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Elution behavior of insulin on high-performance size exclusion chromatography at neutral pH

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ABSTRACT

The pharmacopoeia protocol for HP-SEC of insulin, using an acidic non-physiological eluent, does not represent insulin's association state in the formulation. This study aimed to evaluate insulin's elution behavior in HP-SEC in a "physiological" (aqueous, neutral pH) eluent, using on-line UV absorption and multi-angle laser light scattering detection. The effect of insulin concentration and association state in the formulation (monitored by circular dichroism) and eluent composition (zinc ion, arginine) on its elution behavior was assessed. We showed that the elution behavior of insulin in "physiological" HP-SEC is affected by both dynamic association–dissociation of insulin molecules and insulin–column interactions. Insulin molecules re-equilibrated in the HP-SEC eluent, making its elution behavior practically insensitive to the association state of insulin in the formulation. Zinc ions in the eluent promoted association of insulin to hexamers, whereas arginine overruled the effect of zinc ions and induced on-column dissociation of insulin to dimers and monomers. Combined results from "physiological" and compendial HP-SEC were shown to provide a better view of the aggregation state of heat-stressed insulin than either of the single methods. The insights obtained with this study are crucial for a proper evaluation of HP-SEC data of insulin.

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1. Introduction

Insulin is a peptide hormone which is used for the treatment of type 1 and advanced type 2 diabetes. Depending on solution conditions and insulin concentration, native insulin usually exists as a mixture of monomers and various quaternary (e.g. dimeric, tetrameric and hexameric) structures [1,2]. Monomeric insulin is the predominant form in acidic (pH < 2) environment, as well as at neutral pH at low insulin concentrations (<0.1 μM) [1,3,4]. In the absence of zinc ions, the insulin dimer is the most prevalent species at neutral pH. Unless the insulin concentration is high enough (millimolar range), zinc ions are essential for hexamerization of insulin molecules [5]. The insulin zinc hexamer is stable over a pH range of 5–8 and exhibits enhanced stability in comparison to monomeric insulin possibly because of limited conformational flexibility and reduced accessibility of reactive sites [4,6,7]. Most commercial insulin preparations are formulated in neutral pH solutions in which the insulin molecules mainly exist in hexameric form [1].

The presence of nonnative insulin aggregates (hereafter referred to as aggregates) in pharmaceutical preparations is a risk factor for unwanted immune responses, which may lead to a decrease in therapeutic efficacy of insulin [8,9]. In the United States and European Pharmacopoeias, high-performance size exclusion chromatography (HP-SEC) is selected as the standard method for detection of insulin aggregates in commercial insulin preparations [10,11]. HP-SEC enables simple, rapid and high throughput analytical methodology with good precision for detection of protein aggregates in the formulation, but also has its own drawbacks [12]. In particular, when using HP-SEC for analytical purposes, one should be aware that aggregates may be destroyed or created during the analysis due to changes in solvent and protein concentration, and interactions with the column material [12–15]. In the pharmacopoeias, an acidic HP-SEC eluent composed of a mixture of 1 g/L L-arginine aqueous solution:acetonitrile:glacial acetic acid 65:20:15 (v/v/v) is prescribed for the analysis [10,11]. Given that insulin is mostly formulated at neutral pH and its association state is strongly dependent on pH, the insulin species measured using the acidic eluent specified by pharmacopoeias may not represent those originally present in the formulations. Specifically, the acidic, acetonitrile-containing mobile phase promotes dissociation of insulin molecules [1,10,11]. This may apply not only to native

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self-assembly states of insulin molecules (e.g. dimers, tetramers, and hexamers) but also to non-covalent insulin aggregates. Conversely, acetonitrile in the mobile phase could enhance unfolding and, subsequently, induce insulin aggregation. Using a spectroturbidimetry method coupled with a photodiode array detection, dispersions of 0.6–5 mg/mL insulin in a solution containing 15% v/v acetic acid and 20% v/v acetonitrile led to insulin of molecular weight of ca. 11–17 kDa [16]. When the amount of acetic acid was increased to 20% (v/v) in the absence of acetonitrile, only the insulin monomer (5.8 kDa) was detected. In some studies, the pharmacopoeia method was modified so that the amount of acetonitrile in the eluent was lowered to 4% (v/v) [17,18]. Under these operating conditions, however, insulin was shown to also elute mainly as monomer [18].

In comparison to the pharmacopoeia protocol, HP-SEC analysis with neutral pH eluents has been used occasionally for quantification of insulin and its aggregates [7,19,20], but the influence of the elution conditions on the chromatographic behavior of insulin was not reported. On the one hand, as HP-SEC at neutral pH does not suffer from acid-induced dissociation of insulin molecules, the analysis may better represent the distribution of insulin molecules in the formulation. On the other hand, using insulin and insulin related proteins, Klyushnichenko and Wulfson [21] demonstrated that weak adsorption of proteins onto silica based column materials occurred during the separation at neutral pH solution. A deeper understanding of the chromatographic behavior of insulin would enable a better interpretation of HP-SEC of insulin.

