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Effect of high shear rate on stability of proteins: kinetic study

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Abstract

Size-exclusion chromatography (SEC) was used to monitor the time–course of protein degradation induced by high shear rates during the formulation and manufacture of controlled-release pharmaceutical dosage forms. SEC with multi-angle laser light-scattering (MALLS) detection was used to characterize the aggregation products, determining their absolute molecular weight. A stability-indicating method was developed and validated to obtain reliable drug degradation data. The results obtained according to the ICH guidelines confirm that the system and methods proposed are suitable for their intended use. The degradation kinetics are influenced by the type of protein and the effect of the shear rate on their stability. Reversible pseudo-first order degradation kinetics were observed for bovine β-lactoglobulin, whereas for human (HSA) and bovine serum albumin (BSA), a monomer–dimer transition was observed, independently of the rate of shear. However, trimer formation was also observed for HSA, especially at high shear rates. The kinetic model may thus be described as a two-step process: a monomer–dimer, and dimer–trimer transition.

Keywords: Protein stability; Size-exclusion chromatography; Degradation kinetics; Shear rate; Validation

1. Introduction

Proteins as drug substances differ from conventional chemical drugs in such properties as size, shape and conformation, multiplicity of functional groups, amphotericity, physical form of bulk drug, and heterogeneity of structure. Clearly, proteins present greater complexity than traditional drugs.

Protein denaturing involves disruption of the higher order structure, such as their secondary and tertiary structure. It may be reversible or irreversible and be caused by temperature ($T > 50\ ^{\circ}C$), pH extremes, shear forces, surface area changes, surfactants, buffers, ionic strength, freeze–thaw cycles, organic solvents, and exposure to interfaces or denaturing chemicals [1] often leading to aggregation and precipitation phenomena.

Denaturation also typically involves reversible or irreversible unfolding, depending on various factors [2]. It has been known for many years that proteins are also often susceptible to aggregation and precipitation phenomena upon shaking [1], it

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being generally accepted that the pathway leading to aggregate formation is related to the conformational changes a protein molecule undergoes when exposed to an interface. For most formulations in solution this is the air–water interface. In this case, aggregation could occur during shaking because a protein adsorbs, then unfolds at the air–water interfaces generated by shaking, thereby exposing the hydrophobic amino acids, which are normally located on the interior surface. The exposed hydrophobic amino acid side-chains of one molecule interact with those of another to form aggregates [2]. Shear can also induce denaturing or aggregation of peptide and proteins, although the exact mechanism is not known and may be complex [3–5]. Thus, both reversible and irreversible inactivation has been observed, depending on the rate of shear [3,4].

In some of the earlier literature it was assumed that shear could directly disrupt or distort the molecules, while more recent work seems to indicate that shear does not directly denature the molecules, but rather accelerates the turnover of air–liquid interfaces, which causes denaturation and/or aggregation [1]. For example, 70% of porcine growth hormone was lost by aggregation when a 0.5 mg/ml solution was vortexed for just 1 min [6]. Similar behavior was observed with other proteins and peptides [2,7–9].

Protein formulation and manufacture often employ processes in which the molecule is subject to shear forces, e.g. mixing, flow (both in solution and powder form), filtration, and passage through pumps [10–14]. Empirical modeling of data obtained on protein shearing shows that in many cases there is a logarithmic relationship between loss in activity and shearing time [1]. Conformational changes and accelerated aggregation have also been attributed to high shear rates [15].

Due to the complexity of protein drugs, a widening range of analytical methods must be applied in protein characterization and the development of stability-indicating assays. Several techniques for studying the effect of heat, shear, surface phenomena, and solvent additions on the protein native state have been used [16]. However, all such methods should have the capability to detect, quantify and distinguish different forms of the active ingredients and their degradation products.

Chromatography, in either reversed-phase or size-exclusion separation modes, is a powerful tool in evaluating both the degradation profile and purity of proteins [17]. RP-LC is a technique where organic modifiers are normally used in the solvents, causing denaturation of the proteins. Hence, RP-LC is suitable mainly to monitor the chemical degradation of proteins.

