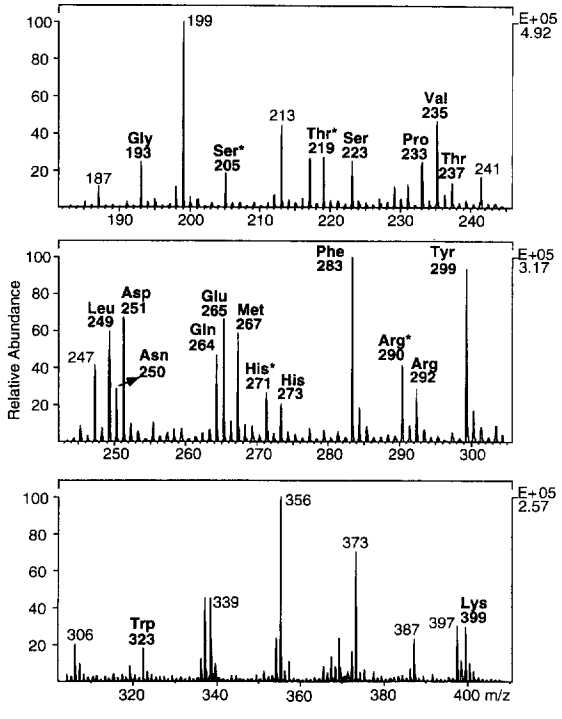


Figure 6. Positive ion electrospray mass spectrum of 50-fmol/μL PTH amino acid derivative mixture of Gly, Ser, Pro, Val, Thr, Leu, Asn, Asp, Gln, Glu, Met, His, Phe, Arg, Tyr, Trp, and Lys PTH derivatives in 5-mM lithium triflate MeOH/methylene chloride (1:1 v/v) solvent at the flow rate of 0.1 μL/min. Each PTH derivative is designated with the standard amino acid three-letter code. Ser* and Thr* designate dehydroserine and dehydrothreonine. His* and Arg* designate forms for those two derivatives that have mass values two units lower than expected for the PTH derivative.

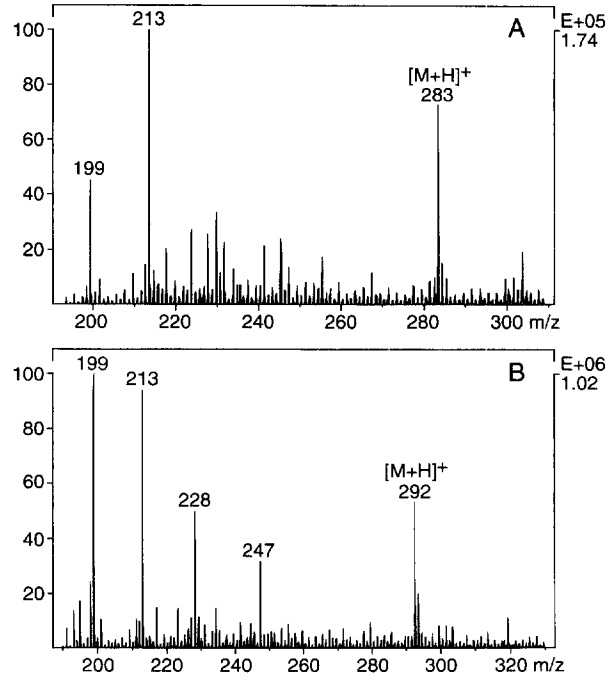


Figure 7. Positive ion electrospray mass spectra of (**A**) 50-fmol/μL solution of PTH-Phe and (**B**) 50-fmol/μL solution of DMAP-TH Phe in 5-mM lithium triflate MeOH/methylene chloride (1:1 v/v) solvent at a flow rate of 0.1 μL/min.

