Insulin Aspart (Asp^{B28} Human Insulin) Derivatives Formed in Pharmaceutical Solutions

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Received November 20, 2001; accepted February 4, 2002

Purpose. To isolate and identify the main insulin aspart (Asp^{B28} human insulin) derivatives formed in pharmaceuticals (pH 7.4 at 5°C), to estimate rates of formation, and to determine their biologic potencies.

Methods. Insulin aspart derivatives have been isolated by reversed-phase high-performance liquid chromatography (RP-HPLC), and identified by RP-HPLC, peptide mapping, amino acid analysis, mass spectrometry, and N-terminal amino acid sequence analysis.

Results. The main derivatives formed were $isoAsp^{B28}$, $isoAsp^{B3}$, Asp^{B3}, and $desPhe^{B1}$-N-oxalyl-Val^{B2} insulin aspart. At 5°C, the rate constants were 0.00028/month for $isoAsp^{B28}$ and $isoAsp^{B3}$, 0.00024/month for Asp^{B3}, and 0.00013/month for $desPhe^{B1}$-N-oxalyl-Val^{B2} derivatives of insulin aspart. Unexpectedly, the rate of isomerization of B28 was high compared to the rate of B3 deamidation at both 5°C and 45°C. The N-terminal and especially the C-terminal of the B-chain are highly flexible, which may explain the high rate of $isoAsp^{B28}$ formation and that deamidation of Asn^{B3} occurs. All the derivatives had full *in vivo* biologic potencies.

Conclusion. Except for $isoAsp^{B28}$ insulin aspart, the main derivatives formed in pharmaceuticals of insulin aspart and human insulin at pH 7.4 are similar. They are all fully active *in vivo*. In proteins, flexibility of the polypeptide chain seems more important than sequence in the formation of succinimides.

KEY WORDS: Asp^{B28} human insulin derivatives; deamidation; isomerization; $isoAsp^{B28}$ human insulin; Asp^{B28}, $desPhe^{B1}$-N-oxalyl-Val^{B2} human insulin.

INTRODUCTION

The aim of treatment diabetes mellitus is to achieve normoglycemia 24 h per day and, thereby, to prevent or delay the development of late chronic complications (1,2). This is difficult to achieve with human insulin preparations, especially at meal times when insulin absorption is too slow due to the natural tendency of human insulin to self-associate to hexamers. In an attempt to make the absorption rate profile of injected insulin similar to the normal postprandial insulin response, fast-acting monomeric insulin analogues have been developed. Two are available for patients: Asp^{B28} human insulin, called insulin aspart and marketed as NovoRapid® (Novo Nordisk, Bagsvaerd, Denmark) (3,4); and lyspro insulin (Lys^{B28}, Pro^{B29}), called lispro insulin and marketed as Humalog® (Eli Lilly and Company, Indianapolis, Indiana) (5). Substitution of Pro^{B28} in human insulin with Asp^{B28} in insulin aspart removes an important contact between Pro^{B28} and Gly^{B23} at the insulin-monomer interface, resulting in less of a tendency to self-association for insulin aspart than for human insulin (6). Therefore insulin apart is more readily absorbed into the systemic circulation (4,6,7), leading to improved postprandial glycemic control, greater convenience by permitting patients to inject insulin close to meals, and more flexibility in meal composition (1,7,8).

Human insulin as well as its analogues may undergo a number of covalent processes during purification, storage, and delivery (9–16). The purpose of this work has been to isolate and identify the main derivatives formed in an insulin aspart pharmaceutical solution (pH 7.4 during storage at 5°C for 24 months), to estimate rates of formation, and to determine their biologic potencies. The derivatives were isolated from a batch stored at 45°C for 27 days.

The most common reaction in human insulin is deamidation, which can occur in aqueous solution under both acidic and neutral conditions. During storage under acidic conditions, insulin preparations deamidate primarily in Asn^{A21} forming Asp^{A21} (12,14,15). In neutral solutions insulin deamidates at Asn^{B3}, resulting in the formation of Asp^{B3} and $isoAsp^{B3}$ as the main products (12,16). Initially, a cyclic imide (succinimide) is formed by deamidation of asparagine residues. The imide subsequently hydrolyzes, generating a mixture of L-isoAsp and L-Asp along with small amounts of D-isoAsp and D-Asp formed by racemization (12,16–19). The presence of the isoAsp derivative can be demonstrated by the failure of the Edman degradation during sequencing (20). In addition to deamidation reactions, insulin also can undergo covalent cross-linking, forming covalent dimers and high-molecular-weight transformation products (10–13). These processes are relatively slow in insulin pharmaceutical solutions (pH 7.4 at 5°C) compared to the deamidation pathways (10).

MATERIALS AND METHODS

Insulin aspart and NovoRapid® were obtained at Novo Nordisk A/S. All biochemical agents, inorganic salts, and other chemicals were of analytical grade or purer.

Three batches (01, 02, and 03) of an insulin aspart solution (600 nmol/ml, 100 IU/ml, 1.5 mg/ml phenol, 1.72 mg/ml metacresol, 16 mg/ml glycerol, 7 mM disodium phosphate dihydrate, 10 mM sodium chloride, and 19.6 μg/ml = 3 Zn^{++}/hexamer, pH 7.4) were stored at 5°C for 24 months.

One batch (04) of an insulin aspart formulation (300 nmol/ml, 50 IU/ml, 3.0 mg/ml phenol, 16 mg/ml glycerol, and 8.2 μg Zn/ml = 2.5 Zn^{++}/hexamer, pH 7.4) was stored at 45°C for 27 days.

Isolation

As described below in detail, the derivatives were isolated from batch 04 by repetitive isocratic and gradient reversed-phase high-performance liquid chromatography (RP-HPLC) and finally desalted by RP-HPLC on LiChrosorb RP-18 columns (Merck, Whitehouse Station, New Jersey) using a Waters (Milford, Massachusetts) HPLC system and UV de-

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ABBREVIATIONS: insulin aspart, Asp^{B28} human insulin; MW, molecular weight; RP-HPLC, reversed-phase high-performance liquid chromatography; Asp^{B3} I, V8 fragment I of Asp^{B3} insulin aspart; MW, molecular weight.

