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Short communication

Studying the formation of aggregates in recombinant human granulocyte-colony stimulating factor (rHuG-CSF), lenograstim, using size-exclusion chromatography and SDS-PAGE

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The stability of proteins is a subject of intense current interest. Aggregation, as a dominant degradation pathway for therapeutic proteins, may cause multiple adverse effects, including loss of efficacy and immunogenicity. In the present study, the formation of aggregates in lenograstim under physiological conditions was monitored. For this purpose, a simple and selective size-exclusion high-performance liquid chromatography method for detection and separation of aggregates from intact protein was developed. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis was performed under reducing and non-reducing conditions to determine the nature of aggregate bond formation. Using both techniques, the presence of a low aggregate content attached *via* disulfide bonds was detected.

Keywords: lenograstim, aggregate formation, size-exclusion high-performance liquid chromatography, sodium dodecyl sulphate-polyacrylamide gel electrophoresis

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With the development of recombinant DNA-technology, the DNA-derived drugs represent a new therapeutic concept for human diseases (1). Two forms of recombinant human granulocyte colony-stimulating factor (rHuG-CSF) are currently commercially available for medical treatment, non-glycosylated protein (filgrastim) and glycosylated protein lenograstim, with molecular mass of 19.6 kDa (2). Glycosylation has been reported to confer many advantages over non-glycosylation, including improvement of physical stability (3–6) and higher biological and pharmacological potency (5, 7, 8).

A common phenomenon of protein instability and a dominant degradation pathway for therapeutic proteins is formation of aggregates (9). It is encountered routinely during refolding, purification, sterilization, shipping and storage processes (10, 11). Ag-

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gregation is rapidly emerging as a key issue underlying multiple deleterious effects for protein-based therapeutics, including loss of efficacy and immunogenicity (10).

Size-exclusion high-performance liquid chromatography (SEC-HPLC) has been a workhorse for detecting and quantifying protein aggregation (11). In conjunction with other techniques, *i.e.*, matrix assisted laser desorption/ionization-time of flight (MALDI--TOF), capillary isoelectric focusing (CIEF), sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the obtained results can be verified.

Many published studies for monitoring protein aggregation are available (12–15) and some of them referred to determination of aggregates formation in filgrastim (10, 11, 16, 17). However, to our knowledge, the SEC-HPLC method for determination of intact protein and detection of aggregates of lenograstim has not been reported in the literature.

The aim of our study was to assess the formation of aggregates in lenograstim under physiological conditions and to develop a simple and selective SEC-HPLC method for detection and separation of aggregates from intact protein, lenograstim. Also, we performed SDS-PAGE under reducing and non-reducing conditions in order to get a deeper insight into the nature of the aggregation mechanism.

EXPERIMENTAL

Chemicals and reagents

The lenograstim [recombinant human granulocyte-colony stimulating factor derived from Chinese hamster ovary cells (rHuG-CSF)] reference material was supplied by Ray Biotech (USA) as sterile lyophilized powder.

All chemicals were of HPLC grade or analytical grade. Orthophosphoric acid and sodium chloride were purchased from Alkaloid (Macedonia). All reagents for SDS--PAGE were obtained from Bio-Rad (USA). HPLC-grade water was used for all analyses.

SEC-HPLC

A Waters HPLC system (USA) was used, equipped with a Waters 600 pump, Waters 996 photodiode array detector and Millennium 32® software for data handling.

The experiments were carried out on size exclusion Fractogel® EMD BioSEC column, superformance 600–16 mm (Merck, Germany). The HPLC system was operated isocratically at ambient temperature using a mobile phase, which was composed of phosphoric acid (pH 2.5; 0.1 mol L^{-1}) containing 150 mmol L^{-1} NaCl, run at a flow rate of 2 L min^{-1}, and with UV detection at 215 nm. NaCl was added to the mobile phase to avoid non-specific interactions between the proteins and the matrix. The mobile phase was filtered using a 0.45-μm filter and was degassed before use. Samples were injected through a Rheodyne injector valve with 200-μL sample loop. All determinations were carried out in triplicate.

