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RESEARCH PAPER

Purification and Identification of High Molecular Weight Products Formed During Storage of Neutral Formulation of Human Insulin

Christian Fogt Hjorth • František Hubálek • Jonatan Andersson • Christian Poulsen • Daniel Otzen • Helle Naver

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ABSTRACT

Purpose To identify High Molecular Weight Products (HMWP) formed in human insulin formulation during storage.

Methods Commercial formulation of human insulin was stored at 37°C for 1 year and HMWP was isolated using preparative size exclusion chromatography (SEC) and reverse phase (RP) chromatography. The primary structure of the isolated species was analysed using liquid chromatography mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS). To test the hypothesis that amino groups of insulin are involved in HMWP formation, the HMWP content of various formulations spiked with amine compounds or formulations of insulin with modified amino groups was measured.

Results More than 20 species of HMWP were observed and 16 species were identified using LC-MS. All identified species were covalent dimers of human insulin linked *via* A21Asn and B29Lys, formed *via* the formation of an anhydride intermediate at A21Asn. Two types of HMWP were identified, with the covalent link in the open or closed (succinimidyl) form. Some species also contained single deamidation at B3 or the desPhe(B1)-N-oxalyl-Val(B2) modification. Reduced rate of HMWP formation was observed after addition of L-lysine, L-arginine or piperazine or when insulin analogues with methylated N-terminals and side chain amines and A21Gly mutation were used. Formulations of human insulin without zinc and *m*-cresol were found to contain a different pool of HMWP.

Conclusions HMWP formed in formulation of human insulin at pH 7.4 with zinc and *m*-cresol consists primarily of covalent dimers linked *via* A21Asn and B29Lys. Insulin formulation properties determine the amount and identity of formed HMWP.

KEY WORDS High Molecular Weight Products (HMWP) · human insulin · mass spectrometry · reverse phase chromatography · size exclusion chromatography

ABBREVIATIONS

HI	Human insulin
HMWP	High Molecular Weight Products
LC-MS	Liquid chromatography mass spectrometry
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
RP	Reverse phase (chromatography)
SEC	Size exclusion chromatography
TCEP	Tris(2-carboxy-ethyl)phosphine
V8	Endoproteinase Glu-C V8 enzyme

INTRODUCTION

Physical and chemical stability of proteins is a great challenge in the development of pharmaceutical proteins as these can have a great impact on biological potency and safety—the latter being antagonized by increased immunogenicity of the degraded protein/peptide (1–3). Proteins and peptides can be subject to a variety of chemical changes during production and storage, including oxidation, deamidation, racemization, transamidation, disulphide bridge scrambling and formation of cross-links to other molecules (4). Variable biological properties of such degradation products have been reported, ranging from increased immunogenicity of oxidated human interferon alfa and beta aggregates (5,6) and formation of isoaspartic acid (7) to almost unaffected biological potency of racemization of aspartic acid to isoaspartic acid in certain locations of insulin aspart (8). The immunogenic potential of protein aggregates and their potency, as a result of physical or

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C. F. Hjorth (✉) • F. Hubálek • J. Andersson • C. Poulsen • H. Naver
Diabetes Protein Engineering, Novo Nordisk A/S
Novo Nordisk Park, 2760 Måløv, Denmark
e-mail: cfth@novonordisk.com

C. F. Hjorth • D. Otzen
Interdisciplinary Nanoscience Center (iNANO), Aarhus University
Gustav Wieds Vej 14, 8000 Aarhus C, Denmark

chemical degradation, is discussed heavily between regulatory bodies, academia and industry (9–13). Development of stabile protein formulations and characterization of potential degradation products is thus a crucial part of modern drug development.

Human insulin (HI) is a peptide composed of 51 amino acids forming two peptide chains (A- and B-chain) linked together by two disulphide bridges. Covalently linked aggregates of insulin, as a consequence of chemical degradation, is referred to as High Molecular Weight Products (HMWP) and is defined by a pharmacopoeia assay (14). While diabetic patients are generally not exposed to high levels of HMWP in insulin pharmaceutics, exposure to low levels cannot be prevented as they inevitably form during use and storage of insulin products.

Insulin HMWP consists of a pool of so far poorly characterised species, but has been found to have altered biological properties (15–19).

Characterisation of human insulin HMWP formed in various different formulations has previously been described in the literature. Brange *et al.* (15) identified A- to B-chain dimers and A- to A-chain dimers in various neutral preparations of porcine insulin, including a zinc free solution, zinc crystalline suspensions and an isophane insulin protamine preparation. Amino acid analysis indicated that the dimers were formed by a reaction of the N-terminal of the A- or B-chain of one monomer with a side chain amido group in the A-chain of another monomer. Brems *et al.* (20) found that storage of neutral formulations of zinc free insulin containing m-cresol and glycerol at 50°C resulted in disulphide scrambling oligomers as the major degradation product. Darrington and Anderson have described how zinc free human insulin in acidic solutions (pH 2–5) formed dimers *via* A21Asn and B-chain N-terminal during storage at 35°C (21,22). Darrington and Anderson also established the link between the formation of an A21Asn anhydride intermediate and crosslinking reactions in insulin, while DeHart *et al.* (23) found that the in-chain asparagine cyclic imide intermediate in model peptides was involved in both deamidation reactions and cross linking to neighbour peptides. Torosantucci *et al.* investigated the formation of insulin cross-linking by metal-catalyzed oxidation mechanisms involving tyrosine (24).

Since the studies of insulin HMWP done by Brange *et al.* in the beginning of the 1990's, mass spectrometry has developed into a powerful tool for the analysis of proteins and chemical degradation products. While Darrington *et al.* used mass spectrometry to identify crosslinks of insulin they did not work with pharmaceutically relevant formulations of insulin. Brange *et al.* characterized HMWP in various pharmaceutical formulations of insulin (human and porcine), but did not use mass spectrometry to identify the primary structure of HMWP. Jiskoot and Friess and coworkers developed novel gelfiltration analysis methods in order to isolate and characterize HMWP species of insulin and other peptides (25,26).

This study aimed at isolating HMWP from marketed formulation of human insulin, identifying the chemical nature of HMWP and its pathway of formation and investigating the dependence of formulation properties on the composition of HMWP. This was achieved using various preparative and analytical chromatographic methods and mass spectrometry. The study found that HMWP formed in neutral zinc formulation of human insulin is composed of covalent dimers linked *via* A21Asn and B29Lys. Absence of zinc and *m*-cresol resulted in a different pool of HMWP, emphasizing the influence of formulation properties on the composition of HMWP formed during storage.

MATERIALS AND METHODS

All chemicals were of analytical grade or purer.

Incubated Human Insulin

Actrapid®/Novolin® R 100 U/ml (0.6 mM, 0.33 mM zinc 28 mM m-cresol, 1.6% (*w*/*v*) glycerol, pH 7.4) was acquired from Novo Nordisk A/S. The formulation was incubated in vials at 37°C for 1 year to accelerate HMWP formation. The formulation had been stored for 1 year at 5°C before acquisition. A small volume of an identical formulation stored for 4 years at 5°C was also acquired.

Synthesis and Formulation of Human Insulin Analogues

Two insulin analogues were synthesized (Fig. S1) (Protein Engineering, Novo Nordisk A/S, Denmark).

One gram of human insulin (analogue 1) or A21Gly mutated insulin (analogue 2) were each dissolved in 10 ml dimethylformamide and 15 ml 0.2 M aqueous citric acid was added and pH was adjusted to 5.5. 0.5 g $NaCNBH_3$ and formaldehyde (35% in water, 0.54 ml) were added and the mixtures were gently stirred at room temperature for 1 h and acidified (pH 1.5) with 1 N HCl. The desired permethylated insulins were purified by HPLC (AXIA, 5 μ, C18, 110 Å, 250 × 30 mm, Phenomenex, Torrance, CA) using two buffers: A: 0.1% TFA in MQ water, B: 0.1% TFA in acetonitrile. A gradient of 0–40% B over 50 min was employed. Pure fractions were lyophilised to afford 0.22 g hexamethyl human insulin and 0.39 g hexamethyl A21Gly human insulin, respectively.

The purity was above 95% for both analogues as determined by LC-MS.

The analogues were formulated in the Actrapid® vehicle (0.33 mM zinc, 28 mM m-cresol, 1.6% (*w*/*v*) glycerol, pH 7.4) in concentrations of 0.6 mM insulin analogue. Samples were

adjusted to pH 7.4 using NaOH and HCl and sterile filtered through a Milipore 0.22 μM filter in a LAF bench. The vials were encapsulated using a rubber membrane and metal cap. Samples of 10 ml were prepared for each analogue and stored in vials for up to 3 months at 37°C. A sample of human insulin (Actrapid®, previously stored at 5°C for 1 year) was stored for up to 3 months at 37°C and used as control.

Analytical Size Exclusion Chromatography (Quantification of HMWP Pool)

Analytical size exclusion chromatography (SEC) was carried out using an Acquity UPLC instrument (Waters Corporation, Milford Massachusetts, USA) employing an Acquity BEH 125 column (1.7 μm, 4.6×300 mm, Waters Corporation). The flow of a dissociating buffer (15% (*v*/*v*) acetic acid, 20% (*v*/*v*) acetonitrile, 0.65 g/L L-arginine (240 mM) in Milli-Q water) was set to 0.250 ml/min for 20 min at room temperature. Absorbance was measured at 276 nm.

