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Stability Study of Human Serum Albumin Pharmaceutical Preparations

ALEXIS OLIVA, ANA SANTOVEÑA, MATIAS LLABRES AND JOSE B. FARIÑA*

Departamento Ingeniería Química y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de La Laguna, 38200 Tenerife, Spain

Abstract

The influence of temperature on the stability of human serum albumin (HSA) pharmaceutical preparations has been studied by size-exclusion high-performance liquid chromatography with multi-angle laser-light-scattering detection and by particle-size analysis.

The behaviour of HSA in two pharmaceutical preparations stored at different temperatures (40, 55 and 70°C) followed the same pattern—an increase in the relative percentage of dimer (MW 132 000 Da) and aggregate (MW > 200 000 Da), and then a decrease in the concentration of all species and, finally, sudden protein coagulation. These results suggest a time- and temperature-dependent process. At 70°C, monomer only was detected for both preparations; the amount remaining was 83 and 72% for formulations A and B, respectively. Analysis of size-distribution curves also seems to confirm these results. Initially, three distributions were observed with length–volume mean diameters ($d_{l,v}$) of 1·67, 10·6 and 57 μm. After 80 days at 55°C, only two distributions were observed, with $d_{l,v}$ of 3·07 and 76 μm. An additional study using pure HSA at different concentrations (0·3, 2·5, 5 and 10% w/v) and storage at 75°C was performed to determine the influence of the concentration of auxiliary substances and of the HSA. Only when the HSA concentration was 0·3% w/v did the remaining fraction of HSA fit a Prout–Thompkins nucleation model. Initially three distributions with mean sizes of 2, 20 and 40 μm were observed whereas at the end of the assay only one distribution, mean size 129 μm, was seen. The methodology used enabled us to separate the HSA degradation products and to determine the absolute molecular weight of albumin monomer and dimer.

It is possible to conclude that the degradation mechanism for the formulations studied is complex, and that it is possible to fit the degradation data to Prout–Thompkins kinetics only when the concentration of HSA is low enough (0·3% w/v).

Albumin is the most abundant plasma protein. Human serum albumin (HSA) is used commonly for the treatment of hypovolaemia and of diseases including chronic renal failure and liver cirrhosis (Narazaki et al 1996). Currently the application of albumin in surgery and shock trauma consumes more than 100 tons of this protein per year in North America alone (Henderson 1996).

Because of its capacity to bind a wide variety of exogenous and endogenous substances, albumin has been used to remove toxins, for drug delivery and for coating in-vivo devices (Carter & Ho 1994; Kurtzhals et al 1996).

Correspondence: J. B. Fariña, Departamento Ingeniería Química y Tecnología Farmacéutica, Facultad de Farmacia, Universidad de La Laguna, 38200 Tenerife, Spain.

On the basis of stability considerations, protein drugs belong to a special class because of their complex structure and degradation pathways. Degradation of protein can involve chemical modification via bond formation, yielding a new chemical entity, and physical instability, which refers to changes in their secondary, tertiary or quaternary structure (Oliva et al 1997). Physical instability or denaturation of a protein often involves unfolding of the molecule. The unfolded protein is susceptible to further inactivation by aggregation, which might be induced by exposure to an air-water interface, to heating, shaking or lyophilization, or by addition of phenolic compounds (Costantino et al 1994; Katakam et al 1995; Oliva et al 1996).

Aggregation usually leads to reduced bioactivity and immunogenic reactions, possibly because of increased molecular weight and unacceptable physical characteristics, such as the turbidity and opalescence, which gives the product undesirable properties (Maa & Hsu 1996). Like most large proteins, albumin also undergoes aggregation, the storage temperature being the main induction factor (Jordan et al 1994; Katam & Banga 1994).

Techniques such as size-exclusion high-performance liquid chromatography (SEHPLC), high-performance capillary electrophoresis (HPCE), mass spectrometry, light scattering and analytical ultracentrifugation have been used for determination of protein molecular weight (Oliva et al 1997). The European Pharmacopoeia (1997) recommends an assay based on liquid chromatography with UV-Vis detection to determine the presence of HSA polymers and aggregates. The method stipulates that the sum of the peak areas corresponding to those species, divided by two, should be less than 5% of total area of all peaks.