SDS-PAGE

SDS-PAGE was carried out on a Bio-Rad Mini Protean Cell electrophoresis system (USA). SDS-PAGE was performed under reducing and non-reducing conditions on 12% separating gel and 4% stacking gel by the standard Laemmli procedure (18) in order to separate both covalent and non-covalent aggregates from intact monomer. Under reducing conditions, a protein sample was prepared with Laemmli sample buffer and was heated to near boiling for 10 minutes in the presence of reducing agent 2-mercaptoethanol. Preparation of the protein sample under non-reducing conditions was done in the same way except that no reducing agent was added. Following electrophoresis, due to higher sensitivity, gels were stained with Silver stain Plus Kit (Bio-Rad, USA) according to the vendor's protocol.

Preparation of standard solution of lenograstim and sample solution with induced aggregates

The standard solution of lenograstim (50 μg mL^{-1}) was prepared by dissolving it in water.

Aggregation of lenograstim occurred under conditions that do not differ greatly from physiological, in the absence of chemical denaturants. The sample solution was prepared by dissolving lenograstim in phosphate buffered saline (pH 6.9) at the same concentration as the standard solution (50 μg mL^{-1}) and was incubated 5 days at 37 °C on a heating block.

RESULTS AND DISCUSION

Aggregation may compromise the stability as well as the biological activity of protein drugs. For protein drugs, the presence of aggregates of any type (soluble/insoluble, covalent/noncovalent or reversible/irreversible) is typically considered to be undesirable because aggregates may lead to loss of efficacy and may cause adverse effects in patients. However, the levels of soluble aggregates such as dimers and trimers, which are acceptable, are not well defined (19).

From the analytical point of view, SEC-HPLC is one of the most sensitive and selective techniques that can separate and analyze aggregates.

In this study, the formation of aggregates in lenograstim under physiological conditions (phosphate buffered saline pH 6.9 at 37 °C for 5 days) was monitored. For this purpose, a SEC-HPLC method for detection and separation of aggregates from intact protein, lenograstim, was developed. Eluent of different pH values (2.5 and 7.0) was tested. Satisfactory results were obtained under chromatographic conditions given in Experimental. It was found that the use of SEC with low pH eluent provides a rapid means for determining the existence and formation of aggregated species.

For the determination of aggregates, any peak from the chromatographic run that was eluted before intact monomer was defined as aggregates.

The chromatograms of standard solution (a) and sample solution (b) presented in Fig. 1 have shown that under the proposed chromatographic conditions monomeric protein and aggregate are completely separated from each other in accordance with their molecular masses. The presence of aggregate was indicated by the appearance of a distinct peak in the chromatogram at retention time shorter than the retention time of intact monomer – lenograstim (Fig. 1b). The obtained retention times were approximately 58.24 min for intact protein and 17.82 min for aggregate.

Peak areas in the chromatograms (Fig. 1) were used to quantify the amounts of monomer and aggregate in incubated sample. The percent of monomeric protein present in incubated sample was calculated by dividing the measured peak area by the peak area for an unincubated sample of lenograstim (standard solution) and multiplying the result