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tection at 214 nm. Several runs were made at each step, and the collected fractions were analyzed for content and purity before pooling the derivative containing fractions of interest. As described for each derivative, elution was performed with a mobile phase consisting of two of the following buffers prepared in Milli-Q water: (A) 7.7% (w/w) acetonitrile, 2.8% (w/w) sodium sulfate, and 0.4% (w/w) o-phosphoric acid, pH 3.6; (B) 42.8% (w/w) acetonitrile; (C) 7.7% (w/w) acetonitrile, 2.8% (w/w) sodium sulfate, 0.4% (w/w) o-phosphoric acid, and 0.03% (w/w) ethanol amine, pH 2.6; (D) 5.5% (w/w) acetonitrile, 2.0% (w/w) sodium perchlorate monohydrate, 0.6% (w/w) o-phosphoric acid, and 0.3% (w/w) triethylamine, pH 2.3; (E) 50.3% (w/w) acetonitrile, 1.0% (w/w) sodium perchlorate monohydrate, 1.1% (w/w) o-phosphoric acid, and 0.2% (w/w) triethylamine, pH 2.3; (F) 0.1% (v/w) trifluoroacetic acid; and (G) 53.9% (w/w) acetonitrile and 0.1% (v/w) trifluoroacetic acid.

Asp^{B3} and $isoAsp^{B3}$ as well as $isoAsp^{B28}$ insulin aspart were isolated in the same initial separation on a LiChrosorb RP-18 (7 μm, 25 × 250 mm) column at 35°C with a flow rate of 6 ml/min and an initial mobile phase of 60%/40% of A/B, a gradient was applied 5 min after injection (40–48% B at 0.1% min, 48–48% B for 15 min, 48–80% B at 6.4% min). $IsoAsp^{B3}$ insulin aspart was rechromatographed as described for the initial separation. Asp^{B3} insulin aspart was purified by four repetitive separations using the conditions for the initial separation, except that the derivative was eluted using different isocratic conditions to obtain optimal separation between Asp^{B3} and some Asp^{A21} insulin aspart with elution at 53%/47% of C/B, then 55%/45% of A/B, then 55%/45% of C/B, and finally, 56.5%/43.5% of C/B.

$DesPhe^{B1}$-N-oxalyl-Val^{B2} insulin aspart was isolated and rechromatographed once on the same column and with the same flow rate as described above but with a temperature of 40°C and elution of the derivative at 45%/55% of D/E. The derivative was further purified in two repetitive separations on a LiChrosorb RP-18 (7 μm, 10 × 250 mm) column at 40°C with a flow rate of 4 ml/min and elution at 45%/55% D/E. Finally, the isolated material was applied to a LiChrosorb RP-18 (5 μm, 4 × 250 mm) column at 35°C with a flow rate of 1 ml/min, applying an initial mobile phase of 80%/20% of A/B and a gradient elution 10 min after injection (20–40% B at 2%/min, 40–42% B at 0.06%/min, 42–42% B for 15 min).

The derivatives were individually desalted on the LiChrosorb RP-18 (7 μm, 25 × 250 mm) column at 40°C with a flow rate of 6 ml/min, applying a gradient elution with an initial mobile phase of 100% F and a gradient (0–100% G at 0.5%/min). The desalted derivatives were vacuum rotor-evaporated to dryness and stored at –80°C.

Purity and Content

The pharmaceutical solutions (batches 01–04) and each derivative were analyzed for purity and content by an RP-HPLC method on a LiChrosorb RP-18 column (5 μm, 4 × 250 mm) with a flow rate of 1 ml/min at 35°C and detection at 214 nm using a Waters HPLC system. The initial mobile phase was 58%/42% of A/B, and a gradient was applied 30 min after injection (42–80% B at 7.6%/min, 80–80% B for 5 min, 80–42% B at 38%/min, and 42–42% B for 9 min). Samples were acidified by adding 4 μl of 6 N HCl per milliliter prior to application. A volume of 10 μl of each sample or reference solution was subjected to analysis. The purity of each derivative was determined as a percentage of the total absorbance area eluted after the preservatives. Assuming that each derivative had the same absorption coefficient as insulin aspart, the content was determined by the detected area of each component relative to a standard curve of an insulin aspart reference.

$DesPhe^{B1}$-N-oxalyl-Val^{B2} insulin aspart coelutes with insulin aspart in the method described above. Therefore, this derivative and the pharmaceutical solutions (batches 01–04) also were analyzed for purity by a different RP-HPLC method. This method differed by using a temperature of 45°C and an initial mobile phase of 44%/56% of D/E, with a gradient applied 10 min after injection (56–80% E at 2.4%/min, 80–56% E at 24%/min). A sample volume of 5–200 μl containing 0.12–6 nmol of the derivative was acidified and subjected to analysis. The purity was determined in a similar manner as described above.

Quantitative Amino Acid Analysis

The quantitative content of the purified derivatives was analyzed after the hydrolysis of a known amount of protein with 6 M HCl for 24 h at 111°C (21). The amino acids were separated by cation exchange chromatography followed by postcolumn ninhydrin derivatization and VIS-detection using a Beckman 121MB Microcolumn System (Ramcon, Birkeroed, Denmark). Quantitation was performed against a calibration standard (Pierce no. 20088H).

Peptide Mapping

Each derivative was digested with *Staphylococcus aureus* V8 protease using a methodology equivalent to the USP method for human insulin (22), in which the enzyme selectively cleaves at the C-terminal side of glutamic acid (Glu). However, formation of pyroglutamyl in residue Gln^{A5} in fragment I (I) was reduced by changing the following: the digestion volume was reduced to 580 μl; the sample was digested for 3 h at 37°C; and the digestion was quenched with a volume of 60 μl of 1M sodium sulfate and 0.25 M sulfuric acid.

The peptide fragments underwent chromatography by RP-HPLC on a Zorbax RP-C8 column (7 μm, 4.6 × 250 mm; Phenomenex, Torrance, California). The mobile phases were (H) 7.9% (w/w) acetonitrile, 0.5% (w/w) ammonium sulfate, and 0.5% (w/w) sulfuric acid, and (I) 33.1% (w/w) acetonitrile, 0.6% (w/w) ammonium sulfate, and 0.5% (w/w) sulfuric acid. The initial mobile phase was 95%/5% H/I, which allows improved resolution of fragment IV (IV), otherwise the gradient was the same as in the USP method (22). The chromatogram represents a fingerprint of the molecule, where the identity is obtained by comparing the retention times of the individual fragments with those of an insulin aspart reference standard from Novo Nordisk A/S.

