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Journal of Chromatography B, 658 (1994) 213–221

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Femtomole detection of amino acids and dipeptides by gas chromatography–negative-ion chemical ionization mass spectrometry following alkylation with pentafluorobenzyl bromide

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First received 3 August 1993; revised manuscript received 29 April 1994

Abstract

Amino acids and di- and tripeptides were derivatized by extractive alkylation using pentafluorobenzyl bromide (PFBBr) followed by reaction with heptafluorobutyric anhydride (HFBA) and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). Good chromatographic separation and the formation of intense diagnostic ions were observed for the derivative when examined using gas chromatography–negative-ion chemical ionization mass spectrometry (GC–NICI-MS). Of the 20 amino acids investigated, only Arg and Glu could not be detected by this method. Also, dipeptides which included neutral amino acid residues were derivatized with more success than those containing either acidic or basic residues. Each of the amino acids or dipeptides formed one major derivative with the exception of Asn which formed two derivatives with either one or two HFB groups. This derivatization method was optimized with respect to the reaction temperature, reaction time, and choice of derivatizing reagents. Recoveries of derivatized [^{3}H]-labeled Phe, Lys, and Thr were 76, 55, and 34%, respectively. Linearity was observed from 10 to 2000 pg of Ala per vial; selected-ion monitoring provided a detection limit of less than 150 fg with a signal-to-noise (S/N) ratio of 80 to 1. This method has proven to work well with urine samples and shows great promise for the detection of small peptides at low levels.

1. Introduction

Many therapeutically used peptides posses potent analgesic properties and thus, are administered at low doses such that they are present at low concentrations in biological fluids. Therefore, highly sensitive and specific analytical methods are essential for pharmacokinetic and disposition studies of peptides. Established methods for quantitation of peptides involve either radioimmunoassay (RIA), bioassay, reversed-phase high-performance liquid chromatography (HPLC), or mass spectrometry (MS). Although RIA is a useful tool to quantitate low concentrations of peptides, this technique ex-

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SSDI 0378-4347(94)00247-3

hibits problems with cross-reactivity, lack of specificity and inability to provide information about the amino acid sequence [1]. Bio-assays also lack specificity and result in high variability for the response measured. Quantitation of low levels using HPLC requires peptide derivatization methods which include the use of dimethylaminonaphthalene-5-sulphonyl chloride [2], fluorescein isothiocyanate [3], and naphthalene-2,3-dicarboxaldehyde/cyanide [4] as well as post-column derivatization with fluorescamine [5]. There are numerous problems associated with these derivatizing reagents, especially fluorescent products resulting from side reactions. Moreover, techniques like HPLC and RIA do not provide molecular mass information which can be important in characterizing therapeutic peptides.

Considerable attention has been focused on the mass spectral analysis of peptides in biological fluids. Gas chromatography–mass spectrometry (GC–MS) is a well established technique for the determination of amino acids since it yields both quantitative and qualitative information. Previously reported methods for the derivatization of amino acids and small peptides have utilized either pentafluoropropionic anhydride [6], *tert.*-(butyldimethylsilyl)trifluoroacetamide [7,8] or heptafluorobutyric anhydride [9]. These methods employed GC–MS with electron-impact ionization and thus could only detect pmol to nmol levels of peptides in biological fluids. Hayashi et al. [10] described the derivatization of Phe and Tyr with pentafluorobenzylbromide (PFBBr) for analysis by GC–MS, employing selected-ion monitoring (SIM) in conjugation with negative-ion chemical ionization (NICI; also referred to as electron capture ionization). The product showed good chromatographic properties and generated intense ions ideal for achieving specificity and sensitivity down to the low fg level. The work described here extends the method of Hayashi et al. [10] to a broad range of amino acids and small peptides. This method shows great promise for pharmacokinetic studies of therapeutic peptides at low concentration in biological fluids.

2. Experimental

2.1. Reagents

Amino acids, small peptides, anhydrous sodium sulfate (Na_2SO_4), and sodium carbonate (Na_2CO_3) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Tritium labeled Phe (130 Ci/mmol), Lys (95 Ci/mmol), and Thr (19 Ci/mmol) were obtained from Amersham Life Science (Arlington Heights, IL, USA). The derivatizing reagents, pentafluorobenzyl bromide (PFBBr), heptafluorobutyric anhydride (HFBA), pentafluoropropionic anhydride (PFPA), trifluoroacetic anhydride (TFAA), and N,O-*bis*-(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) were obtained from Pierce (Rockford, IL, USA). Solvents employed were HPLC grade and included acetonitrile, acetone, isopropanol, hexane, and ethyl acetate.

