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Determination of protamine peptides in insulin drug products using reversed phase high performance liquid chromatography

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Abstract

This paper describes the establishment and validation of a reversed phase HPLC (RP-HPLC) method for determination of protamine peptides in protamine sulphate raw material and insulin drug products, discusses the analytical results obtained for a number of protracted insulin formulations and the potential of the method in connection with protamine sulphate raw material and final drug product quality control.
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Keywords: Protamine sulphate; Protamine peptides; Insulin drug products; Reversed phase HPLC

1. Introduction

Treatment of diabetes mellitus with insulin normally requires the administration of fast acting insulin at mealtime combined with the administration of a long-acting or protracted formulation of insulin to cover the basal need in-between meals. Three main groups of insulin preparations exist: (1) fast acting preparations with a quick onset and a relatively short duration of action, (2) intermediate or long acting preparations with a fairly flat time action profile and a duration of action lasting for up to 24 h or more, and (3) biphasic insulin preparations being a readymade mixture of fast acting and intermediate to long acting preparations. One of the most widespread principles for protracting the action of insulin is co-precipitation at neutral pH with protamine peptides in the presence of small amount of zinc and phenol and/or phenol derivatives [1]. By this process, an amorphous white precipitate is formed between the net negatively charged insulin molecule and the poly-cationic protamine peptides, which gradually transforms into tetragonal crystals.

Protamine is the generic name of a group of strongly basic peptides present in the sperm or mature testes cell nuclei of fish in salt-like combination with nucleic acids [2]. Commercially available protamines are usually obtained as the sulphate salt, and for insulin formulations, salmine protamine from fish of the family Salmonidae is normally used. Salmine protamine may be classified as a mono-protamine as only one basic amino acid, Arginine, is present. The four major peptides, which constitute almost the entire nitrogen containing material in salmine protamine, have been fully characterized [3] and found to be poly-peptides 30–32 amino acids in length, of which 21–22 residues consists of Arginine. Similar complexing properties with regard to insulin have been found for all four peptides [3].

The prolonged action of insulin following injection of an insulin formulation protracted with protamine is expected to be due to the rate-limiting degradation of the protamine peptides by fibrinolytic tissue enzymes and/or displacement by other cations in the interstitial fluid [1]. Insulin formulations protracted with protamine may be purely long-acting, so-called neutral protamine hagedorn (NPH) formulations where insulin and protamine are brought together in the so-called isophane proportions where the mutual precipitation is complete, or they may be biphasic pre-mixtures of rapid-acting dissolved insulin and long-acting NPH insulin to cover both basal and bolus needs in a single injection.

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Since the complete action profile (onset of action, peak insulin concentration, duration of action, etc.) is very much dependent of the content and quality of protamine peptides in the formulation, it is very important that adequate analytical methods exist to ensure batch consistency within each formulation type. So far, test methods normally used may be regarded as functional tests, verifying complete co-precipitation (NPH formulations) or verifying labeled content of dissolved insulin (biphasic formulations). However, these functional properties may be affected by other factors like the pH and ionic strength of the formulations or by unintended contamination during use of the drug product by a number of compounds like for instance heparin. A method for determination of dissolved insulin in biphasic formulations has been published as candidate for adaptation by the European Pharmacopoeia [4]. Nevertheless a suitable method has not as yet been included in the monograph for biphasic insulin human formulations of the European Pharmacopoeia [5]. Furthermore, the functional tests do not allow the specific investigation of the stability of each of the four major protamine peptides in the protracted insulin formulations during storage. In a recent publication [6] a high performance liquid chromatographic method was described for the determination of protamine sulphate in gel formulations of this substance. However, this method is not capable of separating the individual protamine peptides. Therefore, a RP-HPLC method has been developed and validated to selectively detect and quantitate each of the four major protamine peptides, as well as the total amount of protamine expressed as protamine nitrogen content, in the presence of insulin, preservatives and other formulation excipients. This paper describes the RP-HPLC method for protamine determination and discusses the analytical results obtained for a number of protracted insulin formulations and the potential of the method in connection with protamine sulphate raw material and final drug product quality control.

