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*Research Paper*

# Effect of Ethylenediamine on Chemical Degradation of Insulin Aspart in Pharmaceutical Solutions

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***Purpose.*** To examine the effect of different amine compounds on the chemical degradation of insulin aspart at pharmaceutical formulation conditions.
***Methods.*** Insulin aspart preparations containing amine compounds or phosphate (reference) were prepared and the chemical degradation was assessed following storage at 37°C using chromatographic techniques. Ethylenediamine was examined at multiple concentrations and the resulting insulin–ethylenediamine derivates were structurally characterized using matrix assisted laser desorption ionization time-of-flight mass spectroscopy. The effects on ethylenediamine when omitting glycerol or phenolic compounds from the formulations were investigated.
***Results.*** Ethylenediamine was superior in terms of reducing formation of high molecular weight protein and insulin aspart related impurities compared to the other amine compounds and phosphate. Monotransamidation of insulin aspart in the presence of ethylenediamine was observed at all of the six possible Asn/Gln residues with $Asn^{A21}$ having the highest propensity to react with ethylenediamine. Data from formulations studies suggests a dual mechanism of ethylenediamine and a mandatory presence of phenolic compounds to obtain the effect.
***Conclusions.*** The formation of high molecular weight protein and insulin aspart related impurities was reduced by ethylenediamine in a concentration dependant manner.

**KEY WORDS:** amine; covalent dimer; ethylenediamine; insulin aspart; transamidation.

## INTRODUCTION

One of the most challenging tasks during development of protein pharmaceuticals is to deal with the intrinsic propensity of these macromolecules to undergo unwanted structural changes during manufacture, storage, use etc. A variety of structural changes have been observed for proteins in liquid formulations including covalent changes (chemical degradation) such as deamidation, oxidation, disulphide shuffling, hydrolysis, isomerization and non-disulphide cross-linking as reviewed by Wang (1). Even though insulin has been in therapeutic use for more than 80 years, and significant improvements have been obtained in terms of chemical stability (2), complete elimination of chemical degradation has not been reached (3). The most prevalent insulin degradation pathway is deamidation where the side chain amide group of glutaminyl ($Gln^{A5}$, $Gln^{A15}$, $Gln^{B4}$) or asparaginyl ($Asn^{A18}$, $Asn^{A21}$, $Asn^{B3}$) is hydrolyzed to form the free carboxylic acid (4). Depending on pH, hydrolysis of $Asn^{A21}$ at acidic conditions (via a cyclic anhydride intermediate catalyzed by the protonated C-terminal α-COOH group (5–7)) or $Asn^{B3}$ at neutral conditions (proceeding through a cyclic imide intermediate to form either $Asp^{B3}$ or $isoAsp^{B3}$) are the most dominant pathways of insulin deamidation (4, 8). Formation of similar hydrolytic derivates have been found in formulations of the monomeric insulin analogue $Asp^{B28}$ human insulin (insulin aspart—Fig. 1) in addition to formation of $isoAsp^{B28}$ specific for this insulin analogue (9). High molecular weight proteins (HMWP) represent another group of chemical degradation products where two or more insulin molecules are covalently linked together forming covalent dimers and polymers, respectively. Various pathways including transamidation reactions, reactions with carbonyl impurities from excipients (*e.g.* glycerol aldehyde (10)) or disulphide exchange (11,12) have been suggested to lead to formation of HMWP. Deamidation and formation of covalent insulin dimers involving the $Asn^{A21}$ residue at acidic pH (pH 2–5) has been thoroughly investigated by Darrington and Anderson showing that both degradation products are formed

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**ABBREVIATIONS:** AMPD, 2-amino-2-methyl-1,3-propanediol; BIS-TRIS, 2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; $Da_{Mo}$, monoisotropic mass in Dalton; DesPhe, $desPhe^{B1}$-*N*-oxalyl-$Val^{B2}$; DTT, 1,4-dithiothreitol; GPC, gel permeation chromatography; HMWP, high molecular weight protein; IARI, insulin aspart related impurities; IEC, ion exchange chromatography; MALDI-TOF, matrix assisted laser desorption ionization time of flight; OR, other related impurities; RP-HPLC, reverse phase high-performance liquid chromatography; TCEP, tris(2-carboxyethyl)phosphine; T.E.A., tetraethylammonium.

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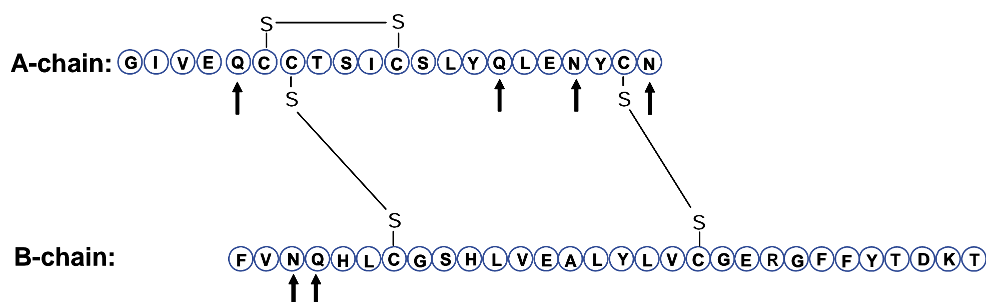


**Fig. 1.** Primary structure of insulin aspart. Each *arrow* indicates a possible residue for ethylenediamine transamidation.