2.2. Derivatization

HFB/PFB/TMS-derivatives

Amino acids, di- and tripeptides were derivatized using a modified version of the method of Hayashi et al. [10]. Standard stock solutions of amino acids or small peptides (1 mg/ml) were prepared in 80% isopropanol with the exception of tyrosine which was dissolved in 0.1 m*M* NaOH, pH 9. The derivatization was performed in 2-ml reaction vials which were previously silanized using 5% dimethyldichlorosilane in toluene. Amino acids or small peptides (1–100 ng) were added to the reaction vial in solution and dried using a stream of nitrogen. Each sample was reacted with 50 μl of HFBA in acetonitrile (1:1, w/w) for 5 min at room temperature followed by evaporation with a stream of dry nitrogen. The dried residue was combined with approximately 4–5 mg of a mixture of equal amounts of anhydrous Na_2SO_4 and Na_2CO_3 followed by 50 μl of 2.5% PFBBr in acetone and allowed to react for 5 min at room temperature. An aliquot (500 μl) of hexane–ethyl acetate (70:30, v/v) was added and then 300 μl of the

supernatant was transferred to another silanized reaction vial. The solvent was evaporated using a stream of nitrogen and the dried residue was reacted for 5 min at room temperature with 25 μl of BSTFA containing 1% TMCS. Excess reagent was evaporated using nitrogen, then the residue was dissolved in 50 μl of ethyl acetate, and analyzed using GC–NICI-MS with SIM. Fig. 1 outlines the derivatization scheme for representative amino acids (Thr and Ala).

Standard curves were constructed using standard solutions of Ala, Phe, and AlaThr against the internal standard, Ile. Data was plotted according to the corresponding peak-area ratio of the standards Ala, *m/z* 284; Phe, *m/z* 360; and Ala-Thr, *m/z* 367 to that of the internal standard Ile, *m/z* 326. The amount (pg) of each standard per vial plotted on the *x*-axis was 0, 4, 10, 20, 40, 100, 200, 400, 1000, 2000 pg. The peak areas were obtained using GC–NICI-MS with selected-ion monitoring.

Other derivatives

Acylation reactions were also performed using PFPA and TFAA in a similar manner to that described above. Comparison of the product yield and sensitivity for use of either HFBA, PFPA, or TFAA were examined through the derivatization of Ala, Phe, Thr, and Lys (25 ng each per vial) using GC–FID.

2.3. Extraction of amino acid derivatives

Tritiated Phe, Lys, and Thr (20 ng; 100 000 dpm per vial) were separately derivatized in triplicate as described above to ascertain the recovery of the complete derivatization method for amino acids containing different functional groups. A comparison was made for the extraction of tritiated derivatives using either hexane, hexane–ethyl acetate (70:30, v/v), or hexane–pentyl alcohol (95:5, v/v). The objective was to determine which solvent would be most suitable for extractive alkylation of a broad range of amino acid derivatives. Underivatized amino acids will not partition into the organic extracting solvent. Radioactivity was assessed after extractive alkylation and at the completion of the derivatization scheme.

2.4. Application of the derivatization method

The derivatization method was evaluated using urine samples (0.5 ml) spiked with either Ala-Phe or Ala-Thr standards (1 mg). Extraction of dipeptides from urine was done using strong cation-exchange (SCX) solid-phase columns pretreated with CH_3OH, H_2O, and 0.05 *M* H_3PO_4, pH 2.0. Urine samples were treated with 2 ml of 0.05 *M* H_3PO_4 which decreased the pH from 5.4 to 2.3, then poured onto the SCX columns and eluted with 2 ml of 25 m*M* Et_3N/formate,