2. Materials and methods

Protamine sulphate (pharmaceutical quality) was obtained from three commercial suppliers. Insulin drug products were obtained from Novo Nordisk A/S (Bagsvaerd, Denmark). All other reagents were of analytical grade.

Determination of protamine peptides was performed by RP-HPLC on a Waters Corp. (Milford, MA) HPLC system, equipped with a three pump gradient formation and column heating capabilities, and UV–vis detection. Samples for

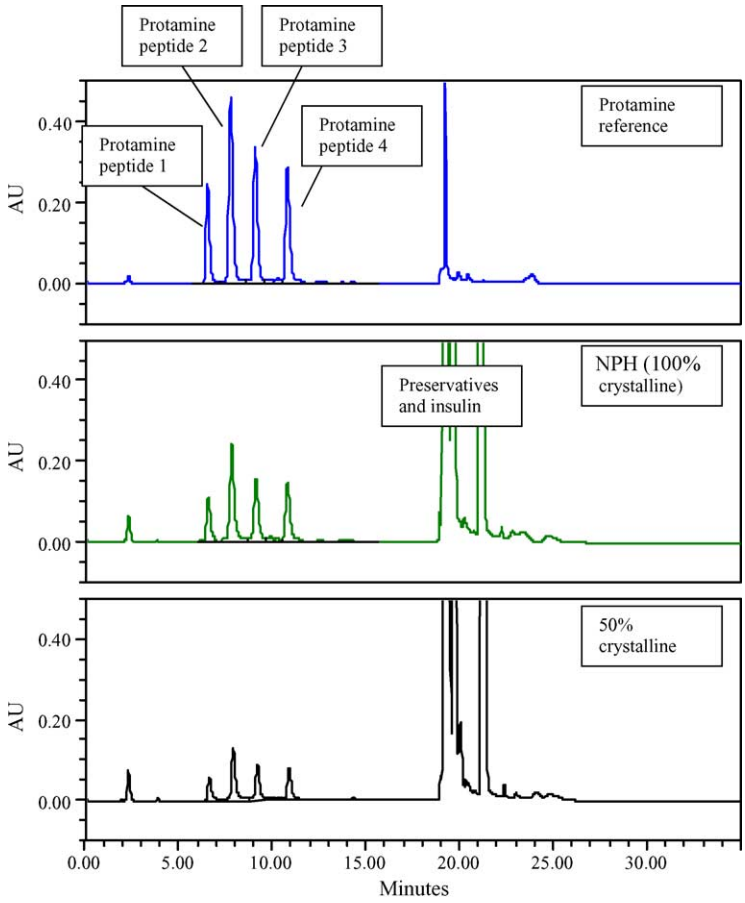

Fig. 1. Chromatogram of the protamine peptide reference solution and two degraded human insulin drug products, analyzed using the RP-HPLC method described in Section 2.

Table 1
Summary of validation of RP-HPLC protamine peptide method

Specificity	No interference from insulin, preservatives or other drug product excipients	
Precision	Protamine N content	Repeatability: R.S.D. = 0.45% Intermediate precision: R.S.D. = 1.26%
	Protamine peptide distribution	Repeatability: S.D. = 0.18% Intermediate precision: S.D. = 0.61%
Linearity/range	Linear over the range 0.0–114.7 μg N/ml with a correlation coefficient (r^2) of 0.99994	
Recovery	Mean recoveries of protamine nitrogen spiked to insulin solutions in levels covering the range indicated above were 98.7–101.4%	
Robustness	The method was found robust for the following variations in analytical parameters—column temperature: ±10 °C, pH of eluent A and B: ±0.2, retention time of protamine peptides (adjusted by introducing minor displacements in the initial shallow gradient): ±2.5 min	