Mass Spectrometry

Intact derivatives and selected V8 fragments were analyzed by electrospray mass spectrometry on a Micromass Platform Mass Spectrometer (Micromass AB, Täby, Sweden) with electrospray interface. Typically, the sample was diluted to 5–30 pmol/μl with 0.5% (v/v) formic acid and 50% (v/v) acetonitrile, and 10 μl of the diluted sample then was ana-

Table I. Rate of Formation and Content for Main Derivatives Formed during Storage of Insulin Aspart Pharmaceuticals, pH 7.4

Component	Rate constant × 10^2/month at 5°C[a]	Content (%) after 27 days at 45°C[b] (n = 6)
$IsoAsp^{B28}$	0.028 (0.025–0.031) $r^2 = 0.94$[c]	20.6 (20.4–20.8)
Asp^{B3}	0.024 (0.022–0.027)[d] $r^2 = 0.99$	17.4 (16.7–18.1)
$IsoAsp^{B3}$	0.028 (0.026–0.030)[d] $r^2 = 1.00$	19.0 (18.7–19.3)
Total B3 deamidation	0.052 (0.049–0.055) $r^2 = 0.99$[c]	—
$DesPhe^{B1}$-N-oxalyl-Val^{B2}	0.013 (0.005–0.021) $r^2 = 0.82$[e]	0.96 (0.93–0.99)

[a] Batches 01, 02, and 03. The values given are the apparent rate constants $k_{obs} \times 10^2$/month (95% confidence interval).
[b] Batch 04. Values given as percentage of total protein content (95% confidence interval).
[c] The rate of formation is based on linear regression analysis from four sets of analysis per batch at time point 0 months and three sets of analysis per batch at time points 3, 6, 9, 12, 18, and 24 months.
[d] The rate of formation is based on linear regression analysis from four sets of analysis per batch at time point 0 months and three sets of analysis per batch at time point 24 months. It is assumed that no Asp^{A21} is formed during storage as Asp^{B3} and Asp^{A21} coelutes in the RP-HPLC method.
[e] The rate of formation is based on linear regression analysis from one analysis per batch at time points 3, 6, 9, 12, 18, and 24 months.

lyzed in positive-ion mode. V8 fragments were desalted by RP-HPLC on a C2 or C18 column prior to mass analysis.

N-Terminal Amino Acid Sequence Analysis

Altered desalted V8 fragments were analyzed by automated Edman degradation (23) on an PerkinElmer Instruments (Shelton, Connecticut) model 471A pulsed liquid protein sequencing system equipped with a Model 140 gradient system.

Biologic Potency

The biologic potency of each insulin aspart derivative was determined by comparison of the hypoglycemic effect of the insulin aspart derivative with the effect produced by an insulin aspart reference standard using the mouse blood glucose assay described in the European Pharmacopoeia (24). The assays adhered to the National Institutes of Health 'Principles of Laboratory Animal Care' (publication 85-23, revised 1985).

RESULTS

The formation of insulin aspart derivatives in the pharmaceutical solution (batches 01–03; 600 nmol/ml, pH 7.4) were analyzed by the two RP-HPLC methods at release and during storage at 5°C for 24 months. The main derivatives were $isoAsp^{B28}$, $isoAsp^{B3}$, Asp^{B3}, and $desPhe^{B1}$-N-oxalyl-Val^{B2} insulin aspart. The rates of formation and the structures of these derivatives are shown in Table I and Fig. 1, respectively. The same main derivatives were formed in a different formulation (batch 04, 300 nmol/ml, pH 7.4) that was stored at 45°C for 27 days (Table I). The content of $isoAsp^{B28}$, $isoAsp^{B3}$, and Asp^{B3} insulin aspart increased dramatically to between 17.4% and 20.6% after storage at the increased temperature.

Chromatograms from the RP-HPLC analyses of the derivatives are shown in Fig. 2. Except for the $desPhe^{B1}$-N-oxalyl-Val^{B2} derivative, which coelutes with insulin aspart, the RP-HPLC method is capable of separating all the other derivatives from insulin aspart. Thus, in contrast to the official pharmacopoeia methods (25,26) in which the B3 deamidation derivatives and insulin aspart coelute, the improved RP-HPLC method allows separations of Asp^{B3} and $isoAsp^{B3}$ from each other and from insulin aspart. The main derivatives elute during the isocratic part, and a number of insulin aspart derivatives, hereafter referred to as hydrophobic derivatives, elute during the gradient phase (Fig. 2).

The expected V8 fragments from insulin aspart are shown in Fig. 1. V8 peptide maps of the derivatives differed from that of insulin aspart only in modified fragments. As shown in Fig. 3, the method is able to discriminate between insulin aspart itself and all of its derivatives. Furthermore, it separates each of the derivatives individually except for the fragments Asp^{B3} I and $isoAsp^{B3}$ I, which coelute. In each peptide map, limited amounts of the intact derivative is, as expected, observed at the end of the gradient as well as some pyroglutamyl of fragment I (I pyroglu) of each derivative (20).

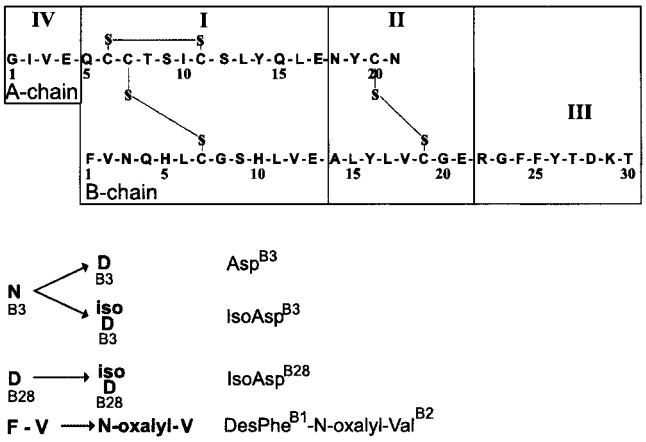

Fig. 1. Insulin aspart and its main derivatives formed at pH 7.4. Peptide mapping of insulin aspart with *S. aureus* V8 protease is shown. The generated fragments are called I, II, III, and IV, respectively. The N-terminal Gln in fragment I may form a pyroglutamyl residue. This fragment I is called I pyroglu (20).

