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Effects of nonionic surfactants on the physical stability of immunoglobulin G in aqueous solution during mechanical agitation

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The objective of this study was to evaluate the influence of nonionic surfactants in the presence of glycine and sodium chloride on the physical stability of immunoglobulin G (IgG) in aqueous solution. Among surfactants suitable for parenteral preparation, Polysorbate 80 (Tween® 80) and Polyoxyl 35 Castor Oil (Cremophor® EL) were selected. The physical stability of IgG in the absence and in the presence of excipients was investigated in aqueous solution during mechanical agitation (concentration of IgG 15%; pH 7.1; temperature 6 ± 2 °C). Suitable concentrations of Tween® 80 and Cremophor® EL were experimentally determined by surface tension measurements at 6 ± 2 °C. Glycine and sodium chloride were used in different concentrations. The influence of the excipients on the physical stability of IgG in solution has been examined by surface tension measurements, protein content assay (Kjeldahl and HPLC) and differential scanning calorimetry (DSC). Based on the results of the investigations, it was found that Tween® 80 and Cremophor® EL, used in experimentally determined critical micelle concentration (cmc), decreased the physical stability of IgG in solution. Tween® 80 and Cremophor® EL in the presence of glycine (1.5 g/l) could stabilize the IgG in solution during mechanical agitation. The comparison of the effects of Tween® 80 and Cremophor® EL on the physical stability of IgG, showed that Tween® 80 had better stabilization effects on IgG in solution under the experimental conditions selected.

1. Introduction

Physical instability or denaturation of a protein often involves unfolding of the molecule. The unfolded protein then adsorbs to surfaces and is susceptible to further inactivation by aggregation with neighbouring molecules. Protein aggregation may be induced by exposure to an air/water interface, heating, lyophilization or reconstitution of lyophilized material [1, 2]. The aggregation pathway proceeds through soluble aggregates to the formation of insoluble aggregates regardless the denaturating stimuli. Agitation induced denaturation and aggregation are mainly due to the adsorbtion of protein at the air/water interface and because of that, one important aspect of successful formulation development is the stabilization at air/water interfaces [3, 4].

The common method of protein solution stabilization is the addition of hydrophilic nonionic surfactants which have been exploited as excipients for their ability to prevent protein penetration and aggregation. Maximum protection occurs at concentrations close to the critical micelle concentration (cmc) independent of the initial protein concentration. Two predominant mechanisms have been proposed for protein stabilization by surfactants: 1) it has been found that stabilization is a consequence of a direct binding of surfactant to the protein that inhibits the intermolecular contacts leading to aggregation, or to a folding intermediate state that acts as a molecular chaperone and foster refolding over aggregation [3, 5]; 2) if a surfactant is significantly more surface active than the protein, it will almost completely block the interface from the protein and provide an essentially hydrophilic surface for protein (hydrophilic heads of the surfactants molecule) which will prevent the protein from adsorbing at the interface and protect it from surface related denaturation and precipitation [4, 5].

Immunoglobulin G was selected as a model protein. It is currently formulated as parenteral solution or lyophilized powder. It has shown a tendency to adsorb at the air/water interface [6]. Shaking induced aggregation of monoclonal antibodies resulted in precipitation, presumably due to unfolding at the air/water interface [4]. Aggregation of IgG in solution during mechanical agitation resulted in two types of aggregates: fibers formed at the interface and the particles formed in the bulk [7]. It has shown a highly pronounced tendency to aggregate when heated to 60 °C at neutral pH [8].

Tween 80 is the only surfactant which is contained in commercial parenteral formulations of IgG [9–11]. The objective of this study was to investigate the influence of other nonionic surfactants on physical stability of IgG in solution and relate it to Tween 80 effect. Among the non-

ionic surfactants approved for parenteral preparations, Cremophor EL was selected [12]. The effects of Tween 80 and Cremophor EL on physical stability of IgG in solution have been assessed using surface tension measurements, HPLC and DSC. By using a number of measuring modes, it is possible to investigate both insoluble and soluble aggregate formations during mechanical agitation and thermal denaturation of IgG in solution and to relate these parameters to the effects of surfactants.

