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Original Article

Biochemistry Section

Biophysical Characterisation of Amadori Modified Human Serum Albumin: A Prognostic Biomarker for Diabetic Complications

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ABSTRACT

Introduction: Glycation of biological macromolecules particularly protein leads to the generation of early and Advanced Glycation End (AGE) products. The interest in early glycation of protein is driven due to the fact of Amadori modified proteins having role in diabetic complications.

Aim: To analyse the biophysical characterisation of Amadori modified human serum albumin as a prognostic biomarker for diabetic complications.

Materials and Methods: This in-vitro experimental study was conducted at Department of Biochemistry, J.N. Medical College, Aligarh Muslim University, Uttar Pradesh, India, from May 2010 to December 2012. The structural characterisation of EGPs was generated by incubating Human Serum Albumin (HSA) with glucose for about a week. The generation of Early Glycation Products (EGPs) of HSA was quantitated as Hydroxy Methyl Furfural (HMF) by ThioBarbituric Acid (TBA) assay and authenticated by boronate affinity chromatography. Moreover, High Performance Liquid Chromatography (HPLC) and Electro Spray Ionisation/Mass Spectrometry (ESI/MS) was carried out to validate the presence of Amadori product formed. Additionally, Circular Dichroism (CD) and thermal denaturation studies were used to investigate the structural changes in Amadori albumin.

Results: Glycated HSA was obtained as detected by the presence of HMF and chromatography peaks. On stratification, the structural perturbation was observed in Amadori HSA. Furthermore, the generation of furosine was also confirmed by obtaining a new peak in the HPLC profile of glycated HSA. The ESI/MS result also substantiated the presence of Amadori products.

Conclusion: The therapeutic strategies that negate the Amadori modification of albumin might be a logical approach in the prevention of diabetic complications.

Keywords: Advanced glycation end product, Amadori adduct, Diabetes, Glycation, Hydroxy methyl furfural

INTRODUCTION

Glycation causes the carbonyl group in acyclic glucose to react with a free amino group in the protein to generate a Schiff base or aldimine intermediate, which then undergoes an Amadori rearrangement to form a stable ketoamine that can cyclise to a ring structure [1]. Schiff's base and fructosamines are called as early glycation adducts or Amadori product [2]. This product is subsequently subjected to a series of condensations with additional amines, dehydrations, and oxidative fragmentation cycles, resulting in a family of heterogeneous chemical substances known as Advanced Glycation End products (AGEs) [3].

In diabetes, excess glucose binds to proteins all over the body, changing their form and properties in ways that have been shown to harm human organ structure and function [4-6]. Moreover, the early and advanced glycation products formed play a prominent role in the development of diabetic complications. It has been shown that both Amadori product as well as Amadori-derived AGEs are involved in the secondary complications of diabetes [7].

In the recent years, the role of AGEs and the structural protein damage induced by it has been extensively researched. However, a little focus has been gained by the EGPs. Amadori adducts have been found to play a key role among the chemicals involved in tissue injury [8-12]. The absorption of LDL in blood arteries may be increased, resulting in atherogenesis [13]. Free radical mediated damage is also increased [14]. These biochemical abnormal changes probably play a role in the pathogenesis of the early functional changes in the diabetic microvasculature.

Albumin is the most abundant protein in circulation, prone to glycation [15]. Glycation induced structural alterations has profound impact on the functional properties of albumin [16]. Evidence suggests that ε-amino groups of lysine residue in HSA are preferred locus for early glycation [17,18]. In patients with type 1 diabetes, elevated levels of Amadori glycated albumin are associated with diabetic nephropathy and retinopathy independently, while it is favourably associated with cardiovascular disease in patients with type 2 diabetes [19,20], pointing towards the fact that this analyte may serve as a biomarker for diabetic complications.

In the present study, Human Serum Albumin (HSA) was incubated with different concentrations of glucose to produce Amadori-rich glycated HSA for about one week. Hydroxy Methyl Furfural (HMF) tested the presence of Amadori product, and boronate affinity chromatography was also used. Furthermore, to authenticate the presence of Amadori-HSA, High Performance Liquid Chromatography (HPLC) was done using furosine as standard. The Amadori-HSA formed was further substantiated by Electro Spray Ionisation/Mass Spectrometry (ESI/MS). Moreover, the structural changes were investigated by CD and thermal denaturation studies. Hence, the present study was conducted to analyse the biophysical characterisation of Amadori modified human serum albumin as a prognostic biomarker for diabetic complications.

MATERIALS AND METHODS

This in-vitro experimental study was conducted at Department of Biochemistry, J.N. Medical College, Aligarh Muslim University, Uttar Pradesh, India, from May 2010 to December 2012. Human serum albumin, and m-aminophenylboronic acid, were purchased from Sigma Chemical company (St. Louis, USA). Furosine was obtained from Polypeptide Laboratories (Strasbourg, France). D-glucose and

sodium borohydride ($NaBH_4$) were obtained from Merck (Darmstadt, Germany). Nitroblue Tetrazolium (NBT) was purchased from SRL Chemicals (India). All other chemicals and reagents used were of highest analytical grade available.

