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Comparison of separation and detection techniques for human growth hormone releasing factor (hGRF) and the products derived from deamidation

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Abstract: Separation of the deamidation products, Asp^8 Leu^{27} hGRF(1–32)NH_2 (MH^+ = 3654) and isoAsp^8 Leu^{27} hGRF(1–32)NH_2 (MH^+ = 3654), from the parent analogue Leu^{27} hGRF(1–32)NH_2 (MH^+ = 3653) was achieved by reversed-phase LC and CE, where the retention order was seen to change from t_r isoAsp^8 hGRF $< t_r$ Asn^8 hGRF $< t_r$ Asp^8 hGRF to t_r Asn^8 hGRF $< t_r$ Asp^8 hGRF $< t_r$ isoAsp^8 hGRF, respectively. Both reversed-phase LC and CE gave adequate separations, limits of detection and standard curves. However, CE was preferred due to shorter analysis time, better separation and a smaller demand for material. Packed capillary LC with ESI–MS was then compared with UV detection. On-line LC–MS was found to offer the most efficient approach to detection and identification of hGRF analogues within a single methodology. Identification of Asn^8 hGRF from the isobaric deamidation products was achieved from analysis of the triply charged states, where the species were separated by 0.5 amu. LC–MS separation and identification of degradation products offers a viable alternative to fraction collection and subsequent sequencing or enzymatic identification methods. The method becomes increasingly useful for such cases as trace degradation product identification, minimal sample availability or instability of resulting degradation products.

Keywords: *Peptide deamidation; capillary electrophoresis; electrospray mass spectrometry; reversed-phase LC.*

Introduction

Proteins and peptides represent unique analytical challenges to scientists in the pharmaceutical industry. Among these analytical challenges are separation, detection and identification of peptide degradation products. Separation of chemical degradation products is often difficult due to small inter-residue modifications that may or may not give rise to changes in the overall structure or charge of the macromolecule. Method acceptability will depend on validation of the quality of separation in minimal elution time, limit of detection and positive identification of all species.

Deamidation of asparagine (Asn) residues represents one of the major chemical degradation pathways for peptides and proteins [1]. Deamidation of Asn residues at neutral and basic pH proceeds through a cyclic imide intermediate formed by intramolecular attack of the backbone nitrogen on the Asn side chain carbonyl. Spontaneous hydrolysis of the cyclic imide yields a mixture of α- (Asp) or β-carboxy (isoAsp) linked aspartate products (Fig. 1). At acidic pH, the reaction involves direct hydrolysis of the amide side chain of Asn to form only the Asp isomer [1–6]. Deamidation of peptides is pharmaceutically relevant in several stages of drug development including bulk drug characterization and stability testing of pharmaceutical formulations because it will lead to loss of biological activity [7–11]. Therefore, reliable analytical methods for identifying trace products with similar properties is of importance.

Previous chromatographic techniques have shown separations of the native and deamidated peptides and proteins, but rarely the separation of isomeric deamidation products. For example, the isomeric deamidation products of adrenocorticotropic hormone (ACTH) could not be resolved using reversed-phase LC, cation-exchange LC or isoelectric focusing [12]. β-Endorphin, γ-endorphin and their N-terminally deamidated products could only be separated by reversed-phase LC under