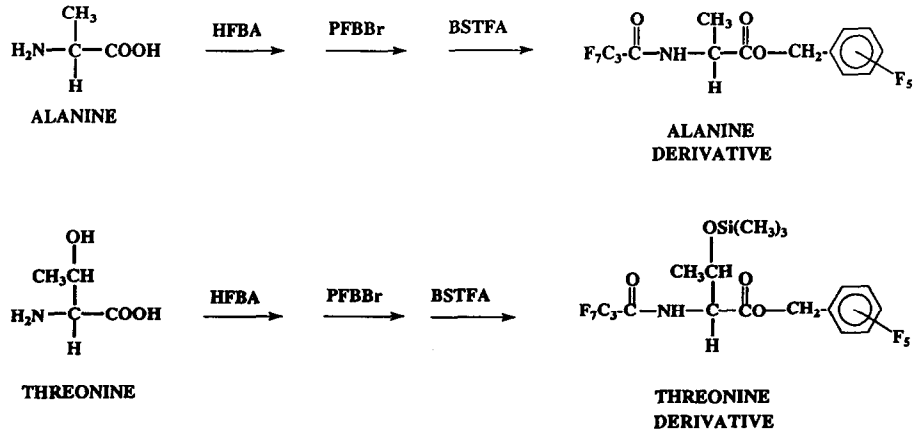

Fig. 1. Derivatization scheme for representative amino acids (Thr and Ala).

pH 9.3. Extraction recoveries of a representative radiolabeled amino acid, [^{3}H]-Phe, from urine (20 ng; 100 000 dpm per tube) was examined using SCX columns. Aliquots of spiked urine samples (0.5 ml) were transferred to silanized reaction vials and treated with a stream of N_2 to remove the solvent. Samples were derivatized for analysis by GC–NICI-MS according to the method described above.

2.5. Instrumentation

GC–FID detection

GC experiments that did not employ a mass spectrometer were performed with an HP 5880 gas chromatograph equipped with a flame ionization detector and a DB-1 (15 m × 0.32 mm I.D.), fused-silica capillary column (J and W Scientific; Folsom, CA, USA). The GC conditions were: injector temperature, 280°C; detector temperature, 300°C; helium flow-rate, 1 ml/min; splitless injection. The initial oven temperature was 75°C; 1 min after injection the temperature was increased at 10°/min to 250°C for amino acids and to 325°C for di- and tripeptides.

GC–NICI-MS

Analyses were accomplished using a Finnigan-MAT Model 4615 mass spectrometer in combination with an INCOS data system. The GC and column conditions were as described above. MS conditions were: electron energy, 70 eV; ion source temperature, 100°C; methane pressure, 0.5 Torr.

3. Results and discussion

Amino acids and small peptides are multifunctional compounds which require derivatization prior to GC–MS analysis. Frequently, the derivatization of these compounds involves alkylation by either methylation, silylation, or PFBBr and acylation by either TFAA, PFPA, HFBA, or silylation [6–10]. Of these derivatizing reagents, PFBBr generates derivatives which when examined by GC–NICI-MS afford selectivity and fmol level sensitivity for quantitation of small peptides [10]. In this study, amino acids and dipeptide derivatives showed good chromatographic separation and yielded intense ions corresponding to either $[M - PFB]^-$, $[M - (PFB + TMSiOH)]^-$, $[M - (HF + TMSiOH)]^-$, or $[M - HF]^-$. Loss of TMSiOH only occurs from dipeptides possessing silylated hydroxyl groups. Reconstructed ion chromatograms for several representative amino acids and a dipeptide (Fig. 2) and a representative mass spectrum of the Ala-Thr HFB/PFB/TMS-derivative (Fig. 3) are shown. The selected $[M - PFB]^-$ or $[M - (PFB + TMSiOH)]^-$ ions were m/z 284 for Ala, m/z 360 for Phe, and m/z 367 for Ala-Thr. Also, a selected-ion chromatogram of approximately 150 fg of Ala, Phe, and Ala-Thr derivatives with a S/N ratio of approximately 80 to 1 for the derivative is shown (Fig. 4). The detection limit for these derivatives was assessed at the lower end of the multiplier voltage scale, thus there is potential to detect even lower levels of the derivatives by increasing the multiplier voltage. Standard curves showed the method to be linear from 10 to 2000 pg (slope = 0.0029; y-intercept = 0.0859; $r^2 = 0.982$).