In principle, HP-SEC separates analytes on the basis of their shape and hydrodynamic volume [14,22] and molecular weight determination of the analytes is possible by using appropriate protein molecular weight markers [23,24]. However, erroneous size estimations may occur due to the non-spherical nature of the selected protein standards or aggregates and protein–column interactions [25]. This problem can be overcome by a combination of multi-angle laser light scattering (MALLS) and UV detection. As the intensity of the light scattered by particles is directly proportional to the molar concentration and molecular mass of the analytes, MALLS detection permits an absolute molecular weight calculation without the use of reference standards and assumptions regarding molecular geometry of the analytes [14,18,26].

This study aims to gain a deeper understanding of the elution behavior of insulin in HP-SEC in a “physiological” (aqueous, neutral pH) eluent, using on-line MALLS and UV detection for absolute mass estimation. In particular, the effects of the association state of insulin in the formulation (dimer and hexamer), eluent composition (zinc ions and arginine) and insulin concentration were evaluated. The best separation conditions, based on the recovery and the ability of the system to maintain the native association state of insulin molecules, were used for detection of insulin aggregates in a heat-stressed solution and the results were compared to the pharmacopoeia method.

2. Materials and methods

2.1. Materials

Recombinant human insulin containing 0.4% (w/w) zinc ions was provided by Schering-Plough, Oss, the Netherlands. Disodium hydrogen phosphate, sodium chloride, zinc chloride, phenol, sodium azide and ethylene diamine tetraacetic acid (EDTA) were purchased from Sigma–Aldrich, Germany. Calcium chloride, anhydrous sodium sulfate, L-arginine and orthophosphoric acid were bought from Merck, Germany. All chemicals were of analytical grade and used without further purification. Deionized water was purified through a Purelab Ultra System (ELGA LabWater Global Operations, UK) prior to use.

2.2. Insulin formulations

Unless other concentrations specified, 1 mg/mL insulin was prepared in sodium phosphate buffer (PB: 69 mM disodium hydrogen phosphate and 45 mM sodium chloride, pH 7.4) containing 61 μM EDTA (PB-EDTA), or PB containing 57 μM zinc chloride, 29 μM calcium and 172 μM phenol (PB-zinc). In these buffers likely insulin will exhibit different association states. In particular, insulin in PB-zinc is most likely in its hexameric form because zinc and calcium ions and phenol promote hexamer formation in the solution [2]. The amount of zinc in the PB-zinc formulation was equivalent to two zinc ions per insulin hexamer. Depletion of zinc ions by EDTA is likely to induce dissociation of insulin hexamers to predominantly dimers in the PB-EDTA formulation. The concentration of insulin was determined by UV spectroscopy, using a molecular weight of 5.8 kDa and an extinction coefficient of 6200 $M^{-1}\,cm^{-1}$ at 276 nm [6].

2.3. HP-SEC

HP-SEC experiments were performed using an Agilent 1200 HPLC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an 18-angle MALLS (DAWN HELEOS, Wyatt Technology Europe) detector. Specifically, the Agilent HPLC system consisted of a 1200 series HPLC pump, degasser, autosampler G1329A, variable wavelength detector G1316A and fluorescence detector G1321A, and an Optilab rEX differential refractometer with extended range detector. As insulin in neutral pH solutions tends to form dimers and hexamers, a TSK3000$_{SWXL}$ column (10 μm silica based column, 300 mm × 7.8 mm from Tosoh Bioscience LLC) which has a cut of range of 10–500 kDa was used for the analysis at room temperature. The HP-SEC eluent comprised 100 mM ammonium acetate buffer, 300 mM sodium chloride, and 0.05% (w/v) sodium azide, pH 6.8. To investigate the effect of zinc ions on the separation, 57 μM zinc chloride was added in the HP-SEC eluent. This zinc ion concentration was equivalent to that used in the PB-zinc formulation. The effects of arginine on the elution behavior of insulin were tested using 5.7 mM L-arginine in the HP-SEC eluent. A flow rate of 0.5 mL/min and an injection volume of 100 μL were used. Chromatograms were acquired using UV absorption at 276 nm and MALLS detection. Under these settings, the void and total column volumes were 6.25 and 14 mL, respectively.

For the investigation of heat-stressed insulin sample, HP-SEC analysis was performed in parallel using neutral and acidic (pharmacopoeia) HP-SEC eluents. To achieve this, the HP-SEC analysis based on the pharmacopoeia method was carried out using a Waters insulin HMPW column, 300 mm × 7.8 mm (Waters Corporation, Ireland). The mobile phase comprised 1 g/L L-arginine solution:acetonitrile:glacial acetic acid in a 65:20:15 volume ratio. A flow rate of 0.5 mL/min and an injection volume of 100 μL were used for the analysis. The HPLC system comprised a Waters 515 HPLC pump, Waters 717 Plus autosampler, Waters 474 scanning fluorescence detector, and Shimadzu SPD-6AV UV–vis detector for analysis.