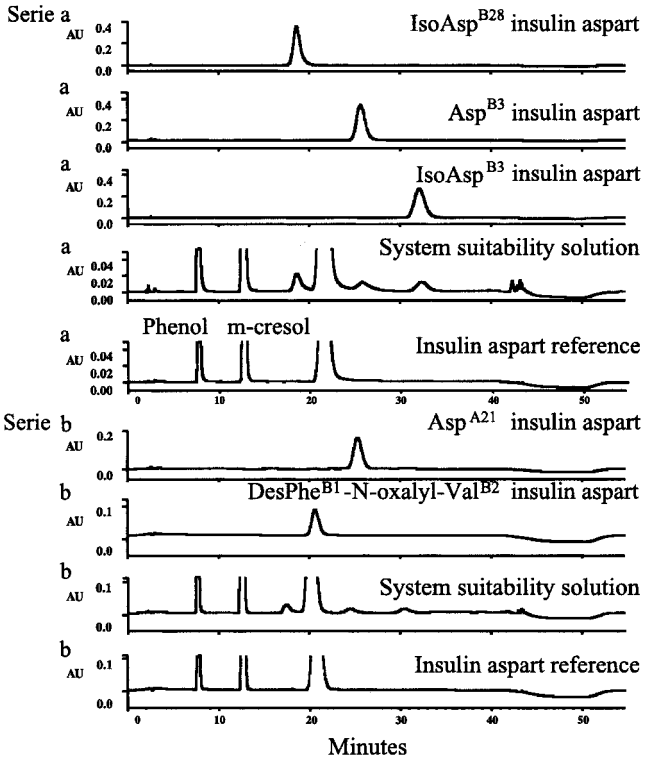

Fig. 2. Purity and content RP-HPLC chromatograms of insulin aspart and its main derivatives. Chromatograms of Asp^{A21} insulin aspart and an aged insulin aspart solution used for system suitability are included for comparison. The absorbance unit (AU) scale varies between chromatograms to facilitate the evaluation of the individual traces. The chromatograms were generated in two series of analyses.

RP-HPLC determined purity and content, with content based on quantitative amino acid analysis and the molecular weight (MW) of each derivative are shown in Table II. The isolated derivatives were >95% pure. Amino acid analyses showed the theoretically expected compositions of each derivative (data not shown). Also, the experimentally determined MWs were in agreement with the theoretically expected MWs within the limits of experimental error (± 1 Da) (Table II). The content of each derivative determined by RP-HPLC and quantitative amino acid analysis was comparable within the limits of experimental error (Table II). This validates the assumption that the absorption coefficient for insulin aspart and each of the main derivatives is the same.

Each generated peptide fragment (I–IV and I pyroglutamyl) of insulin aspart reference material previously had been characterized by mass spectrometry and Edman degradation (data not shown). Therefore, only altered fragments of the derivatives were analyzed. N-terminal amino acid sequences of altered fragments of the derivatives were in agreement with the theoretically expected (data not shown). The isoAsp^{B28} fragment III was blocked in the residue corresponding to B28, indicating the presence of isoAsp^{B28}. The Asp^{B3} fragment I had a phenylthiohydantoin-Asp in the residue corresponding to B3 and the isoAsp^{B3} fragment I was blocked in the residue corresponding to B3. Combining the results shown above identifies the isoAsp^{B28}, isoAsp^{B3}, and Asp^{B3} derivatives.

One derivative showed only two Phe per mole in amino acid analysis compared to three in insulin aspart (data not shown). The MW of this derivative was 75.6 Da less than the theoretical MW for insulin aspart (Table II). Fragment I of this derivative eluted slightly after fragment I of insulin aspart in peptide mapping (Fig. 3), and when analyzed by Edman degradation the B-chain was N-terminally blocked, whereas the correct A-chain was observed. The experimentally determined MW of this fragment was 2894.2 Da. Based on these data and on analogy to a similar derivative of human insulin (27), the suggested structure is desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart (theoretical MW is 2894.2 Da), as shown in Fig. 4.

IsoAsp^{B28}, isoAsp^{B3}, Asp^{B3}, and desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart all had full biologic potency compared to insulin aspart in the *in vivo* mouse blood glucose assay (Table III).

DISCUSSION

A number of insulin aspart derivatives are formed during storage at 5°C. IsoAsp^{B28}, isoAsp^{B3}, and Asp^{B3}, and desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart were shown to be the main derivatives formed at pH 7.4 at 5°C (Tables I and II). The first three mentioned derivatives were also the main derivatives formed at 45°C. The rate of formation of isoAsp^{B28} was surprisingly high for a protein at elevated temperature. The rate of B3 deamidation was somewhat higher than observed in human insulin formulations at 45°C but was much lower than for a peptide (9,18).

The only main derivative formed in insulin aspart drug products but not in human insulin drug products involves the isomerization of Asp^{B28}. Asp in polypeptides is known to be dehydrated and to form a succinimide intermediate, which is subsequently hydrolyzed to yield the isoaspartyl polypeptide (17–19), and isoAsp^{B28} insulin aspart is probably formed by this reaction mechanism (Fig. 5). Asp^{B3} and isoAsp^{B3} insulin aspart are most likely generated by the same reaction mecha-

