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THERMOSPRAY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC–MASS SPECTROMETRIC CHARACTERIZATION OF BIOLOGICAL MACROMOLECULES

I. ANALYSIS OF ACID HYDROLYSATE OF PEPTIDES

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SUMMARY

A method for the routine analysis of phenylthiocarbamyl (PTC) amino acids by thermospray high-performance liquid chromatography–mass spectrometry (TSP LC–MS) is described. Data were acquired on a small dedicated TSP LC–MS system in which the temperature of the vaporizer and ion source block were optimized. PTC-amino acids exhibited unique TSP mass spectra containing sufficient fragment ions to determine structural data. Therefore, using this method the amino acids contained in the acid hydrolysates of unique and modified peptides were able to be positively identified. Additionally, the amino acid composition of peptides as determined by TSP LC–MS in the selected ion monitoring mode corresponded well with the theoretical value. The detection limits for the PTC-amino acids were at the low picomole level.

INTRODUCTION

Within the last few years the use of high-performance liquid chromatography (HPLC) for the analysis of derivatized amino acids obtained from the acid hydrolysis of proteins and peptides has overtaken classical technique used for amino acid analysis as the accepted method of choice. Several reagents including phenyl isothiocyanate (PITC), dansyl chloride, dabsyl chloride, *o*-phthalaldehyde, ninhydrin and fluorescamine have been used[1]. One of the more popular methods involving the use of PITC to form phenylthiocarbamyl (PTC) amino acid derivatives was first demonstrated by Koop *et al.*[2] and later commercialized by Waters Assoc. as the Pico-Tag™ system[3]. The advantages of this method include reaction with primary and secondary amino acids, producing quantitatively stable derivatives, and relatively easy and fast derivatization. A disadvantage is the dependence on retention time data for the qualitative identification of amino acids. The PTC-amino acids all have the same chromophore and, although this is an advantage for quantitation by UV methods, it

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makes identification very difficult. This is also a problem with other derivatization methods because the chromophore is usually contained in the derivatizing reagent and differences in the UV spectra obtained with a diode-array detector are slight.

On-line liquid chromatography–mass spectrometry (LC–MS) has been increasingly used in the past few years. Many of the early studies dealt with the analysis of both underivatized and derivatized amino acids[4–6]. The recent refinements in thermospray (TSP) ion source design, the availability of inexpensive, dedicated mass spectrometer systems and the development of HPLC systems designed for LC–MS have made the routine application of TSP LC–MS to quantitative and qualitative amino acid analysis practical[7]. This paper describes a TSP LC–MS procedure for the analysis of the acid hydrolysate of peptides using currently available commercial equipment. PITC was used for derivatizing the acid hydrolysates prior to TSP LC–MS analysis.

EXPERIMENTAL

Materials

Individual amino acid standards, an amino acid standard mixture (2.5 μmol/ml in 0.1 *M* hydrochloric acid) and angiotensin I were purchased from Sigma (St. Louis, MO, U.S.A.). PTH-amino acids, PITC reagent and 6 *M* hydrochloric acid of protein sequencing grade were obtained from Pierce (Rockford, IL, U.S.A.). N-Methylamino acids were obtained from Peptides International (Louisville, KY, U.S.A.). Rat atrial natriuretic peptides (rANP) were prepared at the Merrell Dow Research Institute. Acetonitrile was an HPLC-grade product from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Water was purified with a Barnstead NANOpure/ORGANICpure system. All other chemicals used were of analytical-reagent grade.

TSP LC–MS

The TSP LC–MS apparatus consisted of a Hewlett-Packard (Palo Alto, CA, U.S.A.) 5970 mass-selective detector mounted in a Vestec (Houston, TX, U.S.A.) 101 TSP interface consisting of both the TSP ion source and associated vacuum systems. Data were acquired and the instrument controlled by a Hewlett-Packard 59970 ChemStation.