analysis were acidified by which the protamine insulin complex is dissociated and dissolved because the net charge of the insulin molecule is changed from negative to positive. Briefly, 100 μl injections of acidified sample solutions (4 μl of an aqueous 6N HCl added per ml of insulin drug product or protamine sulphate stock solution, approx. 0.5 mg/ml) were analyzed on a Jupiter C18 (5 μm, 300 Å, 250 mm × 4.6 mm i.d.) column (Phenomenex, Torrance, CA) maintained at a column temperature of 55 °C. Separated protamine peptides were detected by UV at 214 nm and quantitated against a protamine peptide reference standard with a certified total content of 122 μg nitrogen per ml. The method uses a 3-eluent system (eluent A: sodium phosphate (pH 1.8; 0.1 M), eluent B: acetonitrile–sodium phosphate (0.1 M) (5:95, w/w) (pH* 1.8), eluent C: acetonitrile–water (42.8:57.2, w/w)) to elute the protamine peptides in a shallow acetonitrile gradient from

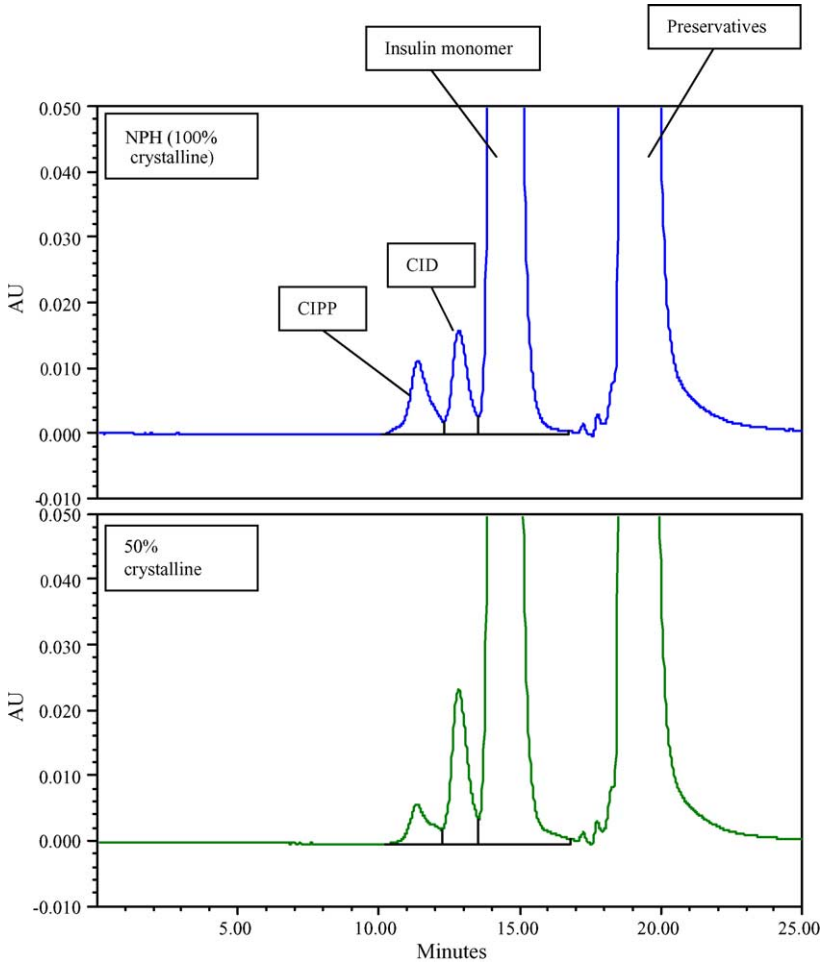

Fig. 2. Chromatogram of a degraded insulin drug product containing protamine, analyzed using a SEC method equivalent to the high molecular weight protein method adopted in the European Pharmacopoeia monograph for insulin [5].

A/B/C = 85/15/0 to A/B/C = 55/45/0 in 15 min. Subsequently the much more retained preservatives and insulin from the formulation are eluted using a high acetonitrile concentration obtained by changing the ratio of A/B/C to 10/10/80 in 1 min and keeping this ratio for 5 min. Total run time was 35 min to allow for the re-equilibration of the column between each run.

