Presented as poster no. P-306-M at 2nd Symposium on the Analysis of Well Characterized Biotechnology Pharmaceuticals (San Francisco, USA, January 4-7, 1998).

Sensitive Asparagine and Aspartic Acid Positions in Biosynthetic Human Growth Hormone (bhGH): Deamidation and Isomerization in Neutral Solutions.

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INTRODUCTION

Together with oxidation and aggregation, *deamidation* and *isomerization* are the most common degradation pathways for proteins in solution, as well as in the solid state. Normally asparagine and glutamine residues are successible to deamidation, and aspartic acid residues to isomerization.

DEAMIDATION:
In the case of an *asparagine* residue, deamidation starts with formation of a cyclic succinimide derivative. Hydrolysis of this cyclic imide residue may result in formation of an aspartic acid residue or an iso-aspartic acid residue.

ISOMERIZATION:
In the case of an aspartic acid residue, isomerization starts with formation of a similar cyclic imide residue as in deamidation. Hydrolysis may result in formation of an aspartic acid residue or an iso-aspartic acid residue.

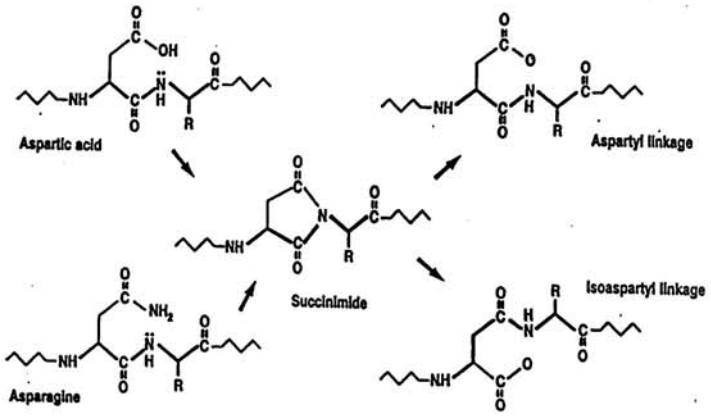
Deamidation:

asn ↔ cyclic imide ↔ asp or iso-asp

Isomerization:

asp ↔ cyclic imide ↔ iso-asp

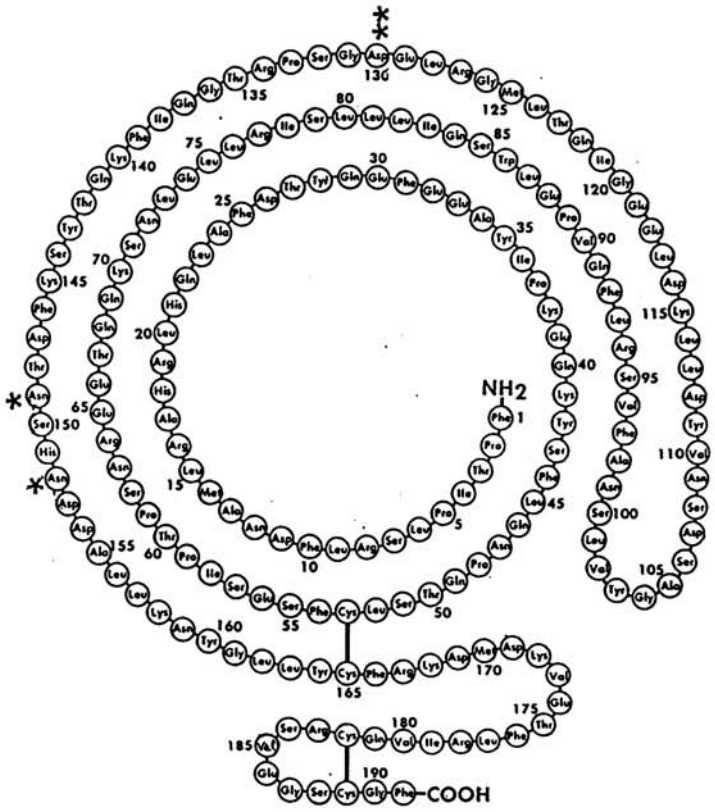
REACTION SCHEME FOR DEAMIDATION AND ISOMERIZATION OF ASPARAGINE/ASPARTIC ACID RESIDUES:



Human growth hormone (hGH) is a 191 amino acid residue protein of major pharmaceutical importance. Several asparagine, glutamine, and aspartic acid residues are present, but their reactivities towards deamidation and isomerization varies, dependent upon the spatial position of the residues in the three dimensional structure of the growth hormone molecule.