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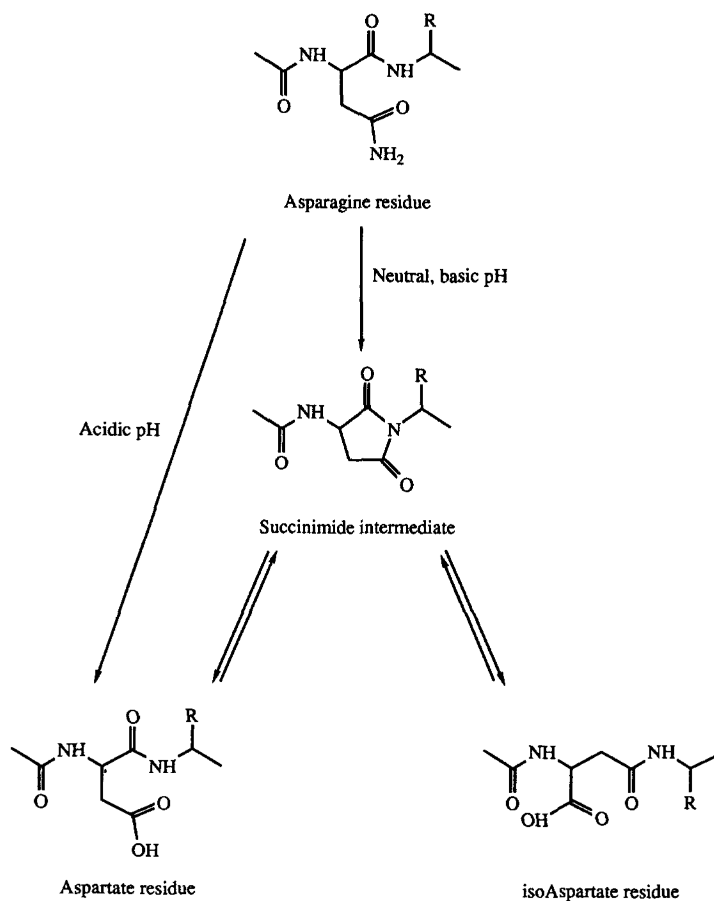

Figure 1
Mechanistic scheme for deamidation of asparagine (Asn) residues. At neutral and basic pH, the reaction proceeds through a cyclic imide intermediate formed by intramolecular attack of the backbone nitrogen on the Asn side chain carbonyl. Spontaneous hydrolysis yields either an α-(Asp) or β-carboxy (isoAsp) linked aspartate product. At acidic pH, the reaction involves direct hydrolysis of the amide side chain.

a stringent ternary solvent system; however, all four endorphin products were separated by capillary electrophoresis (CE) in 16 min [13]. It should be noted that this separation only involved Asn and Asp products, but not the separation of isoAsp and Asp products.

Human growth hormone releasing factor (Leu^{27} hGRF(1–32)NH_2) contains one Asn residue at position 8 which is known to deamidate readily [14, 15]. Conventional reversed-phase LC separation of these products has been previously accomplished [15]. In the present study a standard mixture of Asn^8Leu^{27} hGRF(1–32)NH_2, Asp^8 Leu^{27} hGRF(1–32)NH_2 and $isoAsp^8$ Leu^{27} hGRF(1–32)NH_2 was used to compare the separation and detection capabilities of reversed-phase LC and CE, both with UV detection. Secondly, identification of hGRF deamidation products by capillary LC–MS was compared with UV detection methodology. On-line LC–MS separation and identification was explored as an identification alternative for future kinetic studies. The objective of this study was to compare these methods under practical analytical conditions.

Experimental

Materials

Asn^8 Leu^{27} hGRF(1–32)NH_2 (MH^+ = 3653), Asp^8Leu^{27} hGRF(1–32)NH_2 (MH^+ = 3654) and $isoAsp^8$ Leu^{27} hGRF(1–32)NH_2 (MH^+ = 3654) were obtained from the Upjohn Company (Department of Reproduction and Growth Physiology Research). A standard solution (600 μg) of a mixture of hGRF analogues consisting of $isoAsp^8$–Asn^8–Asp^8 (3:2:1, w/w/w) was prepared, split into three microfuge tubes and lyophilized to dry-

ness. Samples were reconstituted in purified water to 1 mg ml^{-1} for each of the three separation techniques. Acetonitrile, trifluoroacetic acid, citric acid and sodium hydroxide were obtained from Aldrich Chemical Company (Milwaukee, WI, USA).

Reversed-phase liquid chromatography

Chromatography was performed on a Hewlett–Packard 1090 LC (Avondale, PA, USA) with an autosampler. The LC was interfaced with an HP79994A HPLC ChemStation, a HP9153C and a HP9000/300 hard drive. A VYDAC Peptide and Protein C-18 5 μm resin column with 300 Å pore diameter was used (150 mm × 4.6 mm). The separation was achieved using a gradient from 32 to 42% B, 0.5% min^{-1}, where mobile phase A was H_2O–acetonitrile–trifluoroacetic acid (950:50:0.45, v/v/v) and mobile phase B was H_2O–acetonitrile–trifluoroacetic acid (200:800:0.39, v/v/v). Detection was by UV absorption at 215 nm.