Table 2. Fragment ions observed in the CID product ion spectra of PTH amino acid derivatives. Intensities are relative to the base peak in the spectra. Numbers in parentheses are mass-to-change ratio values. The columns labeled a, b, c, and d refer to the PTH ring cleavages indicated in Figure 8

Amino acid	MH^+	Relative intensity (*m/z*) 94	(*m/z*) 136	(*m/z*) 193	a (*m/z*)	b (*m/z*)	c (*m/z*)	d (*m/z*)	e (*m/z*)	MH^+-X[a]	Others (*m/z*)
A	207	56	**100**	—	NA	9 (86)	58 (148)	—	11 (179)	—	(120,147,164,173,189)
C*[h]	279	—	—	—	—	—	—	—	—	**100** (279)	(116,205,251)[b]
D	251	1	—	—	2 (88)	—	—	—	—	**100** (233)	(174,191,205)[c]
E	265	—	—	—	—	—	—	—	—	**100** (247)	41 (84)[d]
F	283	1	1	—	**100** (120)	11 (162)	2 (224)	—	3 (255)	—	(103,132,196)
G	193	35	**100**	NA	NA	—	15 (134)	—	21 (165)	—	(106,133,150,159,175)
H	273	—	—	—	3 (110)	7 (152)	—	**100** (180)	—	—	—
I	249	6	7	4	**100** (86)	5 (128)	2 (190)	—	4 (221)	—	—
K	399	—	—	—	—	—	—	**100** (306)[f]	—	—	**100** (306)[e,f]
L	249	10	9	—	**100** (86)	5 (128)	8 (190)	—	4 (221)	—	6 (189)?
M	267	—	—	—	4 (104)	16 (146)	—	**100** (174)	2 (239)	—	18 (219)[g]
N	250	—	—	—	—	—	—	3 (157)	—	**100** (233)	—
P	233	—	1	NA	**100** (70)	—	—	—	3 (205)	—	—
Q	264	—	—	—	—	—	—	—	—	**100** (247)	—
R	292	—	—	—	—	—	2 (233)	1 (199)	4 (264)	**100** (275)	(112,157,232?)
S	223	—	—	**100**	NA	—	—	—	—	—	6 (118), 1 (146)
T	237	—	—	**100**	—	—	—	—	1 (209)	—	—
T*[i]	219	—	20	—	NA	**100** (98)	13 (160)	65 (126)	1 (191)	—	—
V	235	11	7	1	**100** (72)	3 (114)	2 (176)	—	3 (207)	—	—
W	322	—	1	**100**	1 (159)	—	—	—	—	—	—
Y	299	1	28[f]	**100**	28 (136)[f]	2	2	1	2	—	—

[a]X = NH_3 or H_2O.
[b]See Scheme II.
[c]See Scheme III.
[d]See Scheme IV.
[e]Loss of C_6H_7N from the PITC derivatized side chain.
[f]Two possibilities for the same mass-to-change ratio value.
[g]Loss of CH_3SH from side chain.
[h]C* = carboxymethyl cystene.
[i]T* = Dehydrothreonine.
NA = not available.

lesser (<12%) abundance, but can be used to distinguish Leu from Ile. The Ile derivative loses the sidechain to yield an ion at *m/z* 193 (Figure 9B). This ion is totally absent in the CID spectrum of Leu (Figure 9A). Additionally, there is an ion at *m/z* 189 in the CID spectrum of Leu that is an order of magnitude less intense in the spectrum for Ile. Although we cannot yet confidently assign a structure to the *m/z* 189 ion, it is always present at the same level.

For the His, Lys, and Met derivatives, the dominant product ion is formed by loss of analine from the protonated molecule (Figure 8, cleavage d). For the Lys derivative, the same loss can occur from the PITC derivatized sidechain amino group. For the Ser, Thr, Trp, and Tyr derivatives, the dominant ion is formed by loss of the sidechain (Figure 8, *m/z* 193). Dehydrothreonine is readily formed when Thr is analyzed on the ion trap. The intensity of the ion corresponding to protonated dehydrothreonine is always greater than that for protonated Thr in the normal mass spectrum. For dehydrothreonine, loss of water from the sidechain effectively eliminates the pathway for formation of the *m/z* 93 product ion. Instead, an ion (*m/z* 98) corre-