Amino acid residues of major importance in *deamidation* of hGH: *Asn 149 & *Asn 152

Amino acid residues of major importance in *isomerization* of hGH: **Asp 130



Primary structure of human growth hormone (hGH).

ALTERNATIVE PHYSICO-CHEMICAL SEPARATION PRINCIPLES FOR ESTIMATING ISO-ASP(130) GH

Due to the above mentioned disadvantages of the cation exchange analysis, it was investigated whether

Capillary zone electrophoresis,

Capillary Isoelectric Focusing, or

Hydrophilic Interaction Chromatography

could be used for the separation of growth hormone variants involved in isomerization and deamidation.

Capillary zone electrophoresis

Utilizing conventional techniques, it was possible to separate a degraded GH preparation in one major and two minor components which contained

cyclic imide(130)GH

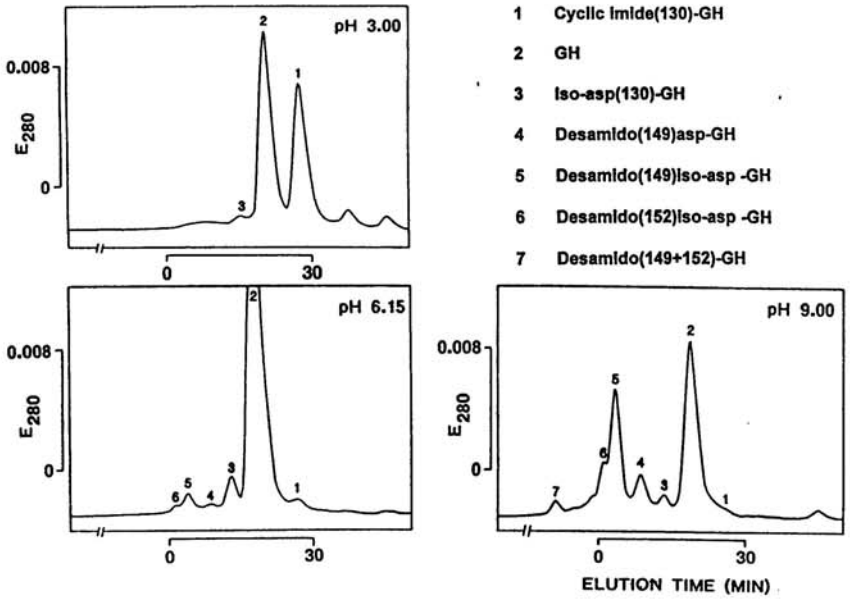
GH

desamido(149)GH + desamido(152)GH.

The fraction containing the two deamidated GH variants was heterogenous, but no separation was obtained.

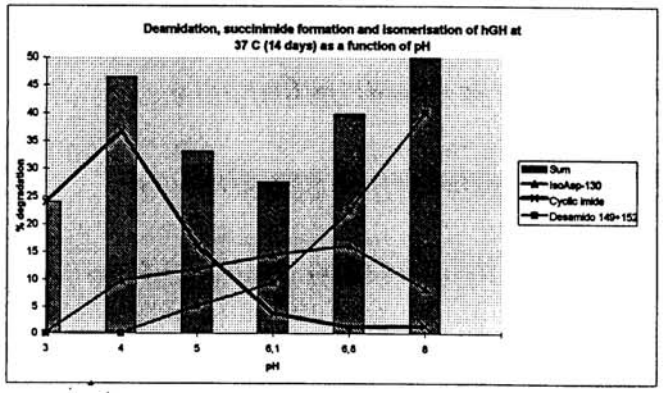
A CZE separation of GH variants comparable to the cation exchange method has not been published in the literature.

