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Development of two high-performance liquid chromatographic methods for the analysis and characterization of insulin and its degradation products in pharmaceutical preparations

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Abstract

Two high-performance liquid chromatography methods either in reversed-phase or as size exclusion separation mode were developed and validated for the analysis of insulin and its degradation products in pharmaceutical preparations. The results show the reliability of the analytical methods for the intended used. The static and dynamic light scattering were used to characterize the insulin and its derivatives. The absolute molecular weight of human insulin monomer and dimer were 5800 and 12400 Da respectively whereas its *z*-average root mean square radius were 21.6±0.4 and 40.5±0.7 nm, respectively. In contrast, the hydrodynamic diameter varied between 2.69 and 5.50 nm, depending of the association behavior of insulin.

Keywords: Insulin

1. Introduction

Insulin is the most important regulatory hormone in the control of glucose homeostasis consisting of 51 amino acids shared between two intramolecular chains and with a molecular weight of 5800 g/mol [1]. Insulin, like other proteins, is not a stable entity but is liable to modification by chemical reactions with molecules in its vicinity. Thus, during storage and use of pharmaceutical preparations insulin is degraded by hydrolytic reactions (deamidated products) or is transformed by formation of intermolecular covalent bonds with other insulin molecules leading to higher molecular weight transformation (HMWT) products [2]. With the development of high-pressure pumping systems providing constant flow-rates and pressure-stable microparticulate packing materials, high-performance liquid chromatography (HPLC) has become available to the protein chemist [3–7].

The recent advances in the on-line combination of size-exclusion chromatography (SEC) with light scattering detection offers an alternative way to investigate the association of macromolecules in solution. Additional techniques such as capillary electrophoresis and matrix-assisted laser desorption/

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ionization mass spectrometry are also potentially beneficial in assessing certain structural features of biopolymers. Indeed, light scattering is one of the few absolute methods available for the determination of molecular mass and structure and certainly is applicable over the broadest range of molecular weights of any method [8]. Thus, weight, number, and *z*-average values for both mass and size may be obtained for most samples by combining light scattering and size-exclusion chromatography.

Once an analytical method has been developed, it must be validated to confirm that the analytical procedure employed for a specific test is suitable for its intended use. There are several reported guidelines for the practical validation of analytical methods [9–11]. However, chemist continues to be faced with the problem that every existing regulation of this type is concerned with the validation of chromatographic methods. The lack of officially endorsed methodologies where sample treatment differs markedly from that of a chromatographic procedure makes validation a process for which no present rules exist [12].

The aim of this work was to separate the degradation products of insulin formed during storage at different conditions of pharmaceuticals preparations using high-performance liquid chromatography either in reversed-phase (RP-HPLC) or as size exclusion (SEC) separation mode. Second, the proposed methods were validated for use as a control method; to this end, the selectivity, precision, linearity and robustness were assessed. Also, the size, shape and absolute molecular weight for the different products were calculated by dynamic and static light scattering.

2. Experimental

2.1. Samples

Pure crystalline human insulin was released by Novo Nordisk (Badvaard, Denmark). Two human insulin pharmaceutical preparations (Novo Nordisk), a regular rapid-acting solution and a long-acting insulin zinc suspension, crystalline, were used. Methylparaben and m-cresol were used as auxiliary substances.

2.2. Analytical methods

The chromatographic system used was a Waters apparatus (Waters, Milford, MA, USA) consisting of a pump, model 600E multisolvent delivery system, 700 Wisp Sample Processor, 490 Programmable Multiwavelenght detector and data adquisition software, Maxima 820. Deionized water prepared with a MilliQ apparatus (Millipore) was used throughout; all other chemicals and reagents were HPLC grade. All solvents were filtered with 0.45 μm (pore size) filters (Millipore). Mobile phase were filtered and degassed.

RP-HPLC was carried out using a modified procedure published elsewhere [13]. The Delta-Pack C-18 column (300×7.8 mm I.D., Waters, Milford, MA, USA) was packed with 5 μm particles of 300 Å pore size, using a mixture (v/v) of 0.2 *M* sodium sulfate buffer (pH 2.3) and acetonitrile (74:26) as mobile phase at a flow-rate of 1.0 ml/min at room temperature and UV detection at 214 nm was used.

The SEC method proposed by Brange et al. [14] was used. Chromatographic conditions were as follows: A protein Pack I-125 column, 15 μm, 60 Å, 300×7.8 mm I.D. (Waters, Milford, MA, USA), using a mixture (v/v) of 2.5 *M* acetic acid, 4 m*M* L-arginine and acetonitrile (96:4) at a flow-rate of 1.0 ml/min at room temperature and detection was performed at 280 nm.