Weight average molecular weight of insulin from MALLS detection was calculated with Astra Software (version 5.3.1.5, Wyatt Technology Europe), using a differential refractive index increment (dn/dc) of 0.185 mL g^{-1}, and the Zimm equation. The molecular weight was calculated using the following equation.

$$\frac{K^{*}c}{R_{\theta}} = \frac{1}{\mathrm{Mw}P_{\theta}} + 2A_{2}c$$

where K^* is the optical constant, c is the concentration of the solute (g/mL), Mw is the weight-averaged molar mass, R_θ is the Rayleigh ratio, A_2 is the second virial coefficient, P_θ is the scattering function. The optical constant, K^*, was calculated as:

$$K^* = 4\pi^2 \left(\frac{\partial n}{\partial c}\right)^2 n_0^2 N_A^{-1} \lambda_0^{-4}$$

where n_0 is the refractive index of the solvent, $\partial n/\partial c$ is the refractive index increment, N_A is Avogadro's number, and λ_0 is the wavelength of the light in vacuum. The molecular weight estimation was carried out using the chromatographic region where both the UV and the MALLS signals were sufficiently high, allowing a reliable molecular weight estimation. Unless otherwise stated, insulin recovery was calculated from injected mass and recovered mass. The injected mass was computed from the injection volume and insulin concentration, whereas the mass recovery was estimated from the integrated UV signals at 276 nm.

2.4. UV spectroscopy

An Agilent 8453 UV–vis spectrometer (Agilent, Waldbronn, Germany) was used for UV measurements. Analysis was performed using a 1 cm pathlength quartz cuvette at 25 °C.

2.5. Near UV circular dichroism (near UV CD) spectroscopy

Near UV CD spectra were recorded from 250 to 320 nm using a Jasco J-815 CD spectrometer (Jasco International, Tokyo, Japan). Analyses were performed in a 1 cm pathlength quartz cuvette at 20 °C using a speed of 50 nm/min, a response time of 2 s, and a bandwidth of 1 nm. Each spectrum was the result of an averaging of 8 repeated scans, background corrected with the corresponding buffer spectrum. The CD signals were converted to molar ellipticity per amino acid residue.

3. Results and discussion

3.1. Association states of insulin in the formulations

The near UV CD signal of insulin has been shown to be sensitive not only to insulin's tertiary structure but also to its quaternary structure, i.e. association state [27,28]. Specifically, the negative magnitude of the signal is indicative of intermolecular interactions in multimeric states, i.e. negative molar ellipticity monomer < dimer < hexamer. In the present study, near UV CD spectroscopy was used to monitor the association state of insulin in each formulation. The CD spectra of 1 mg/mL insulin in PB-EDTA and in PB-zinc are given in Fig. 1a. The CD spectrum of insulin in PB-zinc shows a larger negative intensity compared to the insulin in zinc ion-free formulation. The spectral shape as well as the molar ellipticity of insulin in PB-zinc with a minimum value of -340° cm^2 dmol^{-1} at 275 nm is consistent with the near UV CD signal reported for hexameric human insulin [29–31]. The lower signal intensity of the near UV CD signal of insulin in PB-EDTA suggests that insulin does not form hexamers in this formulation. The CD spectra of insulin in 20% acetic acid and 0.1 M HCl solutions, representing the spectra of insulin in monomer and monomer-