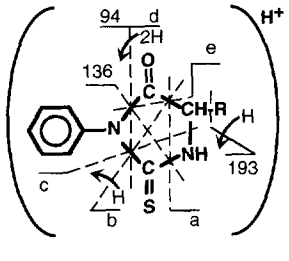
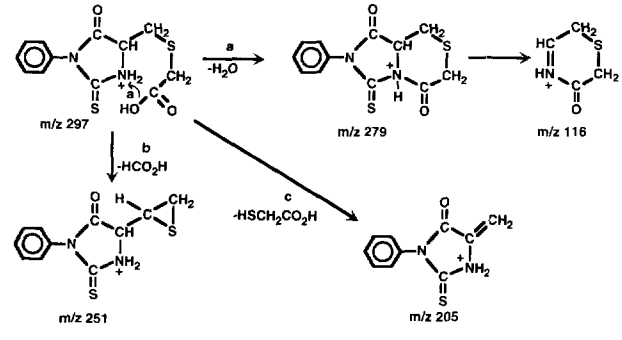
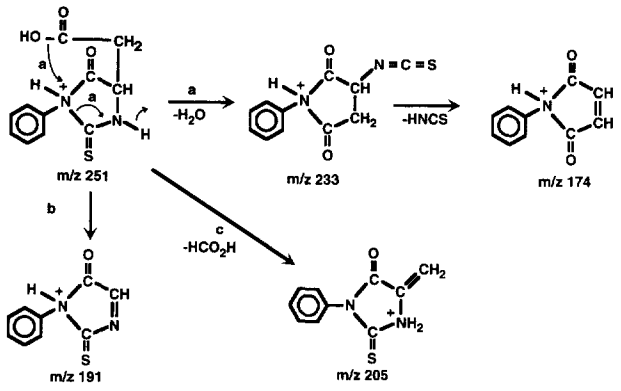


Figure 8. Diagram of ring cleavages observed in the CID product ion spectra for various PTH amino acid derivatives on an ion trap mass spectrometer.



Scheme II

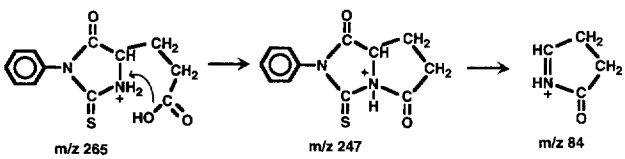


Scheme III

sponding to loss of phenylisocyanate is observed (Figure 8, cleavage b).

Using tandem mass spectrometry and doing the analysis in the SRM mode, the sensitivity of the analysis can be considerably improved. This is illustrated by the analysis of the PTH-Phe derivative where the mass-to-change ratio range from 119 to 123 was scanned in order to obtain the profile of the m/z 120 product ion. The response was linear over the concentration range of 10–1000 fmol (Figure 10A). A constant ion injection time of 300 ms was used and each data point is the average of 50 scans over a 1-min analysis time. The decision to use 50 scans was arbitrary. Even at a concentration of 10 fmol/μL, ten scans yields an excellent profile for the m/z 120 ion and its ^{13}C isotope at m/z 121 (Figure 10B). This represents a total sample consumption amount of 600 attomol with a flow rate of 300 nL/min. Clearly, the sensitivity could be pushed even further for this standard sample. However, it would be a meaningless exercise in the context of analyzing samples from a sequencer where the sensitivity is going to be limited by the chemical background from the sequencer. The full mass range tandem mass spectrum of PTH-Phe at the 10-fmol level (Figure 10C) reveals that only three of the observed ions with intensities >5% of the base peak are derived from PTH-Phe. The remainder are from the decomposition of background ions that fall within the 2 m/z window for precursor ion selection. The practical limits of detection for any of the PTH derivatives can only be determined once work in progress on the interface between the mass spectrometer and the sequencer is completed.