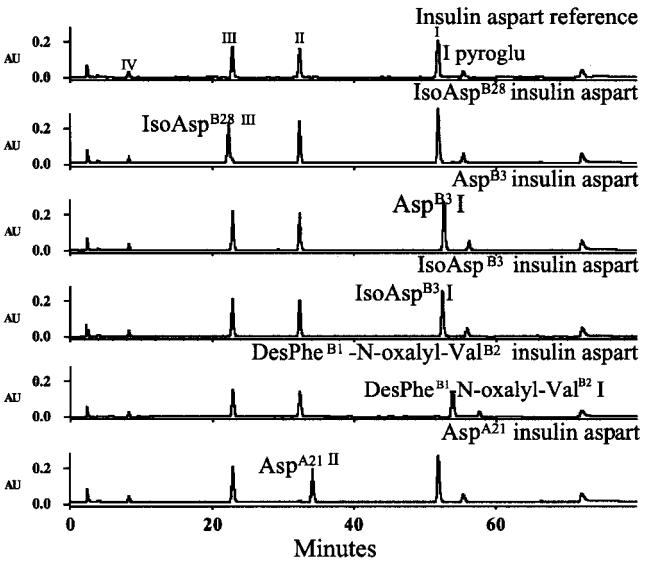

Fig. 3. Chromatograms of peptide mapping of insulin aspart and main derivatives with *S. aureus* V8 protease. The fragment, which differs from that of insulin aspart, is indicated for each derivative. The nomination is described in Fig. 1. A peptide map of Asp^{A21} insulin aspart is included for comparison.

Table II. Results from Physicochemical Characterization of the Main Insulin Aspart Derivatives

Component	Purity[a] of purified component RP-HPLC (%) (n = 3)	Content[a] RP-HPLC (nmol/vial) (n = 3)	Content[a] amino acid analysis (nmol/vial) (n = 2)	Theoretical MW (Da)	Experimental MW (Da) (n = 1)
IsoAsp^{B28}	96.1 ± 0.9	19.0 ± 0.3	18.65 ± 0.01	5825.8	5825.3
Asp^{B3}	98.8 ± 0.1	14.4 ± 0.2	14.2 ± 0.1	5826.8	5826.7
IsoAsp^{B3}	99.8 ± 0.1	20.4 ± 0.2	20.3 ± 0.4	5826.8	5825.9
DesPhe^{B1}-N-oxalyl-Val^{B2}	99.7 ± 0.1 99.0 ± 0.1[b]	4.08 ± 0.06	4.0 ± 0.1	5750.5	5750.2 ± 1.2[c]

[a] Values given as mean ± SD.
[b] Additionally, three vials were analyzed for purity by the method specifically developed for desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart.
[c] Three vials were analyzed.

nisms as the comparable derivatives seen in human insulin formulations (12).

Some Asp^{A21} derivative was present in the drug product at the time of release, which is not surprising since Asp^{A21} human insulin can be formed during freeze-drying (15). Hardly any Asp^{A21} derivative is formed during the storage of drug products of human insulin at pH 7.4 (9,11). Therefore, it is here assumed that the increase over time of the RP-HPLC peak eluting at the position of Asp^{B3} and Asp^{A21} insulin aspart reflects the formation of Asp^{B3} (Table I).

The rate constant for B3 deamidation in insulin aspart at 45°C is approximately 0.40 per month (the sum of Asp^{B3} and isoAsp^{B3} is 0.364 per 27 days; Table I). This rate constant is 2.8 times higher than in a human insulin formulation (0.144 per month) (9). The equilibrium between monomer-dimer-hexamer is less toward hexamer in insulin aspart, and this hexamer is probably less tightly packed than human insulin hexamer. Together this allows for more flexibility in B1–B8 residues in insulin aspart explaining the relatively higher rate of B3 deamidation (6,9). The deamidation rate is not unusual for proteins with Asn in a flexible part of the molecule (28).

The rate of isoAsp^{B28} formation is approximately half of that for isoAsp^{B3} and Asp^{B3}, which is surprisingly high, as in hexapeptides, the rates of formation of succinimide were 13 to 36 times faster for the asparaginyl peptides than for the comparable aspartyl peptides (19). The half-lives of Asn and Asp in an L-hexapeptide (Val-Tyr-Pro-Asn/Asp-Gly-Ala) were 1.4 and 53 days, respectively, at 37°C, pH 7.4 (18). In comparison, the calculated half-lives of Asn^{B3} and Asp^{B28} in insulin aspart at 45°C were approximately 37 and 66 days, respectively (Table I). Assuming that the increase in rate constant from 37°C to 45°C is similar to the increase observed in B3 deamidation in human insulin of 2.8 (9) calculated half-lives of Asn^{B3} and Asp^{B28} would be approximately 104 and 185 days, respectively, at 37°C. Thus, the rate of formation of isoAsp^{B28} is approximately four times less than for a hexapeptide, whereas the rate of B3 deamidation is approximately 70 times less than that for a hexapeptide.

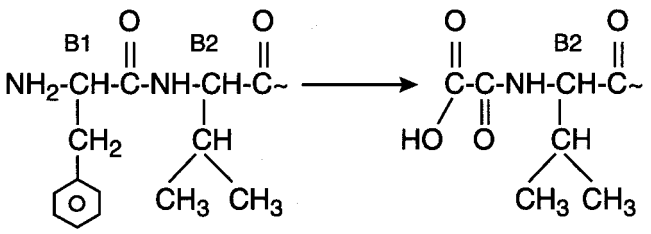

Fig. 4. The chemical structure of desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart.

The sequence in which asparaginyl and aspartyl are present influences the tendency to form succinimide, but sequence cannot explain the high rate of formation of isoAsp^{B28}. IsoAsp tends to form preferentially at Asn-Gly, Asn-Ser, and Asp-Gly sites, and somewhat at Asn-His sites (16,18,19). Deamidation/isomerization is observed in Asn^{B3}-Gln^{B4} in human, pork, and beef insulin (12), and in insulin aspart, and isomerization is observed in Asp^{B28}-Lys^{B29} in insulin aspart. Neither of these sequences would be considered especially prone to succinimide formation.