The sample treatment was as follows: Pure human insulin samples were prepared by direct dilution with 0.05 *M* HCl over a range of concentrations of 2–8 μg/ml and analyzed the same day. In the case of pharmaceutical preparations, 1.0 ml homogeneous samples were withdrawn from vials and samples were stored at 2–8°C until analysis. Insulin suspension samples were isolated by centrifugation, 1850 *g* for 20 min (Econospin, Dorvall Instruments Du Pont). Supernatants were removed and the dry residues were diluted with 0.05 *M* HCl. Samples containing insulin in solution were diluted with 0.05 *M* HCl to obtain concentration values within the calibration range. Unless otherwise indicated, all samples were analyzed in triplicate.

2.3. Light-scattering

2.3.1. Static light-scattering

A MiniDawn (Wyatt Technology, Santa Barbara, CA, USA) multi-angle laser light scattering (MALLS) instrument coupled with SEC system was used. The MiniDawn measures the light scattered from the eluant out of the chromatography column at three different angles (45, 90 and 135°) simultaneously, providing three chromatograms per sample. A Waters Model 410 refractive index detector (DRI) was used in series with the MiniDawn. The 90° detector was calibrated using toluene, and the other detectors were normalized using a protein solution.

The column and other chromatographic conditions were identical with those used for SEC. Data collection from the miniDawn and DRI detectors was controlled by Wyatt Technology's Astra Program. The differential refractive index detector was calibrated with sodium chloride standard, operated at room temperature. A 200 μl sample of each solution was injected onto the system and data collection and analysis was performed using Astra software.

The insulin suspension sample treatment was as described earlier. To samples containing insulin in solution, 300 μl Zn-Acetate 0.01 *M* was added for each ml of sample, and they were allowed to stand at temperature range 2–8°C for 48 h until precipitation of insulin and its derivatives was completed. Supernatants were removed and the dry residues were appropriately diluted with the mobile phase.

2.3.2. Dynamic light-scattering

The diffusion constants and particle size distribution for insulin samples were measured by dynamic light-scattering in the SDP mode, using Coulter® Model N4MD (Coulter Electronics, FL, USA). The intensity autocorrelation function of 633 nm laser light-scattering at 90° was calculated with a delay time of 2 μs per channel. All measurements were carried out at room temperature (20°C). Insulin solution (1 ml) was diluted directly with the SEC mobile phase. Insulin suspension samples were isolated by centrifugation, supernatants were removed and the dry residues were diluted with the SEC mobile phase. In both cases, the samples were filtered through a 0.22 μm Millipore filter directly onto the scattering tubes.

2.3.3. Data analysis

2.3.3.1. Dynamic light scattering. The scattered light intensity may be described in terms of the autocorrelation function [15]

$$G(\tau) = \sum_t I_s(t) I_s(t+\tau) \quad (1)$$

where $I_s(t)$ is the intensity at a specific time and $I_s(t+\tau)$ at a later time, τ. At very short values of τ, $I(t) \sim I(t+\tau)$, so that $G(\tau)$ approximates the mean value of I^2, whereas at high τ values, there will be no correlation between the I values.

In polydisperse systems, the autocorrelation function contains a sum of exponentials [16]

$$G(\tau) = \left(\sum_i G_i \, e^{-D_i Q_t^2} \right)^2 \quad (2)$$

where G_i is the relative intensity scattered, D_i is an average diffusional coefficient of the ith particle and Q_t is the size of the scattering vector.

Assuming that the particles are spherical, the hydrodynamic radius (R_h) can be calculated through the Stokes–Einstein equation [17]

$$R_h = \frac{K_B \cdot T}{6\pi \cdot \eta \cdot D} \quad (3)$$

where K_B is Boltzman's constant, T is the absolute temperature, η is the solution viscosity, and D is the diffusion coefficient.