through the same cyclic anhydride intermediate catalyzed by the protonated C-terminal α-COOH group (p$K_a$~3.6) (5). The partitioning of the cyclic anhydride intermediate to deamidated *versus* covalent dimer products has been suggested to most likely reflect the competition between a nucleophil attack of the cyclic anhydride by water or a second insulin molecule, respectively (6). The proposed transamidation pathway at neutral pH involves a nucleophil attack of an amine group (*e.g.* N-terminal $–NH_2$) of one insulin molecule to the side chain carboxyamide group (Asn or Gln residues) of another insulin molecule (8). An unequal propensity of the two N-terminal amine groups of the A and B chain and the six Asn/Gln residues to form covalent insulin dimers by the transamidation route has been observed in pH neutral insulin formulations showing a preference for the N-terminal of the B-chain and three of the four A-chain amido groups ($Gln^{A15}$, $Asn^{A18}$, $Asn^{A21}$), in particular $Asn^{A21}$ (8, 13). A transamidation pathway has also been suggested for the formation of covalent insulin–protamine reaction products between the N-terminal amino group of protamine and insulin side chain amide groups in protamine-containing insulin preparations (3). However, more recent data suggest a cross-linking reaction between the imidazole group of $His^{B5}$ or $His^{B10}$ and the guanidino group of protamine arginine residues (14). Cross-linking reactions of insulin involving reactive glycerol impurities have been reported and suggested to involve primarily aldehyde impurities (10, 12). Aldehydes can react with amino groups under the formation of a Shiff base compound, which upon Amadori rearrangement can form an aldoamine compound, which can further react with other amino groups leading to covalent cross-linking (15). The biological properties of the different insulin degradation products have been examined and the various desamido forms have been found to possess full or nearly full biological potency compared to native insulin (9). On the other hand, substantial reductions in biological potency have been observed for covalent dimers and polymers (15% and 1.5% of native insulin potency, respectively) (13) and they have been associated with increased immunogenic and/or allergenic potential compared to the native insulin molecule (16–18) as well as increased circulating insulin immunoreactivity in type 1 diabetic patients (19,20). Hence, based on the biological properties of the different types of insulin degradation products, focus on reduction or preferably elimination of formation of covalent dimers and polymers (HMWP) must have high priority during development of pharmaceutical insulin formulations. Taking into account that covalent insulin dimers and polymers are primarily formed via pathways involving amino groups (transamidation or reactions with carbonyl impurities) at pH-neutral formulation conditions, we have examined if the presence of different non-insulin amino groups in form of amine compounds can interfere with these reactions and in turn affect the extent of covalent dimer and polymer formation in insulin formulations.

## MATERIALS AND METHODS

### Insulin Aspart Formulations

Insulin aspart formulations of the following standard composition were prepared: 0.6 mM insulin aspart (Novo Nordisk A/S, Bagsværd, Denmark), 1.5 mg/ml phenol, 1.72 mg/ml metacresol, 16 mg/ml glycerol, buffer (7 mM disodium phosphate dihydrate or amine buffer), 10 mM sodium chloride, 19.6 μg/ml zinc ~3 $Zn^{2+}$/hexamer, pH 7.4. All excipients were of USP/Ph Eur or analytical grade and the following amine compounds were examined: AMPD [2-amino-2-methyl-1,3-propanediol, ≥99%, Sigma-Aldrich], glycinamide [glycinamide hydrochloride, ≥99%, Sigma-Aldrich], ethylenediamine [ethylenediammonium dichloride, ≥99%, Merck], imidazole [imidazole, ≥99.5%, Sigma-Aldrich], BIS-TRIS [2,2-Bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol, ≥99.0%, Sigma-Aldrich] and T.E.A. [tetraethylammonium chloride, ≥99%, Sigma-Aldrich]. To test the effect of the amine compounds, formulations were stored in glass vials/cartridges at 37°C and samples were pulled at predefined time points and analyzed according to the analytical methods described below.

### Gel Permeations Chromatography (GPC)

The content of HMWP (sum of covalent dimer and polymers) was determined using a Waters Insulin HMWP column (7.8×300 mm) with a flow rate of 0.7 ml/min at ambient temperature and detection at 276 nm. Elution was performed at isocratic conditions with the following mobile phase prepared in Milli-Q water: 0.07% (*w/w*) L-arginine, 15% (*w/w*) glacial acetic acid, 15% (*w/w*) acetonitrile. Samples were acidified with 4 μl 6 M HCl per ml prior to injection (100 μl~60 nmol insulin aspart). The amount of covalent dimer and polymer was determined as the absorbance area of the corresponding peaks given in percentage of the total absorbance area of insulin aspart peaks. Equal response factors of native and non-native insulin aspart molecules were assumed. The GPC method is equivalent to the analytical method used for quality control of Novo Nordisk marketed insulin aspart pharmaceuticals (NovoRapid®/NovoLog®).

### Reverse Phase High-performance Liquid Chromatography (RP-HPLC)

Samples were analyzed for content of insulin aspart related impurities (IARI) using a LiChrosorb RP C18 column (5 μm, 250×4 mm) with a flow rate of 1 ml/min at 35°C and detection at 214 nm. Elution was performed with a mobile phase consisting of the following prepared in Milli-Q water: (A) 7.7% (*w/w*) acetonitrile, 2.8% (*w/w*) sodium sulfate, 0.3 (*w/w*) *o*-phosphoric acid, pH 3.6; (B) 42.8% (*w/w*) acetonitrile. Gradient: 0–35 min isocratic with 57%/43% of A/B: 35–40 min linear change to 20%/80% of A/B; 40–45 min isocratic with 20%/80% of A/B; 45–46 min linear change to 57%/43% of A/B and 46–55 min isocratic with 57%/43% of A/B. Samples were acidified with 4 μl 6 M HCl per ml prior to injection (10 μl~6 nmol insulin aspart). The amount of IARI was determined as the percentage of the total absorbance area eluted after the preservative agents and not representing native insulin aspart (main peak), desamido or isoAsp forms (9). Equal response factors of native and non-native insulin aspart molecules were assumed. The RP-HPLC method is equivalent to the analytical method used for quality control of Novo Nordisk marketed insulin aspart pharmaceuticals (NovoRapid®/NovoLog®).

### Ion Exchange Chromatography (IEC)

An IEC system using a Dionex DNAPac PA100 column (250×4 mm) with a flow rate of 1.0 ml/min at 30°C and detection at 276 nm was used for quantification and isolation of insulin–ethylenediamine derivates. Elution was performed with a mobile phase consisting of the following in Milli-Q water: (A) 0.012% (*w/v*) monobasic potassium phosphate, 0.1% (*w/v*) disodium phosphate dihydrate, 48.8% (*w/v*) ethanol, pH adjusted to 7.6 with o-phosphoric acid; (B) 1.32% (*w/v*) ammonium sulfate in buffer A. Gradient: 0–30 min linear change from 100% A to 86%/14% of A/B; 30–35 min linear change to 10%/90% of A/B; 35–37 min isocratic with 10%/90% of A/B; 37–38 min linear to 100% A, 38–45 min isocratic with 100% A. An injection volume of 60 μl was used (~approximately 36 nmol insulin aspart). The absorbance area of each insulin aspart related peak was given in percentage of the total insulin aspart peak absorbance areas. Equal response factors of native and non-native insulin aspart molecules were assumed.

For further characterization, sampling of the main peak and peak 1–4 was done in glass test tubes containing 10 μl of 200 mM octyl-β-D-glucopyranoside in Milli-Q-water. An insulin aspart formulation of standard composition containing 7 mM ethylenediamine and stored at 37°C for 8 weeks was used for this purpose.