Size-exclusion chromatography (SEC) can provide information as to the levels of aggregation and fragmentation in a pharmaceutical protein, and combined with light-scattering detection offers an easy, accurate and reliable alternative technique to investigate the association of macromolecules in solution [19]. As the intensity of the scattered light is measured at different angles, the radius of gyration and the molecular mass can be measured simultaneously, requiring no calibration. Both are independent of elution volume [18]. An additional benefit of this calculation lies in the molecular mass being simply that of the dissolved protein solution. This can reveal whether the protein is present as a monomer, dimer, or higher aggregation state.

The aim of this work was to study the effect of shear rate, as usually applied during preparation of sustained released systems, on stability of proteins using SEC. The experimental design enabled us to monitor and characterize this process kinetically. β-Lactoglobulin, and human and bovine serum albumin (BSA) with nominal molecular weights of 18 400 and 66 000 Da, respectively, were used as model proteins.

2. Materials and methods

2.1. Materials

Bovine β-lactoglobulin and human serum albumin were purchased from Sigma (St Louis, MO, USA). BSA was purchased from Merck (Darmstadt, Germany). All other chemicals and reagents were analytical or HPLC grade.

2.2. SEC analytical method

The chromatographic system used was a Waters apparatus (Milford, MA, USA) consisting of a pump, 600E multisolvent delivery system, 700 Wisp sample processor, a Shodex C-18 Column (8 × 300 mm, Waters), 490E Programmable multiwavelength detector and data acquisition software, MAXIMA 820. De-ionized water prepared with a Milli-Q apparatus (Millipore) was used throughout. All solvents were filtered with 0.45 μm (pore size) filters (Millipore) and degassed.

The mobile phase was phosphate-buffered saline (300 mM NaCl, 25 mM phosphate, pH 7.0) at a flow rate of 1.0 ml/min at room temperature, and detection was performed at 214 nm. For human serum albumin, the mobile phase was 120 mM NaCl, 25 mM phosphate, pH 7.0. The column and other chromatographic conditions were identical to those described above.

2.3. Static light-scattering measurements

A miniDawn (Wyatt Technology, Santa Barbara, CA, USA) multi-angle laser light-scattering (MALLS) instrument coupled with an SEC system was used. The miniDawn measures the light scattered by the eluent from the chromatography column at three different angles (45, 90 and 135°) simultaneously, providing three chromatograms per sample. A Waters Model 410 differential refractive index (DRI) detector 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 chromatographic conditions were identical to those used for SEC. A differential index of refraction (dn/dc) of 0.186 ml/g was assumed for the analyzed proteins [20]. Data collection from the miniDawn and DRI detectors was controlled by Wyatt Technology's ASTRA program. The DRI detector was calibrated with sodium chloride standard, operated at room temperature. A 200 μl sample of each solution was injected into the system and data collection and analysis were also performed using ASTRA software.

2.4. Shaking studies

Standard solutions of 25 μg/ml for β-lactoglobulin and 400 μg/ml for HSA and BSA prepared in phosphate-buffer (pH 7.4) and shaken using an Ultraturrax T25 (Janke Kunkel Ika ®labortechnik) device at different shear rates (8000–20 500 rpm) for 3 min, the surface conditions being the same for all samples. Aliquots were taken every 30 s and analyzed immediately in duplicate unless otherwise stated.

3. Results

3.1. Validation of SEC methods

As a preliminary step, the proposed analytical methods were validated for each protein used in this study according to the International Conference on Harmonization (ICH) guidelines [21]. For this, the specificity, precision, accuracy, linearity, detection and quantitation limits, and robustness were determined.

3.1.1. Specificity

For β-lactoglobulin, a single peak with elution volume 10.27 ml was detected, the absolute molecular weight being 22 500 Da with a CV of 2.45%, slightly higher than the molecular weight provided by the manufacturer (18 400 Da). This could be due to the incorrect dn/dc value.