2.3.3.2. Static light-scattering. The weight-average molecular weight and the z-average mean square radius $\langle r_g \rangle^2$ can be determined from the expanded form of the Rayleigh equation:

$$\frac{K \cdot c}{R(\theta)} = \frac{1}{M_w \cdot P(\theta)} + 2A_2 c \quad (4)$$

where K is an optical constant incorporating the solvent's refractive index, the solute's dn/dc, e.g. the refractive index increment of the solute, the wavelength of incident light in vacuum, and Avogadro's number; c is the solute concentration; A_2 is the second virial coefficient; $R(\theta)$ is the excess Rayleigh

scattering at angle θ; M_w is the weight-average molecular weight and $P(\theta)$ is the scattering function where θ is the scattering angle. For molecules smaller than about $\lambda/20$, where λ is the wavelength of the scattered light in solution, $P(\theta)$ can be expressed as

$$P(\theta) = 1 - \frac{16\pi^2}{3\lambda^2}\langle r_g\rangle^2 \sin^2(\theta/2) \quad (5)$$

where $\langle r_g\rangle^2$ is the mean square radius of molecules. For larger molecules, more terms containing higher powers of $\sin^2(\theta/2)$ are required [8]. The mean square radius $\langle r_g\rangle^2$ is proportional to the low-angle derivative of $P(\theta)$ with respect to $\sin^2(\theta/2)$. For any given slice, $R(\theta) \propto P(\theta)$ as long as c is small enough that the A_2 term in Eq. (4) can be ignored.

Molecular weights are calculated across all increments of a chromatogram using the Debye plot of $K \cdot c/R(\theta)$ versus $\sin^2(\theta/2)$.The reciprocal weight average molecular weight $1/M_w$ is calculated from the intercept of Eq. (4) in the limit as θ and $c \rightarrow 0$. Examining the slope near $\sin^2(\theta/2) = 0$ of the graphically extrapolated zero concentration variation, one may derive the *z*-average square radius. It should be clearly evident that the $\langle r_g\rangle^2$ of a molecular solution can generally be obtained independent of dn/dc, M_w or even c if it is sufficiently small [8].

2.4. Degradation studies

Two human insulin pharmaceutical preparations were stored at different temperatures between 40 and 60°C with variations less than ±1°C on a continuous motion system at 100 rev./min (Ika-vibrax Schot-Ibérica) and without shaking. Samples were taken at different times, depending on the storage conditions.

3. Results and discussion

3.1. Validation of rp-hplc method

3.1.1. Linearity

To validate the analytical method, seven standard solutions were prepared using pure human insulin at concentrations of 2, 3, 4, 5, 6, 7 and 8 μg/ml. Each sample was analyzed four times. To quantify human insulin we used the total area of peak. The analysis of variance (ANOVA) of linear regression confirmed the linearity of the method used through the rejection of the null hypothesis of deviation of the linearity for a significance level of 0.05 ($\alpha = 0.05$); the coefficient of variation of predicted concentrations was 2.6% [18]. The equation of the regression line was:

$$\text{Area} = (-61956 \pm 4436) + (93643 \pm 824) \cdot C;$$

$$r = 0.9990 \ (n = 28)$$

and the root mean square error (S_{xy}) was 8718.

3.1.2. Precision

3.1.2.1. Repeatability. To calibrate the RP-HPLC system and to monitor its performance we analyzed an insulin solution sample daily as standard. The estimated area value for standard concentration was 618901 with a C.V. of 1.4%. The upper and lower limits for the control chart were established at ±2SD of this value, taking as standard deviation the value obtained from variance of analytical method (Fig. 1A). The RP-HPLC system precision was assessed using an insulin solution sample, which was analyzed in triplicate on different days under same conditions (same analyst, apparatus, identical reagents and short interval of time). The repeatability was <1% for the peak area.

3.1.2.2. Intermediate precision. The intermediate precision expressed as the variability between days was evaluated jointly by one-way analysis of variance; the results showed that the inter-assay differences were not significant ($P > 0.05$). The inter-assay precision was better than 0.5%.

3.1.3. Accuracy

RP-HPLC system accuracy was expressed as percent recovery by the assay of known added amount of pure insulin being the mean value of 101% with a coefficient of variation 1.2% ($n = 9$).

3.1.4. Detection limit

The detection limit based on the standard deviation of the response and slope [11] was 0.3 μg/ml.