### Matrix Assisted Laser Desorption Ionization Time-of-Flight Mass Spectroscopy (MALDI-TOF)

Fractions collected from IEC separation containing native insulin aspart (IEC main peak) or the distinct insulin–ethylenediamine derivates (IEC peak 1–4 eluting before main peak) were subjected to determination of molecular mass using a 4800 (TOF/TOF) MALDI mass spectrometer (Applied Biosystems). Additionally, the same fractions were analyzed following reduction of the Cys residues using tris(2-carboxyethyl)phosphine (TCEP) in 100 mM ammonium bicarbonate (pH 8; 30 min at 50°C) or following reduction and alkylation of the Cys residues using 1,4-dithiothreitol (DTT) and 4-vinylpyridine (Sigma Aldrich), respectively. Prior to mass analyses, all samples were desalted by applying 20 μl on an in-house packed column based on selfpack POROS 20 RS material (Applied Biosystems) followed by elution with 5 μl α-cyano-4-hydroxy-cinnamic acid matrix solution prepared in 50% acetonitrile and 0.1% trifluoroacetic acid. 0.5 μl desalted sample was applied sample target for mass analysis. Mass spectra of the samples were acquired on the mass spectrometer operating in delayed reflection mode with acceleration voltage at 20 kV. MS/MS analysis was performed on the reduced and/or alkylated samples on peaks with masses corresponding to A- or B-chain ethylenediamine derivates of insulin aspart.

## RESULTS

### Effect of Different Amines on Insulin Aspart Degradation

The degradation of insulin aspart in the presence of 7 mM of different amines in terms of formation of covalent dimer and polymers as well as IARI was examined following 3 months of storage at 37°C. The results were expressed as a ratio (in percentage) to the amount of degradation products formed in an insulin aspart formulation containing 7 mM phosphate. Depending on type of amine and type of degradation product, both decreased and increased degradation (*i.e.* degradation score in Fig. 2 below or above 100%, respectively) was observed compared to formulations containing phosphate buffer. The largest overall degradation was observed with BIS-TRIS followed by AMPD, however, ethylenediamine was found to be a superior amine compound with respect to improvements in formation of covalent dimer and polymer as well as IARI (Fig. 2). The effect of variable concentrations of ethylenediamine on reduction of HMWP and IARI formation was examined ranging from far-below (0.007 mM ethylenediamine) to far-above (14 mM ethylenediamine) 1:1 molar stoichiometry to the 0.6 mM insulin aspart. An insulin aspart formulation with 7 mM phosphate buffer instead of ethylenediamine was included for reference purposes. A clear reduction in HMWP formation rates with increasing concentration of ethylenediamine was observed reaching an almost complete elimination of HMWP formation at 7 and 14 mM (Fig. 3a). Typical GPC chromatograms are presented in Fig. 3b + c, visualizing the clear effect of ethylenediamine *versus* phosphate (7 mM) on the content of HMWP following 12 weeks of storage at 37°C. In terms of IARI formation, similar ethylenediamine-concentration dependence was observed, however, complete elimination was not observed even at the highest ethylenediamine concentrations (Fig. 4a). Typical RP-HPLC chromatograms are presented in Fig. 4b + c, visualizing the clear effect of ethylenediamine *versus* phosphate (7 mM) on the content of IARI following 12 weeks of storage at 37°C. In addition, no peaks attributable to new insulin aspart degradation products seems to have appeared in the GPC (*i.e.* eluting before/at the insulin monomer, Fig. 3b + c) or RP-HPLC (*i.e.* eluting between metacresol and IARI, Fig. 4b + c) chromatograms of insulin aspart formulations containing ethylenediamine compared to phosphate.