SEHPLC is probably the most versatile and widely applied method for estimating the molecular weight of a protein in its native form on the basis of its elution position. However, ordinary SEHPLC techniques based on calibration standards are ineffective and often yield erroneous molecular weights if the standards and the sample have different conformations (Dayal & Metha 1994). The development of laser-light-scattering techniques combined with SEHPLC has been a great advance in protein characterization because it is possible to determine molecular weight directly (Wyatt 1993).

In this work we studied the influence of temperature on the stability of commercial preparations of HSA by use of conventional SEHPLC equipment coupled with a multi-angle laser-light-scattering detector to determine the absolute molecular weight of the monomer and its derivatives. A particle size analyser was used to follow the evolution of HSA aggregate size distributions during the assays. The proposed technique enabled us to complement the European Pharmacopoeial method and to characterize the HSA aggregation process.

Materials and Methods

Size-exclusion high-performance liquid chromatography with UV-Vis detection

The HPLC system used in this study was a Waters apparatus comprising a pump, 600E multisolvent delivery system, 700 Satellite Wisp sample processor and 490E programmable multiwavelength detector. Control and results management were performed by the Maxima 820 chromatography program from Waters. Elution was performed at room temperature on a Shodex C-18 column (Waters); the mobile phase was 25 mM disodium hydrogen phosphate and 120 mM sodium chloride, adjusted to pH 7·0, at a flow rate of 1·0 mL min^{-1}. Detection was performed at 280 nm. Deionized water prepared with a MilliQ apparatus (Millipore) was used throughout; all other chemicals and reagents were HPLC grade. All solvents were filtered through 0·45-μm (pore size) filters (Millipore) and degassed.

To validate the analytical method, eight standard solutions were prepared using human serum albumin (HSA) from Sigma (St Louis, MO) at concentrations of 0·1–0·4 mg mL^{-1}. Each sample was analysed in triplicate. To quantify HSA we used the total area of all the peaks. The sizes of the individual peaks, as a percentage of the total area, enabled estimation of the content of HSA monomer and derivatives (dimer and aggregates). Analysis of variance of linear regression of the dependence of the total area of all peaks on HSA concentration 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 ($\alpha = 0{\cdot}05$); the coefficient of variation of predicted concentrations was 1·0% (Hunter & Lamboy 1981). The equation of the regression line was:

$$\begin{aligned}\text{Total area} &= (10416 \pm 4403) \\ &\quad + (1393 \pm 17{\cdot}2)\text{C}\end{aligned} \qquad (1)$$

$$r = 0{\cdot}998 \quad (n = 24)$$

and the root mean square error (S_{xy}) was 8·695.

For pharmaceutical preparations, homogeneous samples (0·5 mL) were withdrawn from vials and stored at 4°C until analysis. Samples were directly diluted with the mobile phase to furnish concentration values within the calibration range.

Size-exclusion high-performance liquid chromatography with multi-angle laser-light-scattering detection

We used the same techniques of size-exclusion chromatography, but with on-line light-scattering and refractive-index detectors, to determine the molecular weight of HSA monomer and its derivatives. The multi-angle laser-light-scattering detector (miniDawn, Wyatt Technology) was placed downstream of the column and upstream of the differential refractive-index detector, a Waters model 410, to avoid the possibility of back pressure

on the differential refractive index cell. To reduce baseline noise a pulse dampener (Alltech Associates, USA) was connected downstream of the pump, to reduce its pulsation, and two 25-mm high-pressure filters with 0·22- and 0·1-μm pores (Millipore) were used for on-line filtration of the mobile phase.

The column and other chromatographic conditions were identical with those used for SEHPLC. A differential index of refraction (dn/dc) of 0·186 mL g^{-1} was assumed (Wen et al 1996). Data collection from the miniDawn and differential refractive-index detectors were controlled by Wyatt Technology's Astra program. The miniDawn instrument incorporates a fixed photodiode detector array capable of measuring scattered-light intensity simultaneously at three angles (45, 90 and 135°). The 90° detector was calibrated by use of toluene according to the manufacturer's instructions. Bovine serum albumin monomer in the mobile phase was used for normalization of the three light-scattering detectors and to enable proper alignment of the light-scattering and differential refractive index signals, a step necessary for calculation of the molecular weight for each data slice.