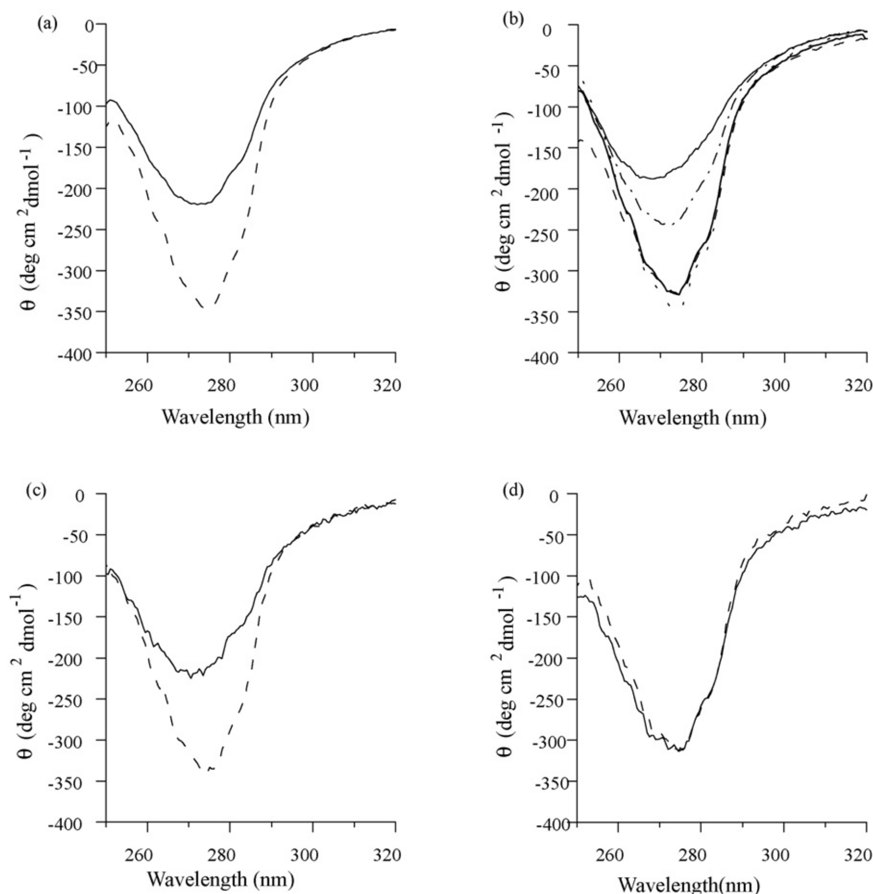

Fig. 1. Near UV CD spectra of 1 mg/mL insulin (a) in (—) PB-EDTA and (––) PB-zinc, (b) in (—) PB, pH 7.4, (. . .) PB, pH 6.8, (––) HP-SEC eluent pH 6.8, (—) 20% acetic acid and (- -) 0.1 M HCl solutions, (c) in PB-EDTA diluted fivefold (to 0.2 mg/mL) in (—) PB-EDTA and (––) HP-SEC eluent containing zinc ions, and (d) in PB-zinc diluted fivefold in (—) PB-zinc and (––) HP-SEC eluent containing zinc ions.

dimer mixture, respectively [32], are presented in Fig. 1b. The close proximity of the spectral shape and molar ellipticity of insulin in PB-EDTA (Fig. 1a) to insulin in 0.1 M HCl implies that insulin molecules in PB-EDTA are predominantly organized as a monomer-dimer mixture [32,33]. Dimer formation of insulin in PB-EDTA likely originates from a depletion of zinc ions from insulin due to zinc ion–EDTA complex formation.

3.2. Initial association state of insulin does not affect its elution behavior in HP-SEC

Theoretically, the elution time of analytes in HP-SEC is inversely related to their hydrodynamic diameter. Insulin in PB-zinc is, therefore, expected to elute prior to insulin in PB-EDTA, as hexamers migrate faster through the column than dimers and monomers. Based on the fact that insulin hexamer is stable over a pH range of 5–8 and a previous "physiological" HP-SEC method used by Rosa et al., the HP-SEC eluent chosen for the current analysis was a 100 mM ammonium acetate solution buffered at pH 6.8 buffer [7,20], which enables the investigation of zinc ions as an additive in the HP-SEC eluent. Furthermore, 300 mM sodium chloride and 0.05% (w/v) sodium azide were added to the HP-SEC eluent to minimize electrostatic interactions between insulin and column materials and to prevent bacterial growth during the analysis, respectively. The association state of insulin in the ammonium acetate HP-SEC eluent was found to be similar to that in PB (pH 6.8 or pH 7.4), as derived from the near UV CD spectrum (Fig. 1b), indicating that insulin in these solutions was mainly in its hexameric state.

To investigate the effect of association state of insulin in the formulation on the elution behavior, 1 mg/mL insulin in PB-EDTA and PB-zinc was analyzed using the zinc ion-free HP-SEC eluent. Chromatograms show broad, asymmetric, and tailing peaks with a peak maximum near 23 min, regardless of the association state of insulin in each formulation (Fig. 2a). Recovery of insulin from both formulations was similar and amounted to approximately 95% under these separation conditions (Table 1). MALLS detection indicated that insulin of an average molecular weight of approximately 21 and 25 kDa was eluted from PB-zinc and PB-EDTA, respectively (Table 2). These estimations were between the molecular weight of insulin dimer (12 kDa) and hexamer (36 kDa). It is likely that multiple association states (monomer, dimer, tetramer, and hexamer) coexisted on the column during separation; however, dimers and hexamers were most likely the dominant species under this solution condition [3]. From the near UV CD spectrum, the molar ellipticity indicated that 1 mg/mL insulin in neutral, zinc ion-free solutions existed mainly in its hexameric state (Fig. 1b). The smaller proportion of hexameric insulin during HP-SEC can be understood by a ∼30-fold decrease in insulin concentration upon dilution in the HP-SEC eluent, which favors dissociation of the insulin hexamers to tetramers and dimers (see the following section).

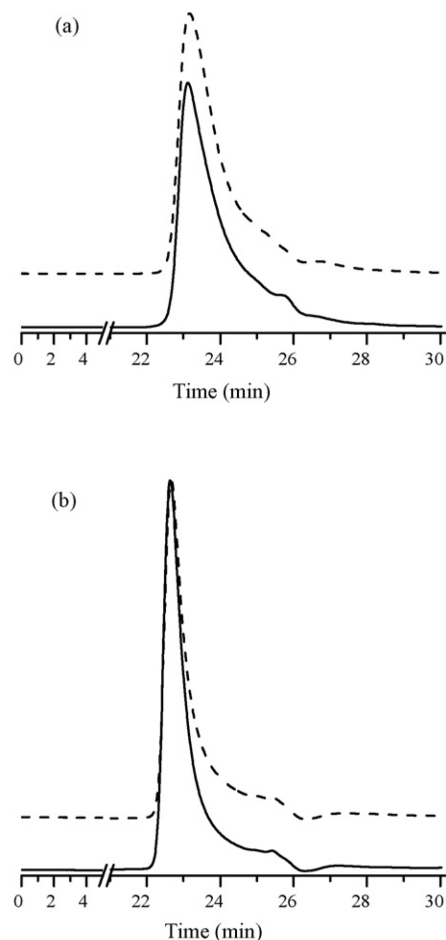

Fig. 2. Size exclusion chromatograms of 1 mg/mL insulin in (—) PB-zinc and (−−) PB-EDTA eluted from (a) zinc ion-free and (b) zinc ion-containing HP-SEC eluents.