Following development of the RP-HPLC method for protamine analysis, as briefly described in Section 3, the method was validated for the following parameters: specificity, precision (evaluated in a combined testing including triplicate determinations of two different insulin drug products over six separate analytical runs), linearity, range and recovery (evaluated by spiking insulin solutions with increasing amount of a protamine sulphate stock solution).

Determination of the covalent insulin protamine product (CIPP) formed during storage [7], especially at accelerated storage conditions, was performed by using a size exclusion chromatographic (SEC) method equivalent to the high molecular weight protein (HMWP) method adopted in the European Pharmacopoeia monograph for insulin [8].

3. Results and discussion

The analytical RP-HPLC method for the determination of protamine peptides in insulin drug products was developed based on the procedure described by Hoffmann et al. [3]. A column wash with high acetonitrile concentration was included to elute preservatives and insulin, which bind very tightly to the analytical column under the conditions for obtaining suitable chromatographic separation of the protamine peptides from each other and from other drug product excipients. Further method optimization focused on obtaining reproducible results with regard to column performance, retention time and area of protamine peptides, etc. A representative chromatogram for a protamine containing insulin solution and the protamine peptide reference standard is shown in Fig. 1 and the results from the method validation are summarized in Table 1, documenting that the method is suitable and fit for the purpose. In the chromatogram, the protamine peptides elute well-separated from insulin, preservatives and other formulation excipients as four major peaks, each representing one of the four predominant protamine peptides, and a limited number of small, closely eluting peaks, consisting of other minor peptides from the protamine source. When quantitating the protamine nitrogen content, all peaks eluting between 4 and 15 min were integrated and quantitated against the corresponding area from the reference sample. For the protamine peptide distribution, the proportion of each of the four major peptides was calculated. Due to the insulin part in the CIPP, this complex is highly retained in the RP-HPLC method and elutes together with insulin and preservatives during column wash with high acetonitrile concentration. Thus any CIPP formed in an insulin formulation will reduce the content of free protamine nitrogen as determined by the RP-HPLC method.

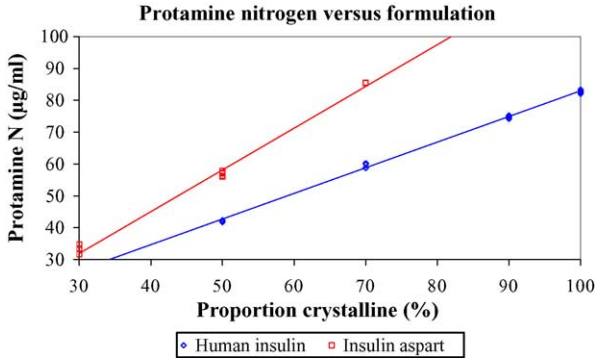

Fig. 3. Protamine nitrogen content, determined by RP-HPLC at initial release, of human insulin and insulin aspart drug products with various content of insulin-protamine crystals.

In the SEC method, the CIPP and the covalent insulin dimer (CID) elute prior to the insulin monomer due to the larger molecular masses and hydrodynamic volumes, while preservatives elute later than the void volume of the column due to a weak hydrophobic interaction with the stationary phase. Despite a smaller molecular mass, CIPP elutes prior to CID due to the relatively large hydrodynamic volume caused by the linear structure of the protamine peptide moiety of the CIPP. A representative chromatogram of a degraded insulin drug product containing protamine is shown in Fig. 2. As detection is performed at UV 276 nm, unbound protamine