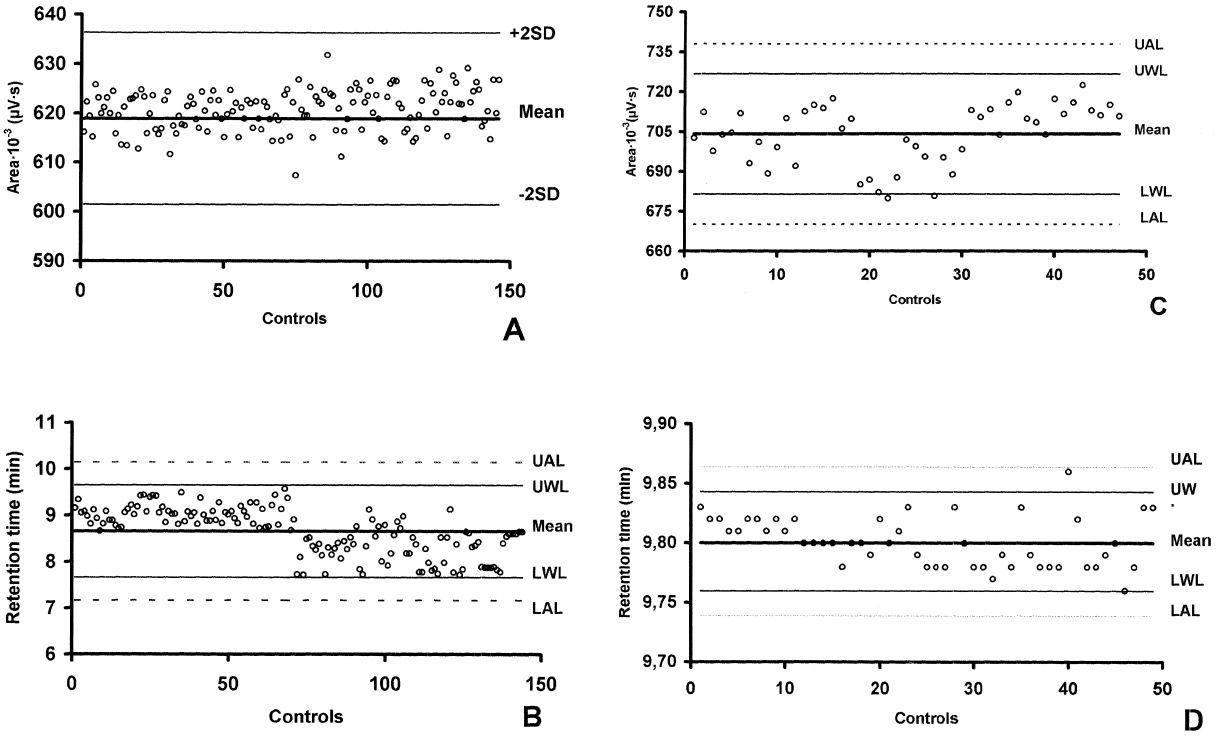

Fig. 1. Control charts for RP- (A, B) and SEC- (C, D) HPLC methods in function of the control parameters, peak area (A, C) and retention time (B, D), respectively. In Fig. 1B a sudden change from sample ca. 67 can be observed related with a column change (new column and different batch) and with the previous quality control, procedure applied by the manufacturer. (UAL and LAL: upper and lower actions limits; UWL and LWL: upper and lower warning limits).

3.1.5. Quantitation limit

The quantitation limit based on the standard deviation of the response and slope [11] was 0.9 μg/ml.

3.1.6. Robustness

A Robustness test was performed to examine the effect operational parameters on the analysis results. The flow-rate (1.0±0.05 ml), injection volume (50.6±1.0 μl), temperature (20.7±1.59°C), pH (2.3±0.2), mobile phase composition (74±0.5 for sulfate buffer, 26±0.75 for acetonitrile, v/v) and column performance over time were determined to confirm the method's robustness. Retention time and insulin peak area was used as control parameters. If the parameters are within a previously specified tolerance, the method guaranteed to perform in such a way to provide quality results every time [19]. Therefore, the acceptance limits should be defined to indicate when the method was out of the limits previously established as statistically acceptable. In the case of peak area, the limits were established between the upper and lower limit of the control chart. In contrast, we established the use of two sets of limits for the retention time: Conventional limits, usually 3×Sigma, called action limits, and the inner limits, called the warning limits, usually 2×Sigma. After 10 standard analysis the mean and standard deviation of the data were calculated and a control chart was constructed [20]. The graph was continuously updated, plotting new points as they were generated as part of this analytical method and continuously recalculating the mean and standard deviation (Fig. 1B). A mean value of 8.66 min for the retention time with a standard deviation of 0.50 min ($n=50$) was obtained being the coefficient of variation (C.V) of 5.7%. If one or both parameters are outside the specified limits, the chromatographic

conditions (for example, flow-rate, relative mobile phase composition) and the column performance, specially, the tailing factor and column efficiency have to be checked and, it necessary, corrections actions have to be taken. Fig. 1(A, B) give an idea about the method robustness. The peak area (Fig. 1A) and retention time (Fig. 1B) hold between the action limits even thought different columns lots were used. The sudden change observed from sample ca. 67 in control chart for retention time is directly related with a column change (new column and different batch) and in the information given by the manufacturer related with the quality control procedures applied.