The differential refractive-index detector was calibrated over a linear concentration range (1–5 mg mL^{-1}) with five evenly spaced sodium chloride standards prepared by direct dilution from a 10-mg mL^{-1} solution.

To validate the SEC-multi-angle laser-light-scattering method, several proteins (β-lactoglobulin, ovalbumin, bovine and human serum albumin, all from Sigma, St Louis, MO) were analysed in triplicate on different days. The coefficient of variation (CV) obtained on the same day (intra-assay precision) was <2% for the weight-average molecular weight; higher values were obtained for number-average molecular weight, but none exceeded 5%. To complement the information available we performed one-way analysis of variance to determine if there were differences between days; the results showed that the inter-assay differences were not significant ($P > 0.05$ always). The inter-assay precision was always better than 2·5%.

To calibrate the SEC-light-scattering method and to monitor its performance we analysed a bovine serum albumin standard (0·3 mg mL^{-1})—the standard was analysed each working day and the monomer molecular weight was determined. We established the use of two sets of limits; the outer limits, sometimes called action limits, are the conventional limits, usually 3 × sigma, and the inner limits, the warning limits, usually 2 × sigma (Grant & Leavenworth 1988). After 10 standard analyses the mean and standard deviation of the data were calculated and a control chart was constructed. 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.

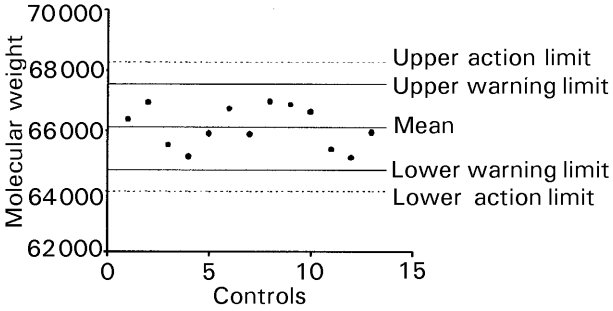

Figure 1. Control chart for size-exclusion high-performance liquid chromatography with multi-angle laser-light-scattering detection, constructed using a bovine serum albumin standard (0·3 mg mL^{-1}) with a nominal molecular weight of 65 000 Da.

We obtained a mean value for the weight-average molecular weight of 66 100 Da with a standard deviation of 736 (n = 13), the coefficient of variation (CV = standard deviation/mean) was 1·11%. Figure 1 shows the control chart for the method, indicating the action and warning limits.

Particle-size measurement

The particle-size distribution of HSA was measured with a Coulter Multisizer II; the samples were suspended in mobile phase. The results are presented as the length-volume mean diameter ($d_{l,v}$, μm).

Stability studies

In this study, we used two commercial samples of human albumin dosage forms suitable for injection, formulation A and formulation B, containing 20% total albumin. Sodium caprylate and sodium acetyltryptophanate were used as auxiliary substances. Both preparations were stored at 40, 55 and 70°C with variations less than ± 1°C. Samples were taken after different times, depending on the storage conditions.

Four solutions were prepared from pure HSA (Sigma) at concentrations of 0·3, 2·5, 5 and 10% (w/v) in mobile phase and stored at 75°C.

Results and Discussion

As a preliminary step, the analytical method was validated for HSA. With this method it is possible to separate the HSA monomer and dimer in the