A likely explanation for the high rate of isoAsp^{B28} formation is the flexibility of the polypeptide chain and the C-terminal flanking residue. In proteins, succinimide formation

Table III. Results from the *in Vivo* Mouse Blood Glucose Assay

Component	Combined bioassay data relative to an insulin aspart reference[a] Average (%)	(95% confidence limits)
Insulin aspart (142X1496.2)	94.9	(84.7–106.0)
IsoAsp^{B28}	92.3	(76.5–110.3)
Asp^{B3}	97.4	(85.7–110.8)
IsoAsp^{B3}	98.3	(87.3–110.6)
[b]DesPhe^{B1}-N-oxalyl-Val^{B2}	106.3	(100.4–112.6)

[a] The data are combined from several experiments in χ^2 tests to generate homogenous combinations with $P > 0.05$. All purified derivatives except for desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart were analyzed relative to insulin aspart reference batch A51001. Another insulin aspart batch (142X1496.2) was analyzed as a sample for comparison. The amount of derivative in each assay was based on RP-HPLC-determined content.
[b] DesPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart was analyzed relative to the insulin aspart batch 142X1496.2.

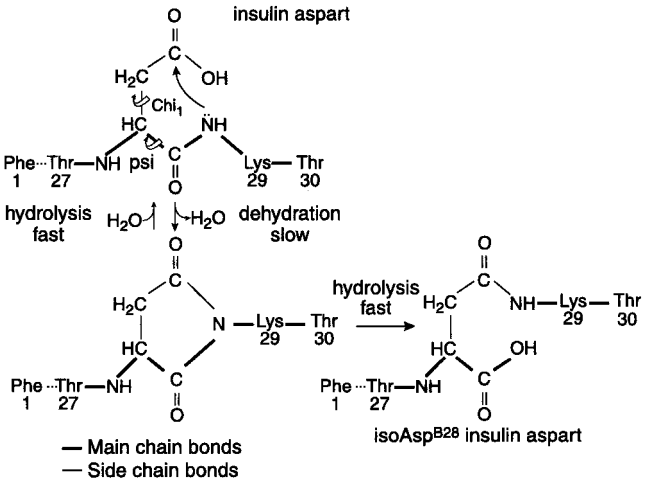

Fig. 5. Proposed reaction mechanism for formation of isoAsp^{B28} insulin aspart.

tends to occur preferentially in regions where the polypeptide chain is highly flexible because the peptide nitrogen atom needs to be in a position to attack the side-chain carbonyl of the Asn or Asp residue, which requires that the dihedral torsion angles psi (defining the rotation around the α-carbon/peptide carbonyl carbon bond) and chi_1 (defining the rotation around the α-carbon/β-carbon bond) approach values of about –120° and 120°, respectively (12,17,18,28). In addition, the value of chi_2, defining the rotation of the acid or amide group, should be about 90° or –90° to minimize steric repulsion from the oxygen and nitrogen side-chain atoms. In proteins, the overall structure of the main polypeptide chain constrains the psi value, and the generally fixed positions of the side chains similarly constrain the chi_1 and chi_2 values (12,17) (Fig. 5).

The insulin hexamer consists of three dimers associated around two zinc ions, and the conformation of the insulin molecules in hexamers can adopt two different states, known as the T and R states, referring to an extended and α-helical conformation, respectively. The more stable R-state hexamer is obtained in the presence of phenol and cresol, the binding of which in hydrophobic pockets causes B1–B8 to form α-helices, stabilizing the structure (1). The natural tendency of insulin to form dimers and hexamers is significantly reduced in insulin aspart, but this molecule can be induced to form R zinc hexamers in the presence of phenol and metacresol (6). The dihedral torsion angles have been measured for B3 and B28 in insulin aspart crystallized in the presence of phenol or metacresol, respectively, and for B3 in human insulin crystallized in metacresol (data not shown). None of the angles are correct for the formation of succinimide, so this does not in itself explain the high rate of formation of isoAsp^{B28}. However, both Asn^{B3} and Asp^{B28} are present on the surface in highly flexible parts of the molecule (6,12). Based on nuclear magnetic resonance studies of insulin aspart, it is known that the R hexamer structure in solution is very similar to the crystal structure and that Asp^{B28} leads to increased conformational flexibility in the B-chain C-termini compared to human insulin (6). Besides, the side chain of Lys^{B29} is protruding into the solution, allowing great flexibility. The vibrational freedom of the side chain and main chain is high for B28, allowing rotation around the α-carbon/peptide carbonyl carbon bond and the acid group, and thereby allowing the correct psi, chi_1, and chi_2 values (6). This flexibility in the C-terminal residues might explain the high rate of formation of isoAsp^{B28}. Although the N-terminal residues are on the surface of the insulin hexamer in the R hexamer, the B1–B8 residues have switched into an α-helix, which mainly is buried within the hexamer, stabilizing the molecule with respect to B3 deamidation (12). In insulin aspart, the monomer-dimer-hexamer equilibrium will be less toward hexamer than in human insulin (4,6), allowing the increased formation of B3 deamidation relative to human insulin, as already has been explained, but still the N-terminal residues are relatively less flexible than the C-terminal residues.

In human insulin and in insulin aspart, no formation of succinimide is observed in Asn^{A18}-Tyr^{A19}, which is probably due to a combination of Tyr being a bulky residue and the fact that Asn^{A18} is present in a rigid region of the molecule (12). There are other examples of the structural constraints being more important than the sequence effects, suggesting that succinimides may form from both aspartyl and asparaginyl residues in proteins. In glucagon, succinimide is formed more at Asp^{9}-Tyr^{10} than at Asn^{28}-Thr^{29} (29). In recombinant human growth factor hormone, succinimide is formed at rates comparable to peptides in Asp^{130}-Gly^{131}and Asn^{149}-Ser^{150}, both of which are present in the flexible part of the molecule but not in Asn^{99}-Ser^{100}, which is in a rigid region of the molecule (16,19,28). In calmodulin, succinimide is formed at Asp^{2}-Gln^{3} and Asp^{78}-Thr^{79}, which are present in the more flexible part of the molecule, whereas six Asn residues do not form succinimide (16,19,28). Consequently, data from human insulin, insulin aspart, and other proteins suggest that the flexibility of the polypeptide chain in proteins is more important in the formation of succinimide than the sequence itself in which asparaginyl or aspartyl is present. Also, it confirms that it is difficult to predict the rate of succinimide formation in proteins from the knowledge of the sequence alone, as observed by others (16,19,28,29).