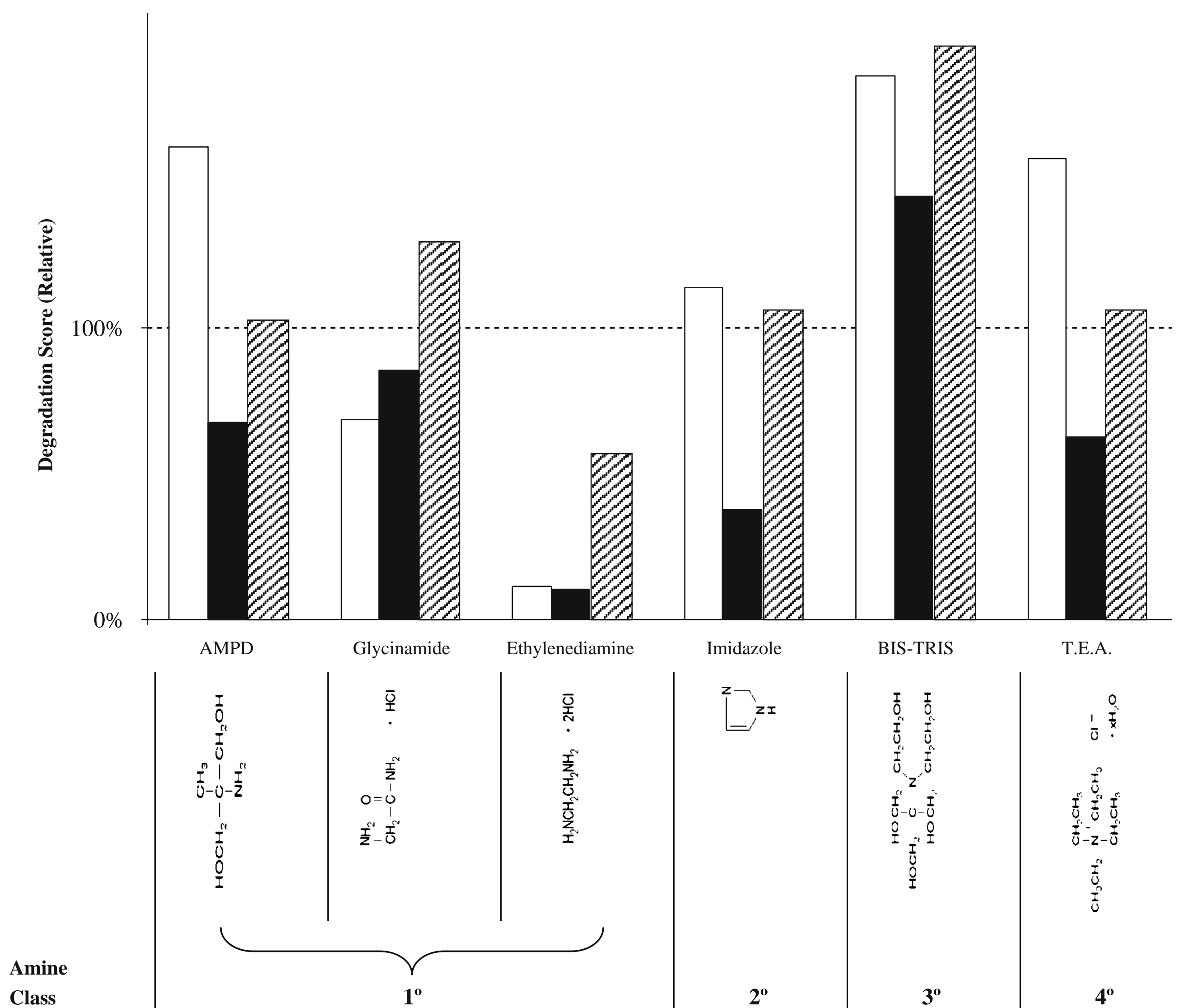


**Fig. 2.** Effect of different amines on formation of insulin aspart degradation products. The results are expressed as a degradation score (percent) defined as the amount of degradation product formed following 3 months of storage at 37°C in the presence of 7 mM amine relative to the amount formed in the presence of 7 mM phosphate. Covalent dimer (*open bars*), covalent polymer (*filled bars*) and IARI (*hatched bars*).

### IEC of Insulin–Ethylenediamine Derivates

The absence of additional peaks on the GPC and RP-HPLC chromatograms does not rule out the existence of new insulin aspart degradation products formed in the presence of ethylenediamine as these might co-elute with the native insulin or some of the well-known degradation products during the chromatographic separation process. To investigate this issue further, a third chromatographic method (IEC) based on a different separation principle (ion exchange chromatography) was used to examine the degradation profile of insulin aspart formulations containing ethylenediamine or phosphate. Chromatograms from the IEC method are presented in Fig. 5, showing four additional peaks eluting prior to the main peak in the chromatogram of insulin aspart formulation containing ethylenediamine compared to phosphate. Additionally, significant lower contents of other related impurities (OR) and desPheB1-*N*-oxalyl-ValB2 (DesPhe) were found in the formulation containing ethylenediamine compared to phosphate.

### Effect of Glycerol, Phenol & Metacresol

Ethylenediamine, in comparison with phosphate, reduce formation of HMWP, IARI, desPhe$^{B1}$-*N*-oxalyl-Val$^{B2}$, and OR but increase formation of the substances corresponding to peak 1–4 on the IEC chromatograms (Table I).

The presence of glycerol in the phosphate formulation increase formation of HMWP, IARI, desPhe$^{B1}$-*N*-oxalyl-Val$^{B2}$ and OR; but a similar effect of glycerol could not be observed in the presence of ethylenediamine. Additionally, formation of desPhe$^{B1}$-*N*-oxalyl-Val$^{B2}$ was only observed in the phosphate formulation containing glycerol and virtually eliminated in the absence of glycerol (Table I). The results suggest that the formation of desPhe$^{B1}$-*N*-oxalyl-Val$^{B2}$ is related to the presence of glycerol, and that ethylenediamine is capable of interfering with the underlying mechanisms leading to an almost complete elimination of desPhe$^{B1}$-*N*-oxalyl-Val$^{B2}$ formation even in the presence of glycerol. In the absence of glycerol, where formation of HMWP is believed to proceed predominantly through the