Isolation of HMWP formed in stored formulations of insulin or analogues in analytical scale was carried out using a Waters Alliance HPLC instrument with a Waters Insulin HMWP column (7.8×300 mm) using a dissociating buffer as detailed above. The flow was set to 1 ml/min for 17 min and absorbance was measured at 276 nm.

Analytical Reverse Phase Chromatography (Quantification of HMWP Species)

Analytical reverse phase (RP) chromatography was carried out using an Acquity UPLC instrument (Waters Corporation) equipped with an Acquity CSH Phenyl-hexyl column (1.7 μm, 1.0×150 mm, Waters Corporation). Two buffers were prepared in Milli-Q water; A: 10% (*v*/*v*) acetonitrile, 0.1 M phosphate (Na_2HPO_4/NaH_2PO_4), pH 7.0. B: 80% (*v*/*v*) acetonitrile. The flow was set to 0.350 ml/min. A linear gradient was used to develop the separation: 10–22% B 5 min, 22–30% B 90 min, 30–80% B 2 min and 80–10% B 2 min. The column was equilibrated for 4 min in starting conditions and absorbance was measured at 215 nm with a column temperature of 30°C.

Preparative Size Exclusion Chromatography (Isolation of HMWP Pool)

Forty milliliter incubated human insulin formulation was concentrated in centrifugal filter tubes to a volume of 7.5–10 ml before injection (Amicon Ultra, Ultracel® 3K, 15 ml, Regenerated cellulose, 3000 Da filter cut-off, Merck Milipore Ltd, Tullagreen, Carrigtwohill Co. CORK IRL). Increasing the concentration of the sample did not change the composition of monomer and covalent dimer in the sample (data not shown). The SEC system was composed of an Äkta Purifier (GE Healthcare Europe GmbH, Brondby, Denmark) employing a Superdex 30 column (34 μm, 50× 950 mm, GE Healtchare Europe GmbH). Elution was carried out using a buffer prepared in Milli-Q water: 2.5 M acetic acid, 20% (*v*/*v*) acetonitrile, 100 mM NaCl, 16 mM L-arginine. The column was washed with 0.2 column volumes (CV) of the buffer before injection and eluted with 2.0 CV at a flow rate of 5 ml/min at room temperature. Absorbance was measured at 276 nm. The resulting fractions containing HMWP were stored at −18°C.

Preparative Reverse Phase Chromatography (Isolation of HMWP Species)

The system was composed of an Äkta Explorer (GE Healthcare Europe GmbH) connected to an XBridge BEH C18 column (5 μm, 10×250 mm, GE Healthcare Europe GmbH). Preparative SEC fractions (described above) were diluted 1:1 with Milli-Q water to an acetonitrile concentration of 10% (*v*/*v*) and loaded onto the column at 1.6 ml/min. Elution was carried out using buffers prepared in Milli-Q water; A: 0.09 M phosphate (Na_2HPO_4/NaH_2PO_4), 10% (*v*/*v*) acetonitrile, pH 3.6. B: 80% (*v*/*v*) acetonitrile. Elution was carried out with a flow rate of 2.5 ml/min at room temperature and absorbance was measured at 215, 254 and 280 nm. An initial test run to evaluate the number of HMWP species in isolated SEC fractions was carried out using a gradient of 25–30% B over 6 h. For preparative purification of HMWP, 100% A was used for 2 CV followed by a gradient of 0–22% B at 0.4%/min before the start of the HMWP elution gradient (specified below). The HMWP elution gradient was continued for 50–60 CV (6–8 h) and fractions were collected throughout the elution period. Isocratic elution at 32% B was used to flush remaining insulin related peptide of the column and was followed by a short wash at 80% B. The protein washed off the column was stored at 5°C and used for the next run. A total of five consecutive runs were carried out to isolate the major HMWP species. The gradients used for each of the five HMWP elution sessions were as follows: Run 1: 22–23.3% B at 0.0035%/min. Run 2: 23.3–23.7% B at 0.0017%/min. Run 3: 23.8–25.0% B at 0.0032%/min. Run 4: 25.8–27.0% B at 0.0030%/min. Run 5: 27.0–28.8% B at 0.0058%/min. In total, more than 20 peaks were separated of which 14 peaks were characterized using LC-MS/MS.

LC-MS (Identification of Molecular Mass)

1.5–3 ml of preparative RP fractions containing HMWP species were concentrated using a centrifugal membrane filter (Amicon Ultra, Ultracel® 3K, 4 ml, Regenerated cellulose, 3000 Da filter cut-off) to a volume of 300–500 μL. The sample was analyzed using an ESI source LTQ Orbitrap (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Waters

UPLC system (Waters Corporation). The UPLC system employed a CSH C18 column (1.7 μm, 1.0×150 mm, Waters Corporation) and two solvents; A: 0.1% trifluoric acid (TFA) in Milli-Q water. B: 0.1% TFA in acetonitrile. The flow was set to 0.07 ml/min using a gradient of 5–55% B over 30 min followed by column wash. The instrument was calibrated according to manufacturer's protocol and analysis was performed in positive mode using a scan range of 200–2000 *m/z*, source voltage of 4000 V, source current of 4 μA, capillary voltage of 20 V and a capillary temperature of 325°C. Results are presented as the monoisotopic mass.

LC-MS/MS (Identification of Primary Structure)

1.5–3 ml of selected preparative RP fractions containing HMWP species were concentrated using a centrifugal membrane filter (see above) to a volume of 300–500 μL. 100 μL of the concentrated sample was transferred to a UPLC vial. The samples were treated with endoproteinase Glu-C V8 enzyme (V8, Sigma-Aldrich, St. Louis, MO, USA) to digest peptide bonds N-terminal to glutamic acid residues. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, Sigma-Aldrich) was added to reduce disulphide bonds. To each sample V8 enzyme was added to a concentration of 10 μg/ml. The sample pH was adjusted to pH 7.4–7.8 by addition of 0.5 M Na_2HPO_4 buffer pH 8.5 (measured using pH paper). The sample was incubated for 17 h at 37°C and a solution of TCEP (100 mM, 0.5 M Na_2HPO_4 buffer pH 8.5) was added to a concentration of 4.8 mM before incubation for additional 30 min. Each sample was desalted using a C4 ZipTip (Millipore Corporation, Billerica, MA, USA) into 50% acetonitrile. The sample was evaporated to 50% volume using a vacuum centrifuge (EZ2, Genevac Ltd., Ipswitch, UK). Formic acid was added to the sample to a final concentration of 0.05% (*v/v*). Four standards were prepared using the procedure described above but excluding the ZipTip desalting procedure and solvent evaporation (600 nmol/ml formulations similar to Actrapid®: human insulin, human insulin + V8, human insulin + V8 + TCEP, human insulin + TCEP and Milli-Q water + V8). The samples were analyzed using an LC-MS/MS setup employing an Easy nLC system (Thermo Fisher Scientific) with a self-packed C18 column (300 mm, 1.9 μm, Reprosil, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany). Separation was achieved by a linear gradient of acetonitrile with 0.1% formic acid at a flow rate of 250 nL/min. The nLC system was coupled to an ESI source Q-Exactive MS (Thermo Fisher Scientific). Analysis was performed in positive mode using a mass range of 300–1750 *m/z*, spray voltage of 2500 V, spray current of 1–2 μA and a capillary temperature of 350°C. Theoretical MS/MS fragmentation of insulin and insulin HMWP was done using GPMAW 9.13 (Lighthouse Data, Odense, Denmark). Results are presented as the monoisotopic mass.

NanoSpray MS and MS/MS (Analysis of Zinc-Free and Scavenger Formulations)

Each sample was desalted using a C4 ZipTip into 50% acetonitrile containing 0.1% formic acid. Samples were prepared in capillary needles and lightly centrifuged before analysis. Samples were analyzed using an ESI source LTQ Orbitrap (Thermo Fisher Scientific) mounted with an off-line nanoES inlet (Thermo Fisher Scientific) using a static air pressure plunger. The following settings were used: spray voltage 1.0–1.4 kV, capillary voltage 20 V, capillary temperature 80°C, tube lens voltage 100–140 V, scan range 800–2000 *m/z*, daughter ion scan range 250–1200 *m/z*, HCD:32–35%.

Insulin Formulations with HMWP Scavengers

690 mM stock solutions of L-arginine, L-lysine, piperazine and imidazole were prepared in Milli-Q water. pH was adjusted to pH 7.4 using NaOH and HCl and solutions were sterilized by filtration (Milipore 0.22 μM filter). Vials were filled with two ml of Actrapid® and 60 μL of scavenger stock solution was added to each vial. Samples were adjusted to pH 7.35–7.45 using NaOH and HCl. The final concentration of scavenger compound was 19.6–20.0 mM and insulin concentration of 570–580 μM. Two vials were added 60 μL of Milli-Q water as control samples. The sample vials were encapsulated using a rubber membrane and metal cap. The samples were stored for 3 months at 37°C. The samples were prepared in a LAF-bench.