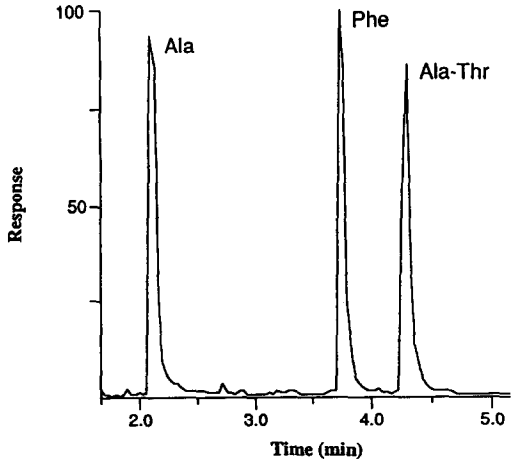

Fig. 2. Reconstructed ion chromatogram for derivatized Ala, Phe, and Ala-Thr analyzed by GC–NICI-MS using a wide mass range scan. GC conditions were as follows: column, DB-1 (15 m × 0.32 mm I.D.); injector, 280°C;; splitless injection; column temperature, 150°C (1 min) to 190°C (1 min) at 10°/min to 250°C at 30°/min. Approximately 3 ng of each derivative was injected (based on the recovery of ^{3}[H]-Phe derivative).

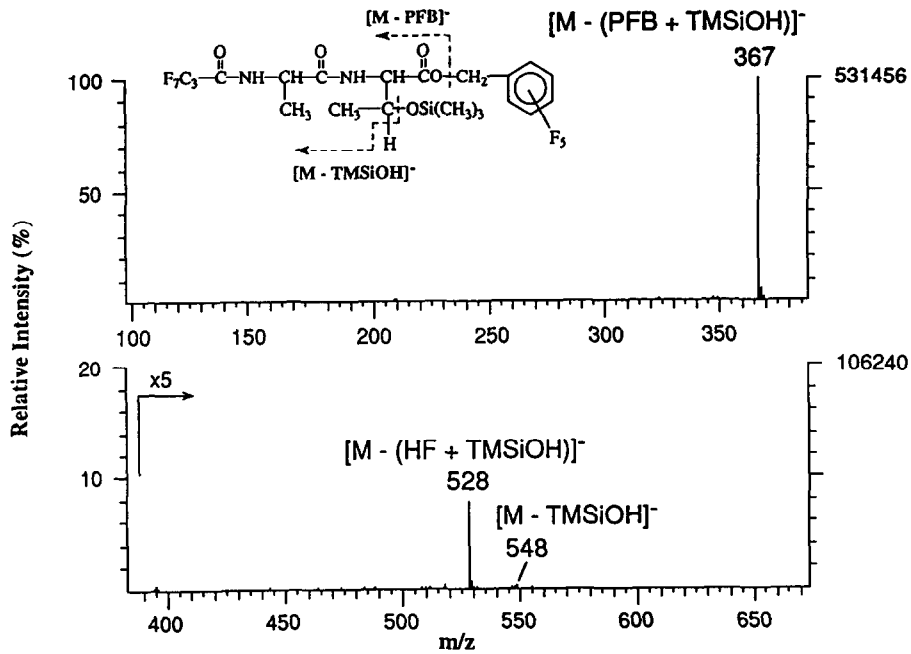

Fig. 3. NICI mass spectrum of the HFB/PFB/TMS-derivative of Ala-Thr.

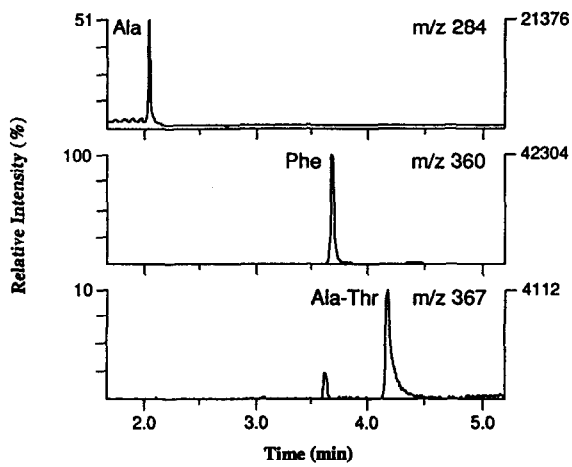

Fig. 4. Reconstructed selected-ion chromatogram for subpg levels of Ala, Phe, and Ala-Thr analyzed by GC–NICI-MS with selected-ion monitoring. The ions monitored were: *m/z* 284, Ala $[M-PFB]^-$; *m/z* 360, Phe $[M-PFB]^-$; *m/z* 367, Ala-Thr $[M-(PFB+TMSiOH)]^-$. GC conditions were as follows: column, DB-1 (15 m × 0.32 mm I.D.); injector, 280°C; detector, 300°C; splitless injection; column temperature, 150°C (1 min) to 190°C (1 min) at 10°/min to 250°C at 30°/min. Approximately 150 fg of each derivative was injected (based on the recovery of ^{3}[H]-Phe derivative).