The HPLC system consisted of a 600-MS multi-solvent delivery system (Waters Assoc., Milford, MA, U.S.A) that was designed with a hydraulic system to optimize flow stability and included a high-pressure switching valve to change from direct loop injection to separations using a column. Manual injections were done using a Waters Assoc., UK6 injector. A Waters Assoc. 490-MS programmable multi-wavelength detector with a high-pressure flow cell was installed in series between the column and the TSP vaporizer, allowing UV data to be obtained at the same time as the LC–MS data. In addition, a Waters Assoc. 590 programmable pump was used to add buffers via a post-column device to enhance response in the thermospray process. Chromatography was performed at 35°C using a Waters Assoc. Nova-Pak C_{18} column (15 cm × 3.9 mm I.D.), preceded by an Alltech C_{18} guard column (2 cm × 2 mm I.D.), with gradient elution using acetonitrile–0.05 *M* ammonium acetate at a flow-rate of 1 ml/min. The detailed mobile phase composition and the gradient elution used for the separation of PTC-amino acid standard mixture are described in Table I. The column

TABLE I
LINEAR GRADIENT FOR THE SEPARATION OF PTC-AMINO ACID STANDARDS

Time (min)	*Mobile phase A**	*Mobile phase B***
0	100	0
2	100	0
12	70	30
17	70	30
31	20	80
32	0	100
50	0	100

* 0.05 *M* Ammonium acetate in water.
** 0.05 *M* Ammonium acetate in water–acetonitrile (50:50).

effluent was mixed, through the use of a mixing tee located between multi-wavelength detector and TSP interface, with 0.5 *M* ammonium acetate at a flow-rate of 0.2 ml/min. The amount of sample injected was 10 μl (0.5 nmol).

The optimum temperature for the thermospray vaporizer was determined to be 162°C for the initial gradient conditions (100% mobile phase A). The vaporizer temperature was then gradually adjusted, through a pre-set automatic gradient compensation controller, to 154°C when the gradient elution reached 100% mobile phase B. The ion source block temperature (desolvation chamber) was maintained constantly at 280°C.

Acid hydrolysis of peptides

Acid hydrolysis was performed using the procedure described in the operator's manual of Waters Pico-Tag workstation. Culture tubes (6 × 50 mm) made of Pyrex (Corning, NY, U.S.A.) were used as sample containers, pre-cleaned with 6 *M* hydrochloric acid and heated at 120°C. Peptide samples (25 nmol) were hydrolyzed *in vacuo* for 24 h at 110°C in 6 *M* hydrochloric acid containing 1% phenol. All drying steps were carried out in a Speed Vac concentrator (Savant Instruments, Farmingdale, NY, U.S.A.) without heat.

PITC derivatization

Derivatization was performed essentially as described by Bidlingmeyer *et al.*[3]. The dried amino acid standards and acid hydrolysate of peptide were dissolved in 10 μl of coupling buffer (absolute ethanol–water–triethylamine, 2:2:1). The solutions were dried and then derivatized with 20 μl of fresh coupling reagent (absolute ethanol–water–triethylamine–PITC, 7:1:1:1). After reaction for 30 min at room temperature, the derivatized samples were dried and dissolved in 500 μl of mobile phase A for immediate TSP LC–MS analysis.

RESULTS AND DISCUSSION

Although several LC methods have been developed for the separation of PTC-amino acids[8–10], the buffers used in the mobile phase were either non-volatile or varied in concentration while performing the gradient elution, thus making them

unsuitable for TSP LC–MS work. In this study, ammonium acetate was selected as a mobile phase buffer as it is volatile and can also be used as a reagent to induce TSP ionization. The concentration of the buffer was maintained constant throughout the gradient elution, which is essential for obtaining a stable baseline. A post-column device was used to adjust the concentration of ammonium acetate, independent of that needed for separation, to be about 0.1 *M*. This has been reported to be the optimum region to perform TSP ionization[11].