Zinc Free Formulations of Human Insulin

Samples were prepared as 0.6 mM human insulin, 1.6% (*w/v*) glycerol and adjusted to pH 7.4 using NaOH and HCl, corresponding to the Actrapid® formulation excluding zinc and *m*-cresol. The vials were encapsulated using a rubber membrane and metal cap. Two samples of 10 ml were prepared for each analogue and stored at 5°C and 37°C respectively. Samples were prepared in a LAF-bench.

RESULTS

Isolation of HMWP from Incubated Human Insulin

HMWP was isolated from incubated human insulin (see Materials and Methods) by using a preparative SEC method developed on the basis of the analytical SEC assay described in the Ph.Eur. (14). A typical SEC chromatogram of stored Actrapid® is shown in Fig. 1, with HMWP and insulin monomer eluting prior to *m*-cresol (Fig. 1, peak A). The Ph.Eur.

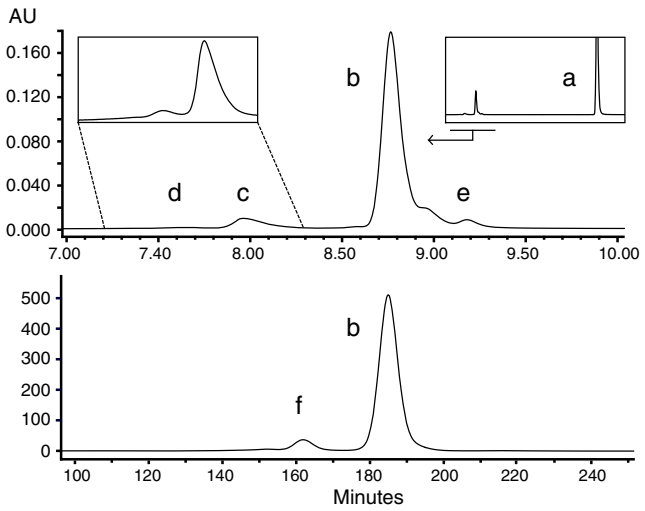


Fig. 1 Size exclusion chromatography of incubated human insulin using an analytical (*top*) and preparative (*bottom*) method. *m*-cresol (*a*); Insulin monomer (*b*); HMWP main peak (*c*); HMWP minor peak (*d*); other degradation products (not HMWP) (e); HMWP main peak used for further characterisation (*f*).

assay defines HMWP as all peaks eluting prior to the insulin monomer peak (Fig. 1, peak B). HMWP of incubated human insulin is composed of a main peak (Fig. 1, peak C, 90% of total HMWP) and a minor peak with a broad shoulder (Fig. 1, peak D, 10% of total HMWP). The total amount of HMWP comprised 5% of the total protein content in the formulation, measured using analytical HPLC-SEC and calculated as area under curve relative to an insulin standard of known concentration. The main peak isolated using preparative SEC (Fig. 1, peak F) was used for further analysis and characterization. There were no major differences detected by analytical RP-UPLC between the observed peak patterns for HMWP fractions isolated using either the preparative (Fig. 1, peak F) or analytical (Fig. 1, peak C) SEC methods, although small differences in peak-to-peak ratios were found (Fig. S2). The HMWP peak pattern of the incubated formulation was similar to formulation stored for 4 years at 5°C when analysed using a similar RP method (data not shown). The preparative SEC method was thus well suited for isolating human insulin HMWP on preparative scale as the isolated pool of HMWP was representative of the pool of HMWP identified using the Ph.Eur. assay. The collected HMWP fractions retained >90% of the original HMWP content after storage at −18°C for 30 days followed by 2 freeze/thaw cycles (data not shown).

Separation of Individual HMWP Species

The HMWP pool isolated using preparative SEC yielded >20 HMWP peaks after separation using a preliminary RP method (Fig. S3). The final preparative RP method was distributed over five consecutive runs to allow for better peak separation (Fig. 2) and produced a similar number of peaks as observed in the preliminary experiment. Fourteen of the isolated HMWP peaks were identified using LC-MS and LC-MS/MS, while the HMWP content of some fractions was too small to allow for full LC-MS identification.

Intact Mass of Human Insulin HMWP (LC-MS)

Intact monoisotopic masses of human insulin HMWP fractions were determined by LC-MS analysis (Fig. 3 and Table I). The masses of species in the major fractions were 11590.31 and 11572.31 Da. Additional fractions contained the same species with single deamidation (+0.98 Da) or N-oxalyl-valyl modification of the N-terminus of the B-chain (−75.08 Da) (8). Non-covalent human insulin dimer has a molecular monoisotopic mass of 11607.28 Da (with intact disulphide bonds). The masses of the majority of the insulin HMWP species was thus 16.97 or 34.97 Da lower compared to the non-covalent insulin dimer.

Identification of the Primary Structure of Human Insulin HMWP (LC-MS/MS)

The primary structure of the insulin HMWP species was determined by incubating the samples with V8 and TCEP before LC-MS/MS analysis. The theoretical V8 + TCEP digestion and reduction of human insulin is shown in Fig. 4. Comparing the total ion current (TIC) chromatograms of an insulin standard and HMWP samples revealed a unique type of V8-peptide in the HMWP samples (Fig. 5). These unique peptides were observed at t=12.4 min, t=25.5 min and t=27.0 min (Fig. 5b). These peptides were later identified to contain covalent links between the A-II and B-III peptides of two insulin monomers (t=12.4 min), A-chain covalently linked to B-III peptide (t=27.0 min) and A-chain covalently linked to B-chain (t=25.5 min). All these unique peptides had a mass corresponding to a 17 Da loss compared to the masses of the constituent A-chain and B-chain pepties. The t=25.5 min and t=27.0 min originate from incomplete digestion of the HMWP. The free A-II and A-III peptides were not found in the HMWP sample or in the insulin reference. The monoisotopic mass of the A-II + B-III peptide (t=12.4 min) was 1610.72 or 1592.70 Da, depending on the intact mass of the HMWP species (Fig. S4). One of the two forms of the A-II-B-III peptide was identified in all of the analysed HMWP species. The masses of the identified A-II-B-III peptides corresponded to a net loss in molecular mass of 17.04 or 35.05 Da compared to the constituting peptides A-II and B-III and thus matches the mass difference between intact HMWP and a non-covalent insulin dimer (Fig. 3). MS/MS data confirmed that part of the A-II-B-III peptide was composed of the C-terminal parts of the insulin A- and B-chain (Fig. S5)

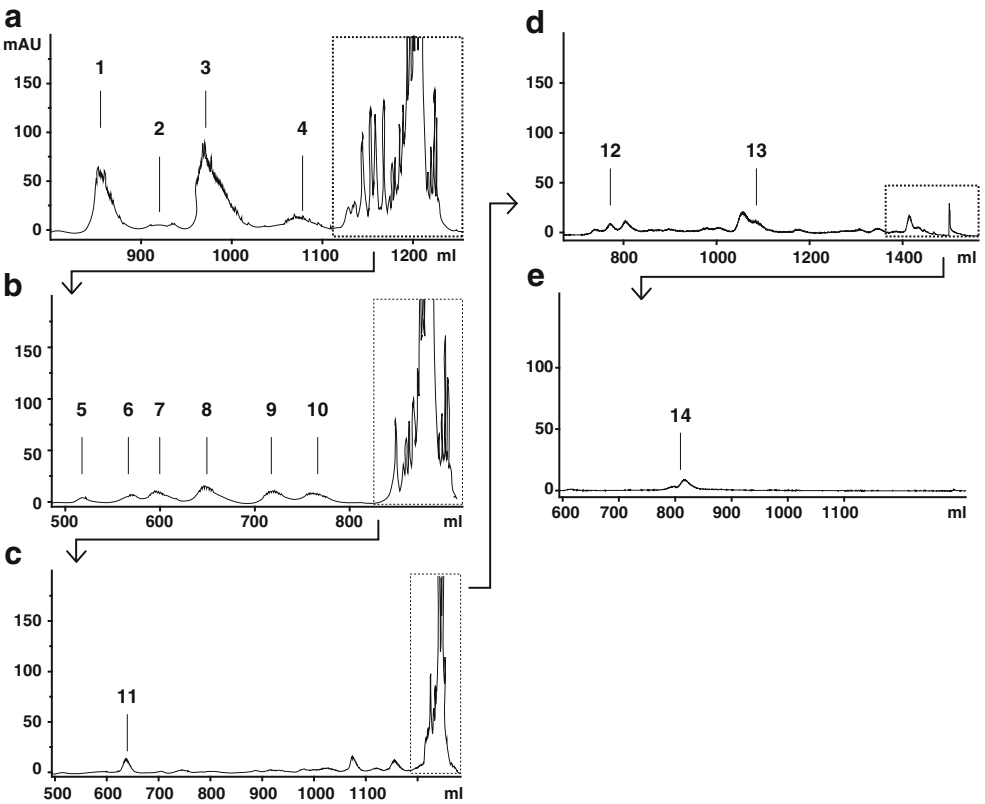


Fig. 2 Preparative RP chromatography (**a**–**e**) of the preparative SEC fraction containing HMWP (Fig. 1, peak f). *Dashed line boxes* mark material washed off the column and re-loaded onto the column for the next chromatographic run. *Numbered peaks* were identified and are listed in Table I.