The relationship between the formation of isoAsp and Asp in proteins is expected to be 60–85% L-isoAsp and 40–15% L-Asp along with small amounts of D-isoAsp and D-Asp (16). However, the ratio of isoAsp^{B3} to Asp^{B3} in human insulin formulations containing phenol at neutral pH has been determined to 1.4, independent of time and temperature (9,12). The ratios of isoAsp^{B3} to Asp^{B3} insulin aspart at 5°C and 45°C were equal within the limits of experimental error (1.17) at 5°C in batches 01–03, and 1.09 at 45°C in batch 04. As phenol and metacresol stabilizes the insulin structures almost equally (30), it is not surprising that the same ratios are found in the four insulin aspart batches that contained different amounts of phenol and metacresol.

In insulin products containing glycerol or mannitol, some (<1%) of desPhe^{B1}-N-oxaly-Val^{B2} insulin is formed at 5°C after 24 months. Although this derivative has not previously been described in detail in the literature, it is a known derivative in human, beef, and pork insulin (27). The rate of formation of the derivative is correlated to the quality of the excipient both for human insulin and insulin aspart batches. Because the batches of excipient with high contents of reducing substances such as α-hydroxy carbonyl compounds seem to enhance the formation of the derivative (data not shown), it is proposed that the reaction mechanism includes an early Maillard reaction between human insulin or insulin aspart

and the reducing substances, leading to the formation of a group of reactive substances (31). In a subsequent Maillard reaction between these reactive substances and human insulin or insulin aspart, an intermediate derivative may be formed that, upon hydrolytic degradation, leads to the formation of the desPhe^{B1}-N-oxaly-Val^{B2} derivative.

The rate constant of desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart formation at 5°C was 0.00013 per month, and after storage of batch 04 at 45°C for 27 days the content was approximately 1% (Table I). The data points at 5°C were limited but fit a linear plot. The formation of desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart is expected to follow first-order kinetics, as it is a reaction between two molecules. The apparent lower temperature dependency of this derivative compared to the other main derivatives (Table I) may be explained by a 2-fold lower concentration of insulin aspart and, most likely, to a lower concentration of reducing substances in batch 04 compared to bathes 01–03.

IsoAsp^{B3} and Asp^{B3} derivatives of human, pork, and beef insulin have full or nearly full biologic potencies (12,32), and comparable data are found for these insulin aspart derivatives (Table III). Also, desPhe^{B1}-N-oxalyl-Val^{B2} and isoAsp^{B28} insulin aspart have full *in vivo* biologic potencies (Table III).

Degradation of Asn and Asp sites via the succinimide pathway can have profound effects on protein activity, and loss of function associated with isoAsp formation has been found with a variety of proteins (16). The change in activity is most likely dependent on where in the molecule the isoAsp formation occurs (16). Also, increased immunogeneity has been associated with isoAsp formation (16). Neither residue B1–B3 nor B28 are directly involved in the binding of insulin to the receptor, and the residues are located in the more flexible parts of the molecule (6,12), which explains why all the derivatives are fully active. Asp^{A21}, Asp^{B3}, and isoAsp^{B3} as well as desPhe^{B1}-N-oxalyl-Val^{B2} derivatives have been present in human insulin drug products for years, and no safety problems have been reported regarding these derivatives in human insulin or insulin aspart drug products.

In the RP-HPLC content and purity method, minor amounts of a few hydrophilic and several more prominent hydrophobic derivatives are observed after storage of the drug products (Fig. 2). In total, an additional formation of approximately 1% of these derivatives was observed, but individually none of them showed an additional formation above 0.3% of the total protein content after storage at 5°C for 24 months.

CONCLUSIONS

The main insulin aspart derivatives formed in pharmaceutical solutions at pH 7.4 and stored at 5°C for 24 months were isolated and identified to be isoAsp^{B28}, isoAsp^{B3}, Asp^{B3}, and desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart. The first three derivatives are most likely formed via a succinimide intermediate. Neither Asn^{B3} nor Asp^{B28} are present in sequences that are likely to form succinimide, however, the residues, especially Asp^{B28}, are located in flexible regions of the polypeptide chain, which seems to be more important than the sequence itself. This may explain the unexpectedly high rate of isoAsp^{B28} formation for a protein at an elevated temperature.

Except for isoAsp^{B28} insulin aspart, which cannot be formed in human insulin and therefore has not previously been described, these main derivatives also are formed in human insulin drug products. Diabetic patients have used human insulin drug products safely for years. All the derivatives have full biologic potencies. Neither the deamidation and isomerization of Asn^{B3} and isomerization of Asp^{B28} nor the formation of desPhe^{B1}-N-oxalyl-Val^{B2} insulin aspart change the biologic potency of the molecule, most likely because the derivatives are formed in flexible regions on the surface of the molecule, which do not participate in receptor binding.

ACKNOWLEDGMENTS

The authors are thankful to L. Langkjær for providing the formulations, C. R. Petersen, M. Hach, J. K. Jacobsen, H. S. Jensen, T. Christensen, A.-M. S. Sørensen, V. Sarossy, N. Leth, K. L. Larsen, G. Norup, D. Højby, M. Lazib, K. B. Hansen, P. C. Agerholm, M. Christensen, G. G. Nielsen, B. Huge-Jensen, M. Lauritzen, A. J. Moody, H. B. Olsen for technical assistance, and to P. Langballe for valuable discussions. Funded by Novo Nordisk A/S.