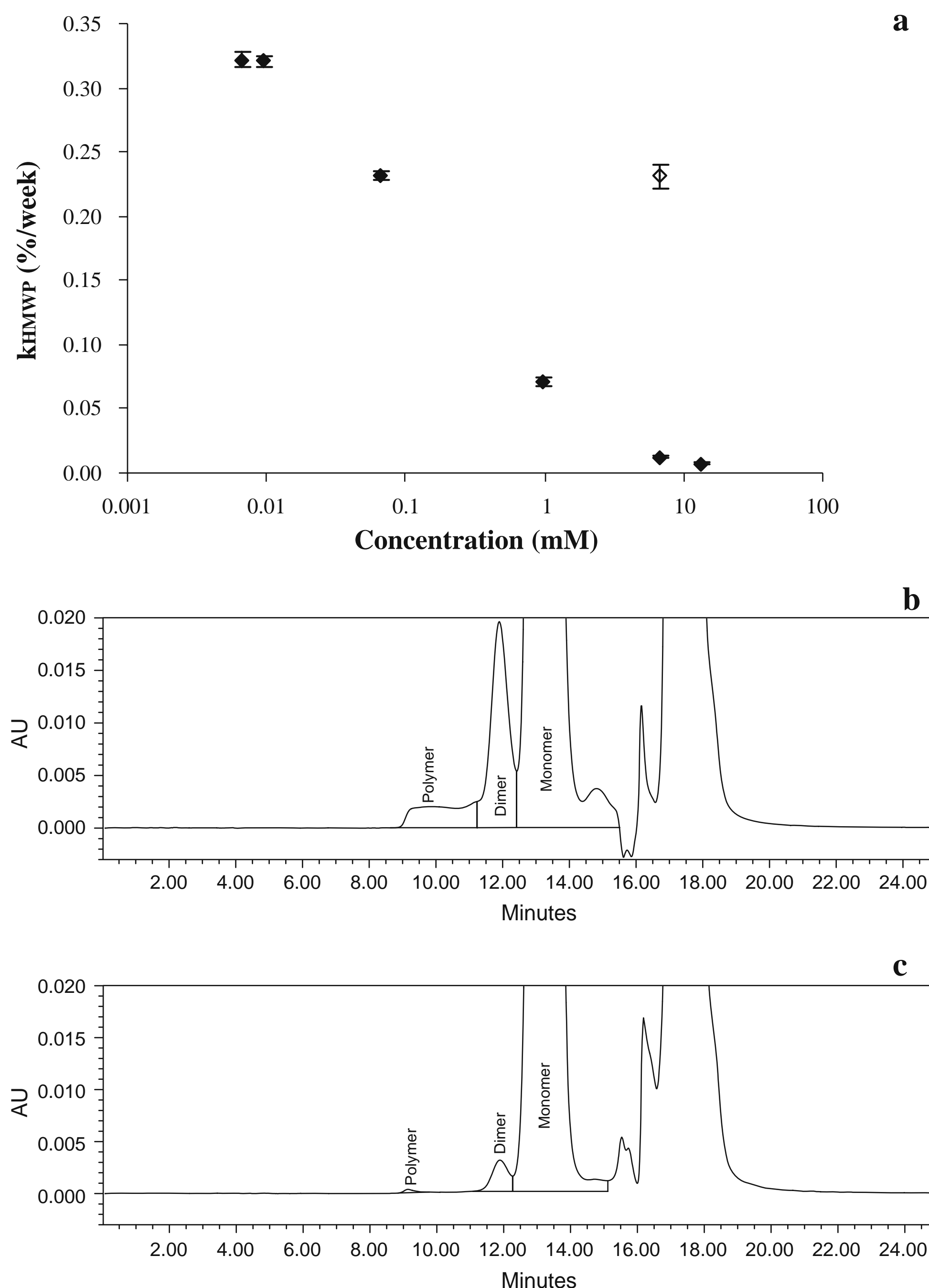


**Fig. 3.** Effect of ethylenediamine concentration on formation of HMWP at pH 7.4. **a** Formation rates of HMWP ($k_{\mathrm{HMWP}}$) *versus* buffer concentration of disodium phosphate dihydrate (*open diamond*) or ethylenediamine (*filled diamonds*) based on data from 0, 6 and 12 weeks of storage at 37°C assuming zero-order kinetics (31). Results are expressed as average formation rate $k_{\mathrm{HMWP}} \pm \mathrm{SE}_{\mathrm{formation\ rate}}$. **b** & **c** GPC chromatograms of samples containing 7 mM buffer following 12 weeks of storage at 37°C; **b** 7 mM disodium phosphate dihydrate; **c** 7 mM ethylenediamine.

transamidation pathway, 0.15%/week HMWP was formed in the 7 mM phosphate formulation and virtually no formation of peak 1–4 was observed (Table I). On the other hand, when phosphate was replaced by 7 mM ethylenediamine, the total formation of peak 1–4 was found to be 0.14%/week and virtually no HMWP-formation was observed (0.01%/week).

Eliminating phenol and metacresol from the formulations caused a substantial increase in formation rates of HMWP and IARI for both ethylenediamine and phosphate formulations (Table I), suggesting that the presence of phenolic antimicrobial agents is mandatory for obtaining the effect of ethylenediamine.

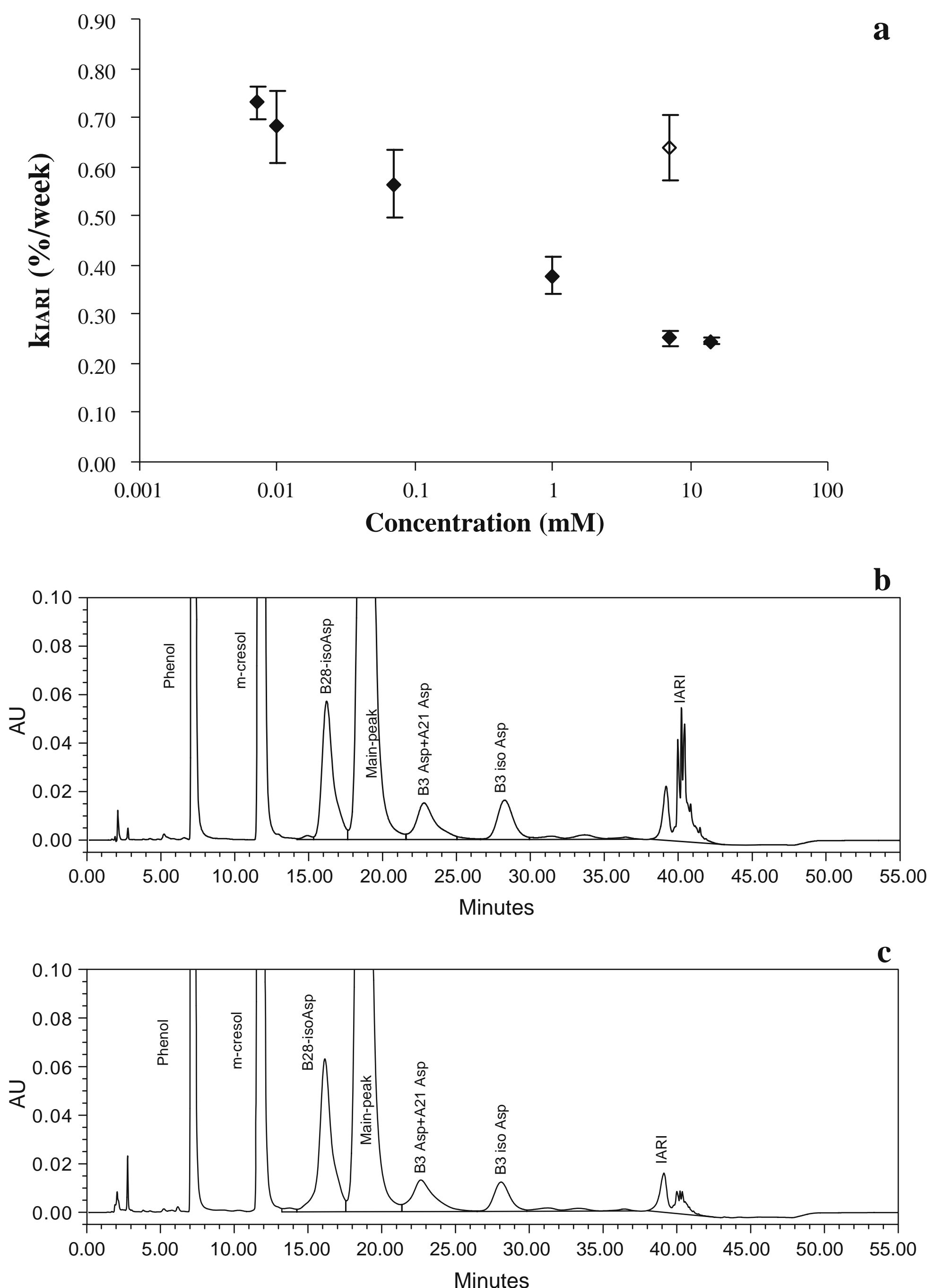


**Fig. 4.** Effect of ethylenediamine concentration on formation of IARI at pH 7.4. **a** Formation rates of IARI ($k_{IARI}$) *versus* buffer concentration of disodium phosphate dihydrate (*open diamond*) or ethylenediamine (*filled diamonds*) based on data from 0, 6 and 12 weeks of storage at 37°C assuming zero-order kinetics (31). Results are expressed as average formation rate $k_{IARI} \pm SE_{formation\ rate}$. **b** & **c** RP-HPLC chromatograms of samples containing 7 mM buffer following 12 weeks of storage at 37°C. **b** 7 mM disodium phosphate dihydrate; **c** 7 mM ethylenediamine.