In the case of the HSA sample, two peaks with elution volume 8.25 ml (peak 2) and 9.10 ml (peak 1) were observed by SEC with UV–Vis detection. Similar behavior was found with BSA: two peaks with elution volume 9.3 (peak 2) and 10.3 ml (peak 1). The following stage was the identification and characterization of the different peaks using SEC with MALLS detection. A key requirement for the determination of absolute molecular weight by light-scattering is the numerical value of dn/dc and a knowledge of the absolute concentration of the sample fraction. A constant dn/dc value of 0.186 ml/g can be assumed if the protein contains no carbohydrates and is independent of amino acid composition [20]. The absolute molecular weight of the HSA was 67 300 with a coefficient of

variation (CV) of 0.96% ($n = 9$) for peak 1, which is in good agreement with the assumed value of 67 000 Da for the HSA monomer, whereas the absolute molecular weight was 132 000 Da (CV = 1.13%) for peak 2, a value close to twice the sequence molecular weight of 67 000 Da. It was, therefore, the dimer species.

For the BSA sample, the absolute molecular weights were 66 100 Da (CV = 1.11%) for the monomer (peak 1) and 127 000 Da with a CV of 2.09% for the dimer (peak 2). With the proposed methodology it is possible to separate the monomer and dimer species in the same short analysis (12 min) to achieve the required resolution and efficiency.

3.1.2. Linearity

For the β-lactoglobulin, a good linearity was observed between the concentration in a range of 5–25 μg/ml and the peak area ($r = 0.984$), confirmed by ANOVA applying the same procedure described earlier ($F_{(4,21)} = 0.40$; $P > 0.05$). The coefficient of variation for the predicted concentrations was 3.77% [22]. The regression line was:

$$\text{Area} = (-14\,093 \pm 8307) + (13\,275 \pm 501)\text{C};\quad r = 0.984\ \ (n = 25)$$

with a root mean square error (S_{yx}) of 17 709.To validate the analytical method, five standard solutions were prepared using pure HSA at concentrations of 100–400 μg/ml. Each sample was analyzed five times. To quantify HSA, we used the total area of all the peaks. The analysis of variance (ANOVA) of the linear regression "total area of all the peaks versus prepared concentration of protein" confirmed the linearity of the method, through rejection of the null hypothesis of deviation from linearity for a significance level of 0.05 ($\alpha = 0.05$). The coefficient of variation for the predicted concentrations was 0.76% [22]. The equation of the regression line was:

$$\text{Total area} = (7941 \pm 4404) + (1282 \pm 17.2)\text{C};\quad r = 0.998\ \ (n = 25),$$

with an S_{yx} of 7526.The sizes of the individual peaks, as a percentage of the total area, enable the content of each species to be estimated, their proportion being constant and independent of the concentration. The values obtained (mean ± standard deviation (S.D.), $n = 25$) were 89.2% for the monomer and 10.8% for the dimer with a relative standard deviation (R.S.D.) of 1.54%.

The same procedure was used with BSA, the concentrations ranging between 5 and 25 μg/ml. The coefficient of variation for the predicted concentrations was 3.19% [22]. The equation of the regression line was:

$$\text{Total area} = (-4825 \pm 16\,399) + (36\,680 \pm 989)\text{C};\quad r = 0.992\ \ (n = 25)$$

and the S_{yx} was 34 642.

The mean percentages of each peak were 90.8 and 9.20% with an R.S.D. of 0.83% for monomer and dimer species, respectively.

3.1.3. Precision

Expressed as repeatability, the HPLC system precision was assessed using a minimum of six determinations of a protein sample at 100% of the test concentration, which was analyzed six times under the same conditions (same analyst, apparatus, identical reagents and short time interval). The repeatability was less than 1.8% for the total area of all the peaks for both albumin samples, except for β-lactoglobulin, with an R.S.D. of 2.10%.

3.1.4. Accuracy

System accuracy was expressed as percentage recovery of a known added amount of each protein by the assay. The mean varied between 98 and 102%, whereas the R.S.D. was in the range 1.8–2.3%.

3.1.5. Detection and quantitation limits

The calculation method is based on the S.D. of the response and the slope of the calibration curve (S) according to the following formulas: LOD = 3.3(S.D./S) and LOQ = 10(S.D./S), where the S.D. of the response is determined from the residual S.D. of the regression line. The LOD and LOQ were always lower than the smallest concentration in the linearity range, except for β-lactoglobulin, with an LOQ of 13.3 μg/ml.

3.1.6. Robustness

This shows the reliability of an analysis with respect to small deliberate variations in the parameters of the method, such as flow-rate (1.0 ± 0.05 ml), pH (7.0 ± 0.2), temperature ($21.0 \pm 1.6\,^{\circ}C$), injection volume (100 ± 1 μl) and column performance over time, which were determined during the validation period.