Capillary electrophoresis

Capillary electrophoresis was performed on an Applied Biosystems (ABI) CE (Pittsburgh, PA, USA) with UV detection at 215 nm. A 70 cm, 50 μm i.d. bare silica capillary was used at +15 kV for 20 min with an average current of 13 μA. Injections were preceded by a 5 min 0.1 N NaOH wash and a 5 min 50 mM citric acid wash. Injections were made by vacuum for 1.5 s. The running buffer was 50 mM citric acid, pH 3.0.

Capillary liquid chromatography

Packed capillary columns, C-4 Aquapore Butyl, 5 μm particle size, 15 cm × 320 μm i.d., were obtained from LC Packings, International (San Francisco, CA, USA). Injections (5 μl) of 0.1 μg μl^{-1} hGRF were made at a flow rate of 5 μl min^{-1}. An ABI 140A Solvent Delivery System with 10 ml syringes, a Spectra-Physics ChromJet integrator (San Jose, CA, USA) and an ABI 783A UV detector at 215 nm was used. On-line electrospray ionization mass spectra were obtained in conjunction with the UV spectra. Separations were obtained using a 0.25% min^{-1} gradient from 25–30% B, where mobile phase A was 0.1% trifluoroacetic acid in water and mobile phase B was 0.1% trifluoroacetic acid in acetonitrile.

Mass spectrometry

On-line microbore LC, with UV detection, was linked to a SCIEX API-III (Thornhill, Ontario) triple quadrupole mass spectrometer. The ion spray needle was maintained at 4800 V relative to the orifice. The flow rate was 5 μl min^{-1} and the orifice potential was 100 V. The mass range was scanned from 400 to 1400 at a rate of 4.22 s $scan^{-1}$ with a 1.0 ms dwell time, where continuum data was collected for each scan.

Results and Discussion

A standard solution of hGRF containing one part Asp^8, two parts Asn^8 and three parts $isoAsp^8$, by weight, was reconstituted in water. The separation, detection and identification of these three analogues was then performed by several methods. The separation was compared by reversed-phase LC and CE, while detection was contrasted using UV and mass spectrometry.

Initially, separation of the three hGRF analogues was compared by reversed-phase LC and CE, both with UV detection at 215 nm. Conventional reversed-phase LC indicated an elution order of t_r $isoAsp^8$ hGRF < t_r Asn^8 hGRF < t_r Asp^8 hGRF (Fig. 2). Six standard concentrations were assayed in duplicate, within each concentration assayed the range was no more than ±14% from the mean value. Peak area values for the three components were averaged and gave a ratio of 2.9:1.7:1.2 ($isoAsp^8$ hGRF:Asn^8 hGRF:Asp^8 hGRF). Standard curves were collected over 2.5 orders

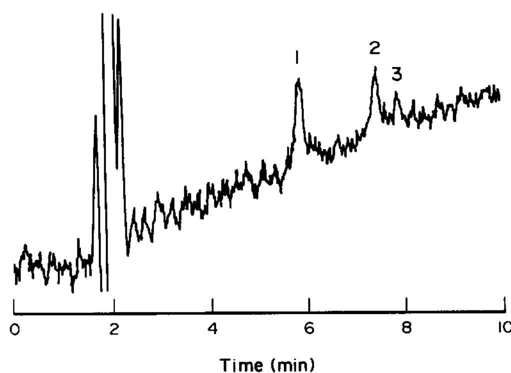

Figure 2
Representative chromatogram of reversed-phase LC limit of detection at 215 nm. The total hGRF loading was 27 pmol for (1) $isoAsp^8$ hGRF, (2) Asn^8 hGRF and (3) Asp^8 hGRF.