REFERENCES

1. J. Brange and Aa. Vølund. Insulin analogs with improved pharmacokinetic profiles. *Adv. Drug Delivery Rev.* **35**:307–335 (1999).
2. The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. *N. Engl. J. Med.* **329**:977–986 (1993).
3. J. Brange, U. Ribel, J. F. Hansen, G. Dodson, M. T. Hansen, S. Havelund, S. G. Melberg, F. Norris, K. Norris, L. Snel, A. R. Sørensen, and H. O. Voigt. Monomeric insulins obtained by protein engineering and their medical implications. *Nature* **333**:679–682 (1988).
4. J. Brange, D. R. Owens, S. Kang, and Aa. Vølund. Monomeric insulins and their experimental and clinical implications. *Diabetes Care* **13**:923–954 (1990).
5. R. D. DiMarchi, R. E. Chance, H. B. Long, J. E. Shields, and L. J. Slieker. Preparation of an insulin with improved pharmacokinetics relative to human insulin through consideration of structural homology with insulin-like growth factor I. *Horm. Res.* **41**: 93–96 (1994).
6. J. L. Whittingham, D. J. Edwards, A. A. Antson, J. M. Clarkson, and G. G. Dodson. Interactions of phenol and *m*-cresol in the insulin hexamer, and their effect on the association properties of B28 Pro→Asp insulin analogues. *Biochemistry* **37**:11516–11523 (1998).
7. J. A. Lutterman, E. Pijpers, P. M. Netten, and L. N. Jørgensen. Glycaemic control in IDDM patients during one day with injection of human insulin or the insulin analogues insulin X14 and insulin X14 (+Zn). In M. Berger and F. A. Gries (eds.), *Frontiers in Insulin Pharmacology*, Georg Thieme Verlag Stuttgart, Thieme Medical Publisher, Inc., New York, 1993 pp. 102–109.
8. P. D. Home, A. Lindholm, and A. Riist. Insulin aspart vs. human insulin in the management of long-term blood glucose control in Type 1 diabetes mellitus: a randomized controlled trial. *Diabet. Med.* **17**:762–771 (2000).
9. J. Brange, L. Langkjær, S. Havelund, and Aa. Vølund. Chemical stability of insulin: 1. Hydrolytic degradation during storage of pharmaceutical preparations. *Pharm. Res.* **9**:715–726 (1992).
10. J. Brange, S. Havelund, and P. Hougaard. Chemical stability of insulin: 2. Formation of higher molecular weight transformation products during storage of pharmaceutical preparations. *Pharm. Res.* **9**:727–734 (1992).
11. J. Brange and L. Langkjær. Chemical stability of insulin: 3. Influence of excipients, formulation, and pH. *Acta Pharm. Nord.* **4**:149–158 (1992).
12. J. Brange. Chemical stability of insulin: 4. Mechanisms and ki-

netics of chemical transformations in pharmaceutical formulation. *Acta Pharm. Nord.* **4**:209–222 (1992).
13. J. Brange, O. Hallund, and E. Sørensen. Chemical stability of insulin: 5. Isolation, characterization and identification of insulin transformation products. *Acta Pharm. Nord.* **4**:223–232 (1992).
14. R. T. Darrington and B. D. Anderson. Evidence for a common intermediate in insulin deamidation and covalent dimer formation: Effects of pH and aniline trapping in dilute acidic solutions. *J. Pharm. Sci.* **84**:275–282 (1995).
15. R. G. Strickley and B. D. Anderson. Solid-state stability of human insulin II. Effect of water on reactive intermediate partitioning in lyophiles from pH 2-5 solutions: Stabilization against covalent dimer formation. *J. Pharm. Sci.* **86**:645–653 (1997).
16. D. W. Asward, M. V. Paranandi, and B. T. Schurter. Isoaspartate in peptides and proteins: Formation, significance, and analysis. *J. Pharm. Biomed. Anal.* **21**:1129–1136 (2000).
17. S. Clarke. Propensity for spontaneous succinimide formation from aspartyl and asparaginyl residues in cellular proteins. *Int. J. Peptide Protein Res.* **30**:808–821 (1987).
18. T. Geiger and S. Clarke. Deamidation, isomerization and racemization at asparaginyl and aspartyl residues in peptides: Succinimide-linked reactions that contribute to protein degradation. *J. Biol. Chem.* **262**:785–794 (1987).
19. R. C. Stephenson and S. Clarke. Succinimide formation from aspartyl and asparaginyl peptides as model for the spontaneous degradation of proteins. *J. Biol. Chem.* **264**:6164–6170 (1989).
20. D. G. Smyth, W. H. Stein, and S. Moore. The sequence of amino acid residues in bovine pancreatic ribonuclease: Revisions and confirmations. *J. Biol. Chem.* **238**:227–234 (1963).
21. S. Moore and W. H. Stein. [117] Chromatographic determination of amino acids by the use of automatic recording equipment. *Methods Enzymol.* **6**:819–831 (1963).
22. The United States Pharmacopoeia. USP 24 Insulin Human, Identification B, Fingerprint Test, The United States Pharmacopeial Convention, Inc., Rockville, Maryland, 2000 pp. 883.
23. P. Edman and G. Begg. A protein sequenator. *Eur. J. Biochem.* **1**:80–91 (1967).
24. European Pharmacopoeia 2nd ed., v. 2.2.3. Assay of insulin, method C, 1984 pp. 3–4.
25. European Pharmacopoeia. Insulin preparations, injectable, No. 1999: 0854. Assay, 2000 pp. 5–8.
26. The United States Pharmacopoeia. USP 24 Insulin Injection, Assay. The United States Pharmacopeial Convention, Inc. Rockville, Maryland, 2000 pp. 882–883.
27. J. M. Olinger. Insulin preparations monograph proposals and rationale for modification. *European Pharmacopoeia Commission, Group of Experts No. 6I, Biological Standardisation Program, Insulin Specifications, Report of the international meeting held 16 February 1995*, Strasbourg, 1995, pp. 12–28.
28. R. M. Daniel, M. Dines, and H. H. Petach. The denaturation and degradation of stable enzymes at high temperatures. *Biochem. J.* **317**:1–11 (1996).
29. I. M. Ota, L. Ding, and S. Clarke. Methylation at specific altered aspartyl and asparaginyl residues in glucagon by erythrocyte protein carboxyl methyltransferase. *J. Biol. Chem.* **262**:8522–8531 (1987).
30. A. Wollmer, B. Rannefeld, B. R. Johansen, K. R. Hejnaes, P. Balschmidt, and F. B. Hansen. Phenol-promoted structural transformation of insulin in solution. *Biol. Chem. Hoppe-Seyler* **368**: 903–911 (1987).
31. F. Ledl and E. Schleicher. New Aspects of the Maillard reaction in foods and in the human body. *Angew. Chem. Int. Ed. Engl.* **29**:565–594 (1990).
32. R. M. Riggin and N. A. Farid. Analytical chemistry of therapeutic proteins. In C. Horváth and J. G. Nikelly (eds.), *Analytical Biotechnology Capillary Electrophoresis and Chromatography*, American Chemical Society, Washington, DC, 1990 pp. 113–126.