3.1.7. Specificity

Hydrolysis of insulin has been studied during storage of various preparations at different temperatures. Insulin degrade rapidly in acid medium at residue Asn A21, while in slightly acid or alkaline medium deamidation takes place at residue Asn B3 [21]. These facts were confirmed when a pure human insulin sample either in acid or alkaline medium was degraded. The separation of these products was carried out by RP-HPLC. Details are given in the analytical methods section. With these conditions, it is possible to separate the insulin and derivatives desamido insulin in the same analysis with a short analysis time, combining a satisfactory resolution ($R_s = 1.8$), value close to that recommended by USP method ($R_s \geq 1.8$) for separation and identification of desamidated derivatives of insulin [22]. Fig. 2 shows the separation of insulin and the respective monodesamido derivatives. The separation is characterized by a good resolution of the insulin and desamidoinsulin peaks, due to the high selectivity ($\alpha = 1.5$), which is especially important for determining the purity and stability of this type of product.

In the case of suspension, the only degradation product detected by RP-HPLC was the desamido B3 insulin at all studied conditions. However, for solution, the main degradation product detected by RP-HPLC was the desamido B3 insulin, whereas in the studies with shaking the desamido A21 insulin [2] was detected.

The aggregation products such as covalent insulin dimer and HMWT products were separated by SEC method proposed by Brange et al. [14] for the

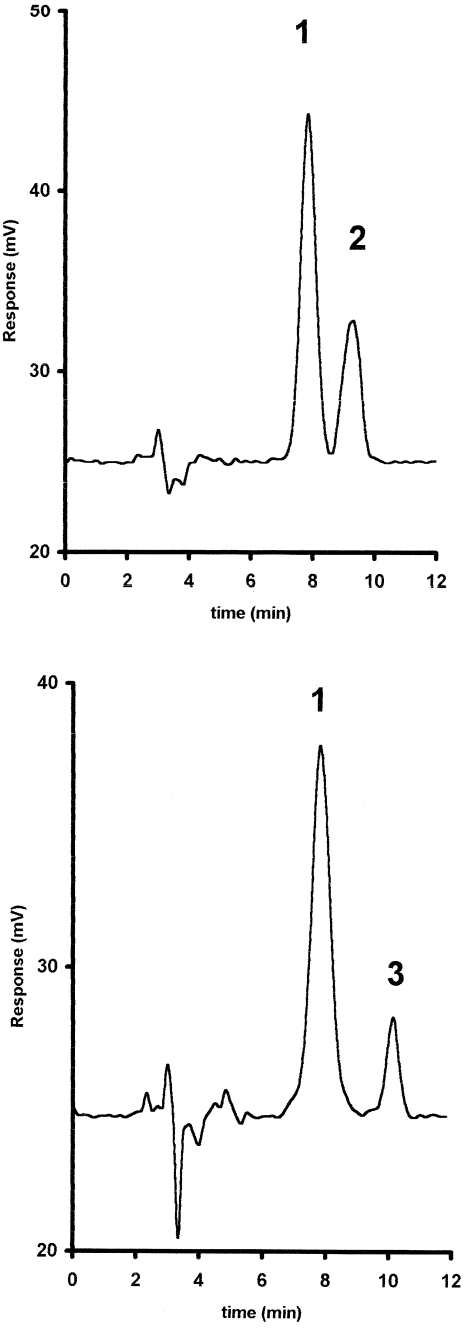

Fig. 2. RP-HPLC separation of insulin and their monodesamido derivatives corresponding to a solution sample stored at 60°C with shaking (Top) ans suspension sample stored at 50°C without shaking (Bottom). Peaks: 1=human native insulin; 2=monodesamido-A21-insulin; 3=monodesamido-B3-insulin.

separation of insulin and covalent insulin dimer using a silica-based support column (Protein Pack I-125, Waters) and acetonitrile and L-arginine as modifiers.

For insulin solution, the covalent insulin dimer was separated by SEC and detected in samples stored at temperatures higher than 40°C with and without mechanical shaking [2]. Samples stored at 60°C and under shaking conditions during 24 h showed another peak corresponding to the higher molecular weight transformations products of insulin [23]. In the case of suspension, insulin aggregation products by SEC were not detected at any time and all conditions [2]. Fig. 3 shows the peak of insulin at 9.8 min and the covalent insulin dimer at 6.5 min corresponding to a solution sample stored at 50°C with shaking. The preservative used in pharmaceutical preparations does not affect the determination by SEC of insulin and its aggregates showing a satisfactory resolution ($R_s \geq 2.0$) and selectivity ($\alpha \geq 1.6$).