Of the 20 amino acids investigated, only Arg and Glu were not detected by this method. Table 1 describes the amino acids which have been successfully derivatized, their retention times and the diagnostic ions formed (either $[M-PFB]^-$ or $[M-HF]^-$). Each amino acid derivative contained at least one HFB group and one PFB group. Asn formed two derivatized products, one which contained two HFB groups, the other which had one. However, Gln and Lys only formed a single derivative which incorporated two HFB groups. Thus, it is evident that the production of multiple derivatives for individual amino acids is negligible as compared to that observed for silylation of amino acids [11].

3.1. Extraction of amino acid derivatives

Recovery of the derivatives after extractive alkylation into either hexane, hexane–ethyl acetate (70:30, v/v), or hexane–pentyl alcohol (95:5, v/v) was examined using tritiated Phe, Thr, and Lys. Hexane–ethyl acetate was somewhat more effective at extracting a broad range of amino acids and small peptide derivatives than

Table 1
Derivatives of amino acids characterized by GC–NICI-MS

Amino acid	t_R[a] (min)	Derivative formed	Diagnostic ions[b]	
			$[M - PFB]^-$	$[M - HF]^-$
Neutral				
Ala	6.6	HFB/PFB	284 (97)	445 (3)
Cys	9.8	HFB/PFB	316 (96)	477 (4)
Gly	7.3	HFB/PFB	270 (92)	431 (8)
Ile	8.4	HFB/PFB	326 (97)	487 (3)
Leu	8.3	HFB/PFB	326 (98)	487 (2)
Met	10.8	HFB/PFB	344 (98)	505 (2)
Phe	11.4	HFB/PFB	360 (96)	521 (4)
Pro	9.5	HFB/PFB	310 (97)	471 (3)
Ser	8.5	HFB/PFB/TMS	372 (97)	533 (2)
Thr	7.9	HFB/PFB/TMS	386 (94)	547 (5)
Trp	12.5	HFB/PFB	399 (98)	560 (2)
Tyr	13.4	HFB/PFB/TMS	448 (97)	609 (2)
Val	8.2	HFB/PFB	312 (99)	473 (1)
Basic				
Asn	8.6	HFB_2/PFB	523 (95)	684 (5)
	9.4	HFB/PFB	327 (97)	488 (3)
Gln	9.3	HFB_2/PFB	537 (96)	698 (4)
His	11.0	HFB/PFB	350 (95)	511 (5)
Lys	13.0	HFB_2/PFB	537 (96)	698 (4)
Acidic				
Asp	9.5	HFB/PFB_2	342 (95)	503 (5)

[a] Absolute retention time for a representative GC–FID analysis; GC conditions were as follows: DB-1 (15 m × 0.32 mm I.D.); injector temperature, 280°C; detector temperature, 300°C; splitless injection; column temperature, 75°C (1 min) to 250°C at 10°C/min.
[b] Relative ion intensity (%) was obtained by GC–NICI-MS and is shown in parenthesis.

hexane or hexane–pentyl alcohol. Extraction recoveries of the derivatives into hexane–ethyl acetate ($n = 3$) were [^{3}H]-Phe, 76 ± 5.0%; [^{3}H]-Thr, 34 ± 9.1%; and [^{3}H]-Lys, 55 ± 1.3%, respectively. Comparable results were obtained when [^{3}H]-Phe, [^{3}H]-Thr, or [^{3}H]-Lys were carried through the entire derivatization procedure. The increased sensitivity afforded by NICI and SIM enables detection of derivatized amino acids and small peptides at low fmol levels even when the extraction efficiency is low.

3.2. Product yield of the derivative

The product yield of amino acid derivatives for Ala, Thr, Phe, and Lys was examined for reaction temperatures of 25, 40, or 75°C and for reaction times of 5, 30, or 60 min for each derivatization step. Quantitative evaluation of each derivative based on the relative area by FID detection showed that increasing the reaction temperature or time did not increase the product yield. The final derivative appeared to be stable at room temperature for approximately 3 days, although it was sensitive to traces of water which resulted in hydrolysis of the derivative. Also, derivatization of the amino terminus using either TFAA or PFPA did not substantially affect the sensitivity of the derivatives formed when measured by GC–MS with NICI and SIM. Contrary to these results, Liberato et al. [12] found significant differences in sensitivity following acylation of Phe using either HFBA, PFPA,

or TFAA, with HFBA providing the highest sensitivity.