A standard solution of β-lactoglobulin was analyzed daily and the peak area used as control parameter. If this is within a previously specified tolerance, the method is guaranteed to provide quality results every time [23]. The upper and lower limits of the control chart were established at ± 2S.D. of the estimated peak area for standard concentration, taking as S.D. the value obtained from the variance of the analytical method. The peak area stayed largely within the limits, otherwise corrective action was taken.

3.2. Effect of shear rate and time

Fig. 1 shows the mean (%) amount remaining for β-lactoglobulin as a function of shearing rate and time detected by SEC. A decrease of approximately 50% in the remaining concentration was observed within 30 s, independently of the shear rate, remaining constant up to the final assay. There was no evidence by SEC of fragmentation or aggregation of β-lactoglobulin upon shaking.

During the formulation and manufacturing stages, the protein may undergo high shear forces that affect its structural integrity and cause denaturation. However, this can be either a reversible or irreversible process, depending on the rate of shear [3,4]. Thus, the results seem to suggest a reversible pseudo-first order degradation kinetics according to the following model:

$$N \underset{k2}{\overset{k1}{\rightleftarrows}} U \tag{1}$$

where N is the protein concentration and U is the concentration of degradation product at time "t".The rate expression for the loss of N is given by Eq. (2):

$$-\frac{d[N]}{dt} = k_1 \cdot [N] - k_2 \cdot [U]; \tag{2}$$

When the initial concentration of U, $[U_0]$, equals zero at $t = 0$, Eq. (2) is integrated to give:

$$[N] = \frac{[N]_0}{k_1 + k_2}(k_1 \cdot e^{-(k_1+k_2)\,t} + k_2) \tag{3}$$

When $t \to \infty$, the protein concentration at the equilibrium, $[N]_\infty$, is given by Eq. (4):

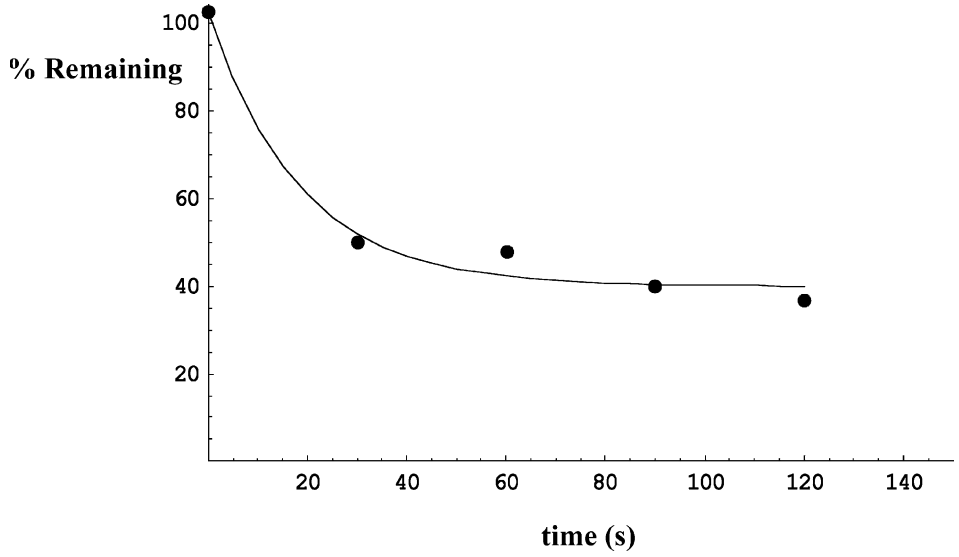

Fig. 1. Amount (%) of β-lactoglobulin remaining vs. shearing time, the rate of shear being 9500 rpm.

$$[N]_{\infty} = [N]_0 \cdot \frac{k_2}{k_1 + k_2} \quad (4)$$

In this case, the NonlinearFit function of the MATHEMATICA 2.1 [24] program was used to fit the data.

However, the goodness of fit was unsatisfactory when the rate of shear was 20 500 rpm, as reflected in the 95% confidence intervals for several estimated model parameters (see Table 1). This was because the kinetic model used was inappropriate and so the fitting process led to incorrect results.