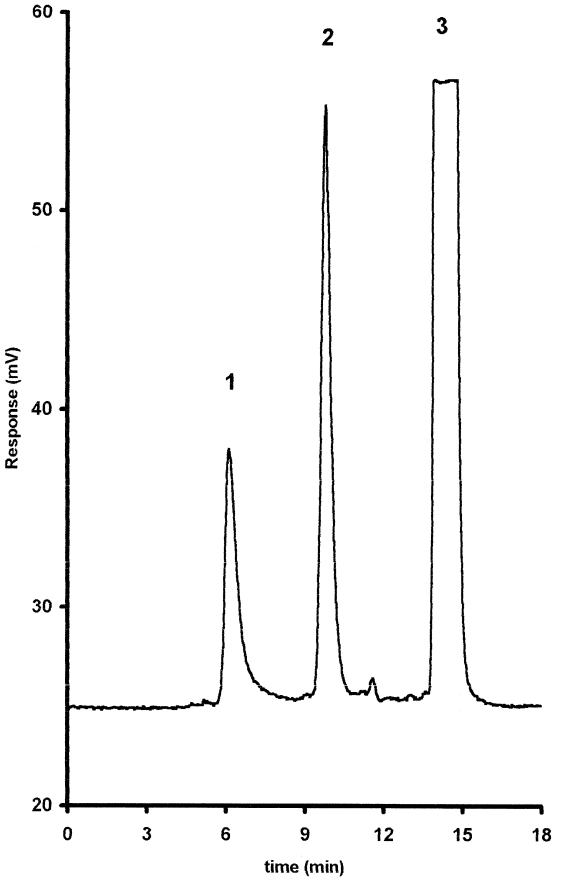

Fig. 3. Separation of insulin and its aggregates on Protein Pack-125 column, with UV–Vis detection at 280 nm of pharmaceutical preparation of human insulin stored at 60°C over 5 days. Peaks: 1=covalent insulin dimer; 2=human native insulin; 3=m-cresol (preservative).

At first, the SEC method was not calibrated since its intended use was initially to separate the different degradation products only and not for quantification analysis. However, to monitor its performance we analyzed a human insulin solution sample each working day and the peak area and retention time were determined. These parameters were used as control for the system suitability test. For both parameters, the action and warning limits were used as criteria to establish the acceptance limits as described earlier. Two control charts were constructed. A mean value of 704131 for the peak area with a standard deviation of 11321 ($n=50$) was obtained; the C.V. was 1.6%, whereas the mean retention time was 9.80 min with a C.V. of 0.2%. Fig. 1(C,D) shows the control charts for the SEC method, indicating the action and warning limits.

Light scattering techniques are traditionally used to determine molecular weights and sizes. Fig. 4 shows the chromatograms obtained from SEC with multi-angle laser light-scattering detection of a human insulin dimer sample. Fig. 5 presents the Debye plot of $KC/R(\theta)$ vs. $\sin^2(\theta/2)$ for the human insulin monomer peak. This Debye plot permits the extraction of molecular weight and root mean square radius since the slope of this line is directly proportional to the mean square radius while the intercept (at very low concentration) yields the molecular weight directly (see Eq. (4)). The second virial coefficient is essentially zero.

A key requirement for the determination of molecular weights by light scattering is the numerical value of dn/dc and knowledge of the absolute concentration of the sample fraction. Soren Hvidt [24] obtained a value of dn/dc of 0.183 ml/g for porcine insulin samples, a value very close to that proposed by Wen et al. [25], who assumed a value of dn/dc of 0.186 ml/g for all those proteins independent of amino acid composition that do not contain carbohydrates. Table 1 summarizes the results obtained for the human insulin monomer and dimer,