of magnitude (27.4 pmol–13.7 nmol total hGRF) and were fit to

$$P = mC + b, \quad (1)$$

where P represents peak area values for each hGRF analogue and C represents total hGRF loading. The equations expressing the standard curves for each hGRF analogue are listed in Table 1 and gave a fit of no less than $r^2 = 0.999$. A limit of detection of 27 pmol, total hGRF mixture was found by conventional reversed-phase LC. The limit of detection for hGRF was defined by the Asp^8 analogue peak, which was the species in lowest concentration. This converted to a limit of detection of 5.6 pmol Asp^8 hGRF analogue. Separation of these species could not be achieved on a standard C-18 reversed-phase column (80 Å), however, baseline separation was easily achieved with the large pore sized C-18 stationary phase (300 Å).

The same standard solution of hGRF analogues was assayed by CE, with UV detection, to compare the ease of separation, limit of detection and elution time. The elution order was seen to change to t_r Asn^8 hGRF $< t_r$ Asp^8 hGRF $< t_r$ $isoAsp^8$ hGRF (Fig. 3). This change in elution order was attributed to alterations in the predominant separation mechanism. Reversed-phase LC retention mechanisms consisted primarily of hydrophobic interactions between the analytes and the stationary and mobile phases, while the predominant CE separation mechanism was based on the net charge status of the analytes and their respective electrophoretic velocities. The pH of the running buffer was much less than the pI of hGRF (pI 10.9) in order to minimize adsorption to the capillary walls. The acidic pH allowed protonation of the peptide and the bare silica so that peptide repulsion occurred, minimizing mixed retention mechanisms.

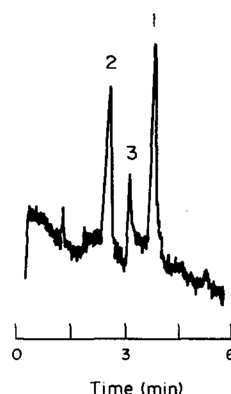

Figure 3
Representative electropherogram hGRF separation showing the CE limit of detection at 215 nm, where the total hGRF loading was 205 fmol for (1) $isoAsp^8$ hGRF, (2) Asn^8 hGRF and (3) Asp^8 hGRF.

Four concentrations were assayed, in duplicate, where the relative standard deviation in peak area for each concentration was no more than ±4%. The injection at the limit of detection was the only exception, and had a range of 20% of the mean peak area value. Deterioration of the peak area accuracy was expected at low loadings due to an increase signal to noise ratio and operation of the detector at the limits of its performance. Peak area values from CE results were averaged for the three species and gave a ratio of 2.9:1.9:1.0 ($isoAsp^8$ hGRF:Asn^8 hGRF:Asp^8 hGRF). Again, good linearity ($r^2 = 0.999$) for the analogue standard curves was found over 1.5 orders of magnitude (4.1–0.2 pmol total hGRF) (Table 1). A limit of detection of 205 fmol total hGRF mixture, or 35 fmol Asp^8 hGRF analogue was found.

The difference in analogue peak area ratios between CE and reversed-phase LC may be due to the difference in peak shape and the subsequent integration parameters. Capillary electrophoresis showed very sharp peaks and the resulting peak area ratios were very close

Table 1
Linearized fit of hGRF data according to equation (1), where all equations had no less than $r^2 = 0.999$

hGRF analogue	Linearized standard curves for hGRF analogues	
	Reversed-phase LC	CE
Asn^8 hGRF(1–32)NH_2	$P = 1.85 \times 10^5C - 1.23 \times 10^4$	$P = 332.20C - 9.42$
$isoAsp^8$ hGRF(1–32)NH_2	$P = 2.88 \times 10^5C - 2.38 \times 10^4$	$P = 580.15C - 36.50$
Asp^8 hGRF(1–32)NH_2	$P = 0.99 \times 10^5C - 0.75 \times 10^4$	$P = 229.71C - 23.42$

to the expected value. However, by reversed-phase LC, the peaks were much broader and the integration parameters may have recognized the peak at a later time, skewing the peak areas ratios. The variation in the limits of detection for LC and CE were, in part, caused by the difference in the detection cell pathlength. The reversed-phase LC cell pathlength was 6 mm, while the CE detection cell pathlength was the diameter of the glass capillary, 50 μm. Therefore, the CE detection limit was limited by the relatively fewer protein molecules in the detection cell at a specific point in time, suggesting that relatively more concentrated solutions are usually used for CE when compared to reversed-phase LC, since CE requires much smaller injection volumes of material. The smaller population of detectable protein molecules also explains the increased standard deviation for CE at the limit of detection. Ultimately, both separation method detection limits were limited by UV detection, whereas fluorescence detection of a suitable derivative would have yielded a much lower detection limit.