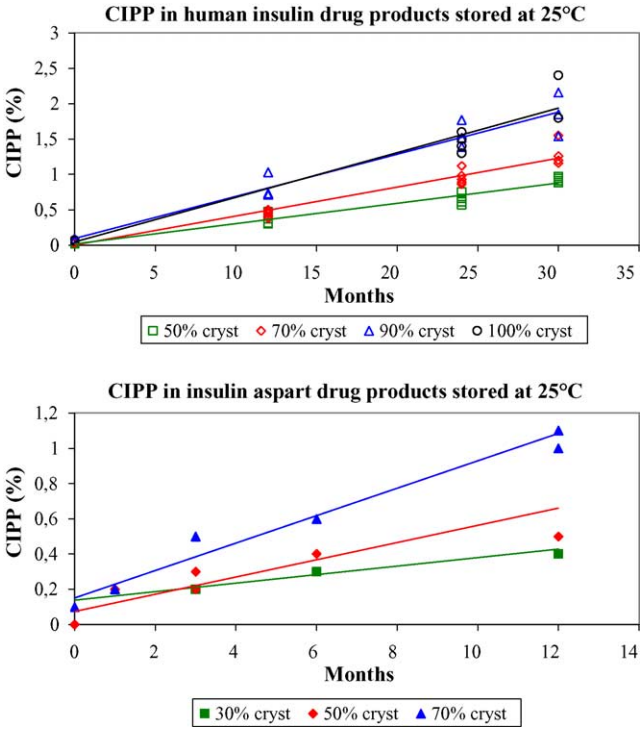

Fig. 4. Analytical results for the CIPP content in protamine containing insulin drug products stored at accelerated conditions.

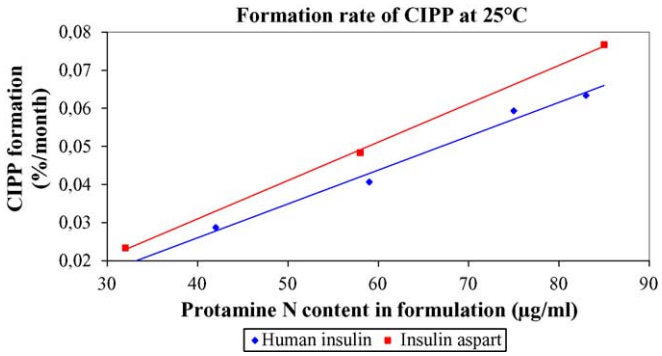

Fig. 5. CIPP formation rate at 25 °C in human insulin and insulin aspart drug products with different contents of protamine, shown as the average protamine nitrogen content at initial release.

peptides and the protamine part of CIPP are not detected using this method.

A total of approximately 250 insulin drug product samples were analyzed for protamine nitrogen content and protamine peptide distribution by the RP-HPLC method and for HMWP content using the size exclusion method as described in Section 2. The samples consisted of human insulin NPH and biphasic drug products (50–90% crystalline part) and insulin aspart (B28Asp human insulin analogue) biphasic drug products (30–70% crystalline part) stored for up to 30 months at 5 °C (recommended storage conditions for insulin drug products) or 25 °C (accelerated storage conditions) or stored for up to 3 months at 37 °C (severe storage conditions, only insulin aspart drug products). All drug products had a labeled content of insulin of 600 nmol/ml.

