Review

Glycated proteins: Clinical utility and analytical approaches

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The binding of glucose to serum proteins in a non-enzymatic reaction occurs proportionately to the prevailing serum glucose concentration during the lifespan of each protein. The non-enzymatic reaction of proteins with reducing sugars results in the formation of glycated proteins that are indicative of the relative amount of reducing sugars present. Hemoglobin, albumin, lipoproteins and other tissue proteins can be non-enzymatically glycated. Hence, estimation of these glycated proteins can be used for the estimation of average glycemic status. Spectrophotometric, chromatographic and immunoassays techniques are used for the detection and quantitation of these glycated proteins.

Key words: Glycated protein, reducing sugars, detection, quantitation.

INTRODUCTION

Protein glycation is a non-enzymatic process initially discovered in 1990s by Louis Camille Maillard who observed chemical modifications to amino acids when heated in the presence of reducing sugars, a process often referred to as the Maillard reaction (Maillard, 1912). This non-enzymatic glycation is a posttranslational modification in which a reducing sugar condenses with protein amino groups at the N-terminus or on lysyl side chains. The reaction is initiated with the attachment of the aldehyde or ketone carbonyl group of the acyclic saccharide to a free amino group in the protein, forming a Schiff base. Such an aldimine intermediate undergoes an amadori rearrangement to form 1-amino-1-deoxyfructose derivative forming a stable ketoamine linkage, which in turn can be cyclized to a ring structure (Lacinová et al., 2010). The cyclic amadori product then undergoes multiple dehydration and rearrangements, leading to the formation of advanced glycation end products (AGEs) (Nakayama et al., 1999). The formation of AGE further can lead to metabolic syndrome, clustering of several metabolic abnormalities (Miller and Adeli, 2008). This metabolic complication has been indicated to arise due to different factors including the use of antiretroviral treatment in HIV patients (Tesfaye et al., 2014).

Non-enzymatic glycation follows mass action kinetics, where by the amount of glycated product is proportional to the concentration of reactants, of which the ambient glucose concentration is a major determinant of the

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forward reaction rate. The second main determinant is the duration of a protein’s exposure to an elevated glucose concentration, which relates to both its residence time in the circulation and episodes of hyperglycemia (Schleicher and Wieland, 1986). Hyperglycemia is associated with a defect in insulin secretion, insulin action, or both. Insulin deficiency in turn leads to diabetes mellitus with disturbances of carbohydrate, fat and protein metabolism (Megerssa et al., 2013).

In vivo, the amount of any glycated protein maintained in a steady state and is influenced by time-averaged glycemia, the rate of glycation, and the rate of the protein’s degradation or removal from the circulation. During the process of glycation (Figure 1), glucose adducts can continue to form until equilibrium is reached, and a new population of the protein is subjected to the pertinent reaction kinetics as an older population is physiologically removed (Schleicher and Wieland, 1986). In persistent hyperglycemic patients, hemoglobin (Hgb), albumin, lipoproteins and other tissue proteins are non-enzymatically glycated to a great extent and are hallmark marks of diabetes mellitus. Hence, glycated hemoglobin (GHb) and glycated albumin (fructosamide) are widely accepted as older indicators of metabolic control in diabetic patients (Kobayashl et al., 1990).

**CLINICALLY RELEVANT GLYCATED PROTEINS**

**Glycated hemoglobin**

In adults, Hgb is usually constituted of HgbA (97%), HgbA2 (2.5%) and Hgb F (0.5%). HgbA, in turn, is made up of four polypeptide chains, two α and two β chains (Brutis et al., 2006). Glycation occurs at several amino acid residues of the different variants of hemoglobin (HgbA, HgbA2 and HgbF) and results in a product called glycated hemoglobin (GHb). Chromatographic analysis of HgbA reveals a number of minor species HgbA1a, HgbA1b and HgbA1c that are collectively known as fast hemoglobin. HgbA1c is the specific amadori product of glucose conjugated with valine at the N-terminal of both β chains of HgbA. This product accounts for approximately 80% of HgbA1 and about half of the total GHb (Benjamin and Sacks, 1994). Other glycohemoglobin species products have glucose linked to an ε-amino group of one or more of their lysine residues on their α or β chain (Cohen and Clements, 1999).