Fig. 1 shows a total ion chromatogram of PTC-amino acid standards obtained by TSP LC–MS. As noted by others[8–10], derivatization of amino acids with PITC produced reagent-related byproducts such as the peaks R shown. The other byproducts (not shown in Fig. 1) were eluted between 35 and 45 min. In fact, byproduct R eluting at 23.2 min was not observed with UV detection and could be easily eliminated by drying the sample in the Speed Vac for about 20 min. Byproduct R eluted at 25.7 min was detected by both total ion and UV detection and exhibited an $[M + H]^+$ ion at *m/z* 181.

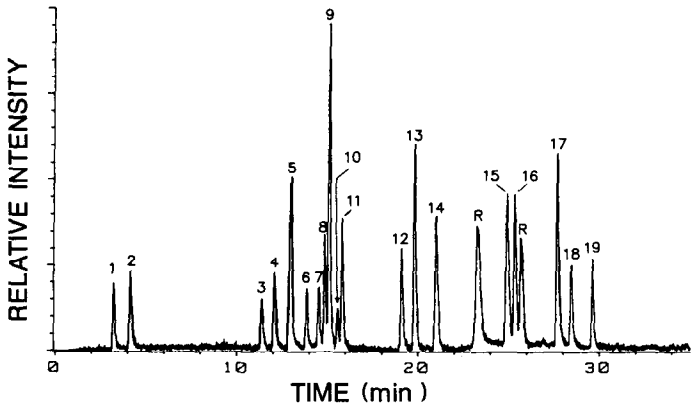

Fig. 1. Total ion chromatogram (*m/z* 125–500) of PTC-amino acids obtained by TSP LC–MS. For identification of peaks 1–19, see Tables II and III. Peak R = reagent-related byproduct. For chromatographic conditions, see Table I.

The TSP mass spectral data for the PTC-amino acids are given in Tables II and III. Note that Lys and Orn formed a bis-derivative with PITC and therefore yielded more fragment ions than the other PTC-amino acids. Owing to intense background ions present in the low mass range, the mass spectra acquired were restricted to be above *m/z* 125. Two fragment ions, which are not listed in Table II, were also found to be significant in the TSP mass spectra of some PTC-amino acids. One was the protonated phenylthiourea ion (*m/z* 153) and the other was the $[M + H - PITC]^+$ ion.

The data in Tables II and III indicate that each PTC-amino acid exhibited a unique base peak and characteristic fragment ions that reveal structural information. Accordingly, the amino acids obtained from acid hydrolysis of peptides can be positively identified. Except those of Asp, Glu, Gly, Lys, Orn, Pro, Ser and Thr, the base peaks of the PTC-amino acid studied all correspond to the $[M + H - H_2O]^+$ ions.

The base peaks of Asp and Glu (acidic amino acids) correspond to the $[M + H - H_2O - HCOOH]^+$ ions; Ser and Thr (hydroxyamino acids) correspond to the $[M + H - 2H_2O]^+$ ions; Lys and Orn (basic amino acids) correspond to the $[M + H - PITC - H_2O]^+$ ions; and Gly and Pro correspond to the $[M + H - H_2S]^+$ and $[M + H - H_2O - C_6H_4]^+$ ions, respectively. Because of the thermal instability of PTC-amino acids, the $[M + H]^+$ ions were found to be fairly weak or completely absent in the spectra. It was also noted that the abundance of the fragment ions in the TSP mass spectra was sensitive to the temperatures of the vaporizer and the ion source block. Therefore reproducible temperature control at these two positions is very critical in order to obtain a reproducible TSP mass spectrum.