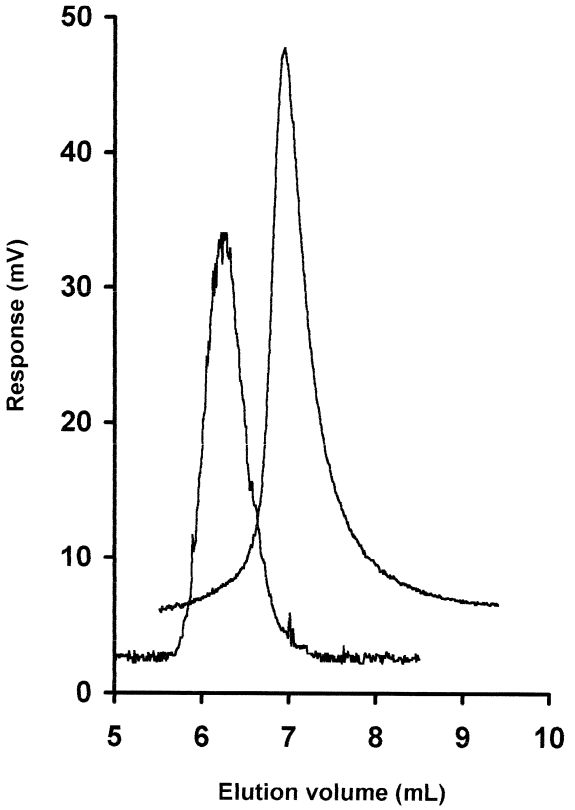

Fig. 4. Chromatograms obtained from size-exclusion chromatography with multi-angle laser light-scattering detection of covalent insulin dimer. (Top, trace differential refractive index (DRI) detector and bottom, trace multi-angle laser light-scattering (MALLS) detector at 90° angle). The delay volume between MALLS and DRI detectors was 0.135 ml. The absolute molecular weight and r.m.s. radius were 12600 Da and 40.1 nm., respectively.

respectively, using different values of dn/dc. The molecular weight can be determined from the ratio of the MALLS and refractive index detectors signal. This is the so called two detector method [25].

$$M_w = K_1 \frac{\mathrm{LS}}{\mathrm{RI}} \quad (6)$$

where K_1 is a function of the instruments calibration constant and dn/dc.

$$K_1 = \frac{K_{\mathrm{RI}}}{K_{\mathrm{LS}}\, \mathrm{d}n/\mathrm{d}c} \quad (7)$$

In case of insulin, the two dn/dc values differ by about 1.6%, which resulting is not significant for molecular weight determination, being relative error close to 3% in the worst case. For others proteins could be more determinant and it will be necessary to know the true dn/dc of a protein. When there is a value uncertainty or the dn/dc value for the studied protein is unknown, as preliminary step, a value of dn/dc of 0.186 ml/g can be used, which permits to obtain an approximated idea of its molecular weight. This fact was confirmed in the molecular weight determination by SEC-MALLS of human serum albumin in pharmaceutical preparations, whereby the variations between the molecular weight calculated and supported by the manufacturer were less than 1% [26]. With other proteins, the variations were higher, although the error could be reduced by introducing the true dn/dc value (data no shown).

For a value of dn/dc of 0.186 ml/g, the absolute molecular weight of the human insulin monomer was 5805 Da, which is in good agreement with the assumed value of 5800 [1], whereas the molecular weight of the human insulin dimer was 12 400 Da, a value close to twice the sequence molecular weight of 5800. In contrast, the z-average r.m.s. radius (r_g) was 21.6±0.4 and 40.5±0.7 nm for human insulin monomer and dimer, respectively (Table 1).

The r.m.s. radius depends on the internal mass distribution of the molecules and is not generally a measure of the molecule's external geometry. For certain linear molecules, the mean square radius is proportional to the square of the molecule's mean diameter, i.e. hydrodynamic diameter. Generally, this is not the case [8]. The results from dynamic light-scattering confirms this fact since the hydrodynamic diameter is calculated through Stoke's law assuming a spherical macromolecule shape. The hydrodynamic diameter of insulin and its derivatives studied are listed in Table 2. In the case of insulin solution, a mean value of 2.69 nm for an insulin monomer and 3.81 nm for insulin dimer were obtained (see Fig. 6). All these values are in reasonable agreement with the earlier published data [24,27]. In contrast, a mean value of 5.50 nm for the insulin suspension was obtained. This result seems to suggest that the insulin is primarily consisting of hexamers due to effect of zinc.