The previously described UV detection limits were then compared with a typical hGRF analogue injection detected by LC–MS. Electrospray LC–MS was performed with a C-4 packed capillary column and simultaneous UV detection. A typical gradient separation of these three species, followed by electrospray MS detection and identification of the three species was performed at a loading of 68 pmol total hGRF. Identification of the hGRF analogue species from the mass spectra was easily achieved at this protein loading. The analogues could have been detected and identified with half the total hGRF loading, however this was not pursued since the total loading compared quite well with the limit of detection for reversed-phase LC.

Initially, separation techniques for peptide deamidation products by CE and LC were discussed. Subsequently, MS and UV detection methods were compared for their detection and identification capabilities. The objectives were to identify the utility of several detection methods for the quality and quantity of information they provide. UV detection identified species absorbing at a given wavelength in a quantitative fashion, while mass spectrometry identified the presence of each analogue species within the LC separation by total ion current and identified the resulting mass of the analogue species present. Ultimately, the detection methods give different types of information and should be used accordingly.

The C-4 packed capillary column did not provide baseline separation of Asn^8 hGRF and Asp^8 hGRF with UV detection, but showed Asp^8 hGRF as a shoulder on the Asn^8 hGRF peak. The elution order was the same as that for the reversed-phase LC separation discussed earlier (t_r $isoAsp^8$ hGRF $<$ t_r Asn^8 hGRF $<$ t_r Asp^8 hGRF). A C-18 packed capillary column was also used with no improvement in the separation. Therefore, it was decided to use the C-4 column separation data, since a C-18 300 Å pore size packed capillary column was not available. However, this separation was sufficient for UV and MS identification purposes. The quality of the separation as detected by UV was better than that observed after the total ion current (TIC) trace, due to dead volume and mixing of the separated species within the glass capillary. Upon processing the scans for each peak region in the TIC, three separate mass assignments were made (Fig. 4). The peptide species at 1219.0 and 1218.5 m/z represent the triply charged states, which corresponded to a protonated molecular weight of 3654 m/z for Asp^8 hGRF and $isoAsp^8$ hGRF, and 3653 m/z for Asn^8 hGRF. Therefore, separation, detection and identification of the Asn-containing peptide from its deamidation products was achieved by LC–MS. Separation and identification of the analogues was performed in one single experiment, at peptide loading levels near the limit of detection for reversed-phase LC. By contrast, a secondary method would have to be used to identify the eluting species when LC or CE was linked to UV detection.

Conclusions

In summary, both conventional LC and CE gave adequate baseline separations, limits of detection and linear standard curves. However, within separation techniques, CE was preferred due to a shorter analysis time, better separation and a smaller demand for material. To date, CE methodology has been used sparingly to separate protein and peptide deamidation products. Motilin deamidation of glutamine (Gln) residues has been explored by CE [16]. Gln^{11} Gln^{14} Motilin was separated from its singly and doubly deamidated