Formation of GHb is essentially irreversible and its concentration in blood depends on both the life span of red cells and blood glucose concentration. As the rate of formation of GHb is directly proportional to the concentration of glucose in the blood, the GHb concentration represents the integrated values for glucose in the preceding 6 to 8 weeks. This provides an additional criterion for assessing glucose control because GHb values are free of day-to-day glucose fluctuations and are unaffected by recent exercise and food ingestion (Brutis et al., 2006).

**Clinical use of HbA1c**

HbA1c level reflects the blood glucose level consistent with the live span of red blood cell, which is close to 120 days (Nitin, 2010). Hence, higher levels of HbA1c are found in people with persistently elevated blood sugar, as in diabetes mellitus. However, though HbA1c is claimed to
reflected the weighted glucose value over 120 days, practically it is more inclined to show the glucose values in the weeks close to blood collection. In more detail, it can be said that the mean glycemia during the month preceding the HbA1c measurement contributes 50% of the result, whereas the glucose value during the 30-60 days prior to the HbA1c accounts for the 25% and the remaining 25% reflects the glucose level during the whole 60-120 days prior to the measurement. The approximate mapping between HbA1c values and estimated average glucose (eAG) measurements is given by the following equation: 

\[
eAG \text{(mg/dl)} = 28.7 \times \text{HbA1c} - 46.7 \text{or eAG (mmol/l)} = 1.59 \times \text{HbA1c} - 2.59 \quad \text{(Sultanpur and Kumar, 2010).}
\]

In general, high values of HbA1c are used to assess the risk of diabetes complications in addition to the diagnosis of diabetes mellitus. Other applications of HbA1c include prediction of cardiovascular events, even in individuals without diabetes, fasting hyperglycemia and metabolic syndrome (Reddy et al., 2012).

**Limitation of the HbA1c measurement**

Any condition that shortens erythrocyte survival or decreases the mean erythrocyte lifespan (e.g., recovery from acute blood loss, hemolytic anemia) falsely lowers GHb test results regardless of the assay method. Drugs such as aspirin are also reported to falsely lower test results, possibly by inhibiting glycation of Hgb (Reddy et al., 2012). Because human body replaces the red blood cells and the Hgb they contain every 120 days, the HbA1c test cannot be performed more frequently than this 120 day time frame and yield a meaningful result. In addition, the HbA1c test does not directly measure the serum protein glycation (Epinex, 2008).

**Methods for measurement of glycated hemoglobin**

**Specimen for measurement of glycated Hgb:** There are a number of methods to measure GHb including chemical method, high performance liquid chromatography, affinity chromatography and immunoassay. All of these methods require only very small volumes of red cells and there are no special collection conditions. Furthermore, there is no need for fasting before sample collection. The anticoagulant ethylenediaminetetraacetic acid (EDTA) is preferred to collect blood but heparin and fluoride oxalate may also be used (John, 2003).

**Method based on chemical reactivity:** Chemical method of GHb estimation is based on generation of 5-hydroxymethylfurfural (5HMF) from glycol amino groups on Hgb, by heating the GHb in a weak acid. This chemical method measures the total glycated Hgb, that is, it measures not only HbA1c, which is glycated at its N-terminal residue, but also Hgb variants that are glycated at sites other than their N-terminal site. Then, the 5HMF generated is reacted with thiobarbituric acid and quantified colorimetrically. Therefore, method estimates HbA1c as well as HbA1a and HbA1b, and hence, the values obtained are higher than chromatography by 1-2% (Chandalia and Krishnswamy, 2002).

**High-performance liquid chromatography (HPLC):** In this technique, the Hgb solution is treated with endo-proteinase Glu-C in order to hydrolyze its constituent proteins into several peptides, among them are the glycated (HbA1c) and the non-glycated (HbA0) N-terminal hexapeptides of the β-chains. Thereafter, the resulting glycated and non-glycated N terminal hexapeptides of the β-chains are separated from the crude peptide mixture and quantified by HPLC and electrospray mass spectrometry or by HPLC followed by capillary electrophoresis with UV detection. The percentage of HbA1c is determined as a ratio of the glycated to non-glycated β-N-terminal hexapeptides of Hgb (Jeppsson et al., 2002).