N-Methylamino acids had also been examined in order to apply TSP LC–MS to the analysis of the acid hydrolysate of modified peptides. N-Methylated peptides are normally used to improve the biological potencies and to increase the resistance to enzymatic digests. However, the N-methylamino acids obtained from the acid hydrolysis of N-methylated peptides can not be confirmed using conventional PTC-amino acid analysis because of their unusually long retention times and the probable interference by the reagent-related byproducts. However, these modified amino acids can be unambiguously assigned by the TSP LC–MS method.

Unlike primary amino acids, the PITC-derivatized N-methylamino acids exhibited only the $[M + H]^+$ ion in their TSP mass spectra as listed in Table IV. This characteristic had also been observed in the TSP mass spectra of PTH-amino acids (Table V). A comparison of the TSP mass spectra of PITC-derivatized Ile and N-methyl-Ile ([N-Me]Ile) is given in Fig. 2. If N-methyl-Ile and PITC had formed a PTC derivative, it would have exhibited a fragmentation scheme similar to that of PTC-Ile except for the formation of the $[M + H - H_2S]^+$ ion. Instead, it yields only an ion at m/z 263 that corresponds to the $[M + H]^+$ ion of PTH-N-methyl-Ile, suggesting that N-methylamino acids and PITC formed a PTH derivative under the reaction conditions described. This suggestion is also supported by their relatively long retention times (capacity factor, k') compared with those of PTC- and PTH-amino acids. In theory, PTC-amino acids need an acid to initiate the cyclization and form the PTH-amino acid analogs[12]. It could be possible that N-methylamino acids are much more sensitive to trace amounts of residual acid.

Fig. 3 shows the application of TSP LC–MS to the analysis of an acid hydrolysate of an N-methylated rANP which is currently under study as an antihypertensive agent. The sample was chromatographed using the same gradient elution as described for the separation of PTC-amino acid standards. The peak identities were confirmed according to their TSP mass spectra.

Fig. 4 shows the application of TSP LC–MS to the confirmation of a modified dipeptide, ArgΨ[CH_2NH]Ile, obtained from the acid hydrolysis of a modified rANP. The sample was chromatographed using gradient elution with a higher concentration of acetonitrile in the mobile phase. Two peaks (a and b) were found to exhibit identical TSP mass spectra that correspond to the bis-PTC derivative of ArgΨ[CH_2NH]Ile. As L-amino acids were used to prepare the peptide, the formation of the diastereoisomers is probably due to the racemization occurring at the Arg moiety caused by the synthetic route used to prepare that peptide.

TSP LC–MS had also been evaluated for determining the amino acid composition of peptides. The base peaks of PTC-amino acids were used for selected-

TABLE II
TSP MASS SPECTRAL DATA FOR PTC-AMINO ACIDS

No.	*PTC-amino acid*	*Mol.wt.*	*k'*	*m/z (relative intensity, %)*		
				$[M + H]^+$	$[M + H - H_2O]^+$	$[M + H - H_2S]^+$
1	Asp	268	1.6	–	251 (11)	–
2	Glu	282	2.1	–	265 (25)	–
3	Ser	240	5.7	–	223 (10)	–
4	Gly	210	6.1	211 (10)	193 (62)	177 (100)
5	Asn	267	6.5	268 (8)	250 (100)	234 (3)
6	Gln	281	7.0	282 (18)	264 (100)	248 (10)
7	His	290	7.3	–	273 (100)	257 (22)
8	Thr	254	7.4	255 (11)	237 (39)	–
9	Ala	224	7.6	225 (9)	207 (100)	191 (18)
10	Arg*	309	7.8	–	292 (100)	276 (45)
11	Pro	250	8.0	–	233 (37)	–
12	Tyr	316	9.5	317 (7)	299 (100)	283 (8)
13	Val	252	9.9	253 (7)	235 (100)	219 (20)
14	Met	284	10.5	285 (5)	267 (100)	251 (29)
15	Ile	266	12.4	267 (14)	249 (100)	233 (23)
16	Leu	266	12.8	267 (13)	249 (100)	233 (26)
17	Phe	300	13.8	301 (13)	283 (100)	267 (52)

* Fragment ions at *m/z* 293, 275 and 231 were also observed.