Zinc ions are known to mediate hexamerization of insulin molecules [2]; therefore, their role in regulating the chromatographic behavior of insulin in the HP-SEC was also assessed in this study. Addition of zinc ions to the HP-SEC eluent sharpened the insulin peak and shifted it to a slightly shorter retention time, 22.5 min (Fig. 2b). Table 1 illustrates that the recovery of insulin was lowered by approximately 15% upon addition of zinc ions into the HP-SEC eluent. This may be caused by precipitation of insulin in

Table 1
Effect of arginine and zinc ions in the HP-SEC eluent on the recovery of 1 mg/mL insulin in PB-zinc and PB-EDTA formulations[a].

Formulation	Eluent without arginine		Eluent with arginine	
	Without zinc ions	With zinc ions	Without zinc ions	With zinc ions
PB-zinc	95.2 ± 0.5	81.0 ± 0.1	97.2 ± 0.0	50.9 ± 3.1
PB-EDTA	94.9 ± 0.1	74.2 ± 3.0	92.0 ± 0.4	46.1 ± 3.0

[a] Data represent mean ± standard deviation (n = 3 injections); recoveries were calculated based on the UV signal of the insulin peak and the injected mass.

Table 2
Effect of arginine and zinc ions in the HP-SEC eluent on the average molecular weight of 1 mg/mL insulin in PB-zinc and PB-EDTA formulations[a].

Formulation	Eluent without arginine		Eluent with arginine	
	Without zinc ions	With zinc ions	Without zinc ions	With zinc ions
PB-zinc	21.4 ± 0.4	36.1 ± 0.1	8.6 ± 1.8	8.9 ± 0.1
PB-EDTA	24.8 ± 3.0	36.9 ± 0.3	8.9 ± 1.8	9.2 ± 0.2

[a] Data represent mean ± standard deviation (n = 3 injections); the molecular weight was calculated using MALLS detection.

zinc ion-containing solutions [1]. Insulin with an average molecular weight of about 36 kDa was eluted under these separation conditions, consistent with the hexameric state (Table 2). So, also in zinc ion-containing eluent, the elution behavior of insulin is independent of insulin's association state in the applied formulation.

To better understand the association state of insulin upon dilution in HP-SEC eluent, a dilution study was carried out. For this, 1 mg/mL insulin in PB-EDTA and PB-zinc was diluted fivefold in its corresponding formulation buffer, or in zinc ion-containing HP-SEC eluent, and the diluted samples were analyzed by near UV CD spectroscopy (Fig. 1c and d). This dilution factor was selected as it allows the effects of the dilution of insulin into HP-SEC eluent to be qualitatively studied by near UV CD spectroscopy. The molar ellipticity of insulin at 276 nm was found near −220 and $-310^{\circ}\,cm^2\,dmol^{-1}$ upon dilution of insulin in PB-EDTA and PB-zinc, respectively. Considering the spectra of the undiluted samples in Fig. 1a, these values suggest that dilution did not alter the association state of insulin. The molar ellipticity of insulin in PB-EDTA diluted in zinc ion-containing HP-SEC eluent intensified to approximately $-340^{\circ}\,cm^2\,dmol^{-1}$ (Fig. 1c). This number is close to the value obtained upon dilution of insulin in PB-zinc and in zinc ion-containing HP-SEC eluent (Fig. 1a and d), indicating that insulin molecules spontaneously reorganized themselves into a hexameric form, which is the most favorable association state under these solution conditions.

In summary, insulin in a neutral pH solution is known to be in an equilibrium mixture of monomer, dimer, tetramer and hexamer forms [1,3]. The finding that insulin in both formulations was eluted predominantly as dimer–tetramer–hexamer mixture in zinc ion-free HP-SEC eluent and mainly as hexamer in zinc ion-containing HP-SEC eluent mirrors the ability of insulin molecules to rapidly (with respect to the separation time) rearrange themselves into the most favorable association state at a given solution condition. As a result, the HP-SEC behavior of insulin depends strongly upon the composition of the HP-SEC eluent and not on the association state of insulin molecules in the formulation to be analyzed.