### Structural Characterization of IEC Main Peak and Peak 1–4

By MALDI-TOF mass spectroscopy of unmodified as well as reduced and alkylated samples, the IEC main peak sample was found to consist of pure native insulin aspart when comparing with the corresponding theoretical mass values (Table II). On the other hand, unmodified samples of peak 1–4 were found to contain an insulin aspart derivate of approximately 5,865 $Da_{Mo}$ (IA-ETY in Table II) corresponding to a monotransamidation reaction (+43 Da) between ethylenediamine and insulin aspart (5,822 $Da_{Mo}$). No insulin–ethylenediamine derivates representing higher order transamidation reactions (*e.g.* di- or tri-transamidation) were found.

In order to locate if the transamidation reaction involved residues on the A- or B-chain of insulin aspart, samples corresponding to peak 1–4 were modified by Cys residue

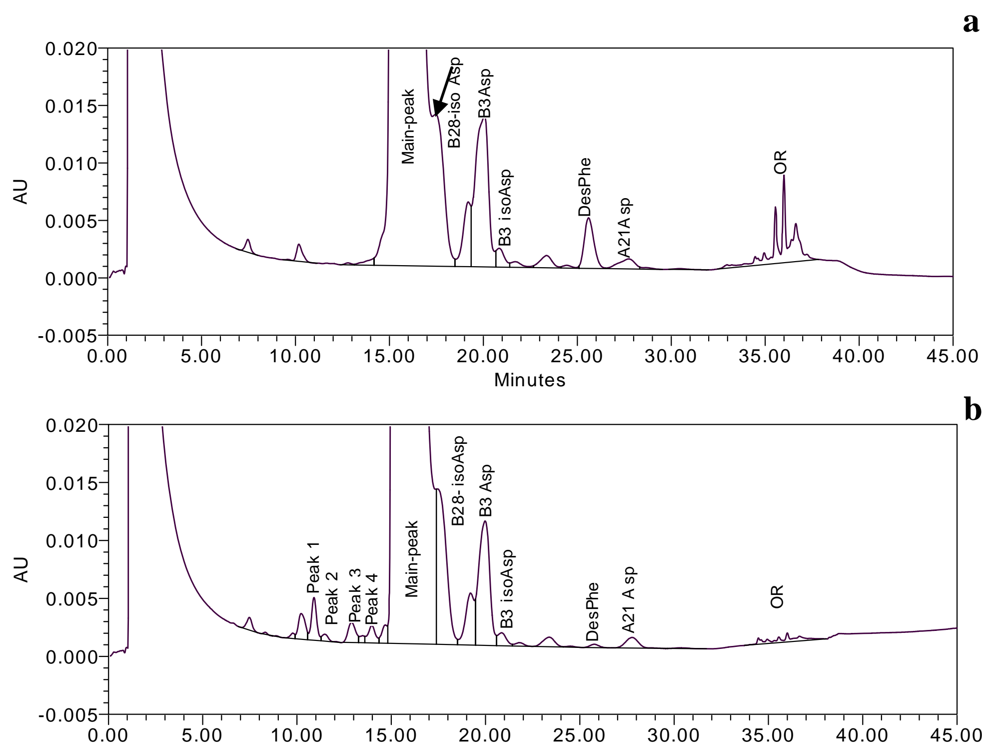


**Fig. 5.** Typical IEC chromatograms of insulin aspart standard formulations containing (**a**) 7 mM disodium phosphate dihydrate or (**b**) 7 mM ethylenediamine following 8 weeks of storage at 37°C. Peak 1, peak 2, peak 3 and peak 4 prior to the main peak are named arbitrarily. Peaks appearing after the $Asp^{A21}$ peak are summed to give OR (other related impurities) which is believed to consist of a heterogeneous group of hydrophobic insulin aspart degradation products.

reduction and/or alkylation; thus breaking the di-sulphide bridges between the insulin aspart A- and B-chain. MALDI-TOF mass spectroscopy of the modified samples showed that peak 1 and peak 2 involved modification of the A-chain only, as these samples contained A-chains with an additional mass of +43 Da compared to the mass of the native insulin aspart A-chain (Table II). Peak 3 and peak 4 were found to represent insulin aspart derivates with one ethylenediamine group attached to either the A-chain or the B-chain (Table II). MS/MS analyses of the modified peak 1–4 samples revealed that peak 1 represented monotransamidation at $Asn^{A21}$; whereas precise determination of the transamidation site for peak 2–4 samples was not possible.

## DISCUSSION

Due to the impaired biological properties of covalent insulin dimers and polymers (HMWP) in terms of reduced/absent potency (13) as well as potential immunogenic effects (19, 20), elimination or at least reduction of these species in pharmaceutical insulin formulation must be strived for. Formation of HMWP at pH-neutral formulation conditions proceeds predominantly through reactions with carbonyl impurities from excipients (*e.g.* glycerol aldehyde from glycerol) or through transamidation reactions with the side chain carboxyamide group of Asn/Gln residues (12). Both pathways involve reactions with free amine groups from the insulin molecule (*e.g.* the N-terminal amino groups) and we examined if substitution of phosphate buffer with different amine compounds affected the formation of insulin aspart degradation products at pharmaceutical formulation conditions.

Six amines were examined and variable effects ranging from increased degradation in the presence of BIS-TRIS to reductions and ultimately elimination of HMWP formation in the presence of ethylenediamine were observed. These results demonstrate that the effect of ethylenediamine on formation of HMWP is not a common property of amines in general. The reason for the unique effect of ethylenediamine compared to the other amines is unknown, but probably related to differences in respect to both charge and spacious (sterical hindrance) properties.

A concentration dependant effect of ethylenediamine on the formation rate of HMWP was observed; however, additional kinetic studies are required to outline reliable reaction mechanisms accounting for this concentration effect. As protonation of amino groups suppress their ability to nucleophil attack (21, 22), at least a part of the ethylenediamine amino groups must have been uncharged at the pH-neutral test conditions. This was most probably the case due to the relatively low p$K_a$ of one of the ethylenediamine amino groups (p$K_{a1}$=7.0 and p$K_{a2}$=10.1 (23)) compared to the other primary amines (p$K_a$ of 8.8 and 8.1 for AMPD and glycinamide, respectively (23)). For comparison, p$K_a$ of the insulin $Phe^{B1}$ N-terminal amino group involved in the HMWP-forming transamidation reaction has been reported to be unusual low and very close to the lowest p$K_a$ of ethylenediamine ($Phe^{B1}$ p$K_a$ of 6.9 and 7.2 in (24) and (22), respectively). Despite the mainly uncharged amino groups of the non-primary amines examined at pH-neutral conditions