TABLE III
TSP MASS SPECTRAL DATA FOR $(PTC)_2$-ORN AND $(PTC)_2$-LYS

*Fragment ion**	*m/z (relative intensity, %)*	
	(18) Orn (k' = 14.3)	*(19) Lys (k' = 14.8)*
$[M + H - H_2O]^+$	385 (21)	399 (23)
$[M + H - H_2O - H_2S]^+$	351 (17)	365 (18)
$[M + H - H_2O - C_6H_5NH_2]^+$	292 (51)	306 (45)
$[M + H - H_2O - PITC]^+$	250 (100)	264 (100)
$[M + H - H_2S - PITC]^+$	234 (15)	248 (60)
$[M + H - H_2O - C_6H_4 - PITC]^+$	174 (20)	188 (36)
$[M + H - 2PITC]^+$	133 (55)	147 (65)

* The fragment ions of relative intensity $\geqslant 15\%$ are included.

TABLE IV
TSP MASS SPECTRAL DATA FOR PITC-DERIVATIZED N-METHYLAMINO ACIDS

N-methylamino acid	*k'*	*m/z (relative intensity, %)*
Ala	16.7	221 (100)
Ile	21.3	263 (100)
Leu	21.8	263 (100)
Phe	21.0	297 (100)

$[M + H - 2H_2O]^+$	$[M + H - H_2O - HCOOH]^+$	$[M + H - H_2O - C_6H_4]^+$
–	205 (100)	–
–	219 (100)	–
205 (100)	–	–
–	–	–
–	–	–
–	–	–
–	–	–
219 (100)	–	–
–	–	–
–	–	–
–		157 (100)
–	–	–
–	–	159 (16)
–	–	191 (6)
	–	173 (15)
–	–	173 (16)
–	–	207 (10)

* Fragment ions at *m/z* 293, 275 and 231 were also observed.

TABLE V
TSP MASS SPECTRAL DATA FOR PTH-AMINO ACIDS

PTH-amino acid	*Molecular weight*	*k'*	*m/z (relative intensity, %)*
Ala	206	12.7	207 (100)
Ile	248	18.2	249 (100)
Leu	248	18.5	249 (100)
Phe	282	18.1	283 (100)

TABLE VI
RESPONSE FACTORS FOR PTC-AMINO ACIDS WITH SIM DETECTION

PTC-amino acid	*m/z*	*Response factor*	*PTC-amino acid*	*m/z*	*Response factor*
Asp	205	1.5	Pro	157	1.5
Glu	219	1.8	Tyr	299	1.9
Ser	205	1.7	Val	235	4.6
Gly	177	0.8	Met	267	2.6
Gln (I.S.)*	264	1.0	Ile	249	4.4
His	273	0.5	Leu	249	4.4
Thr	219	1.5	Phe	283	3.4
Ala	207	1.7	Lys	264	1.1
Arg	292	0.3			

* Internal standard.