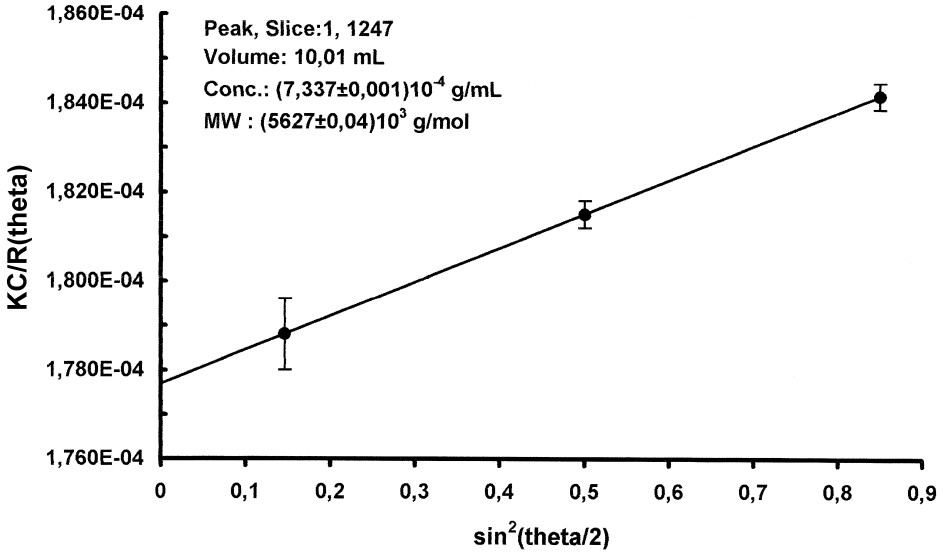

Fig. 5. Debye plot of $KC/R(\theta)$ vs. $\sin^2(\theta/2)$ from size-exclusion chromatography with multi-angle laser light-scattering detection analysis of human insulin monomer peak. Plot shows information from one data slice corresponds to the apex of the peak. Plotted points correspond to the three light scattering detectors for the observed angles of 45, 90 and 135°.

The high temperature prolonged storage or mechanical shaking of insulin solutions can result in high molecular weight aggregates [2], but the results do not show any indications of higher aggregates than dimer, although a broad size-distribution by dynamic light-scattering was observed.

In conclusion, RP- and SEC-HPLC have proved to be valuable tools for the separation of insulin, its monodesamido derivatives and aggregation products in pharmaceutical preparations. The results of the validation procedure show that the methods used are specific, precise, reproducible and robust. The combination of liquid chromatography and light scattering permits a good estimation of the molecular

Table 1
Molecular weights and sizes for insulin monomer and dimer obtained by size-exclusion chromatography with multi-angle laser light scattering detection in function of dn/dc[a]

	dn/dc (ml/g)			
	0.183	0.186	r.m.s. (nm)	radius
Monomer				
M_n	5843±125	5749±101	R_n	20.2±0.4
M_w	5898±115	5805±97	R_w	21.3±0.4
M_z	5872±147	5778±165	R_z	21.6±0.4
Dimer				
M_n	12453±205	12293±101	R_n	39.8±0.2
M_w	12644±402	12405±367	R_w	40.1±0.5
M_z	12820±420	12613±552	R_z	40.5±0.7

[a] M_n, M_w, and M_z are the number, weight and z-average molecular weight and R_n, R_w and R_z are the corresponding average r.m.s. radius.

Table 2
Average diffusion coefficient ($\bar{D}$) and hydrodynamic diameter (R_h) of insulin solution and suspension calculated by dynamic light scattering assuming a spherical shape

Insulin solution	$\bar{D}$ (cm^2/s)	R_h (nm)
Monomer	1.60×10^{-6}	2.69±1.43
Dimer	1.13×10^{-6}	3.81±2.07
Insulin suspension		
Hexamer	7.81×10^{-7}	5.50±1.59

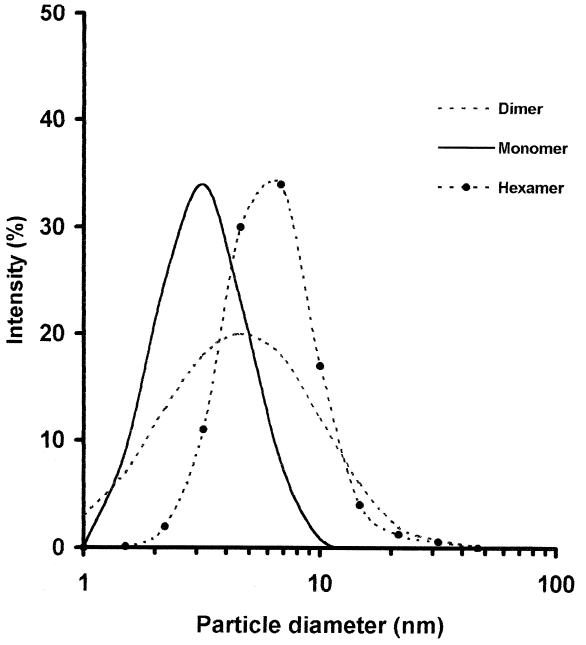

Fig. 6. Particle size distribution for human insulin solution and suspension sample obtained by dynamic light scattering.

weight and size of the monomer and dimer of human insulin.

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