**Table I.** Formation Rates of Insulin Aspart Degradation Products at 37°C

| Phosphate (mM) | Ethylenediamine (mM) | Glycerol (mg/ml) | Phenol & *m*-cresol (mM) | GPC | RP | IEC | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | HMWP | IARI | Peak 1 | Peak 2 | Peak 3 | Peak 4 | DesPhe[a] | OR[b] |
| 7 | 0 | 16 | 16 | 0.24 (0.02) | 0.63 (0.06) | n.d. (–) | n.d. (–) | n.d. (–) | n.d. (–) | 0.12 (0.02) | 0.19 (0.02) |
| 7 | 0 | 0 | 16 | 0.15 (0.00) | 0.44 (0.01) | n.d. (–) | n.d. (–) | n.d. (–) | 0.01 (0.01) | 0.02 (0.00) | 0.11 (0.01) |
| 0 | 7 | 16 | 16 | 0.01 (0.01) | 0.28 (0.01) | 0.06 (0.00) | 0.01 (0.00) | 0.04 (0.00) | 0.03 (0.00) | 0.01 (0.00) | 0.02 (0.00) |
| 0 | 7 | 0 | 16 | n.d. (–) | 0.24 (0.02) | 0.06 (0.00) | 0.01 (0.00) | 0.04 (0.00) | 0.03 (0.00) | n.d. (–) | n.d. (–) |
| 4 | 0 | 16 | 16 | 0.18 (0.02) | 0.64 (0.06) | | | | | | |
| 4 | 0 | 16 | 0 | 2.5 (0.20) | 1.6 (0.14) | | | | | | |
| 0 | 4 | 16 | 16 | 0.01 (0.01) | 0.36 (0.01) | | | | | | |
| 0 | 4 | 16 | 0 | 2.7 (0.15) | 1.3 (0.10) | | | | | | |

Results are expressed as average formation rate in percent per week ($SE_{formation\ rate}$) calculated based on data from 0, 4 and 8 weeks of storage at 37°C assuming zero-order kinetics (31).
*n.d.* No detectable formation during 8 weeks at 37°C
[a] desPhe$^{B1}$-*N*-oxalyl-Val$^{B2}$ insulin aspart
[b] Other related impurities

(p$K_a$ of imidazole, BIS-TRIS and T.E.A. are between 6.5 and 6.9 (23)), only moderate to absent reduction in HMWP formation was observed, probably related to simple sterical hindrance of these more bulky non-primary amines.

At neutral pH, formation of HMWP is believed to occur following a transamidation pathway involving a transamidation reaction between the side chain carboxyamide group of Asn/Gln residues and free amino groups (12). Additionally, in the presence of reactive carbonyl impurities (*e.g.* glycerol aldehyde originating from the glycerol) HMWP will also be formed following a carbonyl-impurity pathway and the carbonyl-impurity-dependant formation of desPhe$^{B1}$-*N*-oxalyl-Val$^{B2}$ will be induced as discussed by Jars *et al.* (9). Removal of glycerol from formulations containing 7 mM phosphate was found to reduce the formation rate of HMWP (from 0.24 to 0.15%/week at 37°C) and almost eliminate formation of desPhe$^{B1}$-*N*-oxalyl-Val$^{B2}$ in consistency with this. On the other hand, when substituting 7 mM phosphate with 7 mM ethylenediamine virtually no HMWP or desPhe$^{B1}$-*N*-oxalyl-Val$^{B2}$ was formed even in the presence of glycerol, which might be an indication of that ethylenediamine, interferes with both the transamidation and the carbonyl-impurity pathway. However, the presence of ethylenediamine caused formation of insulin–ethylenediamine derivates (peak 1–4) as detected by IEC. At transamidation conditions (*i.e.* in the absence of glycerol), the sum of HMWP and peak 1–4 formation rates were almost identical irrespectively of buffer substance (0.16 and 0.14%/week for phosphate and ethylenediamine, respectively). However, HMWP was primarily formed in the presence of phosphate whereas the vast majority of peak 1–4 formation was seen in the presence of ethylenediamine. This equality in HMWP and peak 1–4 formation rates might be a coincidence and more studies are required to elucidate this further. However, it could be speculated whether ethylenediamine shifts the transamidation reaction from involving insulin amino groups resulting in HMWP to ethylenediamine amino groups resulting in insulin–ethylenediamine derivates corresponding to peak 1–4. Following this, it can be further speculated whether the observed concentration dependent effect of ethylenediamine, might rely on a competition between insulin N-terminal amino groups and ethylenediamine amino groups in the nucleophil attack on carboxyamide groups of insulin Asn and Gln residues in the transamidation pathway. Interestingly, a similar competition between nucleophil attack of a cyclic anhydride in the A21$^{Asn}$ residue by water or a second insulin molecule, leading to formation of A21$^{Asp}$ or covalent dimer, respectively, has been suggested to occur at acidic pH (pH 2–5) (6).

Eliminating phenol and *m*-cresol from formulations containing phosphate increased the formation rates of HMWP and IARI several fold, in accordance with what has been published recently (25). As comparable HMWP and IARI formation rates were observed when substituting phosphate with ethylenediamine in the absence of phenol and *m*-cresol, presence of such phenolic substances is apparently required to obtain the benefits of ethylenediamine. The mechanism behind this dependency has to our knowledge not been described in the literature. However, it seems reasonable to suggest that it might be related to the shift in hexamer conformation from the $T_6$ to the $R_6$ state in the presence of phenolic substances, changing the spatial

**Table II.** MALDI-TOF Mass Spectroscopy of IEC Main Peak and Peak 1–4 Fractions

| IEC fraction | Sample modification | Mass ($Da_{Mo}$) Observed | Mass ($Da_{Mo}$) Theoretical | Identification |
|---|---|---|---|---|
| Main peak | None | 5,821.94 | 5,821.61 | IA |
| | Reduced and alkylated[a] | 2,802.25 | 2,802.23 | IA A-chain |
| | | 3,655.77 | 3,655.77 | IA B-chain |
| Peak 4 | None | 5,821.76 | 5,821.61 | IA |
| | | 5,864.84 | 5,864.65 | IA-ETY |
| | Reduced and alkylated[a] | 2,802.25 | 2,802.23 | IA A-chain |
| | | 2,845.29 | 2,845.27 | IA-ETY A-chain |
| | | 3,655.79 | 3,655.77 | IA B-chain |
| | | 3,697.85 | 3,698.82 | IA-ETY B-chain |
| Peak 3 | None | 5,863.85 | 5,864.65 | IA-ETY |
| | Reduced and alkylated[a] | 2,802.26 | 2,802.23 | IA A-chain |
| | | 2,845.31 | 2,845.27 | IA-ETY A-chain |
| | | 3,655.81 | 3,655.77 | IA B-chain |
| | | 3,697.86 | 3,698.82 | IA-ETY B-chain |
| Peak 2 | None | 5,821.85 | 5,821.61 | IA |
| | | 5,864.82 | 5,864.65 | IA-ETY |
| | Reduced and alkylated[a] | 2,802.23 | 2,802.23 | IA A-chain |
| | | 2,845.27 | 2,845.27 | IA-ETY A-chain |
| | | 3,655.76 | 3,655.77 | IA B-chain |
| Peak 1 | None | 5,865.81 | 5,864.65 | IA-ETY |
| | Reduced[b] | 2,425.69 | 2,425.01 | IA-ETY A-chain |
| | | 3,446.58 | 3,445.64 | IA B-chain |