Effect of pH in formulation liquids: A balance between deamidation and isomerization



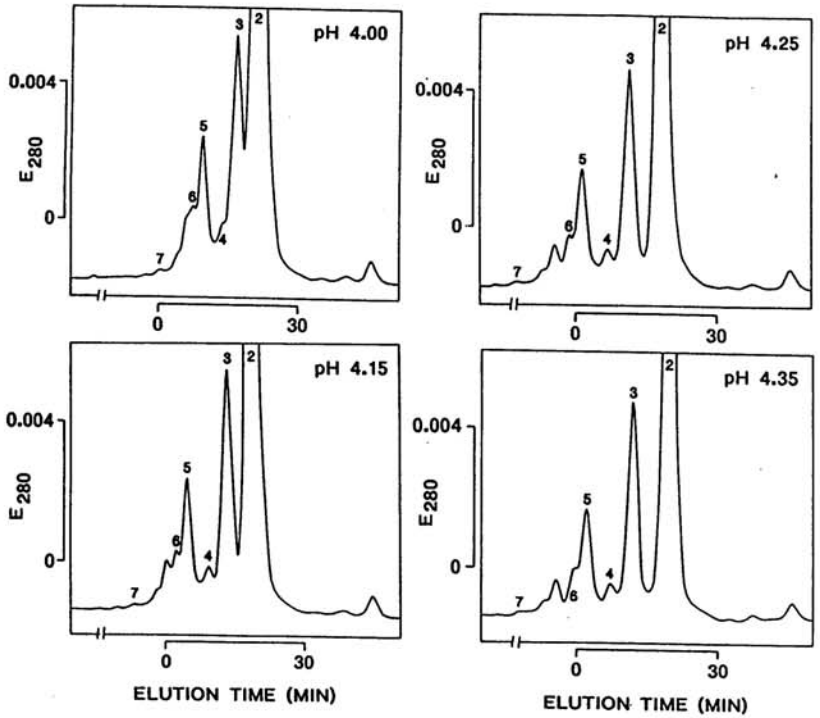
Cation exchange chromatography of GH samples formulated at pH 3.00, 6.15, and 9.00 and thereafter stored at 25 °C for two weeks before analysis. Note the shift from almost total isomerization (pH 3.00) to almost total deamidation (pH 9.00), and the intermediate pH 6.15, where isomerization as well as deamidation can be demonstrated.

Used in combination with other chromatographic techniques, the resultant changes in deamidation and isomerization following variations in the physico/chemical milieu during downstream processing, storage, and formulation, can be predicted.



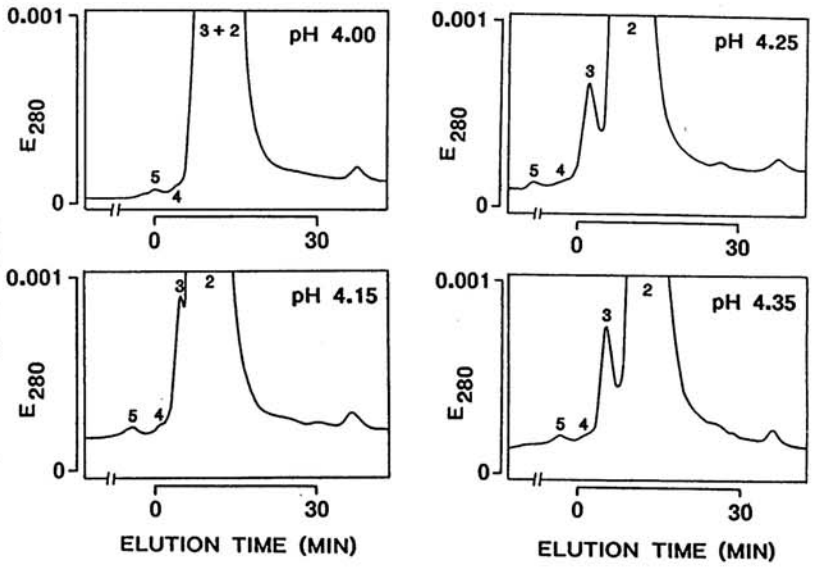
The sum of deamidation, succinimide formation and isomerization seems to be minimal around pH 6. (pH 3 is the lowest of all the pH points tested. However, at this pH, substantial amounts of clipped GH was found to be present, a variant virtually not formed above pH 3).

Effect of variations of the mobile phase pH (high content of iso-asp(130)-GH):



Cation exchange chromatography of a degraded GH sample containing app. 17% iso-asp(130)GH. A PolyCAT A column was eluted at pH 4.00, 4.15, 4.25 and 4.35, respectively with an ammonium acetate gradient in 40% acetonitrile. Peak identification: See previous figure.