At this shear rate the degradation of both albumin samples was total within 30 s, so it was not possible to study the effect of shear rate on stability. We thus decided to reduce the shear rates to 8000, 9500 and 13 500 rpm.

For the BSA sample, independently of the shear rate used, after 2.5 min of shaking, the results indicate a decreased percentage of the monomer remaining in the 5–8% range, with a corresponding increase in the dimer (see Fig. 2). The SEC analysis did not reveal the presence of other aggregation products. The results obtained seem to indicate a reaction with monomer–dimer transition according to the following model:

$$M + M \underset{k_2}{\overset{k_1}{\rightleftarrows}} M_2 \quad (5)$$

In contrast to the previous case, this appears to be a bimolecular reaction. The rate equations for monomer [M] and dimer [M_2] are both first-order non-linear differential equations.

$$\frac{d[M]}{dt} = -2 \cdot k_1 \cdot [M]^2 + k_2 \cdot [M_2] \quad (6)$$

$$\frac{d[M_2]}{dt} = -k_2 \cdot [M_2] + 2 \cdot k_1 \cdot [M]^2 \quad (7)$$

The mathematical treatment of bimolecular reaction kinetics is tremendously complicated. Since no general mathematical formalism for the analytical solution of all differential equations has yet been found, a particular solution is often based on a mere guess confirmed by inserting it into the equation. To confirm a solution it is important to check whether it fulfills all the initial conditions. For this, the NDSolve function from MATHEMATICA was used to resolve the differential equations [24]. This program provides a numerical solution for the degradation rate constants under the established initial conditions (see Table 2).

The HSA showed similar behavior to that observed for BSA when the shear rate was 8000 rpm, although the remaining protein concentration decreased significantly at 9500 and 13 500 rpm, since its content decreased to 61% for the monomer (see Fig. 3) and increased to 25% for the dimer species. A third peak with an average molecular weight 198 000 Da was also detected within 30 s by SEC with MALLS detection (see Fig. 4). This is possibly the trimer species, the average remaining percentage being 14% after 2.5 min shaking.

This result seems to indicate that HSA aggregates at high shear rates, since the dimer and trimer were observed within 30 s of shaking, even duplicating their values in 60 s, e.g. the dimer

Table 1
Estimated model parameters with 95% confidence intervals (in brackets) for β-lactoglobulin as a function of shear rate

Parameters	β-Lactoglobulin		
	9500 rpm	13 500 rpm	20 500 rpm
D_0	99.9 (94.4–105.4)	99.9 (96.8–103.0)	99.9 (86.3–113.6)
K_1	3.92×10^{-2} (2.59×10^{-2}–5.25×10^{-2})	2.45×10^{-2} (2.12×10^{-2}–2.78×10^{-2})	5.99×10^{-2} (-9.9×10^{-2}–2.18×10^{-1})
K_2	2.28×10^{-2} (1.30×10^{-2}–3.26×10^{-2})	1.23×10^{-2} (9.93×10^{-3}–1.47×10^{-2})	5.52×10^{-2} (-9.8×10^{-2}–2.68×10^{-1})
D_{∞} (%)	36.7	33.4	47.9
$t_{(1/2)}$ (s)	11.2	8.8	6.2