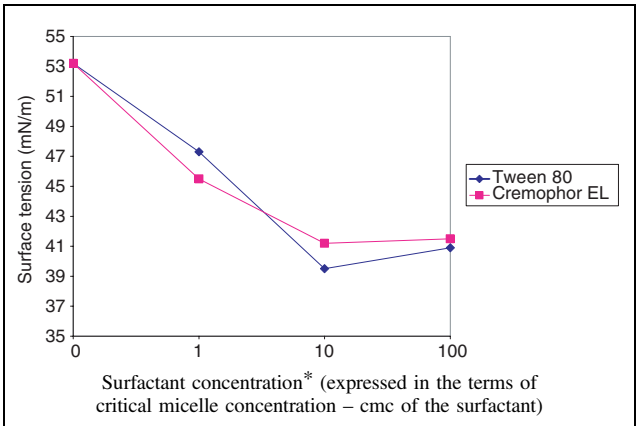
2. Investigations, results and discussion

2.1. Surface tension

Cmc of selected surfactants under experimental conditions were determined based on the plot of surface tension dependence on the surfactant concentrations (Fig. 1). It is known that the inflection point of the plot corresponds to the cmc of surfactants [13, 14]. The inflection points of Tween 80 and Cremophor EL plots corresponded to their 10 cmc under the experimental conditions and were used for the preparation of IgG/surfactant mixtures.

The surface tension measurements showed that the surface tension value of IgG solution were greater than the surface tension value of water (Table 1). The earlier investigation of interfacial shear rheology of human IgG in aqueous solution at air/water interfaces indicated that at pH 7.4 (the isoelectric point [pI] of IgG), the surface adsorption of protein molecules is usually maximum, as the consequent reduction in surface tension caused by the presence of these molecules [6]. The surface tension is relatively low during the formation of spread films, in low protein concentration, whereas in adsorbed films the surface tension rises [15]. Based of these observations, greater surface tension value of IgG solution under the experimental conditions, in comparison with surface tension value of water, was probably due to the high concentration of IgG in solution and multilayer formatting at air/water interface.

By the comparison of the surface tension values of IgG solution and IgG/surfactant mixtures with the surface tension values of surfactant solutions in experimentally determined cmc, both mixtures (IgG/Tween 80 and IgG/Cremophor EL) had a lower surface tension value than IgG solution. The surface tension value of the IgG/Tween 80 mixture ranged between the surface tension values of IgG solution and Tween 80 solution in experimentally determined cmc, while the surface tension value of IgG/Cremophor EL mixture was similar to that of Cremophor EL solution in experimentally determined cmc. Published results of experimental works based on the investigations of mixed protein/surfactant systems at air/water interfaces suggested that in these systems, over the intermediate surfactant concentration region, the surface tension becomes close to that of the pure surfactant and the adsorption of surfactant and the protein at interface are comparable. When the surfactant concentration is close or above the cmc, the surface tension is overlapping with that of the pure surfactant and complete removal of protein from surface is achieved [16]. These results indicated that Tween 80, in experimentally determined cmc and experimental conditions, did not cover the surface in a monolayer and that interface of IgG/Tween 80 mixture was constituted of IgG and Tween 80 molecules together, while Cremophor EL covered the interface in a monolayer.