3.3. Association–dissociation reactions of insulin during HP-SEC influence its elution behavior

Theoretical simulation demonstrated that reversible dimerization of proteins during HP-SEC analysis can cause peak splitting, merging, fronting, and tailing [34]. As the association–dissociation reactions of insulin molecules depend on insulin concentration and eluent composition (in particular the presence of zinc ions), their role on the elution behavior of insulin at neutral pH may be studied by monitoring the chromatographic behavior of insulin applied in various concentrations [1]. For this experiment, 0.5–5 mg/mL insulin in PB-zinc was analyzed using HP-SEC eluents with and without zinc ions.

Fig. 3 shows that the elution of insulin is sensitive not only to the presence of zinc ions in the HP-SEC eluent, but also to the insulin concentration. In particular, the retention time of the peak maximum increases with decreasing insulin concentration (Fig. 3a and b). The influence of insulin concentration on the peak position (Fig. 3a and b) and average molecular weight of insulin (Fig. 3c) is less pronounced for zinc ion-containing HP-SEC eluent. In the case of zinc ion-free HP-SEC eluent (Fig. 3a), the migration of the insulin peak upon decreasing insulin concentration may be understood by re-equilibration of insulin molecules during the HP-SEC separation. Most insulin molecules were eluted as hexamers in zinc ion-free HP-SEC eluent when the insulin concentration was ≥3 mg/mL, as detected by MALLS (Fig. 3a and c). These hexamers were not fully dissociated during the analysis and, consequently, peak spreading during the separation was relatively small. Apparently, the local insulin concentration was sufficiently high to maintain a large proportion of the insulin molecules in the hexameric state during the separation in HP-SEC. However, as insulin migrates through the column, reduction in insulin concentration stimulates dissociation of the molecules, explaining why dimers and possibly monomers were detected in the tail region (Fig. 3a). Dissociation of the hexamers in conjunction with favorable dimer formation upon lowering the insulin concentration was associated with a shift of the insulin peak towards longer retention times as well as lower average molecular weights (Fig. 3a and c). Additionally, Fig. 3a demonstrates that the average molecular weight of insulin reaches its maximum value slightly after the position of concentration maximum in the UV detection, indicating that the self-association of insulin also occurs during the separation, at a rate slightly slower than the separation time. The overall results illustrate that association–dissociation reactions play a vital role in determining the elution behavior of insulin during HP-SEC analysis using a zinc ion-free HP-SEC eluent at neutral pH. The observation that the insulin peak comprises multiple association states of insulin suggests that the association–dissociation reac-

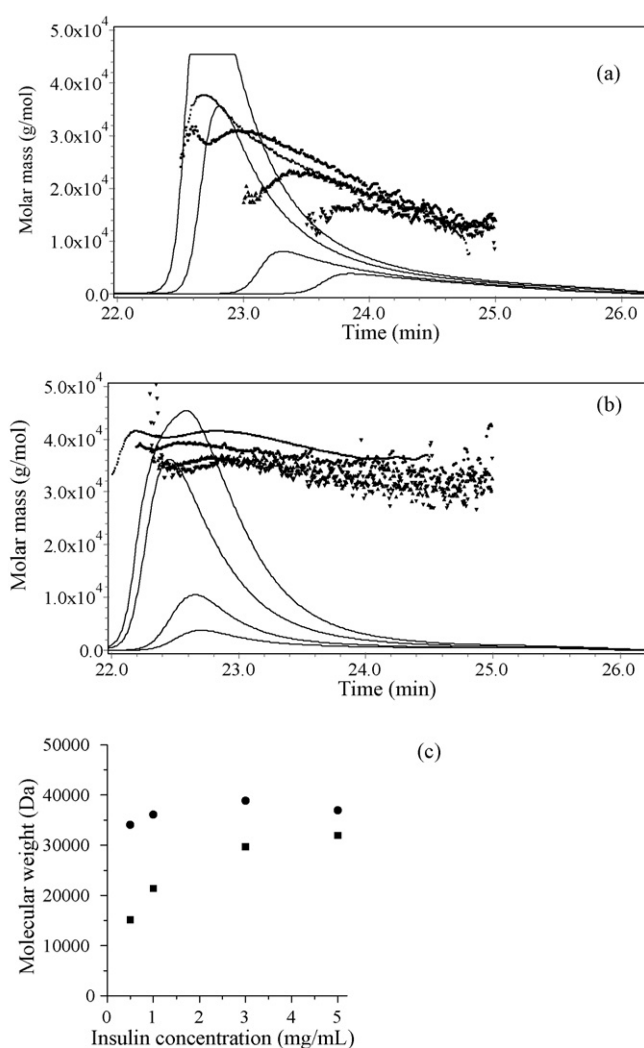

Fig. 3. Size exclusion chromatograms of 5, 3, 1, and 0.5 mg/mL insulin in PB-zinc (from left to right) eluted from HP-SEC eluent (a) without and (b) with zinc ions. The *y*-axis represents molecular weight estimation of (■) 5, (●) 3, (▲) 1, and (▼) 0.5 mg/mL insulin from UV (or refractive index, for the 5 mg/mL formulation) and MALLS detection; (c) average molecular weight of insulin as a function of insulin concentration when the insulin was eluted from HP-SEC eluent (■) without and (●) with zinc ions. The trajectory of the chromatogram used for the calculation was 22.3–25.3 min and 22.0–23.3 min for HP-SEC eluent without and with zinc ions, respectively.

tions of insulin molecules are dynamic, but a bit slower than the timescale of the HP-SEC separation. Without the MALLS detection, the relationship between dissociation of insulin molecules and tailing would have been missed.