A proposed mechanism for the formation of covalent insulin dimers was constructed on the basis of a reactive anhydride intermediate formed at A21Asn, described by Carpenter (27) and Darrington and Anderson (21) (Fig. 6).

The initial formation of the anhydride intermediate would result in a mass loss of 17.03 Da (NH_3). Upon formation of the reactive anhydride intermediate, two possible reactions can occur. Addition of water would result in a deamidation reaction and possibly racemization to D-Asp. A nucleophilic attack on the anhydride intermediate by an amino group would form a covalent cross link between two insulin molecules. The resulting covalent dimer could re-arrange, forming a cyclic ring (succinimidyl) at the A21Asn interface, resulting in a loss of water (−18.01 Da) and a total mass loss of 35.04 Da compared to two insulin monomers. A single-step transamidation reaction without the involvement of the

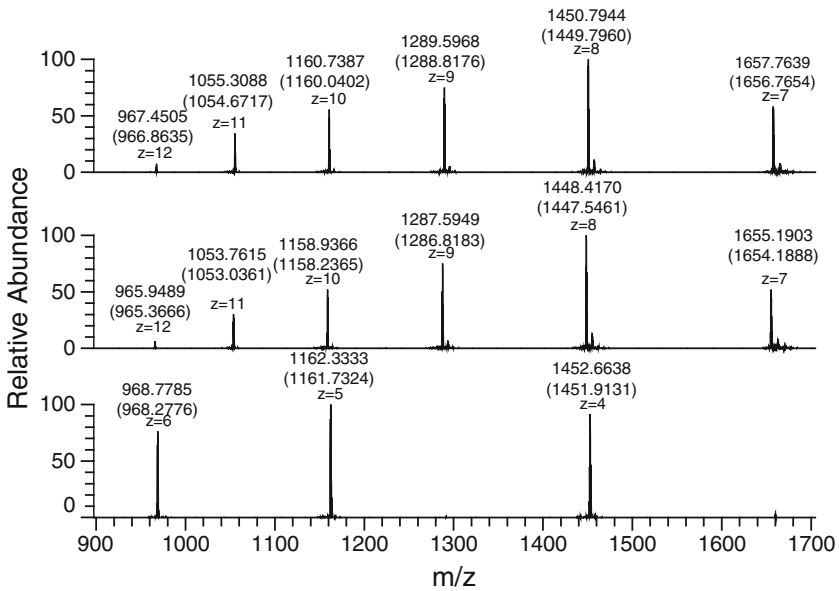


Fig. 3 MS spectra of intact HMWP and human insulin. Spectra are shown for two types of HMWP and a human insulin control in the mass range 900–1700*m/z*. HMWP 11590.31 Da (*top*); HMWP 11572.31 Da (*middle*); Human Insulin monomer 5803.62 Da (*bottom*). Average mass is indicated with monoisotopic mass in *parenthesis*.

Table I Identified HMWP Species Isolated from Incubated Human Insulin Formulation Using Preparative Reverse Phase Chromatography (Fig. 2)

Group[a]	Monoisotopic mass (Da)	Identified primary structure and modifications	Peak # (Fig. 2)	% of total HMWP[b]			
				Species	Group[a]	Type[c]	Sum
A	11590.3	Open	3	23			
			5	0.65			
			10	2.4	33		
			12	1.1			
			13	6.1			
B	11591.3	Open, B3 deamidation	4	4.3		46	
			8	3.1	9.5		
			11	2.1			68
C	11515.1	Open, DesPhe(B1)-N-oxalyl-Val(B2)	6	1.4			
			7	0.85	3.7		
			14	1.4			
D	11572.3	Closed	1	13	13		
E	11573.3	Closed, B3 deamidation	2	3.1			
			4	2.6	9.3	22	
			7	1.4			
			9	2.2			

[a] Groups are based on molecular mass and identified primary structure

[b] Calculated as peak area % (Fig. 2) relative to the total HMWP content (Fig. 1)

[c] Types are based on the structure of the covalent link (open/closed)

A21Asn anhydride intermediate would result in the loss of water (−18.01 Da) and could not alone explain the presence of the −17.04 and −35.05 Da HMWP species.

The covalent link within the A-II-B-III peptide was located to A21Asn and B29Lys by comparing the MS/MS data to the theoretical fragmentation pattern of a proposed structure of the A-II-B-III peptide with the A21Asn in either the extendend conformation or its succinimidyl form (Fig. 7).

A mass difference of 18.01 Da was observed for fragments including the covalent link, supporting the hypothesis of two forms of HMWP with the A21Asn in either the extended conformation or in the succinimidyl form.

Thus, the masses of the majority of the insulin HMWP species were found to be 17.04 or 35.05 Da lower than a non-covalent insulin dimer corresponding to elimination of NH_3 (expected mass loss of 17.03 Da) or NH_3 and H_2O (expected mass loss of 35.04). MS/MS fragmentation of HMWP species were consistent with HMWP species formed by a nucleophilic attack of the B29Lys free amino group on an A21Asn anhydride intermediate. The resulting covalent dimer was found in open and closed states—the latter being formed by elimination of H_2O following the initial formation of the covalent link.

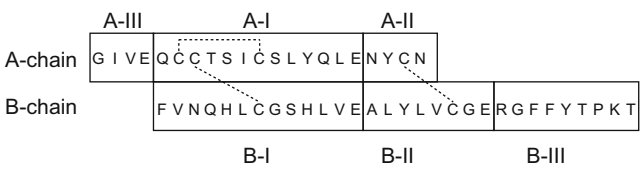


Fig. 4 Theoretical human insulin peptides after digestion and reduction with V8 and TCEP. Peptides labelled according to chain and peptide number (e.g. B-I = B-chain peptide I).

Identification of HMWP Species with B3 Deamidation or DesPhe(B1)-N-oxalyl-Val(B2) Modification (LC-MS/MS)

Some HMWP species also contained deamidations located at the B-I peptide (Fig. S6). The deamidation was found to be located at position B3 on the basis of MS/MS data. The fragmentation pattern of sequences including B3 of the B-I peptide was shifted +1 Da compared to the human insulin reference (Fig. S7).

The DesPhe(B1)-N-oxalyl-Val(B2) modification of the N-terminal end of the B chain was identified in certain HMWP species, resulting in 75 Da loss from the B-I peptide (Fig. S8).

The MS/MS data indicated that CO_2 was lost during fragmentation of the modified B-I peptide in the instrument. Comparing the insulin standard and HMWP sample, the MS/MS data showed a peak pattern with $119 m/z$ ($z=1$) difference between the two samples, corresponding well with the presence of a DesPhe(B1)-N-oxalyl-Val(B2) modification (−75 Da) with an additional loss of CO_2 (43.99 Da) during instrument fragmentation (Fig. S9).

All the identified HMWP species are listed in Table I, with references to their respective position in the preparative RP chromatogram (Fig. 2).

In summary, 16 species of HMWP found in Actrapid® were identified as A21Asn to B29Lys covalent dimers. Two main types exist with the covalent link in either the open or closed form. Furthermore, multiple species were deamidated at B3Asn or having the DesPhe(B1)-N-oxalyl-Val(B2) modification. Several possibilities for isomerisation can account for the observed identical masses as discussed later.

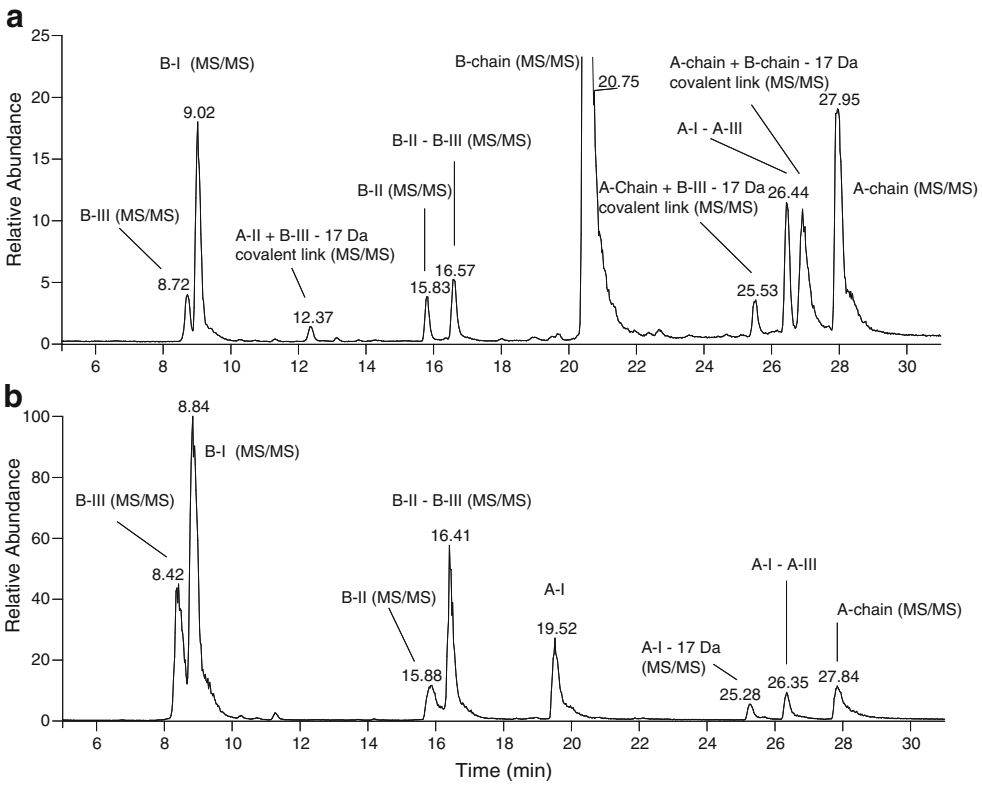


Fig. 5 LC-MS TIC of V8 and TCEP treated HMWP (**a**) and insulin standard (**b**), 5–31 min. Peaks labeled according to V8-fragments shown in Fig. 4. Peaks identified using MS/MS data are labelled accordingly.