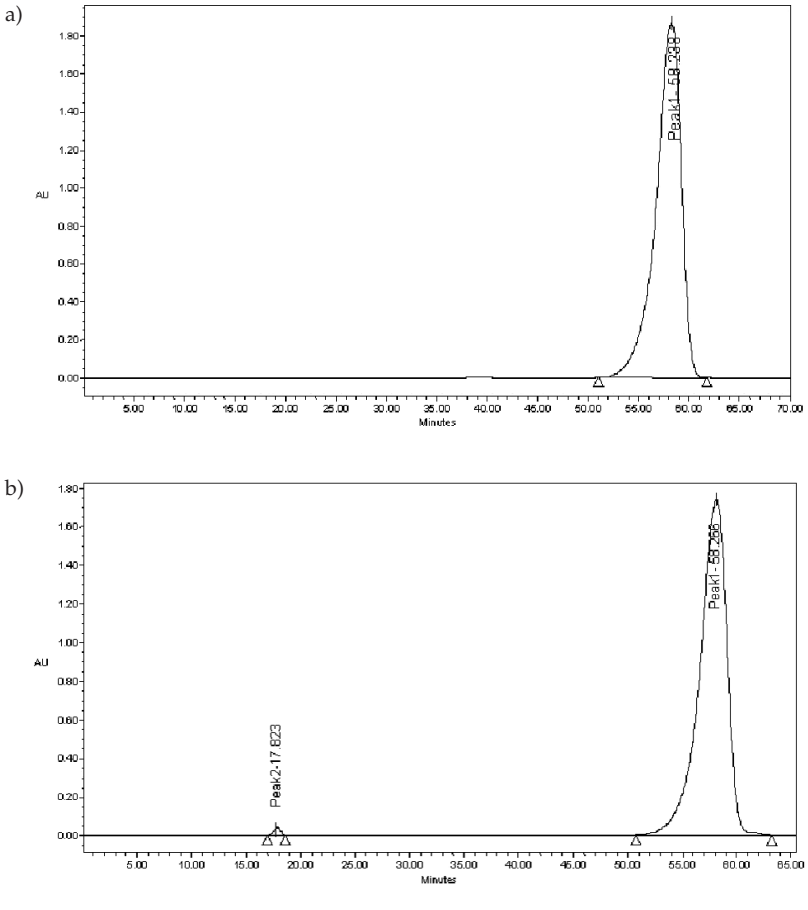

Fig. 1. SEC-HPLC chromatograms of lenograstim: a) standard solution of lenograstim (50 μg mL^{-1}), unincubated (peak 1 – intact protein, monomer), b) sample solution of lenograstim (50 μg mL^{-1}), incubated (peak 1 – intact protein, monomer; peak 2 – aggregate).

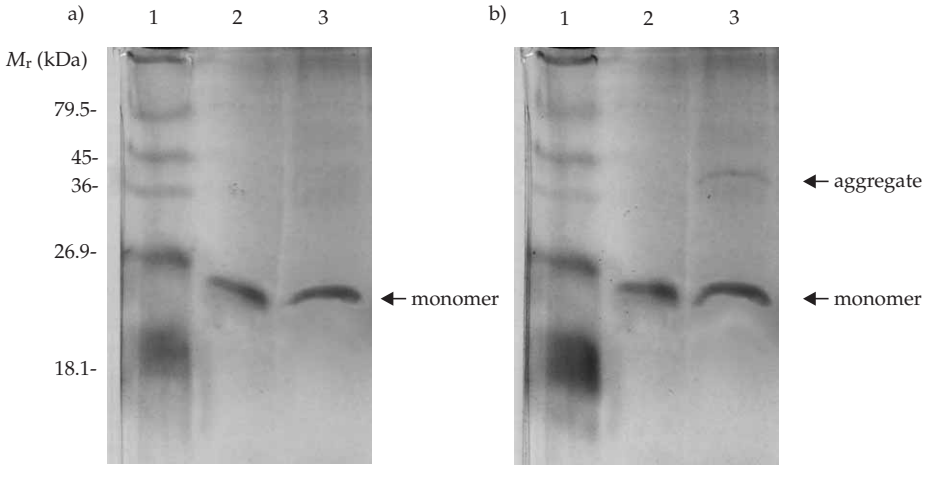

Fig. 2. Silver stained SDS-PAGE gels of lenograstim: a) reduced and b) non-reduced. Lane 1 – molecular mass protein standard; lane 2 – standard solution of lenograstim, unincubated; lane 3 – sample solution of lenograstim, incubated.

by 100. The percent of aggregate was calculated as the difference between the total protein present before incubation (standard solution) and the monomeric protein remaining after incubation (sample solution), based on peak areas in the respective chromatograms. The obtained data shows that during incubation at 37 °C in phosphate buffered saline pH 6.9, 93% of monomeric lenograstim remained after 5 days. The percent of aggregate was only 7%.