Effect of variations of the mobile phase pH (low content of iso-asp(130)-GH):



Cation exchange chromatography of a degraded GH sample containing app. 1.5% iso-asp(130)-GH. A PolyCAT A column was eluted at pH 4.00, 4.15, 4.25 and 4.35, respectively with an ammonium acetate gradient in 40% acetonitrile. Note the much more pronounced effect of the pH changes upon the resolution between iso-asp(130)-GH and GH when only 1.5% iso-asp(130)-GH is present. Peak identification: See previous figure.

A new cation exchange chromatographic analysis.....

Advantages:

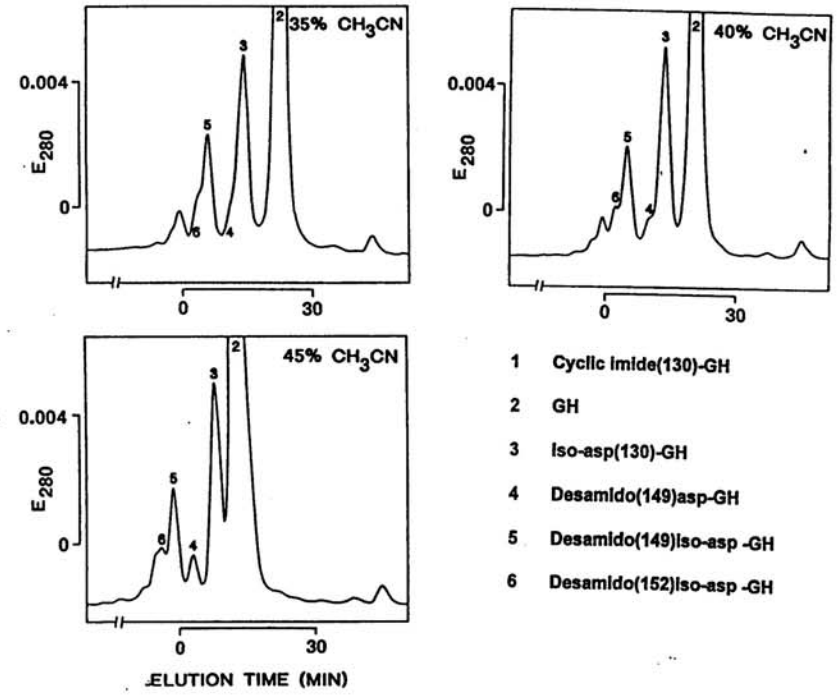
Unrivalled selectivity towards closely related growth hormone variants - the only method available for estimating the content of iso-asp(130)GH in GH preparations

Disadvantages:

Long analysis time (135 min)

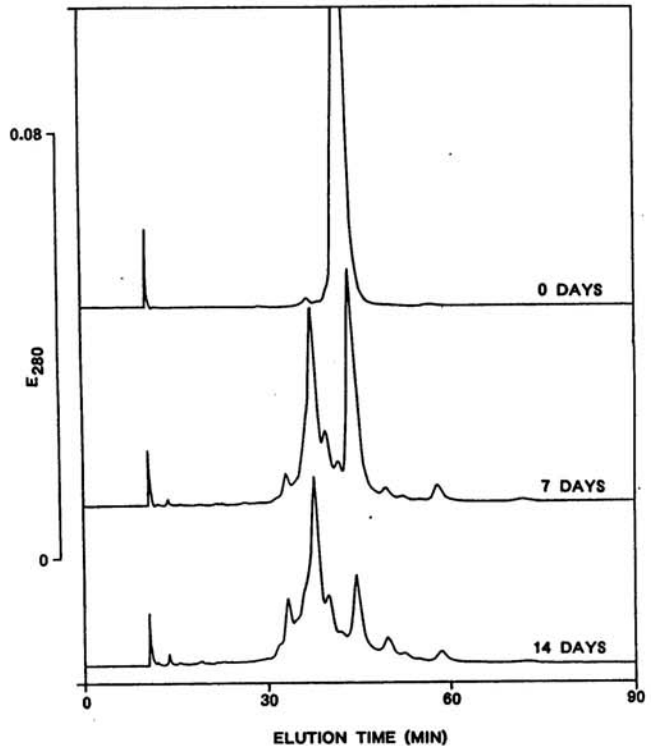
Sensitive towards changes in pH and acetonitrile content

Effect of variations of the acetonitrile content in the mobile phases:



Cation exchange chromatography of a degraded GH sample containing app. 17% iso-asp(130)GH. A PolyCAT A column was eluted at pH 4.25 with an ammonium acetate gradient in 35%, 40%, and 45% acetonitrile, respectively. Note the change in resolution between desamido(149)asp-GH, iso-asp(130)-GH, and GH.