Addition of Amine Scavengers Reduces HMWP Formation

It was tested whether addition of amine compounds to the formulation could reduce the formation of HMWP by competing with the B29Lys side-chain amine for reaction with the A21Asn intermediate. L-lysine, L-arginine or piperazine reduced the rate of HMWP formation in formulations of human insulin during 3 months of storage at 37°C (Fig. 8).

Imidazole did not reduce the rate of HMWP formation compared to the control. The presence of human insulin covalently linked to L-arginine, L-lysine or piperazine was detected by LC-MS (Figure S10). Masses corresponding to

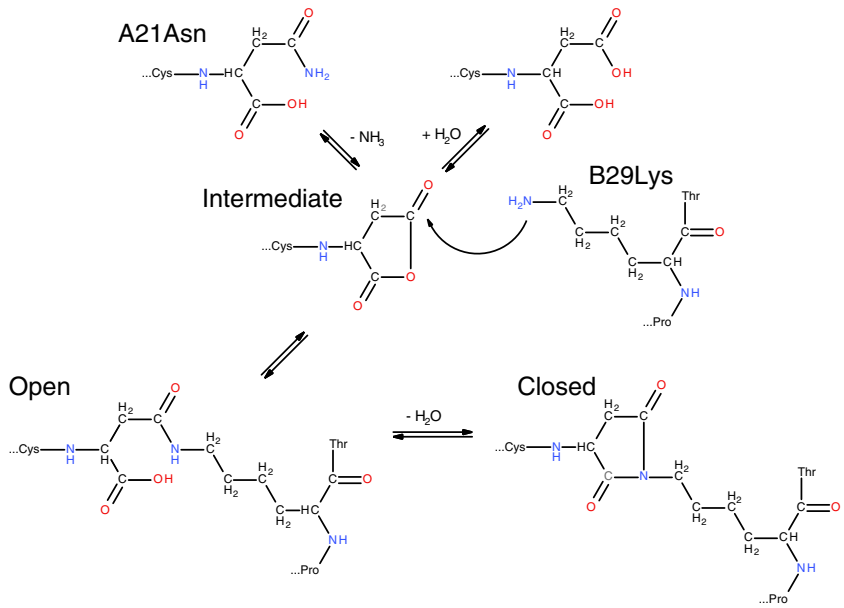
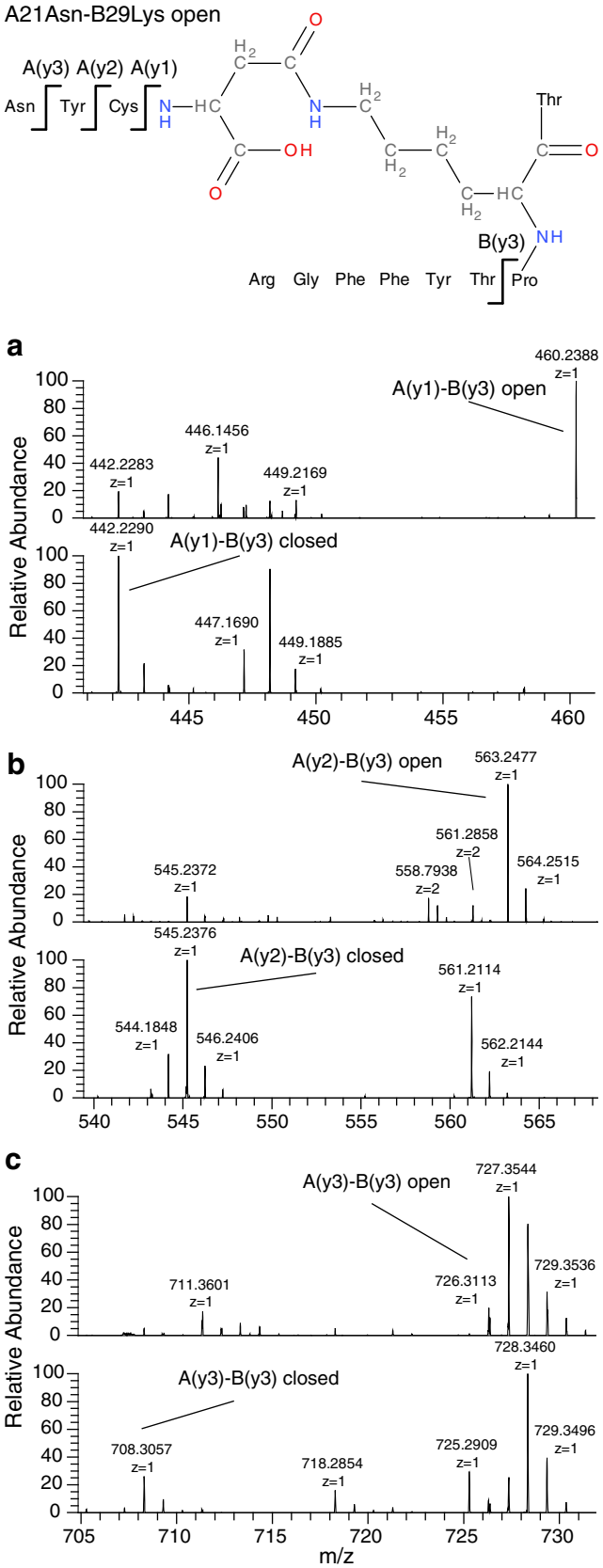


Fig. 6 Proposed mechanism for the formation of a covalent insulin dimer *via* A21Asn and a free amine group, examplified using B29Lys. Only the C-teminal ends of the two involved monomers are shown. The reaction has two possible end products with the A21Asn in either the extended conformation (open) or the succinimidyl form (closed) (modified from (21)).



◀ **Fig. 7** MS/MS fragmentation of A-II-B-III species in its open form, parent ion 537.87 *m/z* (*top*) and closed form, parent ion 531.91 *m/z* (*bottom*) obtained from samples of HMWP with intact mass of 11,590 and 11,572 Da respectively. *Top insert*: proposed structure of the A21Asn to B29Lys covalent link. Mass range 441–461 *m/z* (**a**); 540–568 *m/z* (**b**); 705–731 *m/z* (**c**).

simple transamidation reactions (−18 Da) could not be identified in the LC-MS data.

Nanospray MS and MS/MS was carried out to identify the location of the covalent link between human insulin and L-arginine or L-lysine (Fig. S11). Figure 9 shows the data confirming that arginine and lysine is linked to human insulin at A21Asn, presumably reacting with the anhydride intermediate and thus preventing nucleophilic attack by B29Lys.

Insulin Analogues

Two insulin analogues were synthesized with the objective of creating insulin analogues without the reactive groups (A21Asn and/or free amino groups) suggested to be involved in the formation of the majority of the HMWP species. By protecting the free amino group of the B29Lys side-chain by dimethylation, the nucleophilic attack of the amino group on the A21Asn intermediate is prevented. N-terminals were also dimethylated. Analogue 2 had Asn21Gly mutation in addition to the dimethylations.

Throughout the storage period, the analogues showed a lower rate of HMWP formation compared to the control sample (Fig. 10). The HMWP formation rate was similar for both analogues.

Thus, the observed reductions in HMWP formation rates following addition of amine scavengers or protection of the reactive A21Asn and free amino groups is consistent with the pathways suggested based on the MS/MS data. However, the fact that HMWP formation was not completely eliminated suggests other pathways not involving A21Asn or free amino groups.

Zinc Free Formulations of Human Insulin

As shown in Fig. 11, the formulation without zinc and *m*-cresol displayed a different peak pattern compared to control

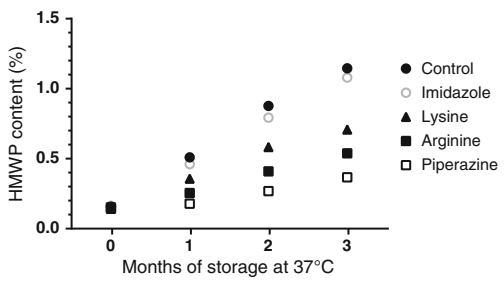


Fig. 8 HMWP content (mol%) of scavenger formulations of human insulin during storage, analysed by analytical SEC ($n = 1$). Actrapid® stored at 5°C for 1 year prior to the experiment was used as control.