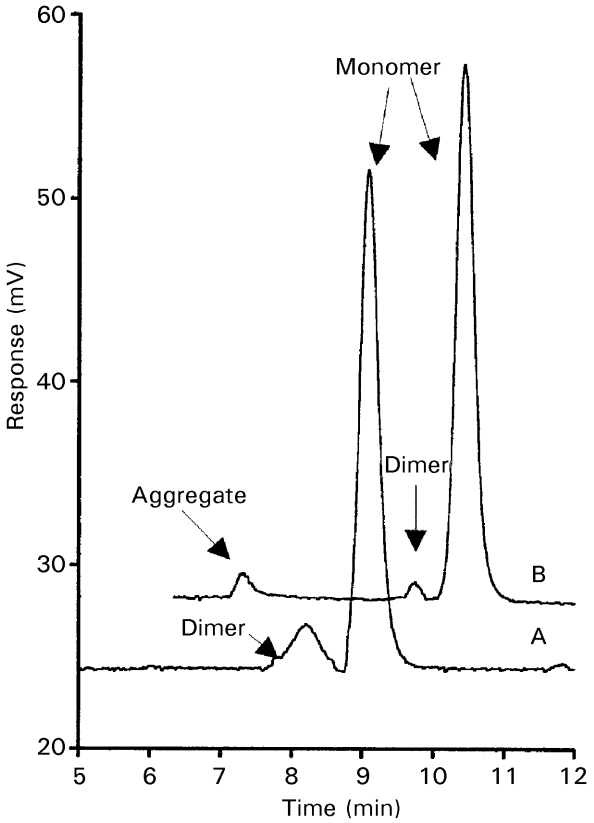

Figure 2. Chromatogram obtained from analysis by size-exclusion high-performance liquid chromatography, with UV-Vis detection at 280 nm, of a pure sample (A) and a pharmaceutical preparation (B) of HSA. The retention times of HSA aggregate, dimer and monomer were 6·1, 8·25 and 9·1 min, respectively.

same analysis with short analysis time (12 min), achieving the required resolution and efficiency. The auxiliary substances present in HSA pharmaceutical preparations do not interfere with the determination.

Figure 2 shows the chromatogram obtained by SEHPLC with UV-Vis detection of the HSA sample. Peaks corresponding to the HSA monomer (elution time 9·1 min) and dimer (8·25 min) were detected, the mean percentages of each peak being 91·5 and 8·5, respectively; the standard deviation was 0·61 (n = 24). Another peak was detected in the commercial human albumin preparation; it was probably that of high molecular weight transformation products of HSA because this peak has an elution time (6·1 min) lower than those of the HSA dimer, as can be seen in Figure 2. The mean percentages of the HSA monomer and derivatives (dimer and aggregates included) for both preparations are summarized in Table 1. The total amounts of HSA in formulations A and B were 20·1 ± 0·3% and 20·6 ± 0·5 (n = 6), respectively, in agreement with the contents declared by the manufacturers. As is apparent from Table 1, the HSA polymer and aggregate content of both HSA pharmaceutical preparations and pure HSA were satisfactory—the sum of the areas of those peaks, divided by two, was lower than 5% of total area of all the peaks, in accordance with the guidelines of the European Pharmacopoeia (1997).

A key requirement for the determination of molecular weights by light scattering is the numerical value of dn/dc and a knowledge of the absolute concentration of the sample fraction. Figure 3A shows the chromatograms obtained from SEHPLC with multi-angle laser-light-scattering detection of an HSA sample. For a protein or complex that contains no carbohydrate, dn/dc is constant ($\approx 0{\cdot}186\ \mathrm{mL\,g^{-1}}$) and almost independent of amino acid composition (Wen et al 1996). The absolute molecular weight of the HSA monomer calculated was 67 000 Da, which agrees quite well with the assumed value of 66 500 for the HSA monomer, whereas the molecular weight of the HSA dimer was 132 000 Da, a value close to twice the sequence molecular weight of 66 500 Da. We could not determine the absolute molecular weight of the aggregate present in HSA pharmaceutical preparations; we believe it to be >200 000 Da, i.e. greater than the exclusion limit of the column used in this study (Figure 3B).

Table 2 lists the degradation products detected in each preparation after storage at different temperatures for different times. At 40°C the concentration of HSA remains constant for both preparations for 90 days—variation is less than 5% of the declared content and differences between the relative proportions of different degradation products at different times were not significant (Figure 4A).

Table 1. Mean percentages, relative to overall peak area, of the peak areas of the HSA monomer and the derivatives.