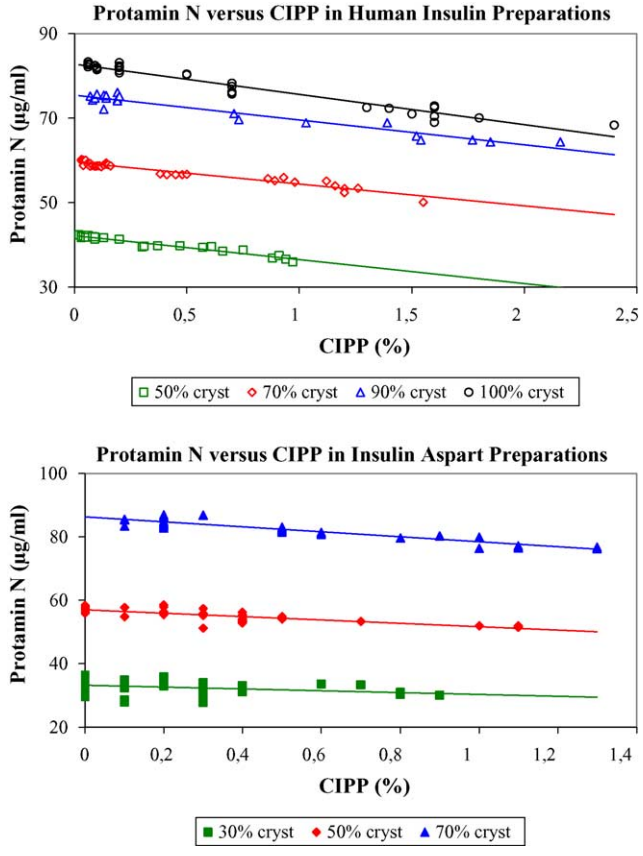

Fig. 6. Protamine nitrogen content in insulin drug products, shown as a function of CIPP determined by SEC, which is used as an index of chemical degradation by storage at recommended, accelerated or severe conditions.

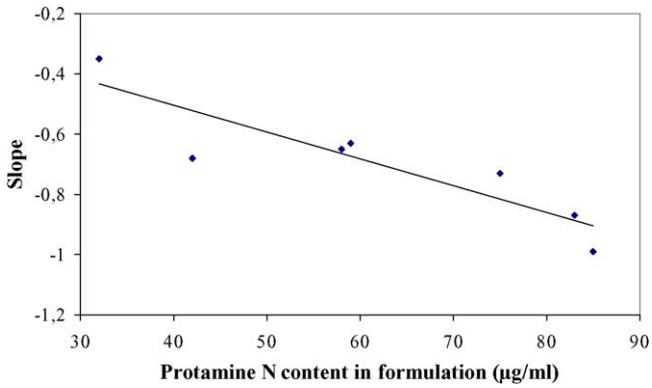

Fig. 7. Change in moles of protamine peptide per mole CIPP formed vs. average protamine nitrogen content in human insulin and insulin aspart drug products at initial release.

The protamine nitrogen content in the different insulin drug products at the time of initial release is shown in Fig. 3. As expected, a linear correlation between the protamine nitrogen content and the crystalline proportion of the drug product was obtained, indicating the potential of the RP-HPLC method for drug product quality control. More protamine is needed for insulin aspart drug products compared to those prepared from human insulin to form the same amount of ionic complexes, as the insulin aspart molecule contains one additional negatively charged group at neutral pH.

The analytical results for the CIPP content in insulin drug product samples stored at accelerated conditions are presented in Fig. 4. The formation of CIPP in samples stored

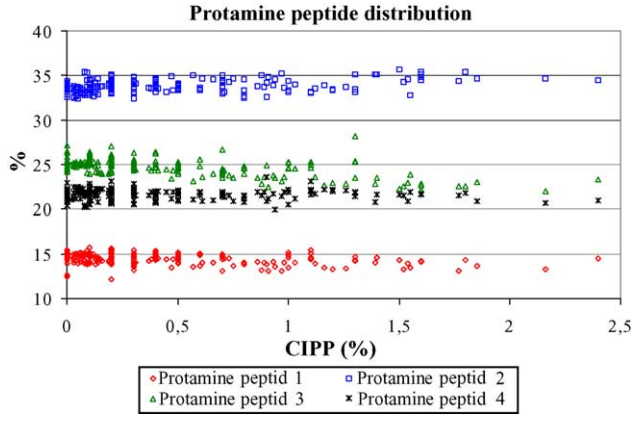

Fig. 8. Protamine peptide distribution of degraded human insulin and insulin aspart drug products. The protamine peptide distribution, determined by RP-HPLC, is shown as a function of CIPP determined by SEC, which is used as an index of chemical degradation by storage at recommended, accelerated or severe conditions. The figure indicates the relative amounts of the four major protamine peptides.