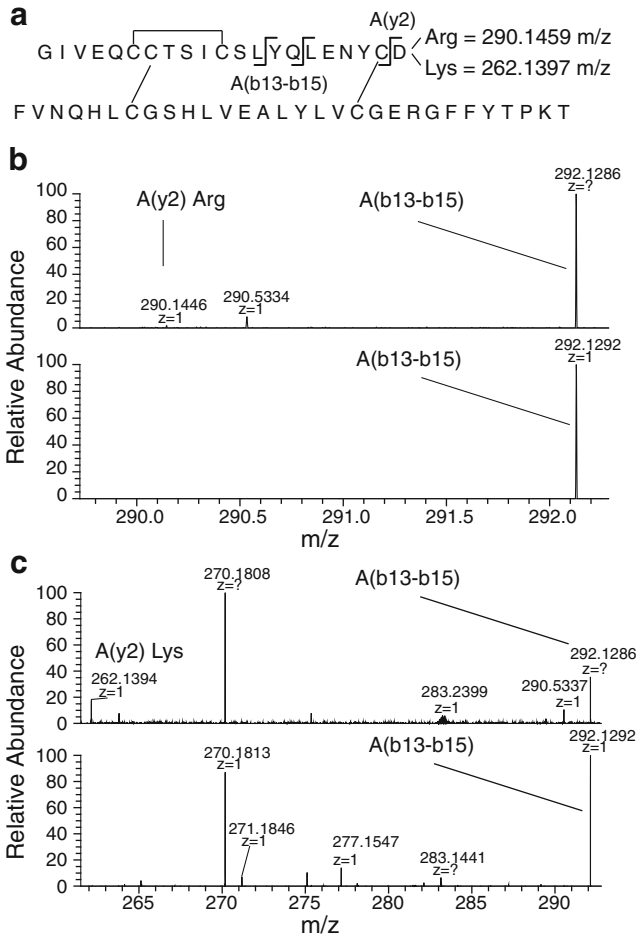


Fig. 9 MS/MS of human insulin linked to arginine or lysine *via* A21 anhydride intermediate (**a**). Data was obtained from un-digested sample of scavenger formulation of human insulin + arginine (**b**) and human insulin + lysine (**c**). Insulin + scavenger (*top*); insulin reference (*bottom*).

formulation. A significant increase in monomeric degradation products was observed (a, t = 26–27 min) and the pattern of HMWP species was also different compared to the control (b). In control samples, the major HMWP species (11590.3 Da, Table I, peak 3) eluted at 56 min. In formulation without zinc and *m*-cresol the peak at 56 min is significantly smaller, but accompanied by an overall increase of other peaks (*e.g.* at 50–54, 60–65 and 74–77 min).

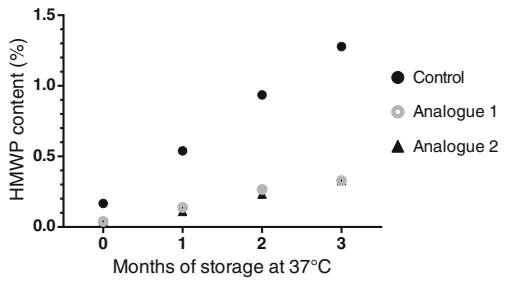


Fig. 10 HMWP content (mol%) of insulin analogue formulations stored for 3 months at 37°C. HMWP content analysed by analytical SEC ($n = 1$). Actrapid® stored at 5°C for 1 year prior to incubation at 37°C was used as control.

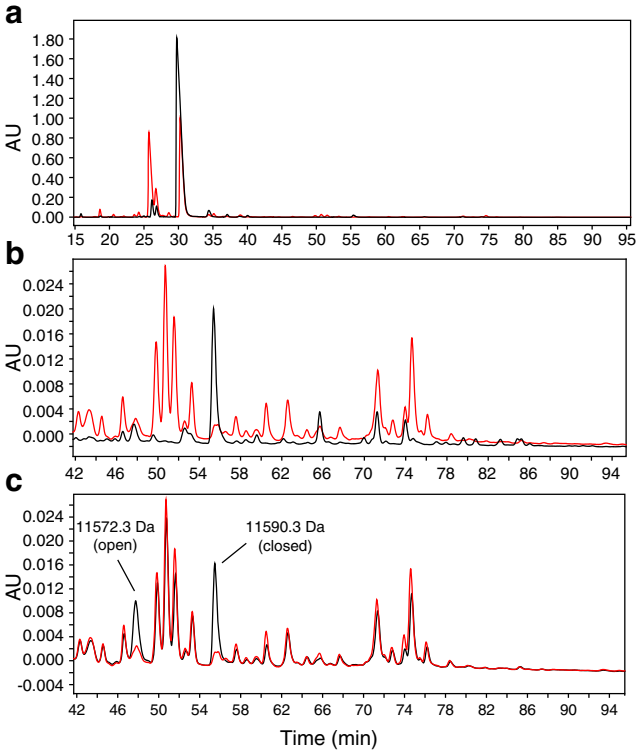


Fig. 11 Analytical RP-UPLC analyses of human insulin formulations with or without zinc and *m*-cresol. (**a**) without zinc and *m*-cresol (*red*), with zinc and *m*-cresol (*black*). (b) without zinc and *m*-cresol (*red*), with zinc and *m*-cresol (*black*), HMWP zoom: 42–96 min. (c) without zinc and *m*-cresol (*red*), without zinc and *m*-cresol spiked with two identified HMWP species (*black*), HMWP zoom: 42–96 min.

Spiking the zinc and *m*-cresol free sample with two identified HMWP species (open and closed form, peak 1 and 3, Table I) resulted in increased peak height at 48 and 56 min, thus confirming the identity of the two peaks (c). This demonstrates that a lack of zinc and *m*-cresol leads to a different pool of HMWP species compared to those found in Actrapid®.

DISCUSSION

Isolation and Characterization of HMWP

Methods for isolating human insulin HMWP on a preparative scale have been developed and detailed mass spectrometry analysis was performed to determine the primary structure of the isolated HMWP species. As HMWP formation at 5°C is slow (<1 mol% after 2 years (14)), detailed characterization of HMWP formed at 5°C is practically impossible (28). Accelerated HMWP formation at temperatures higher than 5°C is required for a full characterization of HMWP involving preparative isolation of HMWP material. Incubating human insulin formulation at 37°C respects the recommendations of staying 10–20°C below the melting temperature of insulin

(29,30) and provided an adequate qualitative representation of the HMWP pool formed during shelf-life at 5°C. The preparative SEC method was successfully up scaled from the described method in Ph.Eur. Although SEC methods using acidic, non-physiological buffer are poor at characterizing non-covalent association states of insulin in formulation (26), the method was fit for the purpose of isolating covalent aggregates of insulin (HMWP). The preparative RP method was successful in separating >20 species of HMWP. This is to our knowledge the first time that individual HMWP species of human insulin have been purified on a preparative scale. The analytical RP method was suitable for determining the content of selected HMWP species across different formulations of human insulin.

HMWP formed in human insulin formulations incubated at 37°C was composed of dimer species covalently cross-linked *via* A21Asn and B29Lys. Two primary forms of these dimers were identified, with A21Asn in either the extended conformation (open form) or as succinimidyl (closed form). Variations involving B3Asn deamidation or DesPhe(B1)-N-oxalyl-Val(B2) modification were also identified. The fact that only A21Asn to B29Lys dimers were observed is surprising compared to previous studies on insulin HMWP by Darrington *et al.* and Brange *et al.*, which identified dimers formed *via* A21Asn and B1Phe (N-terminal) (15,21,22,28). This discrepancy must be related to differences between the studies in terms of formulation conditions at which the insulin was incubated. The primary work on the identification of HMWP by Brange *et al.* was performed on formulations of porcine insulin containing zinc as either crystalline or amorphous insulin suspensions. Brange *et al.* noted that the HMWP formation varied only slightly by the species of insulin, but varied with the composition of the formulation (15). Darrington *et al.* did not work with pharmaceutical formulations of human insulin, but used soluble acidic formulations (pH 2–5) which did not contain zinc. Thus, the formulation investigated in this study differs from previous studies by containing human insulin in combination with zinc as a soluble formulation at pH 7.4. The differences in the chemical structure of HMWP found in this study compared to previous studies are an indication of HMWP formation pathways being very formulation specific. This might be correlated to formulation pH and the effects of zinc and phenolic ligands on the tertiary structure of insulin in hexamer formation, which will be discussed below.