* Values on x axis are expressed in the terms of critical micelle concentration (cmc) of the surfactants at 20 °C (1), ten times greater than cmc of the surfactants at 20 °C (10) and one hundred times greater than cmc of the surfactants at 20 °C (100) independent on their w/v concentrations

Fig. 1: The plot of surface tension values dependence on the surfactant concentration in solution

Table 1: Surface tension values of IgG solution and IgG/surfactant mixtures

Sample	Surface tension (mN/m)		Surfactant concentration (% w/v)
	Sample	Surfactant*	
Sterile water for injection	53.2 ± 2.2	–	0
IgG solution	55.63 ± 1.1	–	0
IgG/Tween 80 mixture	52.97 ± 0.47	39.5 ± 0.5	0.013
IgG/Cremophor EL mixture	39.5 ± 1.35	41.2 ± 0.2	0.09

The surface tension values are means ±S.E., N = 3 at 6 ± 2 °C.
* Surfactant solution in experimentally determined critical micelle concentration – cmc (concentration ten times greater than cmc of the surfactant at 20 °C)

There could be several causes for mixed IgG/Tween 80 system formation at the air/water interface in experimentally determined cmc of Tween 80. One of them could be the unprecise method of cmc determination. This statement could be supported by the fact that cmc within approximately 20% of the inflection point is achieved by the applied method of cmc determination – surface tension measurements [13]. This pointed out that the surface tension measurement is not a highly sensitive method for the determination of cmc. On the other hand it is known that Tween 80 tends to form complexes with some proteins [1]. HPLC, used for characterization of formed aggregates in experimental samples, is one of the methods for separating and analyzing protein/surfactant complexes too, based on their elution [17]. Elution profiles (not presented in this paper) obtained by HPLC runs of experimental samples did not indicate protein/surfactant complex formation. Another reason for the IgG/Tween 80 mixture behavior was found among the observations made on the basis of interfacial shear rheology investigations of protein at their pI. These observations indicated that the shear viscosity and the displacement of protein by surfactant were more difficult as the pI of protein was approached because of the presence of a more strongly cross-linked protein network at the interface [18].

2.2. Effects of excipients on molecular size distribution

Numerous experimental works indicated on aggregate formation during freeze-drying of proteins and storage of lyophilized powders together with the effects of stabilisers on proteins during these pharmaceutical processes [3, 19–24]. As the high polymer content was determined in

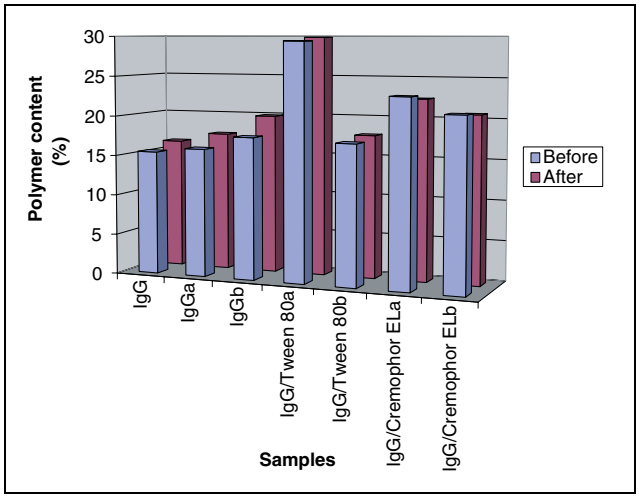


Fig. 2: Polymer content in IgG solution and IgG/PAM mixtures with different concentrations of glycine and sodium chloride before and after mechanical agitation

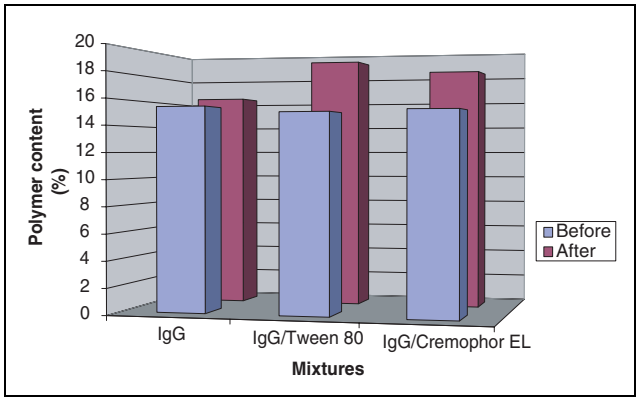


Fig. 3: Polymer content in IgG solution and IgG/PAM mixtures before and after mechanical agitation

IgG solution under the experimental conditions (about 15% w/v) it was probably due the presence of aggregates in lyophilized powder of IgG formed during freeze-drying because of the absence of suitable stabilisers.