The feasibility of analyzing actual sequencer samples was demonstrated by taking the first three cycles from



Scheme IV

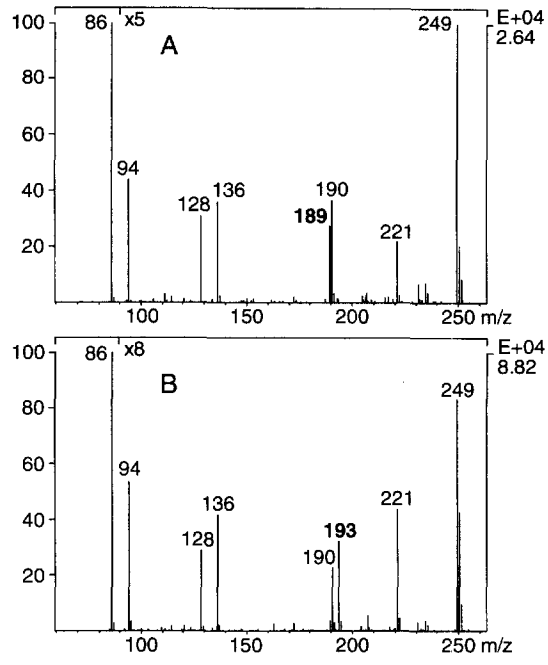


Figure 9. Ion trap CID product ion spectra for (A) PTH-Leu and (B) PTH-Ile.

a sequencing run on a prototype multiple sample sequencer currently under development in our lab and analyzing them by both mass spectrometry and HPLC with UV detection (Figure 11). The principal obstacle to analysis of actual sequencer samples is high levels of amine salts created by the sequencer chemistry that unavoidably contaminates the sample and precludes direct analysis of the PTH-aa derivatives. However, the amine salt (in this instance $Et_4N^+TFA^-$) is easily removed by trapping the PTH-aa derivatives on a short capillary column packed with reverse phase support, washing the column with water, and then eluting the PTH-aa derivatives with the methanol–water–lithium triflate solution. Under these conditions, there is no significant separation of the different PTH derivatives. The same general relative response from cycle to cycle for the first three residues (ALF) of the sample peptide is evident in both the mass spectral (Figure 11A) and HPLC (Figure 11B) analyses. The large amount of carryover of Ala in cycles 2 and 3 and Leu in cycles 3 evident in both the mass spectrometry and HPLC analyses is due to failure of the sequencer to achieve complete conversion.

Conclusions

A method for obtaining high sensitivity analyses of PTH amino acid derivatives has been developed. The method requires that the electrospray potential be applied to an electrode with a large surface area in contact with the sample solution flowing to the needle, and a solvent system that contains lithium triflate as the electrolyte. Although the methodology is similar in

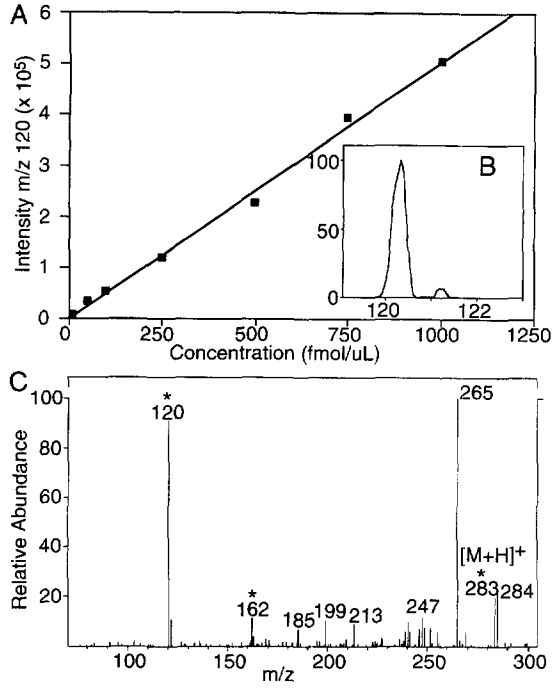


Figure 10. (**A**) Intensity of the m/z 120 product ion from the CID of PTH-Phe as a function of sample concentration. Each data point is an average of 50 scans collected over m/z 119–123 during 1 min. (**B**) Average of ten consecutive spectra from the data set for 10-fmol/μL concentration. (**C**) Full mass range CID product ion spectrum for PTH-Phe for the 10-fmol/μL sample solution. Ions marked with an asterisk are derived from PTH-Phe.

