Technical note

Glycation of polyclonal IgGs: Effect of sugar excipients during stability studies

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A B S T R A C T

A number of intravenous immunoglobulin preparations are stabilized with sugar additives that may lead over time to undesirable glycation reactions especially in liquid formulation. This study aimed to evaluate the reactivity of sugar excipients on such preparations in condition of temperature, formulation and concentration commonly used for pharmaceutical products. Through an innovative LC-MS method reported to characterize post-translational modifications of IgGs Fc/2 fragments, a stability study of IVIg formulated with reducing and non-reducing sugars has been undertaken. The rate of polyclonal IgGs glycation was investigated during 6 months at 5, 25, 30 and 40 °C. High levels of glycation were observed with reducing sugars such as glucose and maltose in the first months of the stability study from 25 °C. Non-reducing sugars presented a low reactivity even at the highest tested temperature (40 °C). Furthermore, a site by site analysis was performed by MS/MS to determine the glycation sites which were mainly identified at Lys246, Lys248 and Lys324. This work points out the high probability of glycation reactions in some commercialized products and describes a useful method to characterize IVIg glycated products issued from reducing sugar excipients.

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1. Introduction

Intravenous immunoglobulin (IVIg) preparations are mainly composed of polyclonal immunoglobulin G (IgGs); these products are purified from plasma and used to treat a number of humoral immune deficiencies associated with recurrent infections and an increasing number of autoimmune disorders. New generation of these therapeutics is presented in a liquid formulation that is more easy-to-use than freeze-dried products. Worldwide marketed IVIg preparations are largely stabilized by sugars excipients [1] (see Supplementary Table 1), which can lead over time to undesirable glycation reactions. Glycation is a non-enzymatic process in which reducing sugars are covalently attached to proteins by a condensation reaction between the aldehyde groups of reducing sugars and primary or secondary amines of amino acids. The reaction results in the formation of an unstable, reversible Schiff base intermediate (aldimine), which may undergo an Amadori rearrangement to form a stable ketaamine (the Amadori product). This reaction of glycation may affect protein structure and function, and may lead to the formation of advanced glycation end products (AGEs) [2].

Glycation of monoclonal IgGs has been investigated in several studies. Glycation reactions were shown to occur during the upstream production process due to the presence of feeding sugars and reactivity was shown to be antibody dependent [3]. Stability studies of monoclonal IgGs long-term stored at 2–8 °C in the presence of sucrose revealed non-significant glycation while this modification was observed at room temperature with dextrose [4]. Glycation of polyclonal IgGs is far less documented, perhaps because of the analytical complexity inherent to these heterogeneous proteins, limiting the use of mass spectrometry. Vrdoljak et al. reported the use of radioactive D-[U-14C]-glucose to monitor in vitro glycation of polyclonal IgGs under pseudo-physiological/diabetic conditions [5]. As expected, they found more glycation of IgGs under pseudo-diabetic conditions than under pseudo-physiological ones. They also found that glycation can occur under long-term storage at 4–8 °C, in the presence of glucose. In 2014, a LC-MS method has been reported to investigate post-translational modifications on the constant fragment (Fc/2) of polyclonal IgGs, generated after IdeS enzymatic proteolysis [6]. Considering the non-enzymatic nature of glycation reactions, glycation sites could be distributed all over the protein sequence. Therefore, the...
constant Fc/2 fragment, which is the only analyzable part of polyclonal IgGs by LCMS, was considered as a reliable marker of the overall IVlg glycation state.

In this study, the formulation of a 5% IVlg product was modified in order to generate different formulations in which the stabilizing sugar was the only variable excipient. Two reducing sugars (maltose and glucose) and two non-reducing sugars (sucrose and trehalose) were used to generate 4 formulations and the glycation level of such formulated IgGs was monitored by LC-MS for 6 months at different temperatures commonly used for stability studies (5, 25, 30 and 40 °C according to ICHQ1A guideline). For the first time, the differential reactivity of sugars on polyclonal IgGs, i.e. the glycation rate, was studied using storage temperatures, formulation and concentration test parameters, chosen to be representative of real life conditions and usages of IVlg.