Glycine and sodium chloride are frequently used in protein stabilisation. Glycine has been used, among others, to inhibit aggregate formation and to increase protein solubility, while sodium chloride may influence protein solubility [25]. In the presence of glycine and sodium chloride, the polymer content in IgG solution was significantly higher in formulation b, which contained a lower content of glycine and a higher content of sodium chloride, in comparison with formulation a (Fig. 2). The aggregate formation in formulation b was probably stimulated both by glycine and sodium chloride concentration: glycine concentration was too low to prevent the aggregate formation and the higher content of sodium chloride allowed stronger intermolecular attractions that resulted in aggregates.

The comparison of polymer contents in IgG solution and mixtures of IgG/Tween 80 and IgG/Cremophor EL after constitution and before mechanical agitation (Fig. 3) indicated that there were no significant differences among them and that Tween 80 and Cremophor EL did not compromise the physical stability of IgG in solution under the experimental conditions. The polymer contents in these samples was not influenced by the different effects of Tween 80 and Cremophor EL on IgG adsorbtion at the air/water interface determined by the surface tension measurements.

The results of the polymer content analysis in IgG solution in the presence of surfactant (Tween 80 and Cremophor EL), glycine and sodium chloride suggested that the polymer content in these samples depended on glycine and sodium chloride concentrations and were higher in formulations a, in comparison with formulations b, independent of the type of surfactant (Fig. 2). Based on the previous observations that Tween 80 and Cremophor EL did not compromise the aggregate formation in IgG solution under the experimental conditions, the higher content of polymers in formulations a was probably due to the high glycine concentration. Less polymer content in formulations b indicated that in the presence of surfactants, lower glycine concentration is prefered.

In the coarse of glycine effects determination on physical stability of IgG in solution dependening on the surfactant type, the comparison of the polymer content in IgG/Tween 80 mixture with polymer content in IgG/Cremophor EL mixture with different concentrations of glycine and sodium chloride was done and compared with the polymer content in IgG solution (Fig. 2). The results of comparison indicated that effects of glycine concentration on aggregate formation were more evident in the presence of Tween 80: polymer content in the presence of Tween 80 and higher glycine concentration (IgG/Tween 80 a formulation) significantly differed from polymer content in IgG solution and IgG/Tween 80 b formulation sample (which contained lower glycine concentration). The polymer content in both formulations with Cremophor EL (a and b) significantly differed (it was higher) from polymer content in IgG solution.

2.3. Effects of excipients on physical stability of IgG in solution during mechanical agitation

The determination of the types of aggregates in all IgG samples after mechanical agitation indicated on the presence of insoluble aggregates only in IgG surfactant mixtures: IgG/Tween 80 and IgG/Cremophor EL.

The comparison of polymer content in IgG solution and IgG/surfactant mixtures after constitution (before mechanical agitation) with the sums of the insoluble aggregates and polymers in the same samples after mechanical agitation indicated the increased polymer content in samples with Tween 80 and Cremophor EL after mechanical agitation (Fig. 3). The comparison of the polymer content in IgG solution and IgG/surfactant mixtures with different concentration of glycine and sodium chloride after constitution and after mechanical agitation indicated significantly increased polymer content only in IgG a formulation (Fig. 2). The stability of IgG b formulation during mechanical agitation, with less content of glycine and more polymer content, in comparison with IgG a formulation, was rather due to the higher polymer content than to the effect of excipients. Based on these results it was evident that selected excipients decreased the physical stability of IgG in solution: surfactants influenced insoluble and soluble aggregates formation during mechanical agitation, while glycine (in dependence of concentration) influenced soluble aggregate formation during sample constitution or mechanical agitation. It could be concluded that adding just a surfactant or just a glycine and sodum chloride could not stabilise IgG in solution during mechanical agitation. The combination of the surfactant, glycine and sodium chloride could increase physical stability of IgG in solution under the same experimental conditions.