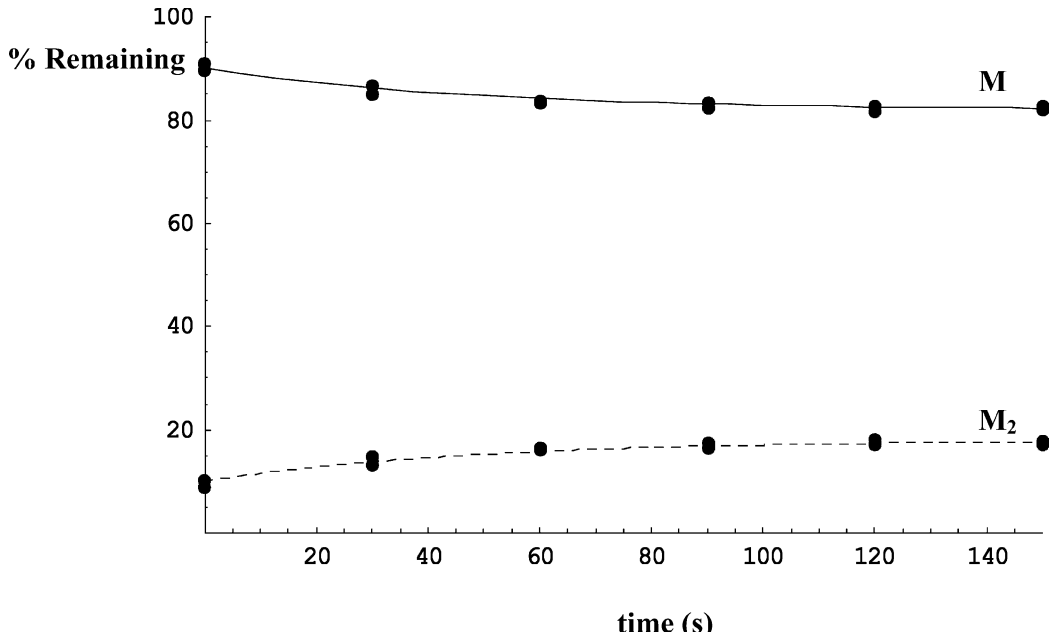

Fig. 2. Amount (%) of monomer (M) and dimer (M_2) of BSA remaining vs. shearing time, shear rate being 9500 rpm. In the equilibrium, the monomer fraction remaining was 84%, whereas for the dimer it was 16%.

percentage rises from 12 to 25%. Therefore, HSA presents a more complex degradation mechanism than BSA, as reflected in the analysis of the degradation versus time curve under the assayed conditions, which suggests the following kinetic model involving a two-step process:

$$M + M \underset{k_2}{\overset{k_1}{\rightleftarrows}} M_2 \tag{8}$$

$$M_2 + M \underset{k4}{\overset{k_3}{\rightleftarrows}} M_3 \tag{9}$$

The rate equations for monomer [M], dimer [M_2] and trimer [M_3] are:

$$\frac{d[M]}{dt} = -2 \cdot k_1 \cdot [M]^2 + k_2 \cdot [M_2] \tag{10}$$

Table 2
Estimated degradation rate constants for BSA and HSA as a function of shear rate

Parameter	BSA			HSA		
	8000	9500	13 500	8000	9500	13 500
k1	1.94×10^{-5}	1.97×10^{-5}	2.66×10^{-5}	1.26×10^{-5}	7.37×10^{-5}	8.99×10^{-5}
k2	1.81×10^{-2}	1.74×10^{-2}	2.35×10^{-2}	7.57×10^{-3}	2.08×10^{-2}	2.93×10^{-2}
k1/k2	1.07×10^{-3}	1.13×10^{-3}	1.13×10^{-3}	1.66×10^{-3}	3.54×10^{-3}	3.07×10^{-3}
k3	–	–	–	–	5.41×10^{-4}	3.65×10^{-4}
k4	–	–	–	–	6.31×10^{-2}	3.82×10^{-2}
k3/k4	–	–	–	–	8.57×10^{-3}	9.55×10^{-3}

For BSA, a monomer–dimer transition was observed, independently of shear rate; whereas for HSA, a kinetic model that involves a two-step process was observed at high shear rates: a double monomer–dimer and dimer–trimer transition. This was supported by SEC with MALLS detection results.