**Affinity chromatography:** Affinity chromatography is a method of separating biochemical mixtures based on specific interaction between receptor and ligand. The method utilizes interaction between the 1,2-cis diol group of GHb and immobilized boronic anions in a column, then separation of GHb from the column is achieved using buffers. First, the non-glycated Hgb elutes directly from the column together with the first buffer; after it the bound hemoglobin, GHB, dissociate by the use of a counter ligand (e.g. Sorbitol). The absorbance of the Hgb fractions can be measured at 414 nm using spectrophotometer, and the percentage of GHB is calculated (John, 2003).

**Immunoassay:** This method utilizes a monoclonal antibody raised against HbA1c, which recognizes the first eight amino acids on the β chain Hgb together with the attached glucose. For the reaction to proceed, first red cells in a sample are haemolysed, the Hgb oxidized and the pH adjusted to optimize binding to a microtitre plate. Then monoclonal antibody-enzyme conjugate bound to the HbA1c is immobilized on the plate while any unbound conjugate is removed by washing. Horseradish peroxidase in the bound conjugate normally reacts with the substrate 3',5',5'-tetramethylbenzidine (TMB) and produces a color. The reaction was then stopped with acid and the color intensity was measured at 450 nm using a microtitre
plate reader. Finally, readings were made by relating to calibrators containing known percentage of HbA_{1c} (John, 2003).

Fructosamine

The term "fructosamine" refers to the sum of all ketoamine linkages between circulating glucose and serum proteins. This substance does not contain fructose; however, the glucose chain, which is linked to the protein amino group in a ketoamine bond, becomes modified to resemble the open-chain form of fructose. All serum proteins can become glycated, and therefore, all glycated serum proteins are fructosamines. Glycated albumin accounts for approximately 80% of measured serum fructosamine (Klonoff, 2000). Serum fructosamine represents an index of intermediate glycaemic state (2-3 weeks) that alert physicians to deteriorating or even improvement in their glycaemic control before that of GHb. However, as it represents glycation of protein (mainly albumin), its value is mainly affected by serum protein concentration and the half-life of the proteins both in normal individuals, and in patients with altered protein metabolism (Mula-Abed and Hanna, 2001).

Assay for fructosamine

There are several methods developed for the determination of fructosamine. A procedure using furosine and HPLC is accepted as the reference method. However, a colorimetric procedure using nitroblue tetrazolium (NBT) has gained popularity due to its speed, reproducibility and ease of automation (Scleicher and Vogt, 1990). This assay suffers from interferences due to uric acid and lipemia. The modified colorimetric method that utilizes addition of uricase and detergent, to remove uric acid and lipemia agrees well with fructosamine determined by use of furosine/HPLC method.

NBT utilizes the principle that fructosamines are reductants under alkaline conditions. Hence, in the presence of the NBT, fructosamines are reduced to formazae, a color product, which can be measured spectrophotometrically. The results are expressed as mmol/L of desoxymorpholino-fructose (DMF), which is the synthetic ketoamine, used as primary standard (Johnson, 1983).

Nevertheless, in the NBT method, the fructosamine concentration falsely increases due to some reducing components contained in serum, e.g., thiol group in the peptides and proteins. These reducing components mainly reacted with NBT pre-incubation of the serum sample to avoid this interference resulting from thiol group which could not be undetected throughout the assay (Xua et al., 2002).

Glycated albumin

Glycated albumin refers to albumin to which glucose has bonded. It usually accounts for 80% of the glycated serum proteins. Its circulating half-life is about 17 days, which is shorter than that of the average red cell life of 120 days. This feature makes periodic determination of glycated albumin an attractive candidate for assessing short to intermediate-term level of the average glucose level (Kumeda et al., 2008).