2.4. Thermal denaturation

Within the experiments carried out using DSC, scans with well separated transitions on the temperature axis were not obtained as the thermodynamic parameters (integrated enthalpy of the unfolding endotherm – ΔH and melting temperature – Tm). Because of that, conclusions on the unfolding of IgG in the presence of excipients were based on the onset temperatures (the beginning of IgG unfolding) and the qualitative comparison of endothermic peaks. Similar attempts of interpreting DSC results have been reported in literature [1].

Data obtained by DSC analysis of experimental samples are presented in Table 2 and Table 3. The comparison of two DSC scans (shape, number of endothermic peaks, onset temperature) of the same sample with the differences in their polymer content before and after agitation, indicated that samples with different DSC scans showed the differences in polymer content during mechanical agitation too and, based on these observations, physical instability of IgG. These conclusions were made in cases of IgG/Tween 80 and IgG/Cremophor EL mixtures, as for IgG a and IgG b formulations. The comparison of their two scans, made within two hours, indicated conformational changes of IgG in solution, caused by the presence of excipients, and formation of more stable conformation of IgG from the first to the second scan. These observations were accomplished by the results of polymer content of the same samples assessed by HPLC. Because of that, DSC scans at the second scan are presented as they depict the more stable conformation of IgG.

Based on the structure, IgG is the multidomain protein. In DSC scan, IgG in solution exhibited a large transition peak in the temperature range of a few degrees of centrigrade (Fig. 4–6). This thermal behavior is characteristic for multidomain proteins. Domain interactions are always strong enough to force an immutable order to the sequence in which domains must unfold [26]. Large transition peaks in DSC scan indicate the presence of intermediates and/or native state aggregation, but conclusion could not be done because DSC data do not fit the models that include aggregation [27]. In this experimental work, DSC and HPLC analysis of samples were compared. Presence of polymers in IgG solution, determined by HPLC analysis, accomplished the DSC data indicating that the intermediates formation during denaturation of IgG in solution resulted in aggregate formation.

Table 2: Onset temperatures of IgG in solution and IgG/surfactant mixtures

Sample	Analysis	Peak number	Onset temperature (°C)
IgG solution	1	1	97.29
	2	1	103.73
IgG/Tween 80 mixture	1	1	100.77
		2	121.21
		3	123.75
	2	1	103.32
IgG/Cremophor EL mixture	1	1	96.53
		2	104.16
		3	119.10
	2	1	88.69
		2	101.92
		3	107.41

Table 3: Onset temperatures of IgG in solution and IgG/surfactant mixtures with different concentrations of glycine and sodium chloride

Sample	Analysis	Peak number	Onset temperature (°C)
IgG a	1	1	103.85
	2	1	81.35
IgG b	1	1	103.00
	2	1	105.30
IgG/Tween 80 a	1	1	106.41
	2	1	106.78
IgG/Tween 80 b	1	1	103.98
	2	1	103.38
IgG/Cremophor EL a	1	1	102.58
	2	1	101.28
IgG/Cremophor EL b	1	1	102.79
	2	1	102.21

Onset temperature of IgG in solution determined by this experimental work was high and was not in the correlation with published results [28]. The study of protein stability during thermal unfolding depending on their concentration, indicated the decreasing protein stability and increasing Tm temperature values along with the increase in protein concentration [29, 30]. As the protein content in IgG solution was high, it could be the explanation for higher onset temperature of IgG under the experimental conditions in comparison with the known values. Other possible cause could be the high polymer content in IgG solution determined by HPLC analysis.