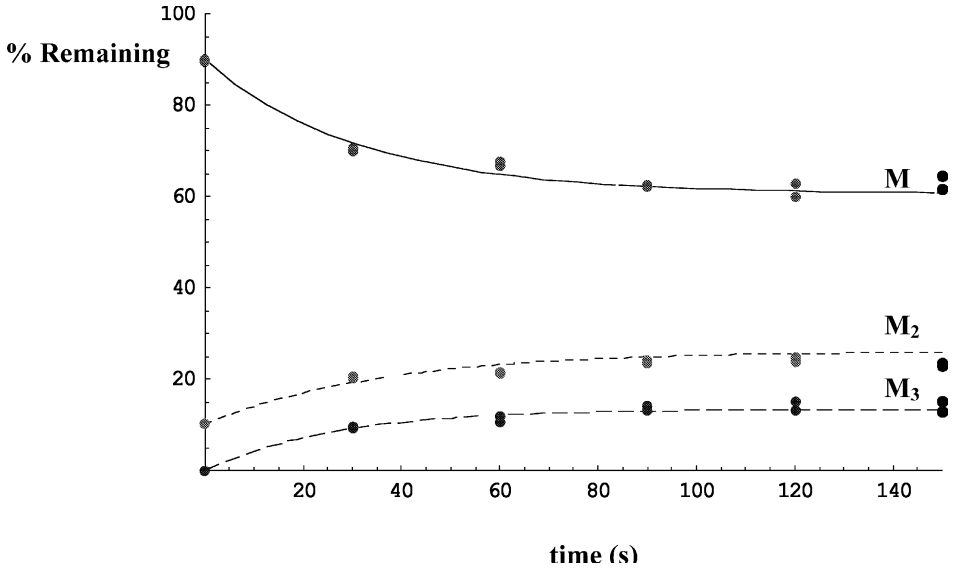

Fig. 3. Amount (%) of monomer (M), dimer (M_2) and trimer (M_3) of HSA remaining vs. shearing time by SEC with UV–Vis detection, at a shear rate of 13 500 rpm. At equilibrium, the fractions remaining were 61% for monomer, 25% for dimer, and 14% for trimer.

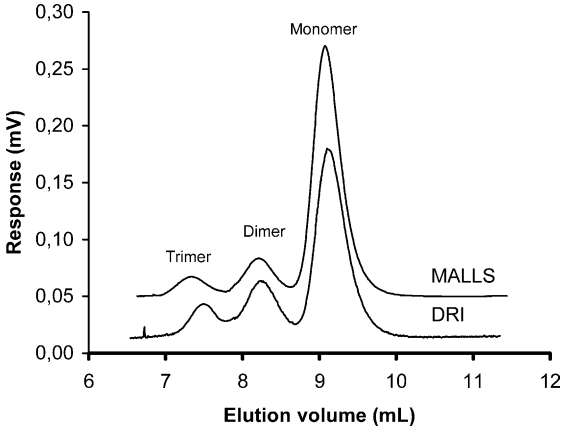

Fig. 4. Chromatograms obtained from an HSA sample (upper, trace MALLS detector, lower trace differential refractive-index detector) subjected to a shear rate of 13 500 rpm for 2 min. The absolute molecular weights were 67 000 Da (monomer), 132 000 Da (dimer) and 198 000 Da (trimer), respectively.

$$\frac{d[M_2]}{dt} = -k_2 \cdot [M_2] + 2 \cdot k_1 \cdot [M]^2 - k_3 \cdot [M_2] \cdot [M] + k_4 \cdot [M_3] \quad (11)$$

$$\frac{d[M_3]}{dt} = -k_4 \cdot [M_3] + k_3 \cdot [M_2] \cdot [M] \quad (12)$$

The NDSolve function was used to obtain the estimated values for each degradation rate constant as a function of the initial conditions (see Table 2).

4. Discussion

To obtain reliable drug degradation data, the development and validation of a stability-indicating method is required. However, once a new analytical protocol has been developed, it must be validated for use in routine analysis; the method can in this way be guaranteed to provide quality results every time. There are several sets of guidelines for the practical validation of analytical methods, e.g. official ones such as those issued by the United States Pharmacopoeia (USP), the ICH, or the Food and Drug Administration (FDA). However, there is no universally accepted procedure for validating a quantitative method by high-performance liquid chromatography. In this

work, we adapted the ICH general guidelines to this purpose [21]. The results obtained in the validation process indicate that the system and method are suitable for their intended use. Thus, the factors considered in validating these procedures included selectivity ($\alpha > 1.1$) and resolution ($Rs > 1.5$) in the identification stage. The precision, accuracy, detection and quantitation limits, and linearity are required in the quantitation stage. The precision expressed as repeatability was lower than 2.09% in the case of β-lactoglobulin, whereas the accuracy of the system varied in the 98.4–101.5% range with an R.S.D. of 2.3% in the worst case.

Once a stability study is initiated, one attempts to use a set of conditions that allow a summary parameter (such as rate constants) to be obtained, by kinetic analysis of a degradation versus time curve under previously specified conditions. A kinetic model is selected to describe the degradation curve, and a rate constant is calculated by fitting the curve to a suitable rate equation according to the assumed model.