If dissociation reactions were the only cause of tailing, addition of zinc ions into the HP-SEC eluent should eliminate the tailing because the dissociation reactions are diminished in this HP-SEC eluent (Table 2). Accordingly, MALLS detection suggested that the average molecular weight of insulin remained stable near 34 kDa over the entire insulin peak for all samples (Fig. 3b). The chromatograms illustrate that the presence of zinc ions in the HP-SEC eluent did not completely eliminate tailing, but reduced it considerably. This effect of zinc ions may be originating from their ability to remove secondary (adsorption) interactions of insulin with sorbent matrices, since insulin hexamers are less surface reactive than tetramers, dimers, and monomers [35]. The retention time of the insulin peak also shifted to greater values upon decreasing insulin concentration, but to a smaller extent compared to that observed in the zinc ion-free HP-SEC eluent. This indicates that insulin–column interactions could be another factor contributing to the tailing of the insulin peak [34,36].

3.4. *Effect of arginine on the elution behavior of insulin in HP-SEC*

Electrostatic and hydrophobic interactions are major causes of non-specific adsorption of proteins to the column material in HP-SEC [21,23,24]. Electrostatic interactions are often reduced by modifying the pH and ionic strength of the HP-SEC eluent. Considering that 300 mM sodium chloride was already present in the HP-SEC eluent and because high salt concentrations could promote hydrophobic interactions and induce protein aggregation [15,24], no attempt was done to further increase the amount of salt in the HP-SEC eluent. Sodium dodecyl sulfate, urea, guanidine hydrochloride, and acetonitrile are often added to the HP-SEC eluent to reduce hydrophobic interactions [12,21,23,24]. Though effective, these additives may induce conformational changes or denature the proteins, making them unsuitable for native HP-SEC separation [21]. Arginine is another additive which has been used to suppress protein–column interactions [12,14,15]. Addition of arginine in the HP-SEC eluent was reported to greatly increase protein recovery and improve separation of soluble oligomers [15]. Although there are concerns that arginine at high concentrations may induce denaturation, at low concentrations arginine can also suppress protein aggregation [37–39]. Therefore, the effects of arginine at low concentration in minimizing protein–column interactions were examined.

Fig. 4 shows that arginine effectively reduced tailing of the insulin peak and its effects were more pronounced in the zinc ion-free HP-SEC eluent. The insulin peak shifted slightly to greater retention times in the arginine-containing HP-SEC eluent (Fig. 4a), indicating formation of lower association states of insulin in arginine-containing solution. A greater shift in the retention time was found in the arginine- and zinc ion-containing HP-SEC eluent (Fig. 4b). Molecular weight calculation from MALLS detection suggested that insulin was in an equilibrium mixture of monomer and dimer in arginine-containing HP-SEC eluents, regardless of the association state of insulin in the formulation and the presence of zinc ions in the eluent (Table 2). The ability of arginine to dissociate insulin oligomers in zinc ion-containing HP-SEC eluent corresponds to its reported ability to suppress intermolecular interactions among protein molecules [37]. In addition to intermolecular interactions, the effect of arginine clearly extends to insulin–column interactions. Given that hydrophobic patches on insulin monomer surfaces are sheltered during self-association into hexamer, insulin monomer may have an increased propensity to interact with column materials [4]. If arginine would only promote dissociation of

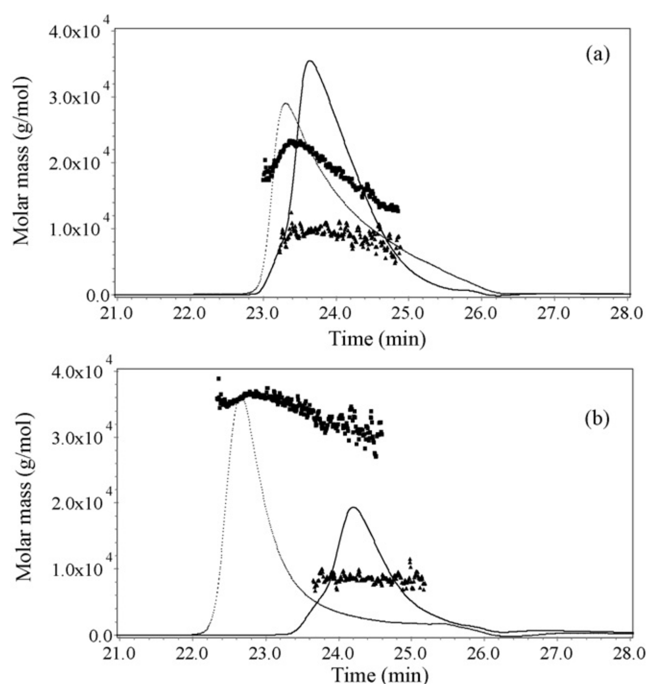

Fig. 4. Size exclusion chromatograms of 1 mg/mL insulin in PB-zinc eluted from (a) zinc ion-free and (b) zinc ion-containing HP-SEC eluents (— and ▲) with and (–– and ■) without arginine.

insulin oligomers and not reduce insulin–column interactions, the tailing of insulin peak would have been increased upon addition of arginine in the HP-SEC eluents. Fig. 4 demonstrates the opposite, as the insulin peak appeared more symmetric in arginine-containing HP-SEC eluents. Hence, it can be concluded that arginine increased peak symmetry by attenuating insulin–column interactions.