DSC scans of IgG in solution in the presence of glycine and sodium chloride showed one asymmetric peak with a two-state transition (Fig. 4). The asymmetric peak in DSC scans usually indicates ligand binding [31]. Also, it is known that glycine tends to adsorb at hydrophobic surfaces of unfolded proteins [32]. Because of these facts, asymmetric peak in DSC scans of IgG solution in the presence of glycine and sodium chloride indicated glycine binding to the hydrophobic areas of IgG during unfolding. Based on the DSC envelope, glycine binding was more evident in formulation a, with higher glycine content. Moving onset temperature of IgG in formulation a (second scan) toward the lower value (Table 3), in comparison with onset temperature of IgG in solution (Table 2), indi-

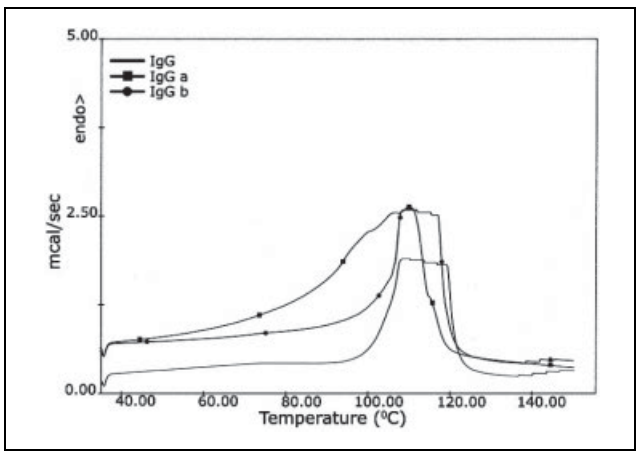


Fig. 4: DSC scans of IgG in solution with different concentrations of glycine and sodium chloride

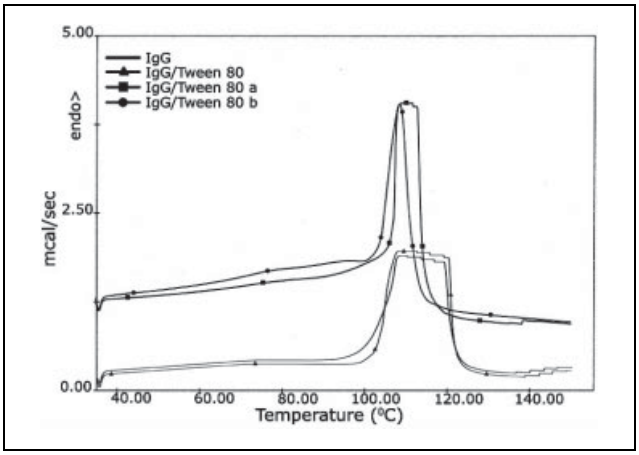


Fig. 5: DSC scans of IgG in solution and IgG/Tween 80 mixture with different concentrations of glycine and sodium chloride

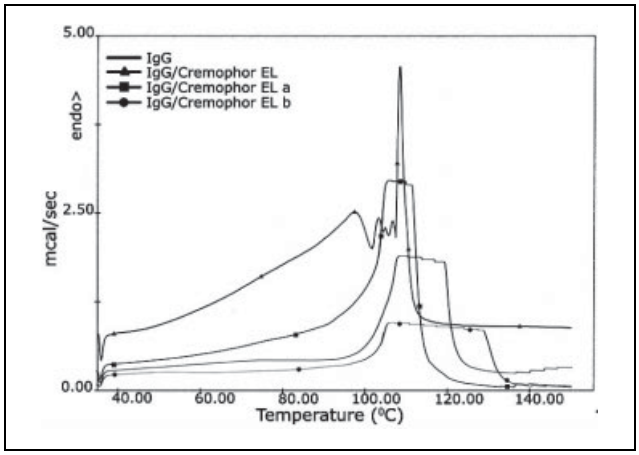


Fig. 6: DSC scans of IgG in solution and IgG/Cremophor EL mixture with different concentrations of glycine and sodium chloride

cated decreased stability of IgG a formulation, as was confirmed by the difference in polymer content before and after mechanical agitation. The relatively stable onset temperatures of two DSC scans of IgG b formulation indicated that this formulation was more stable than IgG a formulation.