*IA* Insulin aspart, *IA A-chain* insulin aspart A-chain, *IA B-chain* insulin aspart B-chain, *IA-ETY* insulin aspart–ethylenediamine derivate, *IA-ETY A-chain* insulin aspart–ethylenediamine derivate on the A-chain, *IA-ETY B-chain* insulin aspart–ethylenediamine derivate on the B-chain
[a] Cys residues reduced with DTT and alkylated with 4-vinylpyridine
[b] Cys residues reduced with TCEP

orientation of the eight first residues of the B-chain N-terminal from an elongated conformation to a more structured α-helix (26–28). The higher flexibility of the N-terminal of the B-chain has been proposed as an explanation for the higher transamidation rate compared to the N-terminal of the A-chain (8). Consequently, changes in the flexibility of the B-chain N-terminal caused by the shift from the $T_6$ to the $R_6$ state could affect the propensity of the B-chain N-terminal to participate in these reactions. Following these speculations, the reactivity of the insulin N-terminal amino groups in the $T_6$ state is expected to be much higher than in the $R_6$ state with the reactivity of the ethylenediamine amino groups in between. Increased stability of the insulin molecules following transition from the $T_6$ to the $R_6$ state is well established and has been discussed elsewhere (28, 29).

The presence of ethylenediamine was found to induce formation of insulin–ethylenediamine derivates that were separated into four unique peaks by IEC (peak 1–4) and subsequent subjected to structural characterization by MALDI-TOF and MS/MS analyses. Based on IEC data (Table I), peak 1 was found to be the predominant derivate formed and further characterization revealed that peak 1 consisted of insulin aspart transamidated with ethylenediamine at the $Asn^{A21}$ residue. These results are in accordance with the preference of $Asn^{A21}$ over the other Asn/Gln residues to be involved in transamidation reactions preferably with the B-chain N-terminal leading to covalent insulin dimers in the absence of ethylenediamine (8, 13). Peak 2–4 were also found to contain transamidation derivates involving the A-chain but the precise residue involved could not be analytically identified. However, as only monotransamidation was observed, and peak 1–4 all contained different transamidation derivates involving the A-chain, transamidation with ethylenediamine must have occurred at all four distinct A-chain Asn/Gln residues (Fig. 1). As peak 1 was found to consist of insulin aspart transamidated in $Asn^{A21}$, the A-chain transamidation derivates in peak 2–4 must involve the remaining three A-chain Asn/Gln residues ($Gln^{A5}$, $Gln^{A15}$, $Asn^{A18}$). The low propensity of $Gln^{A5}$ to be involved in transamidation forming covalent insulin dimers (8, 13) combined with the relatively low peak 2 formation rate in IEC, suggest that the observed A-chain derivate in peak 2 could be ethylenediamine transamidation at $Gln^{A5}$. Following this, the A-chain derivates in peak 3 and peak 4 involve transamidation with ethylenediamine at either $Gln^{A15}$ or $Asn^{A18}$. Additionally, B-chain derivates were found in peak 3 and 4 which must be assigned to transamidation at the two possible transamidation sites of the B-chain ($Asn^{B3}$ and $Gln^{B4}$). The results from the characterization studies are summarized in Table III. In conclusion, monotransamidation of insulin aspart with ethylenediamine was observed at all of the six possible Asn/Gln residues with $Asn^{A21}$ having the highest propensity to react with ethylenediamine.

**Table III.** Insulin Aspart–Ethylenediamine Transamidation Derivates

| Possible site of transamidation | Suggested peak in IEC |
|---|---|
| $Gln^{A5}$ | Peak 2 |
| $Gln^{A15}$ | Peak 3 or peak 4 |
| $Asn^{A18}$ | Peak 3 or peak 4 |
| $Asn^{A21}$ | Peak 1 |
| $Asn^{B3}$ | Peak 3 or peak 4 |
| $Gln^{B4}$ | Peak 3 or peak 4 |

The biological properties such as potency, immunogenic response, receptor binding affinity etc. of the observed insulin–ethylenediamine derivates are to our knowledge unknown and calls obviously for further examinations. Properties similar to native insulin are of course preferable, but even superiority compared to HMWP having reduced/absent potency (13) as well as potential immunogenic effects (19, 20) will introduce an overall benefit for the patient. The major insulin–ethylenediamine derivate (peak 1) was identified to involve the $Asn^{A21}$ residue changing the side chain from $–CH_2CONH_2$ to $–CH_2CONH(CH_2)_2NH_2$. Alterations of the $Asn^{A21}$ residue might affect the biological potency as discussed by Markussen *et al.*, however, it seems like some variation—especially if restricted to amino acid substitutions—is allowable without detrimental loss of potency (21, 30). Thus, based on this it seems reasonable to expect that the peak 1 derivate possess biological potency more similar to native insulin than a covalent dimer where another insulin molecule is covalently attached to the $Asn^{A21}$ residue.

In conclusion, ethylenediamine was found to significantly reduce formation of insulin aspart degradation products (HMWP and IARI). Further studies are needed to elucidate the mechanism behind the observed effect of ethylenediamine; however, data indicating interference of ethylenediamine with both the transamidation and the carbonyl-impurity mediated degradation pathways are provided. A concentration dependent effect of ethylenediamine was found, and at equimolar concentrations (7 mM) an ~18-fold reduction in HMWA formation and a ~3-fold reduction in IARI formation at 37°C was observed compared to phosphate. On the other hand, monotransamidation derivates of insulin aspart with ethylenediamine was observed at all six possible Asn/Gln residues with $Asn^{A21}$ having the highest propensity to react with ethylenediamine. Characterization of these derivates in terms of biological properties such as potency, immunogenic response, receptor binding affinity etc. remains to be done. Presence of phenolic compounds such as phenol and *m*-cresol was found to be mandatory to obtain the benefits of ethylenediamine, probably associated with the structural changes introduced to the insulin hexamer in the transition from the $T_6$ to the $R_6$ state in the presence of phenolic compounds.

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