Chemistry of albumin glycation

Non-enzymatic glycation of albumin occurs at multiple sites. Glucose can attach at Lysine 199, Lysine 281, Lysine 439 and Lysine 525, other minor lysine and arginine residues, and also at N-terminal residues of polypeptides. Lysine 525 accounts for 33% of all glycation. Initial reactions are reversible, but subsequent reactions involving protein unfolding and refolding give rise to irreversible cross-linked rearranged products of glucose with proteins. This results in a stable form of glycated albumin that persists at markedly elevated levels in the plasma of diabetic patient (Iberg and Fluckiger, 1986).

Clinical utility of glycated albumin

Glycated albumin has been recently used as another clinical indicator of glycemic control. It provides a short-term index of glycaemic control, and it is not influenced by albumin concentration (Schleicher et al., 1993). In addition, glycated albumin is not affected by RBC lifespan (Inaba et al., 2007). In diabetic subjects, glycated albumin has strong correlations with glucose and provides a reliable index of glycaemic control over the preceding 2–3 weeks (Takahashi et al., 1995). Glycated albumin concentrations increase and decrease more rapidly with fluctuations in overall glucose as compared to HbA_{1c} and this allows rapid changes to be detected at an earlier stage (Takahashi et al., 2007). It has been revealed that increased levels of glycated albumin are linked to both the presence and severity of cardiovascular disease, and impaired renal function (Pu et al., 2007). Observations of the biological properties of glycated albumin are also related to the pathogenesis of diabetic vascular complications (Amore et al., 2004). Furthermore, glycated albumin reflects endogenous insulin secretion more sensitively than HbA_{1c} (Koga et al., 2010).
Clinical implications of glycated albumin

Glycation of albumin significantly reduces transport ability of albumin because of the conformational change associated with glycation that causes a decrease in binding affinity. As compared to the non-glycated form, the affinity of glycated albumin to bilirubin is reduced by 50% and is also reduced by 20-fold for the long chain fatty acid, cis-paranaric acid. This means that the glycation of serum albumin will have a negative impact on the transportation of serum molecules (Epixen, 2008). In addition, glycation of albumin results in impairment of its protein structure and consequently its antioxidant properties (Singh et al., 2007).

Macrophages in the artery walls recognize glycated albumin via specific receptors and, in turn, trigger activation of endoplasmic reticulum kinase, a potent cell-signaling pathway that activates nuclear factor kappa B (NF-κB), a key player in inflammatory reactions. This also produces potent cytokines like transforming growth factor beta (TGF-β), the corollary being a perpetuation of the inflammatory pathways in the artery wall that characterizes the evolution of the atheroma plaque (Hattori et al., 2002).

Diabetic nephropathy can also be generated by the interaction of glycated albumin with receptors in the mesangial cells, independently of the direct actions of hyperglycemia through an amplification cell signaling cascade, involving protein kinase C and secretion of potent cytokine like TGF-β, a series of deleterious effects occur that produce glomerular dysfunction and albuminuria (Epixen, 2008).

Glycated albumin stimulates the phosphorylation of c-Jun, a component of the transcriptional factor activation protein-1 (AP-1) in retinal glial cells. AP-1 up regulates the mRNA level of cytokine vascular endothelial growth factor (VEGF), stimulating increased levels of VEGF, and proliferation of unregulated capillary growth. When the newly formed capillaries invade the retina, leakage of blood plasma damages the retinal area, inducing macular degeneration. The result is a loss of vision in the central retinal area (Okumura et al., 2007).

Measurement of glycated albumin

The concentrations of glycated albumin can be measured by chromatography, thiobarbituric acid assay and immunoassay. However, these methods have a number of disadvantages, e.g. specimens must be pretreated and procedures are complicated. In order to determine glycated albumin more easily and rapidly, a new enzymatic method or glycated albumin using albumin-specific proteinase, ketoamine oxidase and albumin assay is developed (Kouzuma et al., 2002). The glycated albumin is hydrolyzed to glycated amino acids by proteinase digestion, and ketoamine oxidase oxidizes the glycated amino acids to produce hydrogen peroxide, which is quantitatively measured. Glycated albumin value is calculated as the percentage of glycated albumin in total albumin, which is measured by the bromocresol green (BCG) method (Kouzuma et al., 2004).