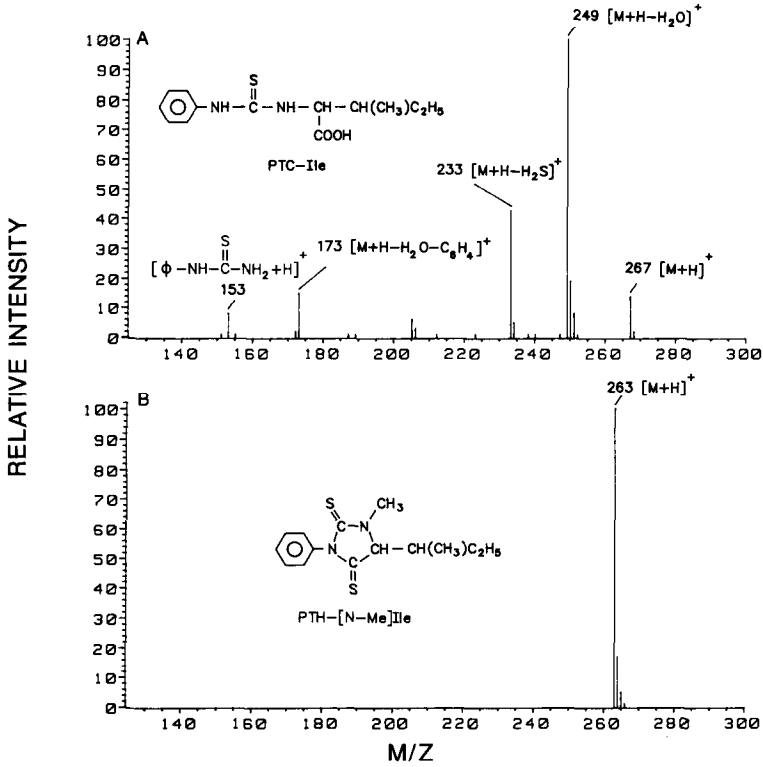

Fig. 2. TSP mass spectra of PITC-derivatized Ile (A) and N-methyl-Ile (B).

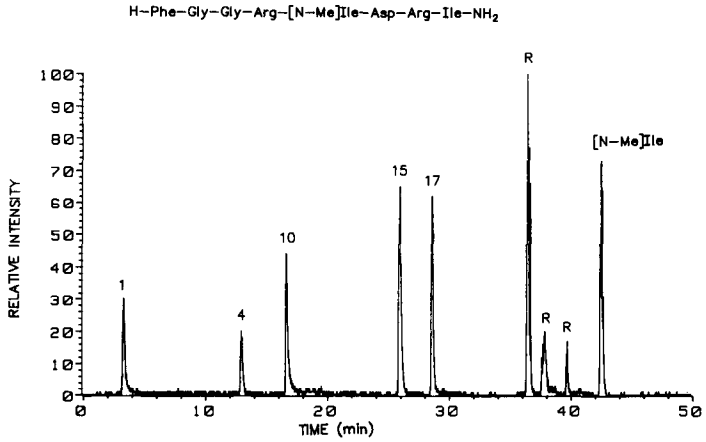

Fig. 3. Total ion chromatogram (*m/z* 125–500) of an acid hydrolysate of an N-methylated rANP. Peaks: 1 = Asp; 4 = Gly; 10 = Arg; 15 = Ile; 17 = Phe; R = reagent-related byproduct. For chromatographic conditions, see Table I.