As presented in Table I, several HMWP species were of identical molecular weight and identical primary structure (*i.e.* A21Asn to B29Lys, open/closed) with possible deamidations at B3 or DesPhe(B1)-N-oxalyl-Val(B2) modifications. HMWP species of identical mass were observed at different retention times in the preparative RP chromatogram (Fig. 2). The elution order of peak 1–4 follows the expected elution behaviour of the open and closed form along with the respective deaminated forms. However, the elution order of peak 5–13 does not exhibit a clear correlation with the identified primary structure. Continuous column bleeding of previously eluted species as a result of gradient interruption and reloading of material can be excluded, as a similar number of peaks were observed in the initial RP test run (Fig. S3) and the analytical RP method (Fig. S2). The secondary/tertiary structure and hydrophobic surface area available for column binding might be influenced by the possibility for different isomeric forms of each HMWP species. The open form of the A21Asn to B29Lys link can exist as either the Asp or isoAsp form, depending on the point of attack by the B29Lys amino group on the two available carbonyl groups in the anhydride intermediate. Darrington and Anderson (31) found that aniline addition to the A21Asn anhydride intermediate favoured the isoAsp derivative (aniline linked to side-chain carbonyl) over the Asp derivative with a ratio of 2.7:1. This is close to the reported 3:1 ratio of IsoAsp/Asp reported for deamidation of in-chain asparagines (32). In HMWP group A, the ratio of peak 3 to peak 13 is 3.8, suggesting that peak 3 is composed of the isoAsp isomer and peak 13 of the Asp isomer. Peak 10 and 12 in group A could be composed of D-Asp/D-isoAsp isomers, but the low content does not allow for more detailed characterization of these species. HMWP group B includes the open form of the covalent dimer link with deamidations at B3. As deamidation at B3 can be located at either one the two involved B-chains, this allows for additional isomeric species in addition to the D/L-Asp/IsoAsp isomerization mentioned above. The same applies for group C, where the DesPhe(B1)-N-oxalyl-Val(B2) modification can be located at either one of the two B-chains.

Group D is composed of a single identified species, with the covalent link in the closed form. Group E is composed of the closed covalent link with single B3 deamidations. The ratio of 2.1 between the open (group A, B and C) and the closed HMWP dimer form (group D and E) shows a higher prevalence of the open form compared to the closed form. A similar mixture of D/L-Asp/IsoAsp species and adducts has been found in lyophilized model peptides including asparagine (23). The wide range of possible isomers and their unknown influence on the secondary/tertiary structure can explain the presence of HMWP species with identical primary structure accompanied by different retention times during preparative RP isolation. The succinimide product of the proposed cross-link reaction (closed form, Fig. 6) could potentially undergo further cross-linking, forming a covalent trimer. Oligomeric aggregates might be present in the formulation but was not detected in the investigated portion of formed HMWP. The potential content of such covalent oligomers would supposedly be too low for purification and detailed identification. Steric hindrance at the succinimide, as being part of an insulin-insulin crosslink, will likely make further cross-linking unfavorable.

Methods for Reducing HMWP Formation

HMWP formation rate was shown to be reduced by addition of scavenger compounds. Addition of L-arginine, L-lysine and piperazine significantly reduced the HMWP formation rate in incubated human insulin. Furthermore, covalent adducts of the scavenger with insulin were identified, consistent with the proposed mechanism of dimer formation and confirmed the proposed pathway *via* the A21Asn anhydride intermediate which is open for nucleophilic attacks by amino groups. L-arginine, L-lysine and piperazine thus act as scavengers by competing with B29Lys for the reactions with the A21Asn anhydride intermediate. The scavengers were added in a molar surplus compared to insulin, mimicking the aniline trapping experiment performed by Darrington *et al.* (21), which established the A21Asn anhydride as the rate-limiting step in HMWP formation and deamidation reactions under acidic conditions. The effect of each scavenger compound on the formation of HMWP cannot be explained exclusively by the pK_a values of each respective compound. As only unprotonated amines can react by a nucleophilic attack on the A21Asn anhydride to form a covalent link, amine scavengers with amine pK_a closest to the pH of the formulation (pH 7.4) should be more reactive. Piperazine (pK_a 9.9, 5.8) and imidazole (pK_a 7.0) should be more reactive than arginine (pK_a 12.5, 9.0) and lysine (pK_a 10.5, 9.0). However, imidazole was not able to reduce HMWP formation and piperazine proved most effective. Other parameters such as steric hindrance could influence the reactivity of each scavenger compound. Amine scavengers such as ethylene-diamine have previously been shown to reduce HMWP formation of insulin aspart (33).

Formulations of two insulin analogues also showed reduced rate of HMWP formation and confirmed the involvement of A21Asn in the cross-linking reaction. Analogue 1 is methylated at N-terminals and at B29Lys, preventing dimerization *via* A21Asn and B29Lys and thereby reducing HMWP formation according to the proposed mechanism of HMWP formation. Analogue 2 was similar to analogue 1 but also included a A21Gly mutation, preventing the formation of the A21Asn anhydride intermediate. The two insulin analogues had reduced HMWP formation during storage compared to human insulin although HMWP formation was not completely prevented. The HMWP content of the analogue formulations (relative to control) was similar to the fraction of insulin HMWP that remains un-identified in this study (30%). It appears likely that the HMWP formed in the insulin analogue formulations are composed of HMWP species similar to the un-identified species of human insulin HMWP formed during storage. The HMWP formed in formulations of analogue 1 and 2 must be explained by mechanisms not involving A21Asn, B29Lys or N-terminals, *e.g.* B3Asn forming a cyclic succinimidyl intermediate reacting with basic amino acids such as Arg and/or His or cross linking *via* disulphide shuffling. Species of dityrosine-based cross-linking by metal-catalyzed oxidation mechanisms is also a possibility (24). Similar results in terms of deamidation rate and dimer formation have been shown for A21-substituted analogues in acidic solutions (34).

Influence of Formulation Properties on HMWP Formation

Altering the Actrapid® formulation to exclude zinc and *m*-cresol resulted in a significantly different pool of HMWP species as observed by RP-UPLC (Fig. 11b). Differences in the HMWP region of RP chromatograms as well as increased formation of monomeric degradation products were observed in samples without zinc and *m*-cresol. The two major species of HMWP found in Actrapid® were no longer the dominating HMWP species in the zinc and *m*-cresol free formulation. Brems *et al.* reported that zinc free formulations of human insulin stored at 45°C formed high molecular weight polymers (disulphide scrambling) as the main component of HMWP in addition to covalent dimers which were not studied in detail (20). No disulphide scrambling dimers were identified in this study, although disulphide scrambling polymers might exist in the HMWP peak not characterized in this study (peak D, Fig. 1).

Hexamer formation, induced and stabilized by zinc and phenolic ligands, is known to stabilize human insulin towards chemical degradation (35–37) and increase thermal stability (30). The insulin hexamer is denoted either R or T depending on the structural conformation of the B1–B8 segment. In the T state, the B1–B8 segment is fully extended. In the R state, induced by the binding of phenolic ligands, the B1–B8 segment adopts an α-helical conformation (38). It is possible that the R_6 state of the human insulin hexamer in Actrapid® both reduces B3 deamidation and reduces the formation rate of HMWP species involving B1Phe, resulting in the pathway involving B29Lys to become predominant. This could explain the presence of B1Phe to A21Asn dimers identified by Brange *et al.* as the zinc-containing suspensions investigated in the study did not contain any phenolic ligands (*e.g. m*-cresol, phenol) but used methylparabene as preservative. The fact that the A21Asn to B1Phe dimers identified by Anderson *et al.* could not be identified in this study can also be explained by differences in the examined formulations, as Anderson *et al.* examined zinc-free formulations of human insulin under monomer/dimer conditions at pH 2–5 (21,22). It can be speculated that monomer/dimer conditions might provide alternative pathways of HMWP formation through collision between monomers and dimers otherwise restricted by a tight hexameric structure in the presence of zinc. The mechanism for asparagine deamidation is pH dependent (39). At pH < 3, deamidation is acid catalyzed resulting in aspartate. At pH >

4, succinimide or anhydride intermediate formation results in a mixture of aspartate and isoaspartate. In this study, the involved asparagine at A21 is located C-terminally and an anhydride is formed as the intermediate step in cross-linking reaction. This study exclusively identified covalent dimers formed *via* an initial loss of NH_3 (17 Da), indicating the formation of an anhydride. Darrington and Anderson established that cross-linking *via* A21Asn to N-terminals declined with increasing pH (pH > 4), as the formation rate of the anhydride intermediate decreases and the nucleophilic attack of the N-terminal or B29Lys amino group reaches a plateau with increasing pH, probably influenced by ionizable neighboring amino acids (22). It is clear, that the effect of pH on anhydride formation and ionization of N-terminals or side chain amines will have an impact on the type of formed HMWP.

Brange *et al.* also reported that HMWP isolated from aged neutral zinc suspensions of crystalline or amorphous porcine insulin was primarily A21Asn to B1Phe transamidation dimers, as shown by PAGE analysis and amino acid analysis (15). This suggests that the physical presence of human insulin in formulation (*i.e.* soluble, crystalline and amorphous) is also a factor influencing the formation of HMWP. A21Asn to B1Phe dimers might be present in the Actrapid® formulation, but at too low levels to allow for identification by LC-MS/MS.