2. Material and methods

2.1. Material

MS grade bovine trypsin was obtained from Roche (Mannheim, Germany). The IdeS endoprotease FabRICATOR and the IgGZERO glycosidase were obtained from Genovis (Lund, Sweden). Dithiothreitol (DTT), Iodoacetamide (IAA), Glucose, Trehalose, Maltose and Sucrose were obtained from Sigma–Aldrich (Saint Quentin Fallavier, France). Acetonitrile (MeCN) was HPLC reagent grade and purchased from JT Baker (Philipsburg, NJ, USA). Trifluoroacetic acid (TFA) was obtained from Merck Biosciences (Darmstadt, Germany). All the aqueous solutions were prepared using ultra-pure water (18.2 MΩ cm resistivity at 25 °C, total organic carbon (TOC) < 5 ppb).

2.2. IgGs formulation and storage

Polyclonal IgGs derived from human plasma (purification based on ethanolic and caprylic acid precipitations) were used to generate 5% liquid IVlg preparations, buffered at pH 6 with sodium hydroxide, in the presence of either 10% w/v maltose, sucrose, trehalose and 5.3% w/v glucose corresponding to an equivalent molar concentration of 292 mM for all sugars. All samples were sterile filtered in laminar flow hood, aliquoted for each time point in 1.5 ml glass vials (1 ml/vial) and stored at 5, 25, 30 and 40 °C for a 6 months stability study. The climatic chambers used during the stability were qualified and temperature controlled.

2.3. IgG enzymatic digestion and reduction

5% liquid IVlg preparations were diluted with ultra-pure water to an IgGs final concentration of 10 g/l. Enzymatic proteolysis and deglycosylation of IgGs were carried out by simultaneously incubating 10 μl of samples during 1 h at 37 °C with 100 UI of IdeS enzyme to generate Fc/2 fragments and 100 UI of IgGZero enzyme to leave only a fucosylated GlcNAc bound to the polypeptide chain. Reduction of digested IgG was performed by adding 35 μl of dithiothreitol (8 M Guanidine- HCI, Tris–HCl 0.1 M, pH 7.5) and 5 μl of a 200 mM DTT solution. The preparation was incubated at 50 °C for 30 min and subsequently analyzed by LC-MS.

2.4. LC-MS analysis

Separation of the Fc/2 fragments was carried out on an Acquity system (Waters, Milford, MA, USA) coupled to a UV detector and an electrospray mass spectrometer (Synapt G2S, Waters, Milford, MA, USA). 20 μg of sample was injected on a Pursuit Diphenyl 150 mm × 2.0 mm column (Agilent, Santa Clara, CA, USA) equilibrated at 70 °C and operated at a flow rate of 200 μl/min. An elution gradient was applied using solvent A (0.1% TFA in water) and solvent B (90% MeCN, 10% water and 0.1% TFA), and after an isocratic elution at 10% B for 5 min, B was raised to 38% in 10 min and to 44% in an additional 48 min. The column was then washed for 4 min at 80% of B and further equilibrated during 9 min at 10% B. The mass spectrometer was operated in the positive resolution mode and data were recorded from m/z 500 to 3000. Protein mass spectra were deconvoluted using the MassLynx software. The capability of the method was addressed by a triple analysis of seven samples presenting glycation rates distributed over the whole range of the analytical procedure. Coefficients of variation lower than 10% were obtained for each point (see Supplementary Table 2).

2.5. Peptide mapping

200 μg of sample was vacuum-dried and dissolved in 25 μl of 8 M urea, and 0.2 M ammonium acetate solution pH 7.2. Reduction was accomplished by adding 10 μl of a 200 mM DTT solution in water and incubating the resulting mixture for 15 min at 55 °C. After cooling at room temperature, 10 μl of a 400 mM IAA solution in water was added and the solution was incubated for 15 min in the dark. After a fourfold dilution with water, digestion with trypsin (1:10, w/w) was performed overnight at 37 °C. The obtained peptides were injected on a Waters CSH C18 column (2.1 × 150 mm, 3 μm) using an Acquity system coupled to an electrospray mass spectrometer Synapt G2-S (Waters, Milford, MA, USA) operating in the positive ion mode. The spray voltage and the source temperature were set at 3 kV and 80 °C, respectively. MS/MS data were acquired with the instrument operating in the data-dependent mode.