	Amount of monomer (%)	Amount of dimer (%)	Amount of aggregate (%)	$\Sigma(M+D)/2^a$ (%)
Pure HSA	91·5 ± 0·61	8·50 ± 0·61	ND	4·25 ± 0·61
Formulation A	94·4 ± 0·20	1·64 ± 0·16	3·94 ± 0·16	2·79 ± 0·12
Formulation B	92·4 ± 0·30	0·96 ± 0·12	6·59 ± 0·14	3·78 ± 0·15

[a]Sum of the peak areas of the polymers and aggregates divided by two. ND = not detected.

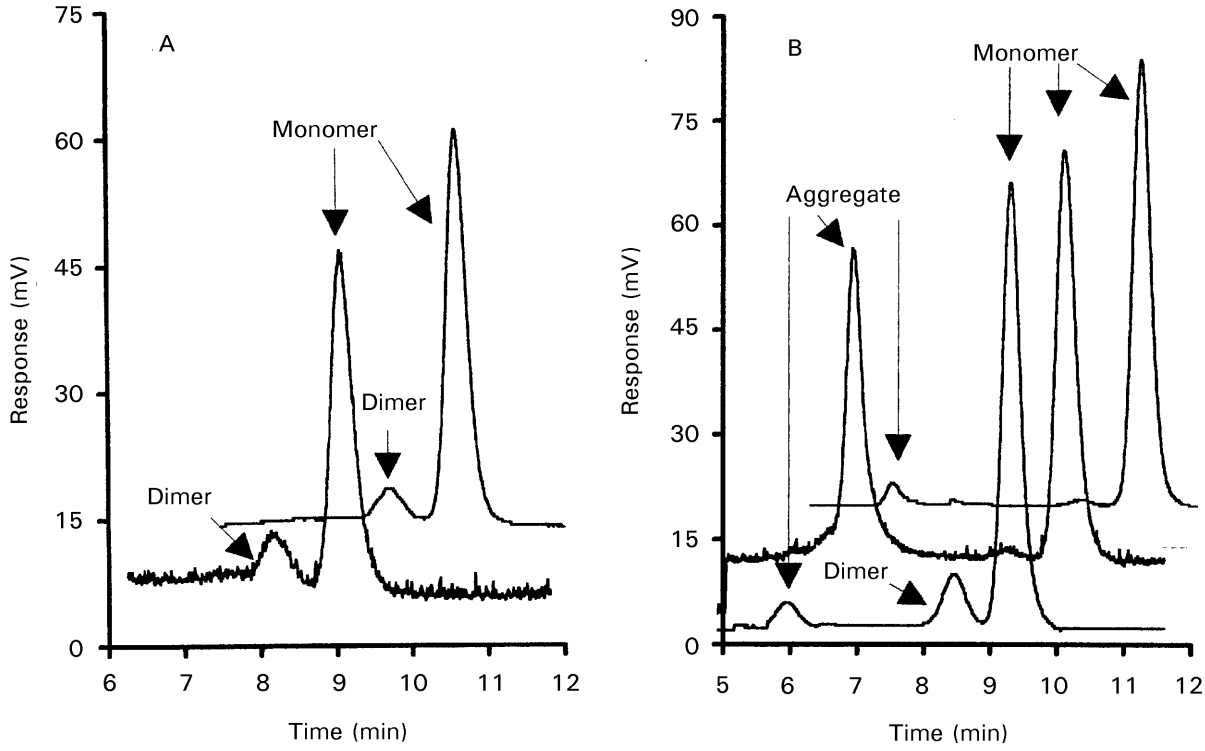

Figure 3. Chromatograms obtained from a pure HSA sample (A, upper trace differential refractive-index detector, lower trace multi-angle laser-light-scattering detector) and a sample of an HSA formulation (B, top trace differential refractive-index detector, middle trace multi-angle laser-light-scattering detector, bottom trace UV-Vis detector) stored at 40°C for 10 days. The absolute molecular weights were 67 000 Da (monomer) and 132 000 Da (dimer). Only for the formulation (B) could the monomer molecular weight be determined; the aggregate molecular weight was outside the column exclusion limits (> 200 000 Da) and the differential refractive-index and multi-angle laser-light-scattering signals were not of sufficient intensity for determination of the absolute molecular weight of the dimer.