Table 1 shows that insulin–column interactions did not cause low insulin recovery; instead they are related to peak tailing, as discussed above. As a result, the effects of arginine on the recovery of insulin are minimal. A combination of arginine and zinc ions in the HP-SEC eluent, surprisingly, reduced the recovery and only 50% of insulin was recovered in the HP-SEC chromatograms.

3.5. *Evaluation of heat-stressed insulin by HP-SEC*

Following the understanding of the factors influencing HP-SEC elution behavior of insulin in neutral pH solutions, a study was performed to evaluate the advantages of determining aggregation of insulin using a more physiological pH solution. Based on the recovery data and the drastic effect of arginine on the association state of insulin, the zinc ion- and arginine-free HP-SEC eluent was selected for this study, in which 1 mg/mL insulin in PB-EDTA was analyzed before and after heating (75 °C, 1 h). The heated sample was centrifuged to remove insoluble aggregates prior to injection into the HP-SEC analysis. For comparison, the analysis was also performed using an acidic HP-SEC eluent as specified in the pharmacopoeias.

Based on the peak area of insulin, both HP-SEC assays provided comparable information when analyzing the unstressed insulin solution (100.0 ± 0.2% and 100.0 ± 0.4% for acidic and physiological HP-SEC eluent, respectively). For the stressed sample, remarkable differences were observed between the two HP-SEC methods. The acidic eluent allowed an estimation of the amount of soluble aggregates (8.0 ± 0.1%), dimers (10.0 ± 0.1%), monomers (59.2 ± 0.1%) and fragmented insulin (15.0 ± 0.1%), which appeared in the chromatograms as separate peaks. Formation of substances of smaller size than insulin monomer has been previously detected upon storage of insulin solution at 37 °C for 14 days [40]. Matrix-assisted

laser desorption ionization mass spectrometry (MALDI-TOF MS) analysis demonstrated that the fragment species represented oxidized and intrachain disulfide bridged B-chain of insulin with a molecular weight of ca. 3.4 kDa. The short exposure to a higher temperature used in our study is likely to promote the formation of the same or similar fragmented species. In the chromatograms from the physiological eluent, however, these insulin species could not be resolved due to continuous re-equilibration during the separation. For this, MALLS detection is indispensable. Therefore, a broad insulin peak representing insulin dimer, monomer and fragments was detected and this accounted for $56.3 \pm 1.6\%$ of the peak area. The non-recovered fraction, presumably reflecting insoluble aggregates, obtained from the acidic eluent was estimated to be approximately $7.9 \pm 0.2\%$, whereas the analysis of the same solution using the physiological eluent yielded around $43.7 \pm 1.6\%$ of non-recovered protein. The striking difference in the estimation of aggregates in the stressed samples from these two HP-SEC conditions may be attributable to greater solubility of insulin in acidic media.

The results from the acidic HP-SEC eluent showed approximately $59.2 \pm 0.1\%$ insulin monomer after stressing, whereas the data from the physiological HP-SEC eluent indicated that insulin (dimer, monomer and fragments) amounted to $56.3 \pm 1.6\%$. So, HP-SEC under acidic conditions may overestimate the amount of 'native' insulin and the HP-SEC assay with the physiological eluent may better represent the 'native' insulin content in the stressed sample.

4. Conclusions

Dynamic association and dissociation of insulin are ubiquitous in physiological pH range solutions. This study demonstrated that HP-SEC elution behavior of insulin (association state, dissociation behavior, elution position and peak tailing) is highly sensitive to eluent composition (pH, zinc ions and arginine), insulin–column interactions and insulin concentration. Re-equilibration of insulin molecules takes place spontaneously during the separation, making the chromatographic behavior of insulin insensitive to the association state of the molecules in the formulation at a given insulin concentration. A combination of UV and MALLS detection in HP-SEC enables the influence of association–dissociation reactions of insulin on the elution behavior to be studied. In zinc ion-free HP-SEC eluent, insulin appears as a broad, tailing peak with multiple association states of insulin molecules. Tailing is caused by spontaneous dissociation of insulin molecules while travelling through the column as well as insulin–column interactions. Addition of arginine into the HP-SEC eluent effectively suppresses the tailing but it also interferes with the association state of insulin during analysis. For HP-SEC of insulin, a combination of the results from acidic HP-SEC and physiological HP-SEC provides a better view of the aggregation state of insulin than either of the individual methods.

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