More than one endothermic peak in DSC scans of IgG in the presence of Tween 80 and Cremophor EL (Table 2) indicated the effects of surfactants on IgG domains and, as a consequence, their independent unfolding. The similarity of the endothermic peaks of IgG in aqueous solution and Tween 80 solution (Fig. 5) at the second scan, indicated the similarity of their physical stability as were confirmed by the similarity in their polymer content (Fig. 3). Adding of glycine and sodium chloride to a IgG/Tween 80 mixture resulted in scans with one asymmetric endothermic peak in both formulations independent of glycine and sodium chloride concentrations. Larger transition in formulation a indicated the presence of intermediates and/or aggregates, and two-state transition in formulation b indicated the absence of intermediates. The comparison of these DSC results with polymer content in the same samples, indicated significantly higher polymer content in formulation a in comparison with polymer content in formulation b (Fig. 2). The correlation of the onset temperatures between two scans of IgG/Tween 80a and IgG/Tween 80b (Table 3), indicated the stability of IgG/Tween 80 mixtures by adding glycine and sodium chloride, that were confirmed by the investigations of the effects of mechanical agitation on their physical stability.

Unlike the effects of Tween 80 on physical stability of IgG in solution and DSC scans, numbers of peaks on DSC scan in the presence of Cremophor EL were increased in the second scan in comparison to the first scan. This could be explained by the presence of the hydrophobic substances in Cremophor EL that probably decreased solubility of IgG and adsorb at hydrophobic surface area of IgG molecule during unfolding allowing domains to unfold independently and preventing self association, that was confirmed by polymer content assessed by liquid chromatography (Fig. 3). By adding glycine and sodium chloride in IgG/Cremophor EL mixture, all DSC scans had one large transition peak (Fig. 6). Associations of these results and results of polymer content assessed by HPLC, which indicated high polymer content, it could be concluded that intermediates formation in IgG/Cremophor EL mixtures with glycine and sodium chloride led to the aggregation.

In conclusion, by dissolving lyophilized powder of IgG in water only soluble aggregates were formed. To stabilize IgG in solution during dissolving, glycine in a concentration of 2.25 g/l was needed, but this glycine concentration could not stabilize IgG during mechanical agitation. Glycine and sodium chloride had no influence on insoluble aggregates formation.

Tween 80 and Cremophor EL did not compromise the physical stability of IgG in solution during dissolving, but influenced insoluble and soluble aggregate formation during mechanical agitation. In the presence of surfactants (Tween 80 and Cremophor EL), glycine and sodium chloride together, the aggregates formation depended on glycine concentration and fewer aggregates were formed with glycine in a concentration of 1.5 g/l. The comparison of the effects of Tween 80 and Cremophor EL in the presence of glycine (1.5 g/l) and sodium chloride (9 g/l) on the physical stability of IgG in solution, showed that the influence of Tween 80 was preferable because of the fewer aggregates formation.

None of excipients did improve the stability of IgG in terms of decreasing polymer content beyond the polymer content in IgG solution. Based on this fact it could be concluded that the most important factor of the physical stability of IgG in solution is the quality of protein starting material with a monomer content as high as possible.

3. Experimental

3.1. Samples preparation

3.1.1. Materials

Lyophylized powder of IgG with no stabilisers (to avoid their effect on the result of the study) were produced in Plasma fractionation Center of the National Blood Transfusion Institute, Belgrade, Yugoslavia. Polysorbate 80 (Tween 80) were obtained from ICI Surfactants, UK and Polyoxyl 35 Castor Oil (Cremophor EL) from BASF Corporation, Germany. Glycine and sodium chloride were purchased from Merck, Germany.

3.1.2. Preparation of surfactant solutions

The surfactant solutions were prepared by dissolving surfactants in sterile water for injection in concentrations that corresponded to their critical micelle concetration (cmc) at 20 °C, based on literature [12], ten times greater than cmc (10 cmc) and one hundred times greater than cmc (100 cmc).