3. Results and discussion

3.1. Characterization of glycated Fc/2

Glycation of polyclonal IgGs was monitored by LC-MS for each temperature on a monthly period. Mass spectrometry provides enough resolution to observe the covalent addition of one or several sugars, under their aldime or ketoamine forms, to the Fc/2 protein backbone. Reversible aldime content can be influenced by sample preparation but in the time window of the present study (over 6 months), stable ketoamines contribute mostly to the total glycation content [7]. This MS based method is thus well designed to follow the total glycation state of IVlg during monthly stabilities. Fig. 1 shows examples of deconvoluted mass spectra obtained for the IgG1 subclass from two distinct formulations, respectively with maltose (A) and glucose (B) and stored during 3 months at 25 °C. Masses of 24,140 Da and 24,108 Da correspond to the Fc/2 fragments of allotypes G1m3 and G1m1, 17 respectively. The mass spectrum of Fig. 1A shows uniform mass additions of +324 Da corresponding to the covalent attachment of the disaccharide and the mass spectrum of Fig. 1B shows uniform mass additions of +162 Da in keeping with the covalent attachment of the reducing monosaccharide. The method is thus fully suitable for the monitoring of glycation due to reducing sugars directly available in the formulation or indirectly through hydrolysis of non-reducing sugars (sucrose or trehalose) into their reducing components.

3.2. Identification of glycated sites

Peptide mapping was used to identify the glycated sites detected after 1 month stability at 40 °C in the presence of glucose or maltose. Three major sites were detected on the Fc/2 of
polyclonal IgGs at Lys246, Lys248 and Lys324. Fig. 2 displays MS/MS spectra of the two identified peptides carrying the abovementioned glycation sites. Fig. 2A shows the MS/MS mass spectrum of [Gly237–Arg255] peptide. The sequence of the peptide is widely covered by y ions and, interestingly, y9 fragments are observed as a mixture of naked, pyrylium and furylium ions, the latter being characteristics of the dissociation pathway of aldohexoses-derived Amadori compounds [8]. After y10 all the fragments were identified as pyrylium and furylium ions exclusively which indicates that the selected precursor ion corresponds to a mixture of glycated species at both Lys246 and Lys248 positions. It can be noticed that this precursor ion gave a single chromatographic peak in spite of its structural heterogeneity. Fig. 2B shows the MS/MS mass spectrum of [Cys319–Lys332] peptide. Fragments b7 to b10 and y11 to y13 were identified as pyrylium and furylium ions, indicating a glycated site at Lys324 position. Finally, these glycation sites were found to be consistent with those already described in the literature for monoclonal antibodies [7]. Other minor glycation sites (in terms of peptide intensity) were also detected on the CH1 domain of IgGs, on Lys143 residue of the heavy chain and on Lys126 and Lys149 residues of the light chain (data not shown).

3.3. Kinetics of IVIg glycation

To monitor glycation, an LC-MS analysis was performed for each temperature, formulation and time point. The peak intensities of the deconvoluted mass spectra were used to determine a percentage of glycated versus non-glycated IgGs. Assuming a similar MS response factor for all the detected species, glycated IgGs were defined as the sum of forms detected with at least one glycation adduct over the total signal. Fig. 3 reviews data collected at 25 °C for the 4 formulations. Surprisingly, formulations using reducing sugars show a high degree of glycation over time at a widely used storage temperature (see Fig. 3A and B) with up to 3 binding sugars detected per Fc/2. On the contrary, glycation is not observed even after 6 months at 25 °C with formulations based on sucrose and trehalose excipients (see Fig. 3C and D). Fig. 4 provides a summary of all the data collected during the study. Analysis of samples at T0 revealed a residual glycation rate of approximately 2.7%, reflecting probably an in vivo baseline glycation level. After 1 month at 5 °C, 25 °C, 30 °C and 40 °C, respectively 3.9%, 22.3%, 37.1% and 85.9% of IgGs Fc/2 were glycated with the glucose excipient, and these values increased to 8.3%, 76.8%, 91.8% and 100% after 6 months. The rate of glycation increased with temperature and followed perfectly the Arrhenius law with a straight line observed by plotting the logarithm of the glycation percentage against inverse temperature (see kinetic rates of glycation in Supplementary Table 3). Furthermore, for each temperature, the time dependence of glycation fitted well a first order kinetic reaction, as expected for the Maillard reaction. At all points, the reactivity of glucose was slightly greater than the one of maltose. This difference of reactivity between glucose and maltose is consistent with the overall knowledge of the Maillard reaction. Indeed, the reactivity of the reducing sugars depends on their size, pentoses being more reactive than hexoses and hexoses like glucose being more reactive than disaccharides like maltose. It can be noticed that only highly glycated
samples obtained after 3 months at 40 °C with glucose displayed a higher content of aggregates (see Supplementary Table 4). Interestingly, even at the temperature of 5 °C, a slight increase of glycation is observed over time with glucose and maltose suggesting that even this low storage temperature is not a guarantee of preventing glycation when reducing sugars are used as excipients. This result is in keeping with Vrdoljak et al. [5], reporting IgG glycation after long term storage at 4–8 °C in the presence of glucose. Instead, formulations based on sucrose and trehalose excipients were relatively stable. No glycation reactions were observed with trehalose even at the highest temperature and the last time point of the stability study. However, with the sucrose excipient, a detectable increase of glycation was observed after 4 months at 40 °C (9.6%) and confirmed with latter time points (18.3% after 6 months storage). The glycation observed with sucrose (adding of a single monosaccharide of +162 Da) is probably the result of an initial hydrolysis of the non-reducing sugar that releases reducing glucose in the media. This result confirms data from the food industry indicating that trehalose is more stable than sucrose.