At 55°C, the formulations behaved differently. A greater decrease in HSA concentration (12%) was measured for formulation B; the percentage of degradation products in formulation A increased by approximately 10%. Dimer was only detected by UV-Vis detection during the first 30 days for formulation B (Figure 4B). At 70°C HSA monomer only was detected in both preparations; the amounts remaining (%) in formulations A and B were 83 and 72%, respectively.

Before coagulation the behaviour of HSA in pharmaceutical preparations stored at different temperatures follows the same pattern—an increase in the amount of dimer and aggregate, then a decrease in the concentrations of all species, and finally a process time- and temperature-dependent sudden protein coagulation. The analysis of size distribution curves seems to confirm this. Initially, three distributions can be observed with $d_{l,v}$ of 1·67, 10·6 and 57 μm; after 80 days at 55°C only two distributions were observed, with $d_{l,v}$ of 3·07 and 76 μm. Figure 5 shows the particle-size distribution obtained for both preparations stored at 70°C. The size-distribution data suggest displacement to higher mean diameters, confirming probable aggregation as a result of a nucleation process.

The results from SEHPLC with light-scattering detection suggest that the absolute molecular weights of different products detected were similar to those obtained with the pure HSA sample. Although the concentration of protein remaining decreased significantly at 55 and 70°C, the data obtained did not follow any known degradation kinetics and, therefore, prediction of stability on the basis of the Arrhenius relationship is inappropriate for HSA pharmaceutical preparations, because the mechanisms of degradation of the protein are complex and could change as a function of temperature (Gu et al 1991; Yoshioka et al 1994). Although the mechanism of degradation is not clear, one possibility is that initial thermal denaturing of the protein might be followed by aggregation; the aggregates might be non-covalent, owing to stabilization by the auxiliary substances used in this type of preparation.

Results from measurement of particle-size distribution seem to suggest that aggregation and reversible flocculation lead to sudden protein coagulation for a given temperature and storage time. The aggregation–coagulation process is in agreement with the process proposed by Glatz (1992). The first process is formation of primary particles

Table 2. Degradation products detected, by size-exclusion high-performance liquid chromatography, in both formulations after storage at different times and temperatures.

Temperature (°C)	Time	% Monomer	% Dimer	% Aggregate	% Remaining
Formulation A					
	0 days	94·4 ± 1·70	1·68 ± 2·31	3·71 ± 1·65	104 ± 1·41
40	90 days	92·1 ± 2·61	3·19 ± 0·46	4·71 ± 2·19	103 ± 0·70
55	80 days	84·3 ± 1·35	8·75 ± 0·65	6·96 ± 0·92	88 ± 1·13
70	9 h	100	–	–	83 ± 2·97
Formulation B					
	0 days	93·1 ± 3·04	1·17 ± 1·46	5·73 ± 1·10	103 ± 2·47
40	90 days	90·8 ± 0·78	3·74 ± 0·37	5·51 ± 1·20	102 ± 0·64
55	80 days	94·7 ± 1·06	–	5·31 ± 1·08	67·6 ± 1·19
70	9 h	100	–	–	72 ± 1·53