3.1.3. Preparation of IgG solutions

The samples were prepared with IgG liophylized powder in concentrations of about 15% w/v. In the first phase of experimental work, IgG solution and IgG/surfactant mixtures were made by dissolving lyophilized powder of IgG in sterile water for injection with and without surfactant (IgG and IgG/surfactant mixtures), in experimentally determined cmc at 6 ± 2 °C. In

the second phase, glycine and sodium chloride, as stabilisers, were added to IgG solution in water and IgG/surfactant mixture in different concentrations (formulation a: glycine 2.25 g/l; NaCl 4.5 g/l and formulation b: glycine 1.5 g/l; NaCl 9 g/l). The dissolving were made by shaking (Heidolph, Unimax 100, GmbH) at 500 r/min at 6 ± 2 °C. pH were adjusted to 7.1 (HCl 0.1 M).

3. 2. Methods of characterisation

3.2.1. Surface tension measurements

The surface tension measurements of Tween 80 and Cremophor EL solutions in different concentrations, IgG solution and IgG/surfactant mixtures were determined by the method of drawing up liquid films (ring method) using a Digital tensiometer, K 10, A. Kruss, Germany. The sample tested was placed in a 100 ml beaker and the Platinum-Iridium ring was positioned on the air/water interface. The surface tension values were recorded after 30 min of equilibration at 6 ± 2 °C. Measurements were made in triplicate. Means (M) $\pm$ standard deviations (S.D.) are given. Based on the results, the plot of the surface tension dependence on the surfactant concentrations were constructed in coarse of determining cmc under experimental conditions.

3.2.2. Investigation of aggregate formation

The aggregate formation was investigated at the air/water interface during mechanical agitation that allowed a continous creation of new interfaces. Aliquots (2 ml) of each sample of IgG solution were placed in 10 ml wide glass bottle to maximize the air/water interface. All samples were shaken for 96 h (Heidolph, Unimax 100, GmbH) at 500 r/min at 6 ± 2 °C. After shaking, samples were transferred to tubes and centrifuged (Cryofuga, Haeraus, GmbH, Hanau) at 12000 g for 30 min at 4 °C. Then, the supernatants were separated from sediment to analyse.

The formation of insoluble and soluble aggregates were assayed by protein content assay (Kjeldahl) in all IgG samples after constitution (mass of native proteins) and in supernants after agitation (mass of soluble aggregates). Determinations were made in triplicate. The amount of insoluble aggregates was calculated as the difference between the means of the mass of protein in samples after constitution and the means of the mass of protein in supernants after agitation (soluble aggregates).

The molecular size distribution of IgG was assayed on samples before and after agitation by HPLC (LC systems, Hewlett Packard). The results were expressed as percentage of the original protein mass after constitution. Determinations were made in duplicate (the means are given).

The effects of excipients on molecular size distribution of IgG in solution were assessed by the comparison of polymer content in samples after constitution. The analysis of variance was used for statistical evaluation with $p = 0.05$ as level of significance.

The effects of excipients on aggregate formation during mechanical agitation were assessed by the comparison of polymer contents in samples after constitution and after mechanical agitation and centrifugation. The Student T-test was used for statistical evaluation with $p = 0.05$ as level of significance.

3.2.3. Thermal denaturation

The conformational changes of IgG during heating were assessed on IgG solution with and without excipients byDSC on a Differential Scanning Calorimeter DSC-4 (Perkin Elmer, Norwolk, CT, USA). Samples were pipetted into an aluminium sample pan, not hermetically sealed, and heated from 35 °C to 150 °C at a scan rate of 10 °C/min. Denaturation was assessed in duplicate within 2 h. DSC analysis were performed simultaneously to the HPLC analysis. The thermodynamic parameters were evaluated from thermograms by means of a Perkin Elmer model 3700 Thermal Analysis Data Station. The mass of proteins in samples were normalized in coarse of the comparison.

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