It is plausible that the identified dimeric HMWP species linked *via* A21Asn and B29Lys are unique to the Actrapid® formulation or general neutral hexameric formulations of human insulin and that other pathways rule the formation of major HMWP species in different formulations of human insulin, *e.g.* without zinc or *m*-cresol. We believe that the structure of human insulin in formulation (*i.e.* dimer/hexamer) is an important factor for establishing the governing HMWP formation pathway. We plan to investigate the tertiary structure of the described HMWP species and their role in hexamer formation in a future study. While the results presented in this study are unique to the investigated formulation, other pharmaceutical proteins subject to deamidation reactions during storage could potentially form covalent aggregates *via* intermediate succinimide or anhydride formation as part of the deamidation pathway. As this study shows, detailed characterization of covalent aggregates requires several preparative and analytical techniques, but provides a better understanding of the complex interplay between formulation properties and chemical stability in pharmaceutical protein formulations.

CONCLUSION

The majority (>68%) of the HMWP formed during storage of human insulin in Actrapid® formulation at 37°C was identified as dimeric transamidation products in a pool consisting of >20 species. The identified species contained B29Lys ε-amino group attached to the deamidated form of A21Asn, where A21Asn was either in extended conformation (open form) or formed a succinimidyl ring (closed form). HMWP species also included AsnB3 deamidations and DesPhe(B1)-N-oxalyl-Val(B2) modifications. Piperazine, L-lysine or L-arginine were found to reduce HMWP formation, by forming an amide bond with the side chain of deamidated A21Asn and thus preventing the side chain of B29Lys to react to the same site. Insulin analogues with methylated N-terminals and Asn21Gly mutation also showed reduced rate of HMWP formation, confirming the proposed mechanism of HMWP formation. Formulation of human insulin without zinc and *m*-cresol resulted in a different pool of HMWP and exemplifies the influence of formulation properties on the HMWP formation pathway in insulin formulations.

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REFERENCES

1. Wang W. Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int J Pharm. 1999;185(2):129–88.
2. Frokjaer S, Otzen DE. Protein drug stability: a formulation challenge. Nat Rev Drug Discov. 2005;4(4):298–306.
3. Hermeling S, Crommelin DJ, Schellekens H, Jiskoot W. Structure-immunogenicity relationships of therapeutic proteins. Pharm Res. 2004;21(6):897–903.
4. Manning MC, Chou DK, Murphy BM, Payne RW, Katayama DS. Stability of protein pharmaceuticals: an update. Pharm Res. 2010;27(4):544–75.
5. Hermeling S, Schellekens H, Maas C, Gebbink MF, Crommelin DJ, Jiskoot W. Antibody response to aggregated human interferon alpha2b in wild-type and transgenic immune tolerant mice depends on type and level of aggregation. J Pharm Sci. 2006;95(5):1084–96.
6. van Beers MM, Sauerborn M, Gilli F, Brinks V, Schellekens H, Jiskoot W. Oxidized and aggregated recombinant human interferon beta is immunogenic in human interferon beta transgenic mice. Pharm Res. 2011;28(10):2393–402.
7. Doyle HA, Gee RJ, Mamula MJ. Altered immunogenicity of isoaspartate containing proteins. Autoimmunity. 2007;40(2):131–7.
8. Jars MU, Hvass A, Waaben D. Insulin aspart (Asp^{B28} human insulin) derivatives formed in pharmaceutical solutions. Pharm Res. 2002;19(5):621–8.
9. Carpenter JF, Randolph TW, Jiskoot W, Crommelin DJ, Middaugh CR, Winter G, *et al.* Overlooking subvisible particles in therapeutic

protein products: gaps that may compromise product quality. J Pharm Sci. 2009;98(4):1201–5.
10. Jiskoot W, Randolph TW, Volkin DB, Middaugh CR, Schöneich C, Winter G, *et al.* Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. J Pharm Sci. 2012;101(3):946–54.
11. Singh SK, Afonina N, Awwad M, Bechtold-Peters K, Blue JT, Chou D, *et al.* An industry perspective on the monitoring of subvisible particles as a quality attribute for protein therapeutics. J Pharm Sci. 2010;99(8):3302–21.
12. Singh SK. Impact of product-related factors on immunogenicity of biotherapeutics. J Pharm Sci. 2011;100(2):354–87.
13. Rosenberg AS. Effects of protein aggregates: an immunologic perspective. AAPS J. 2006;8(3):501–7.
14. Insulin human. European pharmacopoeia. 7 ed. Strasbourg: Council of Europe; 2013
15. Brange J, Hallund O, Sørensen E. Chemical stability of insulin. 5. Isolation, characterization and identification of insulin transformation products. Acta Pharm Nord. 1992;4(4):223–32.
16. Maislos M, Mead PM, Gaynor DH, Robbins DC. The source of the circulating aggregate of insulin type I diabetic patients is therapeutic insulin. J Clin Invest. 1986;77(3):717–23.
17. Schernthaner G. Immunogenicity and allergenic potential of animal and human insulins. Diabetes Care. 1993;16(3):155–65.
18. Ratner ER, Phillips TM, Steiner M. Persistent cutaneous insulin allergy resulting from high molecular weight insulin aggregates. Diabetes. 1990;39(6):728–33.
19. Robbins DC, Mead PM. Free covalent aggregates of therapeutic insulin in blood of insulin-dependent diabetics. Diabetes. 1987;36(2):147–51.
20. Brems DN, Brown PL, Bryant C, Chance RE, Green LK, Long HB, *et al.* Improved insulin stability through amino acid substitution. Protein Eng. 1992;5(6):519–25.
21. Darrington RT, Anderson BD. 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. 1995;84(3):275–82.
22. Darrington RT, Anderson BD. Effects of insulin concentration and self-association on the partitioning of Its A-21 cyclic anhydride intermediate to desamido insulin and covalent dimer. Pharm Res. 1995;12(7):1077–84.
23. DeHart MP, Anderson BD. Kinetics and mechanisms of deamidation and covalent amide-linked adduct formation in amorphous lyophiles of a model asparagine-containing peptide. Pharm Res. 2012;29(10):2722–37.
24. Torosantucci R, Mozziconacci O, Sharov V, Schöneich C, Jiskoot W. Chemical modifications in aggregates of recombinant human insulin induced by metal-catalyzed oxidation: covalent cross-linking via michael addition to tyrosine oxidation products. Pharm Res. 2012;29(8):2276–93.
25. Printz M, Friess W. Simultaneous detection and analysis of protein aggregation and protein unfolding by size exclusion chromatography with post column addition of fluorescent dye BisANS. J Pharm Sci. 2012;101(2):826–37.
26. Tantipolphan R, Romeijn S, Engelsman J, Torosantucci R, Rasmussen T, Jiskoot W. Elution behavior of insulin on high-performance size exclusion chromatography at neutral pH. J Pharm Biomed Anal. 2010;52(2):195–202.
27. Carpenter FH. Relationship of structure to biological activity of insulin as revealed by degradative studies. Am J Med. 1966;40(5):750–8.
28. Brange J, Havelund S, Hougaard P. Chemical stability of insulin. 2. Formation of higher molecular weight transformation products during storage of pharmaceutical preparations. Pharm Res. 1992;9(6):727–34.
29. Hawe A, Wiggenhorn M, Van de Weert M, Garbe JHO, Mahler HC, Jiskoot W. Forced degradation of therapeutic proteins. J Pharm Sci. 2012;101(3):895–913.
30. Huus K, Havelund S, Olsen HB, Van de Weert M, Frokjaer S. Thermal dissociation and unfolding of insulin. Biochemistry. 2005;44(33):11171–7.
31. Darrington RT, Anderson BD. The role of intramolecular nucleophilic catalysis and the effects of self-association on the deamidation of human insulin at low pH. Pharm Res. 1994;11(6):784–93.
32. Geiger T, Clarke S. Deamidation, isomerization, and racemization at asparaginyl and aspartyl residues in peptides. Succinimide-linked reactions that contribute to protein degradation. J Biol Chem. 1987;262(2):785–94.
33. Poulsen C, Jacobsen D, Palm L. Effect of ethylenediamine on chemical degradation of insulin aspart in pharmaceutical solutions. Pharm Res. 2008;25(11):2534–44.
34. Markussen J, Diers I, Hougaard P, Langkjær L, Norris K, Snel L, *et al.* Soluble, prolonged-acting insulin derivatives. III. Degree of protraction, crystallizability and chemical stability of insulins substituted in positions A21, B13, B23, B27 and B30. Protein Eng. 1988;2(2):157–66.
35. Teska BM, Alarcón J, Pettis RJ, Randolph TW, Carpenter JF. Effects of phenol and meta-cresol depletion on insulin analog stability at physiological temperature. J Pharm Sci. 2014;103(8):2255–67.
36. Brange J, Langkjær L. Chemical stability of insulin. 3. Influence of excipients, formulation, and pH. Acta Pharm Nord. 1992;4(4):149–58.
37. Derewenda U, Derewenda Z, Dodson EJ, Dodson GG, Reynolds CD, Smith GD, *et al.* Phenol stabilizes more helix in a new symmetrical zinc insulin hexamer. Nature. 1989;338(6216):594–6.
38. Kaarsholm NC, Ko HC, Dunn MF. Comparison of solution structural flexibility and zinc binding domains for insulin, proinsulin and miniproinsulin. Biochemistry. 1989;28(10):4427–35.
39. Peters B, Trout BL. Asparagine deamidation: pH-dependent mechanism from density functional theory. Biochemistry. 2006;45(16):5384–92.