Glycated lipoproteins

Lipids are important structural and bioregulatory components of human cells, and are transported in blood by the form of lipoproteins (Deric et al., 2006). Like other serum proteins, lipoproteins are non-enzymatically glycated in the presence of glucose (during normal aging and at accelerated rate in diabetes mellitus). Oxidation, accompanying glycation in vivo, further supports chemical modifications (Graier et al., 1997).

Glycation of LDL occurs under hyperglycemic conditions in a time and a glucose concentration dependent way and favors the oxidation of LDL (Figure 2) (Galle and Wanner, 1999). Glycoxidation consists of two related processes oxidation of protein-bound sugars and oxidation of free glucose and its products. Both processes can generate radicals that modify LDL, and hence potentially contribute to the enhanced uptake of such particles by macrophage (Brown et al., 2007). These macrophages, then transform to so called foam cells, the lipid laden cells characteristic for an early atherosclerotic lesion (Deric et al., 2006). Glycation of HDL, which often accompanies hyperglycemic conditions, is associated with decreased paraoxonase levels, and renders HDL more susceptible to oxidation (Hedrick et al., 2000). Oxidized HDL has impaired ability to promote cholesterol efflux, and adverse effects on vascular inflammation (Ansell et al., 2007).

Clinical utility of glycated LDL

LDL glycation is likely to reflect blood sugar variations over a much shorter period. This is because the plasma half-life of LDL is only about 3 days, meaning that the breakdown of glycated protein and the production of fresh unglycated protein occur at a faster rate in the case of LDL, thereby, diluting the glycated LDL and probably minimizing the effects of blood sugar changes that occurred more than 3 days before measurement. Glycated LDL could also be particularly suitable as a measure of cardiovascular risk because it initiates and promotes several critical pathological processes that are involved in vascular disease. An additional advantage of its short term variability is that it could be useful for titrating the dose of glucose lowering treatment over a few days, thereby, allowing rapid control of hyperglycemia (Veiraia
and Wales, 2005).

**Analytical approach for glycated lipoproteins**

Analysis of glycated lipoproteins (glycated LDL and HDL) involves use of HPLC with an affinity boronate column and a gel permeation column. The system consists of three processes. In the first, serum proteins are resolved into glycated proteins and non-glycated proteins by affinity chromatography with the boronate column. In the second, glycated and non-glycated proteins are separately resolved into glycated and non-glycated lipoproteins (LDL and HDL) with the gel permeation column. In the last, the cholesterol content of glycated and non-glycated lipoproteins (LDL and HDL) is measured enzymatically. These three processes are automatically regulated by a system controller. By this method, glycated lipoproteins (glycated LDL and HDL) in a small serum sample can be measured easily within a short period of time (Tanaka, 2001). Glycated LDL and glycated HDL are presented as a percentage of total LDL and total HDL, respectively. The following formula is used to calculate their percentage:

\[
\text{Glycated LDL} \, (\%) = \left( \frac{\text{glycated LDL}}{\text{glycated LDL} + \text{non-glycated LDL}} \right) \times 100
\]

\[
\text{Glycated HDL} \, (\%) = \left( \frac{\text{glycated HDL}}{\text{glycated HDL} + \text{non-glycated HDL}} \right) \times 100
\]

(Tanaka et al., 2006).

**CONCLUSION**

Glucose binds to serum proteins in a non-enzymatic reaction, which occurs in proportion to the prevailing serum glucose concentration during the lifespan of each protein. This non enzymatic reaction is known as glycation, and the product is called glycated protein. Important glycated protein products include: GHb, fructosamine, glycated albumin and glycated lipoproteins. Glycation of proteins affords an index for glycemic control. Moreover, literatures reported that increased glycated proteins are linked to nephropathy, cardiovascular diseases, retinopathy, etc. Several methods such as spectrophotometry, chromatography and immunoassays are presently employed for quantification of different glycated proteins.

**Conflict of interest**

The authors did not have any conflict of interest.

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