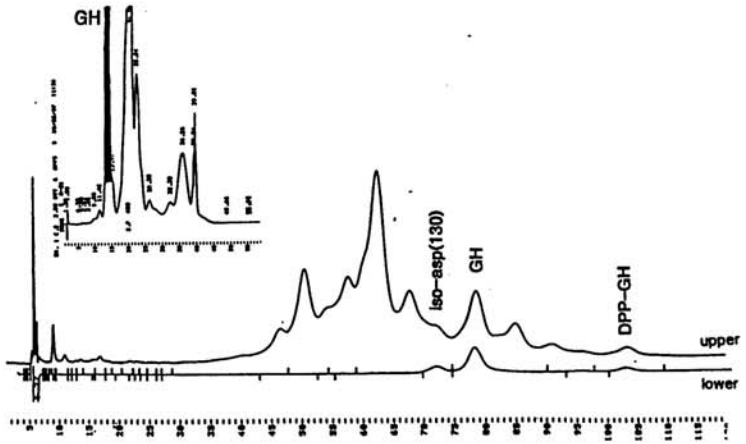
A NEW ANALYSIS FOR hGH VARIANTS: CATION EX-CHANGE CHROMATOGRA-PHY



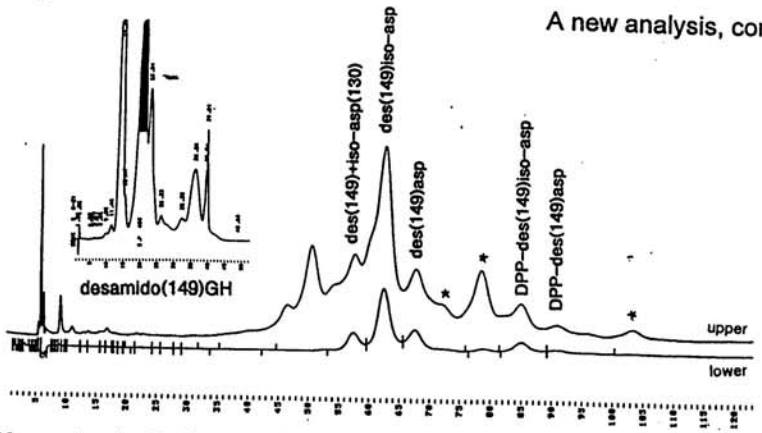
Cation exchange chromatography of a hGH preparation which were incubated at 37 °C in bicarbonate buffer, pH 8,3. A PolyCAT A (PolyLC, Inc.) column was eluted at 30 °C with an ammonium acetate gradient in 40% acetonitrile, pH 4,25.

Identification of the hGH variants:

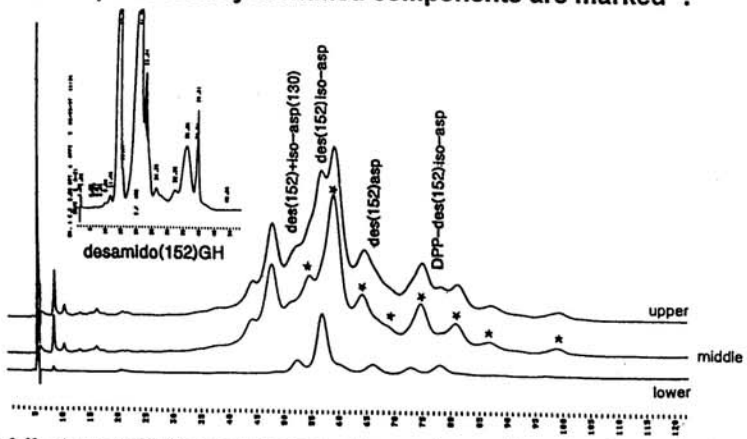
The GH peak, the desamido(149) and the desamido(152) peaks were isolated from the preparative anion exchange chromatography described above. These components were then injected in the cation exchange system, and the resultant peaks were isolated and identified utilizing various alternative chromatographic hGH analyses, mass spectrometry and - especially - tryptic mapping.