Previous studies (10, 11) relating to the aggregation of filgrastim reported that under the same aggregation conditions filgrastim was aggregated readily with 96% loss of monomer.

Hence, the obtained results allow us to assume that lenograstim is more stable regarding aggregate formation than filgrastim. This data is in compliance with the previously reported statements of greater stability of glycosylated rHuG-CSF compared to non-glycosylated rHuG-CSF (3–6).

Further work is being done in order to verify these results and to assess the stability behaviour of lenograstim molecule.

SEC-HPLC proved to be useful and capable of monitoring high molecular mass aggregates and monomeric proteins. But, this method provides information about the total content of soluble aggregates. To get a deeper insight into the nature of the aggregation mechanism of lenograstim, SDS-PAGE was performed, allowing differentiation between covalently and non-covalently linked aggregates (under reducing and non-reducing conditions). Non-covalent aggregation is caused by interactions between the exposed hydrophobic residues of denatured protein molecules. Covalently linked protein aggregates are due to chemical reactions, *e.g.*, β-elimination, disulfide exchange or transamidation (10).

When the standard solution of lenograstim (unincubated sample) was analyzed by SDS-PAGE under both reducing and non-reducing conditions, a heavy and distinct single band with an approximate molecular mass of 20 kDa was observed (Fig. 2). Under reducing conditions, the incubated sample solution of lenograstim showed only a single distinct band corresponding to monomer (Fig. 2a). However, under non-reducing conditions, two bands were observed: one distinct band assignable to monomer and another faint band assignable to higher molecular mass aggregates (Fig. 2b). The lack of higher molecular mass bands for the incubated sample solution of lenograstim under reducing conditions demonstrates that if any soluble aggregates are attached covalently, the bonds must be of disulfide type (15, 19).

A very weak high molecular mass band indicates the presence of trace amounts of aggregate. The semi-quantitative analysis of band intensities by TotalLab software delivered results that are in good agreement with SEC data. Both methods demonstrate a low aggregate content after incubation at 37 °C for 5 days.

CONCLUSIONS

SEC-HPLC was used for monitoring aggregate formation in lenograstim and SDS-PAGE confirmed the nature of aggregate bond formation. The presence of a low aggregate content attached *via* disulfide bonds was detected. Further investigations are being performed to assess the stability behaviour of lenograstim molecule.

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S A Ž E T A K

Proučavanje stvaranja agregata rekombinantnog humanog granulocitnog faktora stimulacije kolonija (rHuG-CSF), lenograstima, pomoću kromatografije isključenjem prema veličini i SDS-PAGE

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Stabilnost proteina vrlo je aktualna problematika. Agregacija, kao dominantni put razgradnje terapijskih proteina, može uzrokovati različite nuspojave, koje uključuju i gubitak učinkovitosti imunološkog sustava. U ovom radu praćeno je stvaranje agregata

u lenograstimu pod fiziološkim uvjetima. Razvijena je jednostavna i selektivna tekućinska kromatografija visoke djelotvornost s isključenjem prema veličini za detekciju i razdvajanje agregata od intaktnog proteina. Pomoću natrij-dodecil sulfat poliakrilamidne gel elektroforeze u reducirajućim i nereducirajućim uvjetima utvrđen je način stvaranja agregata. Pomoću obje tehnike moguće je detektirati prisutnost malih količina agregata koji su spojeni disulfidnim vezama.

Ključne riječi: lenograstim, stvaranje agregata, tekućinska kromatografija visoke djelotvornosti isključenjem prema veličini, natrij-dodecil sulfat poliakrilamidna gel elektroforeza

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