3.3. Derivatization of di- and tripeptides

Peptides containing neutral residues were derivatized more easily than those containing either acidic or basic amino acids. Table 2 shows the results for a variety of dipeptide derivatives, their retention times, and the diagnostic ions formed. The ions produced were $[M - PFB]^-$ and $[M - HF]^-$ for dipeptides not possessing hydroxyl amino acids and $[M - (PFB + TMSiOH)]^-$ and $[M - (HF + TMSiOH)]^-$ for dipeptides with hydroxyl amino acids such as Thr, Ser, Tyr. Dipeptides with hydroxyl groups form ions which exhibit a loss of TMSiOH, in contrast to the diagnostic ions generated from hydroxylated amino acids which showed little loss of TMSiOH. SIM of diagnostic ions at higher m/z values was subject to less background interferences and provided excellent selectivity of the derivative. Dipeptides possessing basic or acidic residues were less successfully analyzed by this derivatization method. In addition, derivatization of tripeptides such as Ala-Leu-Ala, Gly-Gly-Phe, Ala-Gly-Gly, and Gly-Ser-Phe was attempted but was not successful due to either incomplete derivatization, poor extraction of the derivative or their unstable behavior once fully derivatized.

In previous studies, considerable attention was directed toward derivatization methods which involved direct silylation of amino acids. These methods generally resulted in the formation of multiple derivatives due to the variable silylation of nitrogen groups [7]. In an effort to avoid this problem, some investigators have utilized *tert.*-butyldimethylsilyl derivatives which minimized the formation of multiple derivatives by sterically hindering the attachment of other silyl groups [8,11,13]. Although fewer derivatives were formed, these investigators used GC–MS with electron-impact ionization (EI) and thus, could only detect upper pmol levels of the derivative. Dipeptide TMS-derivatives have been examined by EI and chemical ionization (CI) where both exhibited low sensitivity for the TMS-derivative [14].

Increased sensitivity is observed for derivat-

Table 2
Derivatives of dipeptides characterized by GC–NICI-MS

Dipeptides	t_R[a] (min)	Derivative formed	Diagnostic ions[b]	
			$[M - PFB]^-$ or $[M - (PFB + TMSiOH)]^-$	$[M - HF]^-$ or $[M - (HF + TMSiOH)]^-$
Ala-Leu	12.8	HFB/PFB	397 (90)	558 (10)
Ala-Lys	22.8	HFB_2/PFB	608 (90)	769 (10)
Ala-Phe	22.1	HFB/PFB	431 (92)	592 (8)
Ala-Ser	12.0	HFB/PFB/TMS	353 (92)	514 (7)
Ala-Thr	12.8	HFB/PFB/TMS	367 (92)	528 (7)
Gly-Ile	13.6	HFB/PFB	383 (86)	544 (14)
Leu-Ala	12.7	HFB/PFB	397 (90)	558 (10)
Leu-Gly	13.0	HFB/PFB	383 (92)	544 (8)
Leu-Met	15.9	HFB/PFB	457 (92)	618 (8)
Met-Gly	20.3	HFB/PFB	401 (93)	562 (7)
Phe-Val	16.2	HFB/PFB	459 (95)	620 (5)
Tyr-Val	18.2	HFB/PFB/TMS	547 (94)	708 (5)
Val-Thr	13.5	HFB/PFB/TMS	395 (94)	556 (5)

[a] Absolute retention time for a representative GC–FID analysis; GC conditions were as follows: DB-1 (15 m × 0.32 mm I.D.); injector temperature, 325°C; detector temperature, 350°C; splitless injection; column temperature, 75°C (1 min) to 325°C at 10°C/min.

[b] Relative ion intensity (%) was obtained by GC–NICI-MS and is shown in parenthesis; TMSiOH loss is observed for dipeptides containing hydroxyl groups.

ized compounds containing halogen atoms when measured by NICI-MS. Derivatives produced with hexafluoroisopropanol, TFAA, PFPA, HFBA, and PFBBr are highly electronegative and can be detected with high sensitivity by NICI-MS analysis [9]. In previous studies, amino acid pentafluoropropionyl (PFP)-derivatives analyzed by GC–MS with EI rather than NICI could only be detected at nmol levels [6]. However, when NICI-MS analysis was performed on PFP-derivatives of Phe, Tyr, and dihydroxyphenylalanine, diagnostic ions were formed which could be detected at low pmol levels [15]. An even more selective and sensitive reagent for analysis by NICI-MS is PFBBr which when used to derivatize Phe and Tyr formed intense ions, well suited for SIM at fmol levels of the derivative [10].