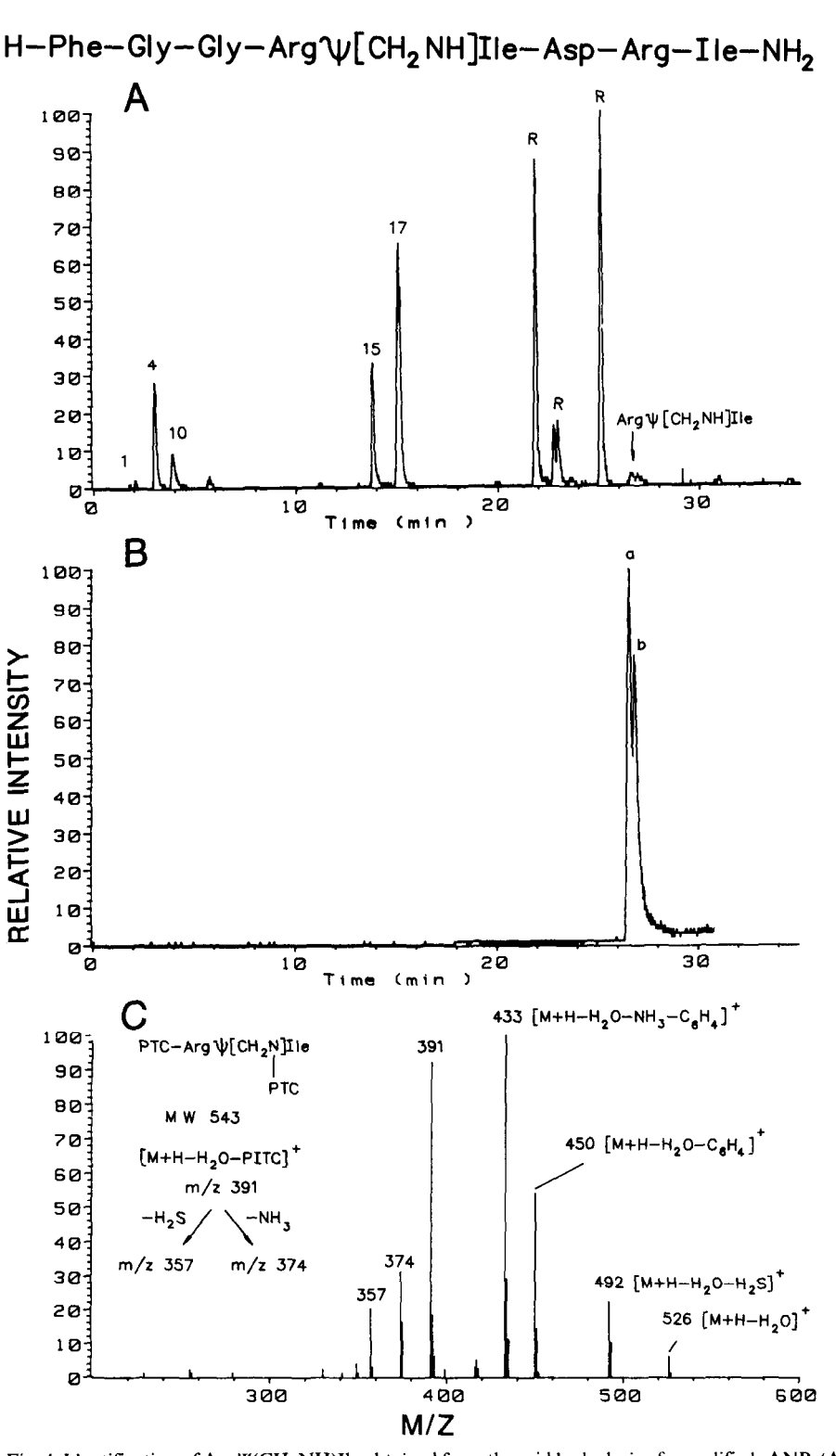

Fig. 4. Identification of ArgΨ(CH_2NH)Ile obtained from the acid hydrolysis of a modified rANP. (A) Total ion chromatogram (m/z 125–600); (B) selected ion chromatogram (m/z = 433); (C) TSP mass spectrum of the bis-PTC derivative of ArgΨ[CH_2NH]Ile. Mobile phase A = 0.05 *M* ammonium acetate in water–acetonitrile (90:10); mobile phase B = 0.05 *M* ammonium acetate in water-acetonitrile (25:75); linear gradient from 100% A to 100% B in 30 min at a flow-rate of 1 ml/min. Peaks: 1 = Asp; 4 = Gly; 10 = Arg; 15 = Ile; 17 = Phe; R = reagent-related byproduct. MW = Molecular weight.

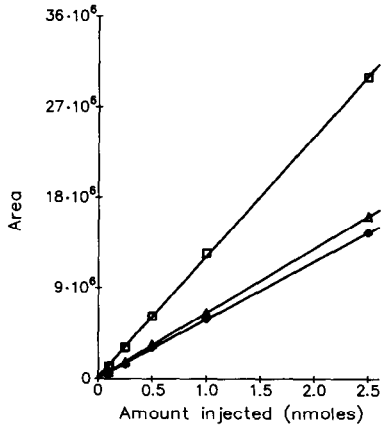

Fig. 5. Linearity of response. △ = Asp; ◇ = Gly; □ = Leu.

ion monitoring (SIM) detection. The peak areas were calibrated using Gln as an internal standard, which was added to the sample prior to PITC derivatization (Table VI).