At this point, the kinetic model is influenced by the nature of the protein and the effect of the shear rate on its stability. The β-lactoglobulin seems to be the protein most susceptible to shaking since a decrease of up to 35% in its initial value was observed, whereas no aggregation into other products was detected by SEC, which could indicate rupture into smaller fragments. The fragmentation of the protein should be visible, even by SEC, unless the protein splits into several low molecular weight fragments. Precipitates were not observed. More conclusive evidence confirming this observation requires careful analysis of the damaged protein by mass spectrometry, but SDS-PAGE could be a much simpler solution. The results indicate that the degradation rate constants increase proportionally with the shear rate, provided it is less than 13 500 rpm. This fact is supported in the parameters for shelf-life and protein fraction remaining at equilibrium (D_∞), which decreased in proportion to the corresponding increase in the rate of shear (see Table 1).

In the case of albumin samples, shaking seems to favor aggregation rather than fragmentation since an increase in the dimer and trimer percentages was observed. For BSA, shaking favors the monomer–dimer transition, independently of shear rate, the average ratio between the rate constants at equilibrium (k1/k2) being constant at 1.10×10^{-3} (see Table 2). However, two different behavior patterns can be seen for HSA as a function of shear rate: (i) Similar to BSA when the shear rate was 8000 rpm, although the ratio between the rate constants at equilibrium was slightly higher (1.66×10^{-3}). (ii) At higher shear rates, the assumed kinetic model involves two steps: the first, a monomer–dimer transition with a ratio between the rate constants (k1/k2) of 3.3×10^{-3}, a value three times higher than that for BSA, which could explain the higher dimer amount detected. After the second step, the formation of the trimer species was detected, the average ratio between the rate constants (k3/k4) was 9.0×10^{-3}, as can be seen in Table 2. Both processes were independent of shear rate.

The degree of aggregation through shaking is influenced by the protein concentration, air–liquid interface area and the primary structure of proteins [4,25,26]. In this study, two of the three factors cited could be considered constants since all assays were carried out at one fixed concentration, and the solvent–air interface was the same for all samples, as the surface conditions were identical. Thus, the different behavior could be due to the structural differences between the two types of albumin. Indeed, studies of solutions of hemoglobin genetic variants exposed to shaking have shown that hemoglobin S and certain others undergo interfacial coagulation far more rapidly and completely than the normal type-A variant. These differences are correlated with differences in the behavior of the mutants at the air–water interfaces and the primary structure of the proteins. Since the mutants vary from the normal type at only one or two positions in the amino acid sequence, it appears that these changes cause a destabilization of the entire structure, which is responsible for the differences observed after shaking [25]. Similar behavior was observed with variants of the tryptophan synthase α subunit obtained by recombinant DNA techniques, where single amino acids were substituted at specific places in the protein chain [26].

It is important to point out that, a priori, the degradation mechanisms observed as a consequence of shear forces cannot be extrapolated to those due to factors such as temperature, pH or shaking, where the degradation generally conforms to simple first-order kinetics. However, more complex kinetics like the Prout-Thompkin nucleation processes have been observed [27].

The method described in this paper could be applied to other proteins of therapeutic interest, since many exhibit such aggregation phenomena during the formulation and manufacturing processes of controlled release pharmaceutical dosage forms [28–30].

5. Conclusions

This study examines the effect of shear forces on stability of proteins as reflected in the different degradation mechanisms observed. β-Lactoglobulin showed a simple kinetic process, whereas both albumin samples presented a monomer–dimer transition. This was supported by SEC results showing the dimer percentage increased in a 5–8% range for BSA against a mean value of 16% for HSA. However, HSA presented a more complex degradation mechanism, especially at high shear rates, since trimer species were detected. This was also supported by SEC and MALLS detection results. This behavior may be attributed to the differences in the primary structure between these types of albumin rather than the protein concentration and the solvent–air interfaces, maintained as fixed factors.

The influence of shear rate on protein stability varies with its magnitude, time of application and the protein structure, as seen in the case of human and BSA (differing in only two amino acids). There is no single kinetic model universally applicable to the degradation processes.

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