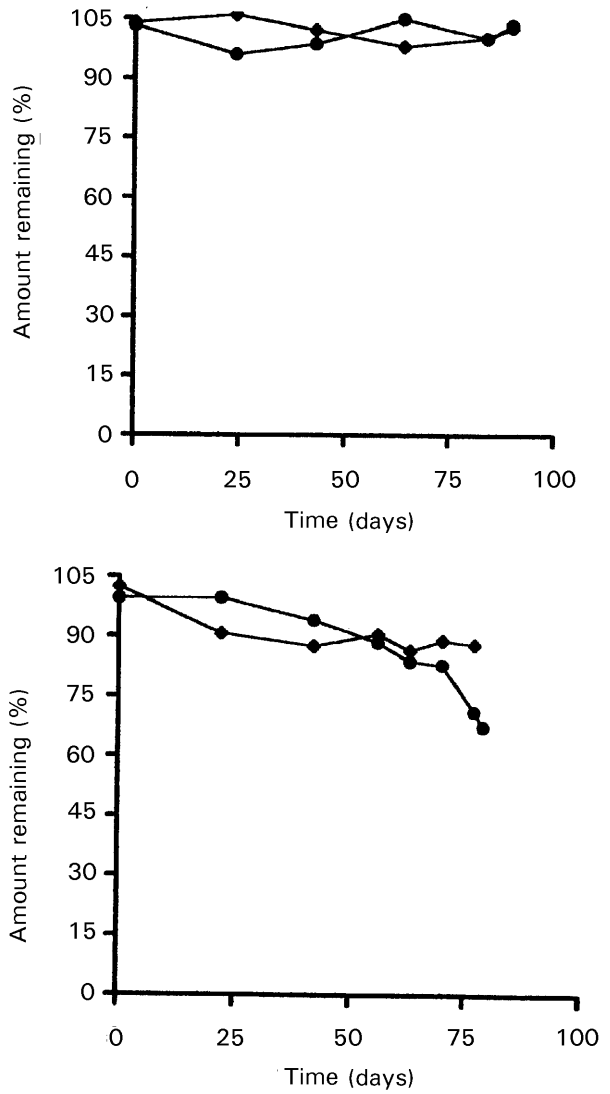

Figure 4. Amount (%) of HSA remaining in pharmaceutical preparations A (◆) and B (●) stored at 40°C (upper) and 55°C (lower). The decrease was greatest for formulation B at 55°C (67·6%).

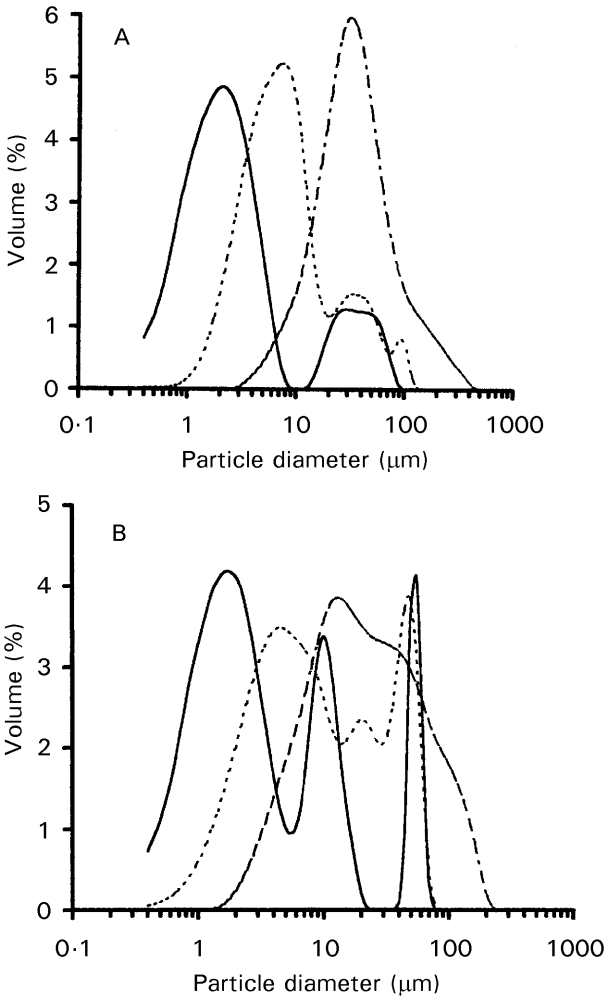

Figure 5. Change of particle size distribution with time for HSA formulations A (—, 0 h, ····, 5 h, – · – ·, 7 h) and B (—, 0 h, ····, 3 h, – · – ·, 7 h) stored at 70°C. For formulation A at 0 h two distributions with mean sizes 2·0 and 40 μm were observed whereas at the end of the assay only one distribution, mean size 40 μm, was observed. For formulation B, at 0 h three distributions with mean sizes of 1·67, 10 and 40 μm were observed whereas at the end of the assay a broad distribution with sizes between 1 and 200 μm was observed.