The precision of the system was evaluated using five consecutive injections of Asp, Gly and Leu standards (0.5 nmol). The average relative standard deviation of the peak areas was 4.3%. Using the same standards the responses of the peak areas were found to be linear in the range of 0.1 to 2.5 nmol, as illustrated in Fig 5. The correlation

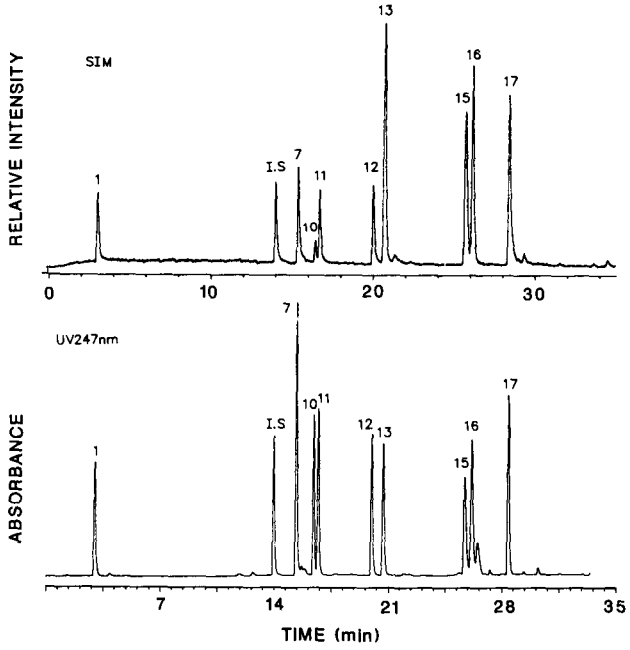

Fig. 6. Chromatograms of an acid hydrolysate of angiotensin I. Peaks: 1 = Asp; 7 = His; 10 = Arg; 11 = Pro; 12 = Tyr; 13 = Val; 15 = Ile; 16 = Leu; 17 = Phe; I.S. (internal standard) = Gln. For chromatographic conditions, see Table I.

TABLE VII
AMINO ACID COMPOSITION OF ANGIOTENSIN I

Amino acid	*Theoretical value*	*SIM value*	*UV (247 nm) value*
Arg	1	1.0	0.7
Asp	1	0.8	0.8
His	2	2.3	1.6
Ile	1	0.7	0.9
Leu	1	0.9	1.0
Phe	1	1.0	1.0
Pro	1	0.7	0.8
Tyr	1	0.7	0.7
Val	1	0.9	0.8

coefficients for these data exceeded 0.9998. The detection limits (signal-to-noise ratio = 2) for PTC-amino acids were found to be about 15 pmol except that for Arg (about 115 pmol).

An example of the determination of amino acids by TSP LC–MS applied to angiotensin I is shown in Fig. 6. The chromatogram obtained with UV detection is also given for comparison. The resulting amino acid compositions determined with SIM and UV detection correspond well with the theoretical values, as shown in Table VII.

CONCLUSIONS

TSP LC–MS is a sensitive and specific technique that can be used for the positive identification of acid hydrolysates of peptides. Therefore, it facilitates the procedure for confirming the structures of both natural and modified peptides whose structures normally can not be confirmed using conventional techniques for amino acid analysis. The quantitative result determined by TSP LC–MS with SIM detection is comparable to that obtained with UV detection. The results of this study reinforce the utility of having a detection system that can be sensitive, specific and quantitative and provide high-quality structural data.

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