Glycation is an undesirable reaction that binds covalently sugar molecules on primary/secondary amine functions distributed all over the protein sequence. This kind of reaction modifies permanently the physicochemical properties of proteins and may induce changes of conformation, stability and activity [9]. Uncontrolled high blood sugar concentration leads to glycation of blood proteins and to various diabetes complications. Vrdoljak et al. [5] estimated

Fig. 2. MS/MS spectra of peptide [Gly237–Arg255] glycated on Lys246 or Lys248 (panel A) and peptide [Cys319–Lys332] glycated on Lys324 (panel B) from IgGs formulated with maltose and stored 1 month at 40 °C.
in vitro, by the use of radioactive D-[U-14C]-glucose, the number of glucose molecules bound per IgG to an average of 3.1 after incubation with glucose in the concentration characteristic for diabetics. In another study, Lapolla et al. [10] ranged from 7 to 28 the number of glucose units condensed on IgGs from badly controlled diabetic patients, using MALDI-MS. The present LC-MS method has the advantage to visually observe the addition of single glucose units to the Fc/2 of IgGs and our data indicate that IVIg products stored with reducing sugars at 25 and 30 °C can reach the same range of glucose addition as diabetics. Kennedy et al. have shown that glycation could restrain the binding of IgGs to their antigens and increase their clearance rate in mice [11]. An increase in permeability of glycated proteins across the blood-nerve barrier has been observed from patients with diabetic polyneuropathy [12] and Brownlee et al. [13] demonstrated a clear increase of trapped IgM and IgG in peripheral nerve myelin from diabetics which has been suggested to have detrimental effects on nerve functions. These reports raise the hypothesis of a possible relationship between the glycation status of pharmaceutical products and their tolerability.

4. Conclusion

Sugars are widely used by pharmaceutical companies for the stabilization of highly concentrated proteins, but to our knowledge, the glycation state of IVIg in pharmaceutical conditions of stability has never been documented so far. This work investigates sugar excipients reactivity on IVIg and describes a MS-based method that is appropriate to monitor routinely the glycation status of IVIg as it is required for monoclonal antibodies (EMEA/CHMP/BWP/157653/2007). Reducing sugar excipients currently used for commercialized products generate significant glycation reactions from 25 °C in a time frame as short as 6 months. The reaction follows the Arrhenius law; as a consequence the glycation effect is minimized at 5 °C but not abolished. The use of non-reducing sugars such as trehalose and sucrose was more successful to avoid glycation. Considering that some commercialized IVIg preparations are formulated with reducing sugars in liquid state [1], our results support strong evidence that glycation reactions are highly probable for these products and that the present LC-MS method is adequate to assess this criteria for the different claimed storage temperatures. As mentioned above, glycation of proteins may alter their physicochemical and biological properties. This study raises the question of possible tolerability issues in relation with the use of reducing sugars especially in the case of chronic impregnation with IVIg.

Conflict of interest

All authors are employees of LFB BIOTECHNOLOGIES or LFB BIO-MEDICAMENTS, subsidiaries of LFB SA group, and commercializing IVIg preparations.
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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejpb.2016.03.016.

References


Fig. 4. Time course of Fc/2 glycation of IgGs stored at 5 °C, 25 °C, 30 °C and 40 °C and formulated with glucose (■), maltose (●), sucrose (▲) and trehalose (●).