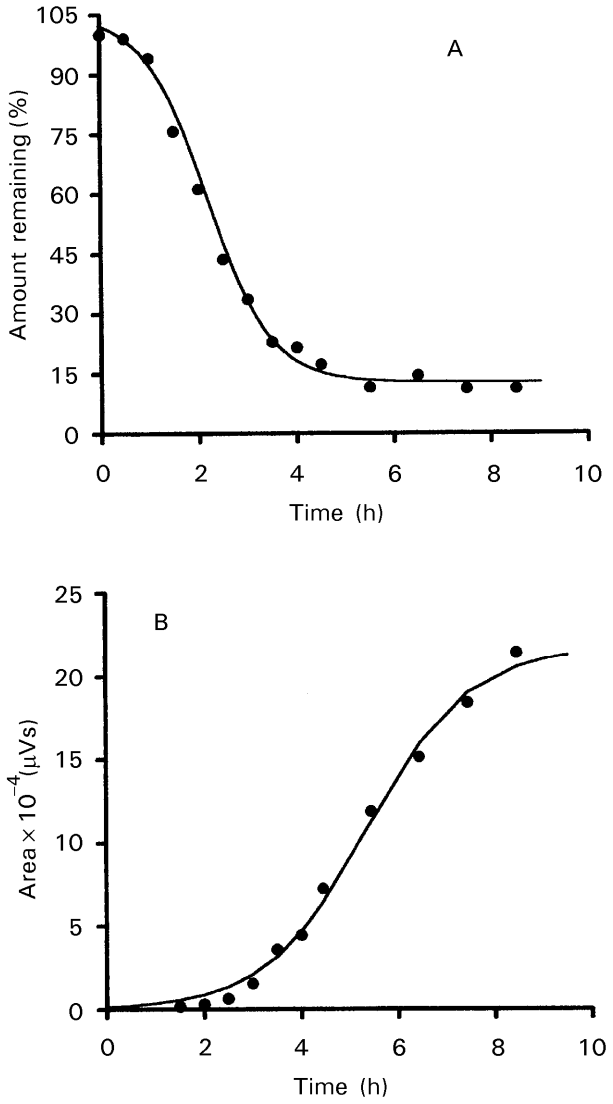

Figure 6. Degradation kinetics of the HSA monomer (A) and formation kinetics of the aggregate (B) for a pure sample of HSA (0·3% w/v) stored at 75°C for 9 h. Data were in good agreement with a Prout–Thompkins nucleation model.

from the protein molecules present in solution; this is followed by the growth of floccules (or aggregates of primary particles) of varying sizes and simultaneous breakage of the floccules as a result of collisional forces. However, the data obtained did not fit any known model because of the limitations of the technique used to determine particle size. To complement the information available we performed an additional study using pure HSA at different concentrations and stored at 75°C to verify the influence of the auxiliary substances and the concentration of HSA in the process mentioned earlier. For the samples at concentrations of 2·5, 5 and 10% (w/v), behaviour was similar to that of the HSA preparations—physical instability was observed with an increase in viscosity and with these samples protein coagulation was quicker.

When the HSA concentration was 0·3%, the fraction of HSA remaining was a better fit to the Prout–Thompkins nucleation model (Figure 6), as is observed with other proteins (Oliva et al 1996). In this study, the HSA dimer was detected during the first 2 h only; later the HSA aggregate was the only degradation product detected. Physical instability was not observed during the assay. The fraction of HSA remaining at time t, and HSA aggregate formation, were calculated by use of equations published elsewhere (Oliva et al 1996); the results are shown in Figure 6. These results were confirmed by measurement of particle size distribution—initially we observed three distributions with mean sizes of 2, 20 and 40 μm; after 5 h two distributions with mean sizes of 3·70 and 76 μm were observed; at the end of the assay (9 h) we observed one distribution only, with a mean size of 129 μm.

In conclusion, complex degradation mechanisms are observed for commercial HSA preparations stored at different temperatures—a decrease in the percentage of HSA monomer, an increase in the amounts of dimer and aggregate, and, finally, protein coagulation. Stability predictions based on the Arrhenius plot are inappropriate, as for the majority of protein preparations. For pure HSA solutions at concentrations (0·3% w/v) one seventh that of the pharmaceutical HSA preparations, the degradation kinetics fitted a Prout–Thompkins nucleation model, as is observed with commercial preparations of human insulin (Oliva et al 1996).

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