3.4. Applications of the derivatization method

The potential applications of this method are the characterization by GC–NICI-MS and quantification of amino acids, dipeptides, or digested peptides in biological fluids. In this work, urine samples spiked with amino acids or dipeptides were chosen to demonstrate the utility of the derivatization method in a biological fluid. A selected-ion chromatogram for a representative derivatized dipeptide, Ala-Phe in urine (20 ng; m/z 431), is shown (Fig. 5). Good peak shape and resolution is shown for 1 mg of Ala-Phe extracted from 0.5 ml of urine. Extraction recovery of [^{3}H]-Phe in urine was 25% when SCX columns were employed. Although a low extraction recovery was obtained, the derivatization method seems to work well in urine and shows great promise at detecting small peptides in biological fluids at low levels.

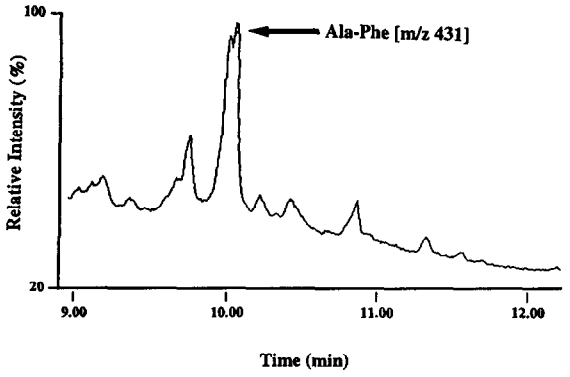

Fig. 5. Selected-ion chromatogram for 20 ng of Ala-Phe analyzed by GC–NICI-MS with selected-ion monitoring of $[M - PFB]^-$; m/z 431. GC conditions were as follows: column, DB-1 (15 m × 0.32 mm I.D.); injector, 280°C; splitless injection; column temperature, 75°C (1 min) to 175°C at 20°/min and then to 300°C at 10°/min.

Dipeptides and amino acid levels in urine can be altered during diseased states such as collagen-metabolism disorders [16,17]. Various dipeptides, Gly-Pro being the major constituent, were present in urine of patients suffering from chronic skin ulceration and oedema [16]. Thus, characterization of amino acids and dipeptides in patient urine may be useful in the diagnosis of certain diseased states.

In addition to amino acids and dipeptides in urine, Liberato et al. [12] explored the utility of quantifying the peptide growth hormone releasing (GHR) factor in plasma by GC–NICI-MS following total hydrolysis and derivatization with PFBBr and HFBA. They examined the liberation of Phe from GHR factor and obtained a detection limit of 100–1000 fg for the stable isotope reference, [$^{13}C_6$]-Phe, although similar limits were not achieved with the unlabeled material due to high background interferences originating from the total hydrolysis of peptides [12]. Peptide cleavage either by total hydrolysis or enzyme digestion results in side reaction by-products which often interfere with the quantitation of peptides. A future objective of this laboratory is to minimize by-product formation thus allowing the quantitation and identification of digested peptide fragments using GC–NICI-MS. This method has been tested using a urine matrix and appears to be widely applicable to the analysis of therapeutic peptides at low levels.

4. Conclusions

A series of amino acids and dipeptides were derivatized by PFBBr, HFBA, and BSTFA to

form derivatives that were characterized by GC–NICI-MS. The most abundant ions formed were $[M - PFB]^-$ for amino acids and either $[M - PFB]^-$ or $[M - (PFB + TMSiOH)]^-$ for dipeptides. These ions were monitored by GC–NICI-MS for quantitation of the derivatives at low concentration; this work demonstrates that a detection limit in the low fmol range can be achieved for the derivative. Applications of the method using urine samples containing dipeptides resulted in the formation of derivatives which displayed good peak shape and were adequately resolved. This method is highly sensitive and selective for peptide analysis and shows great promise for the determination of therapeutic peptides.

Acknowledgements

This work was supported by NIH GM41828. P.C.S. is a recipient of a Pharmaceutical Manufacturers Association Foundation Faculty Development Award.

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