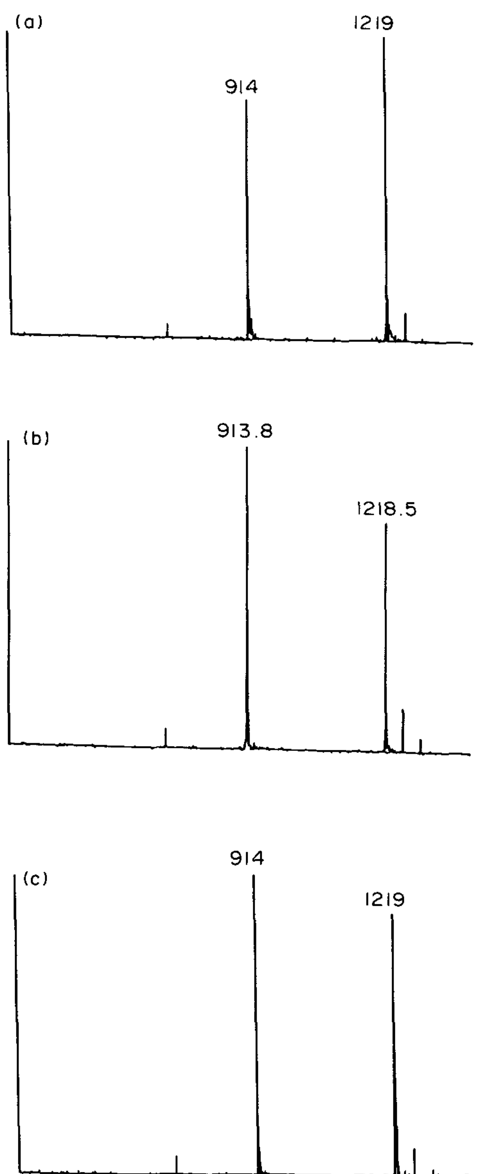

Figure 4
Mass spectra showing the averaged scans for each peak region obtained from the LC–MS separation, where 1219 *m/z* represents the triply charged species. Panel (a) represents the isoAsp^8 region of the TIC, (b) represents the Asn^8 region and (c) represents the Asp^8 shoulder.

products; however the two singly deamidated species, Glu^{11} Gln^{14} and Gln^{11} Glu^{14}, were not successfully separated, nor were any γ-glutamate products. Recently, Asp, isoAsp dipeptides and their D,L, isomers were separated by CE, using anionic β-cyclodextrin derivatives in the running buffer [17]. However, application to larger peptide systems has not yet been addressed.

Packed capillary LC with ESI–MS was then compared with UV detection, and LC–MS was found to offer the most practical approach to detection and identification of the analogues encompassing a single method. Identification of Asn^8 hGRF from the two isobaric deamidation products was achieved from the triply charged states, where the species were separated by 0.5 amu. However, the isomeric deamidation products were not differentiated by mass spectrometry. Identification of the isomeric deamidation products could be achieved by LC–MS with pre-column deuteration, where the resulting mass identification would assign the Asn^8-containing peptide 1 amu below the Asp^8 peptide, and the isoAsp^8-containing peptide 1 amu above the Asp^8-containing peptide [18]. On-line LC–MS was not only useful for difficult separations and differentiation of analogue species by 1 amu, but may also be useful for more complicated, or multiple degradation pathways. Another traditional method for detecting Asp- and isoAsp-containing peptides is by fraction collection and Edman sequencing, where sequencing is terminated for the isoAsp-containing analogue. However, fraction collection followed by Edman sequencing or enzyme digest and sequencing often requires more material, is time consuming and expensive. Therefore, on-line detection and identification of the degradation products by LC–MS provides a viable alternative.

Packed-capillary column LC–MS was preferred over larger analytical columns requiring excessive eluent splitting for on-line MS detection. Such analysis was not acceptable due to the excessive waste of the sample. Conversely, miniaturization of the separation process to CE–MS also presented instrumentation limitations. For example, when the quadrupole mass spectrometer was tuned to discern 0.5 amu difference in mass, the sensitivity required to detect the signal from a typical CE loading was not achieved.

In conclusion, LC–MS separation and identification of degradation products offers a viable alternative to fraction collection and subsequent sequencing or enzymatic identification methods. Electrospray mass spectrometry offered a relatively soft method of ionization which minimized parent ion fragmentation. Thus, large peptides were easily identified without the extensive fragmentation that occurs with other mass spectrometry methods. Furthermore, peptide material demands were radically decreased over enzyme digests or

amino acid analysis identification methods. Therefore, for trace degradation product identification, which cannot be achieved by fraction collection prior to identification, the advent of on-line techniques can be used to alleviate these problems.

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