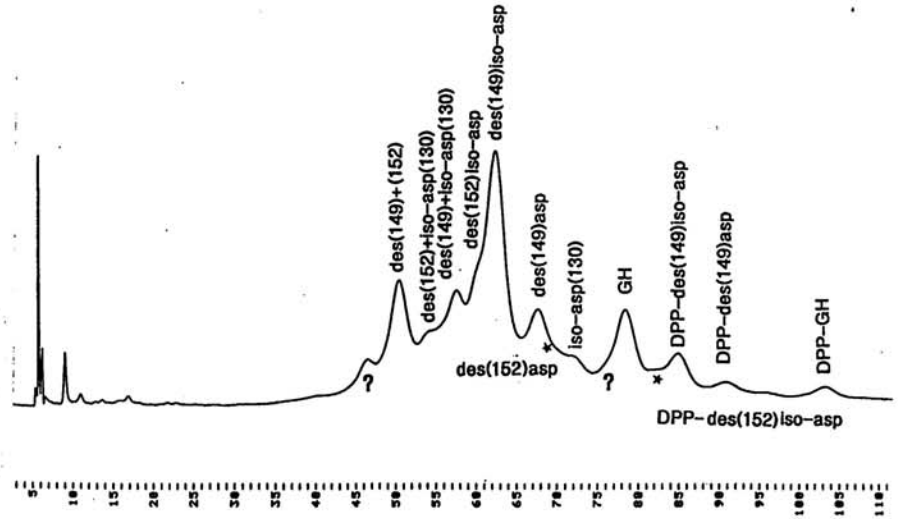
Upper trace: **Cation exchange chromatography of a degraded GH sample (two weeks at 37 °C).** ***Lower trace:*** **Cation exchange chromatography of a "GH fraction" isolated from preparative anion exchange chromatography of the degraded GH sample (see insert). DPP-GH = des-PhePro-1,2 GH, i.e. GH where the first two N-terminal amino acids (Phe and Pro) have been cleaved off, resulting in a residue 3-191 GH molecule.**



Upper trace: **Cation exchange chromatography of a degraded GH sample (two weeks at 37 °C).** ***Lower trace:*** **Cation exchange chromatography of a "desamido 149" fraction isolated from preparative anion exchange chromatography of the degraded GH sample (see insert). Previously identified components are marked *.**



Middle trace: **Cation exchange chromatography of a degraded GH sample (two weeks at 37 °C).** ***Lower trace:*** **Cation exchange chromatography of a "desamido 152" fraction isolated from preparative anion exchange chromatography of the degraded GH sample (see insert).** ***Upper trace:*** **A mixture of 50% original sample and 50% isolated desamido(152)GH. Previously identified components are marked *.**



Cation exchange of a degraded GH sample with identified variants. With the exception of cyclic imide(149)GH and cyclic imide-(152)GH, all variants involved in deamidation of asn 149 and of asn 152, and in isomerization of asp 130, have been identified. Note that "double derivatives" (i.e. deamidated des-PhePro-1,2 GH's, etc.) have been identified, as well. Two minor peaks (marked ?) have not been identified, so far.

Anion-exchange chromatography:

The components detected in this chromatographic analysis have been referred to as "GH", "desamido 149", "desamido 152" and "didesamido 149+152". The variants present in these peaks are:

GH:

GH
iso-asp(130)GH

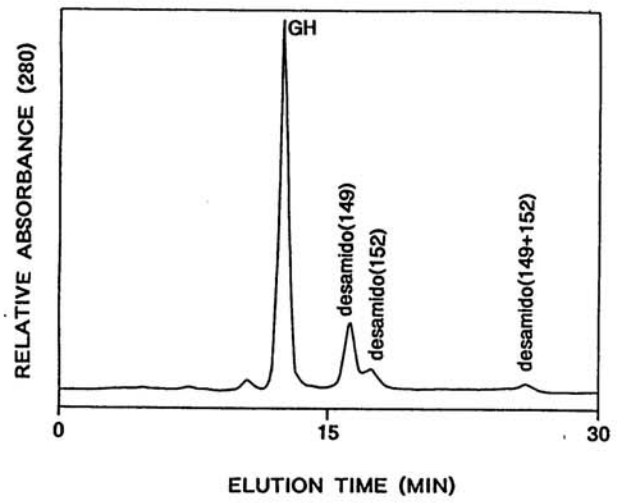
desamido(149):