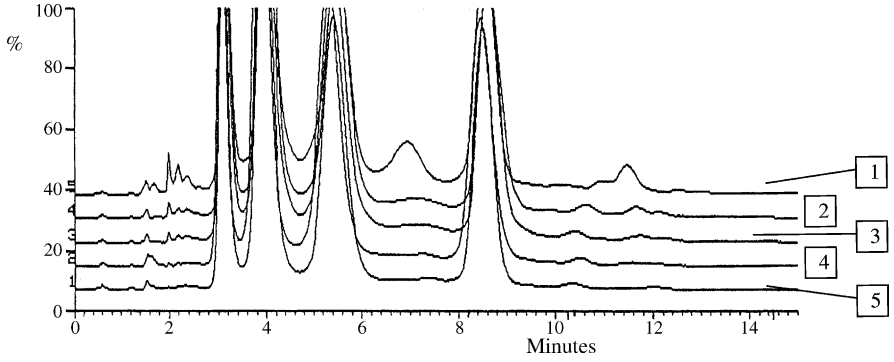

Fig. 9. Comparative RP-HPLC chromatograms of protamine sulphate raw materials from three different commercial suppliers: supplier A (tracks 1–3), supplier B (track 4) and supplier C (track 5).

at recommended storage conditions is only marginal (in average below 0.1% per year) while formation at 37 °C was only determined for insulin aspart drug products, and these data were therefore not included in the figure. Increasing formation rate of CIPP at 25 °C was observed for insulin drug products containing increasing amount of protamine, as shown in Fig. 5. This shows that the formation rate increases linearly within each insulin drug product type (human insulin or insulin aspart) indicating first order kinetics for the covalent reaction between insulin and protamine peptides.

The protamine nitrogen content of all the insulin drug product samples is presented graphically in Fig. 6, where analytical results are shown as a function of CIPP content, used as an index of chemical degradation during storage at recommended, accelerated or severe conditions. During storage, the content of protamine nitrogen in the insulin drug products decreases due to the formation of CIPP, which elutes outside the quantitation window in the RP-HPLC method. The slope for each of the different formulations has been calculated and the results have been expressed as change in moles of protamine peptide per mole CIPP formed. The results are presented in Fig. 7 versus the average protamine nitrogen content of the different formulations at initial release, indicating a changed stochiometry for incorporation of protamine into the CIPP when the drug product contains a low amount of protamine compared to those formulations containing a high amount of protamine. The exact nature of the CIPP complex has not been determined, but matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) investigation of the isolated CIPP fractions from drug products containing the highest and lowest amount of protamine (biphasic insulin aspart drug product with 30% and 70% crystalline part, respectively), have shown the presence of the same molecular masses corresponding to adducts of one molecule of each of insulin aspart and at least three of the four major protamine peptides (data not shown). Further characterization studies will be needed to elucidate this apparent discrepancy in analytical results, where on one hand the chromatographic data suggests that for some drug products, protamine is incorporated into CIPP in a less than one to one molar ratio, whereas the MS data supports a one to one ratio in all cases.

The protamine peptide distribution was determined for all insulin drug product samples, and the proportion of each of the four major protamine peptides was found to be very constant, independent of the chemical degradation, as shown in Fig. 8. This indicates that each of the four major protamine peptides contributes equally to the formation of CIPP. Under normal conditions or even after storage at accelerated or severe conditions, this ratio between the four peptides does not change, indicating a proportionally equal contribution of the four major protamine peptides to the formation of CIPP.

Five protamine sulphate raw material batches were analyzed by the RP-HPLC method for protamine nitrogen content and protamine peptide distribution, and the chromatograms are shown in Fig. 9. As seen, three of the batches from one supplier showed a higher content of peptides or UV-absorbing (214 nm) impurities eluting in between and after the four major protamine peptides. This observation correlated with the finding that more protamine sulphate nitrogen from this supplier was needed in order to obtain an isophane insulin drug product (data not shown). Consequently, the protamine sulphate quality from supplier A was abandoned for insulin drug products at Novo Nordisk.

4. Conclusions

This study has demonstrated the potential of the established RP-HPLC method for quality control of the protamine sulphate raw material and the final insulin drug products. Unlike the functional test methods normally used to ensure the right amount of protamine has been added to the different drug products, this method enables the direct and specific determination of the content of protamine independently of the drug product being tested. In addition, the method allows the purity of the protamine in the in-

sulin drug product to be determined, estimated as the total and relative content of each of the four major protamine peptides.

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