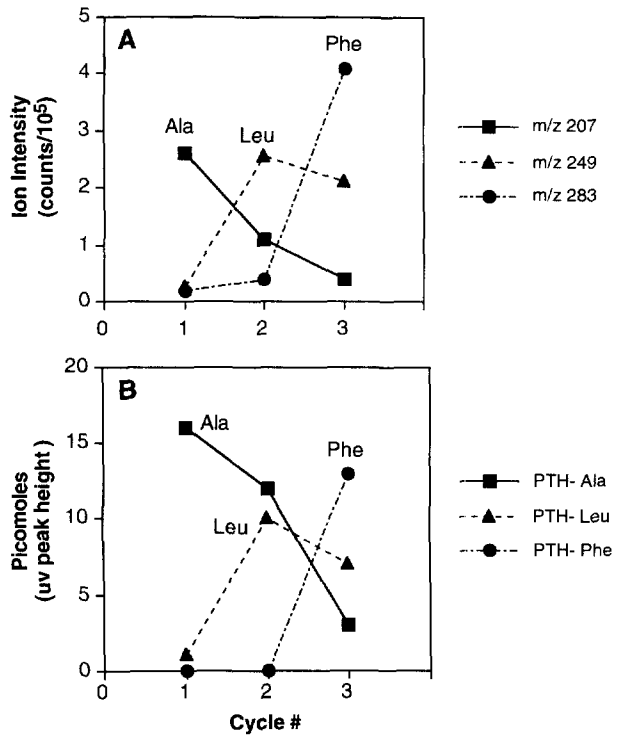


Figure 11. Comparison of the results for the first three cycles from the same sequencer run analyzed by (**A**) mass spectrometry collecting full mass spectra over the range m/z 200–400 and (**B**) HPLC with UV detection at 269 nm. Only the signals corresponding to the first three residues of the peptide ALFHGRVSWAMFPNGK were plotted in each graph.

many respects to that described by Van Berkel and Zhou [13] for the analysis of neutral analytes, there are important differences. Protonated molecules were produced rather than radical cations, and the signal was enhanced if methanol was used in the place of acetonitrile. Significantly reduced flow rates (100–300 nL/min versus 2–5 μL/min) are essential, requiring the use of a microscale needle assembly capable of stable electrospray operation under these conditions. Signal intensity is not enhanced by substituting a platinum electrode for one made of stainless steel. Rather, with the platinum electrode, the analysis is complicated by the formation of adducts derived from oxidation of the methanol solvent. The observed response for the protonated molecule was linear over the range of concentrations from 50 to 1000 fmol/μL with a fourfold difference between the least sensitive and most sensitive derivatives tested. Additionally, when analyzed as a mixture, there was no significant suppression of the signal for any of the derivatives. Ile and Leu could be distinguished using tandem mass spectrometry in an ion trap mass spectrometer to obtain CID product ion spectra. In a SRM experiment, subfemtomole limits of detection were easily achieved for the PTH-Phe derivative. The results of mass spectrometry analysis of actual sequencer samples was shown to be consistent with those obtained by analysis with HPLC and UV detection.

These results significantly enhance the probability that mass spectrometry can be used as an alternative detection system for automated Edman microsequencing. In addition to the potential for greater sensitivity, the speed mass spectral analysis should make it possible to sequence multiple samples simultaneously. Work is currently in progress to interface a mass spectrometer to the multiple sample sequencer currently under development in our lab.

Acknowledgments

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