asp(149)GH
iso-asp(149)GH

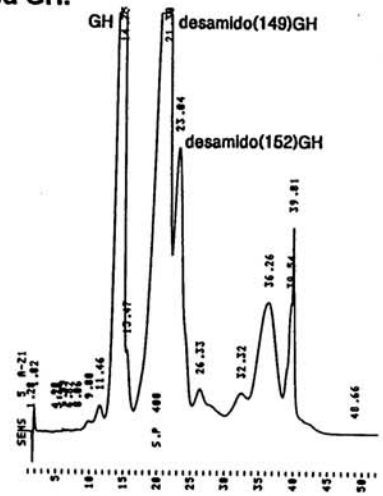
desamido(152):

asp(152)GH
iso-asp(152)GH

Cyclic imide(130)GH is eluted just before the GH peak, whereas the elution positions of cyclic imide(149)GH and cyclic imide(152)GH are unknown.



Analytical Mono Q column (Pharmacia), eluted with an ammonium acetate gradient, pH 5.25. The GH sample contains app. 20 % deamidated GH.



Preparative Resource Q column (Pharmacia) eluted as described above. The GH sample contains app. 80 % deamidated GH.

Degradation studies of hGH in solution, as well as of lyophilized hGH formulations, should therefore - in addition to addressing oxidation, aggregation, etc., involve separation and quantitation of all the deamidation/isomerization variants:

Deamidation:

asp(149)GH	(from asn 149)
iso-asp(149)GH	
cyclic imide(149)GH	
asp(152)GH	(from asn 152)
iso-asp(152)GH	
cyclic imide(152)GH	

Isomerization:

iso-asp(130)GH	(from asp 130)
cyclic imide(130)GH	

ESTABLISHED ANALYTICAL METHODS:

The two established methods for detecting and quantitating deamidation in GH preparations are *PAGE (polyacrylamide gel electrophoresis)* and *anion exchange chromatography.*

PAGE

• Main band	GH
	iso-asp(130)GH
• "desamido GH"	asp(149)GH
	iso-asp(149)GH
	asp(152)GH
	iso-asp(152)GH
• "didesamido GH"	desamido 149+152

PAGE reveals, in addition to a major band, two additional bands, which have been referred to as "desamido GH" and "didesamido GH". The most probable distribution of GH variants in the three bands are given above.

Capillary isoelectric focusing

The separation of degraded GH samples, utilizing isoelectric focusing in polyacrylamide gels, revealed a separation capacity of GH variants which made this separation technique of potential interest.

(See poster P-1608-W, displayed January 7, 1998, 1.00 PM - 2.05 PM at this meeting :

Kirsten Ebbehøj, Benny S. Welinder, Stephen Bayne and Hans H. Sørensen:

"Adequate analytical tools in characterization and Quality Control of Somatropin").

However, it has so far not been possible to reproduce a similar detailed separation pattern when isoelectric focusing was performed in capillaries.

Hydrophilic interaction chromatography (HILIC)

The separation of iso-asp(130)GH from other GH variants is extremely difficult to obtain in separations based upon interaction with the hydrophobic parts of the GH molecule (RP-HPLC, HIC, etc.). Two versions of a complementary technique were therefore applied:

1. **A PolyHYDROXYETHYL A column was eluted with an acetonitrile gradient (80% → 20%) in 10 mM ammonium acetate, pH 4,25.**

2) **A PolyCAT A column was eluted with an ammonium acetate gradient (pH 4,25) in 80% acetonitrile (mixed mode separation IEC/HILIC)**

However, separation of iso-asp(130)GH and GH was not obtained in any of the HILIC variants.

CONCLUSIONS

With the exception of the cyclic imides in position 149 & 152, all GH variants involved in deamidation (in position 149 & 152), and in isomerization (in position 130), have been identified.

"Double" GH variants (i.e. deamidated as well as isomerized GH's, deamidated DPP-GH's) have been identified, as well.

With the two ion exchange analyses we have the tools necessary for predicting the outcome of potential changes in the downstream processing and formulation procedures.

The cation exchange analysis is sensitive and time-consuming. However, the limited separation capacity of capillary electrophoresis, capillary isoelectric focusing, and HILIC (with respect to closely related GH variants) rendered an introduction of these technologies impossible - at present.