Formation of 5-Hydroxymethylfurfural (HMF): The Thiobarbituric Acid (TBA) reaction was used to detect the formation of 5- HMF from the Amadori product of glycated HSA, as described by Ney KAT et al., [21]. A 1 mL of native HSA and modified-HSA samples were mixed with 1 mL of oxalic acid (1 M) and incubated for 2 hours at 100°C. The protein from the assay mixture was then extracted using 40% trichloroacetic acid precipitation. TBA (0.05 M) was added to 0.75 mL of protein free filtrate and incubated for 40 minutes at 40°C. The colour was measured at 443 nm, and the amount of HMF was determined using a molar extinction coefficient of 4×10^4/cm/mol.

Affinity chromatography: The production of Amadori adducts in the glycated-HSA samples was further investigated using Chesne S et al., method of boronate affinity chromatography [22]. Using binding buffer, 10 mL of the separating gel (11 mmol m-aminophenylboronic acid) was equilibrated (0.2 M ammonium acetate, pH 8.8). Binding buffer was used to dilute native and glycated-HSA samples, and 1 mL of the solution was administered per mL of the separating gel. The absorbance of each fraction was measured at 280 nm after 3 mL fractions were collected. The boronate bound albumin (corresponding to the Amadori product) was eluted using the elution buffer after non bound albumin was collected (NaCl 0.15 M, $MgCl_2$ 10 mM, sorbitol 0.2 M, pH 3.4).

Circular Dichroism (CD): A spectropolarimeter was used to investigate glycation-induced conformational/structural changes [23]. By placing samples in a temperature-controlled cell holder attached to Neslab's RTE 110 water bath (temperature precision of 0.1°C), CD profiles were obtained at 25°C. A 3.0 μM was chosen as the protein concentration. The Mean Residue Ellipticity (MRE) was computed using the formula below, and the result was represented in deg cm^2 mol^{-1}.

$$MRE = \frac{CD}{(10\times n\times l\times Cp)}$$

The MRE was calculated according to the formula given above and the result was expressed: in deg cm^2 mol^{-1}

Where, CD is the cell path length in centimetres, 'n' is the number of amino acid residues in human albumin (585), 'l' is the cell path length in centimetres, and 'Cp' is the mole fraction. K2D programme computed the percentage of helix, sheet, and random coil.

Thermal denaturation studies: To investigate the stability of HSA after glucose modification, native and glycated-HSA samples were heated under controlled conditions. The samples' midpoint melting temperature (Tm) was determined [24]. The samples were melted at a rate of 1.0°C/min from 30°C - 90°C. With rising temperature, the change in absorbance at 280 nm was measured.

The following equation was used to compute percent denaturation:

$$Percent\ denaturation = \frac{A_T - A_{30}}{A_{max} - A_{30}}\times 100$$

where,

A_T=Absorbance at temperature T°C

A_{max}=Final absorbance on the completion of denaturation (95°C)

A_{30}=Initial absorbance at 30°C

HPLC detection of furosine: By using a capillary HPLC system (Aligent 1100 series) and a synergi C18 analytical column, furosine (a compound generated by acid hydrolysis of fructosyl lysine) was detected in glycated-HSA. The native and glycated-HSA samples were hydrolysed in 6 N HCl at 95°C for 18 hours prior to loading [25]. A medium-grade filter paper was used to filter the hydrolysates. The following was the general chromatographic setup: The eluents employed were 0.4% acetic acid (Solvent A), 0.2% acetonitrile (Solvent B), each containing 2% formic acid, on a C-18 reverse phase column (4x250 mm with 5 m particle size). The gradient elution strategy was as follows: 0-2% solvent B in the first 5 minutes, 2-6% solvent B in 19 minutes, 6-80% solvent B in 11 minutes, and then 80% to wash the leftover material off the column at a constant flow rate of 0.5 mL/min.

Electrospray Ionisation Mass Spectrometry (ESI-MS): ESI-MS is a potent technique for isolating intact ions from big, complicated species in solution in vacuum [26]. Authors employed an orthogonal Time Of Flight (TOF) mass spectrometer with a standard ESI source (Applied Biosystems Mariner Atmospheric Pressure Ionisation TOF Workstation, Framingham, MA, USA) for this experiment. The nebuliser gas utilised was nitrogen. For direct infusion of the sample onto the mass spectrometer, the instrument was equipped with an integrated syringe pump with a twin syringe rack. The spray tip potential was set at 4,000 V and the sciex heater was set to 350°C. The mass spectrometer was set to positive ion mode and mass spectra were taken over a mass range of 100-800 m/z.

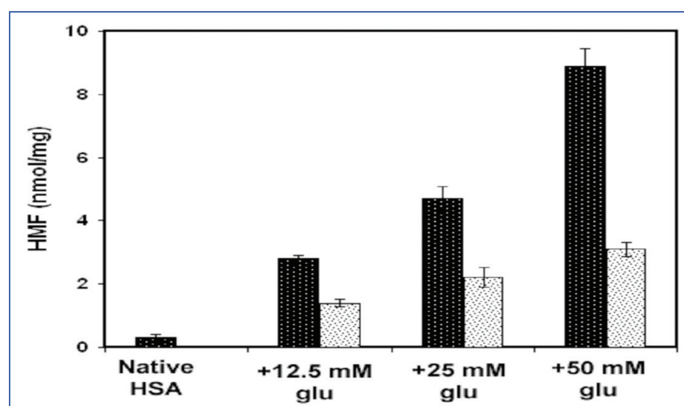
STATISTICAL ANALYSIS

The data are presented as mean±SD. Statistical significance of the data was determined by student's t-test (stat graphics, origin 6.1). A p-value ≤0.05 was considered statistically significant.

RESULTS

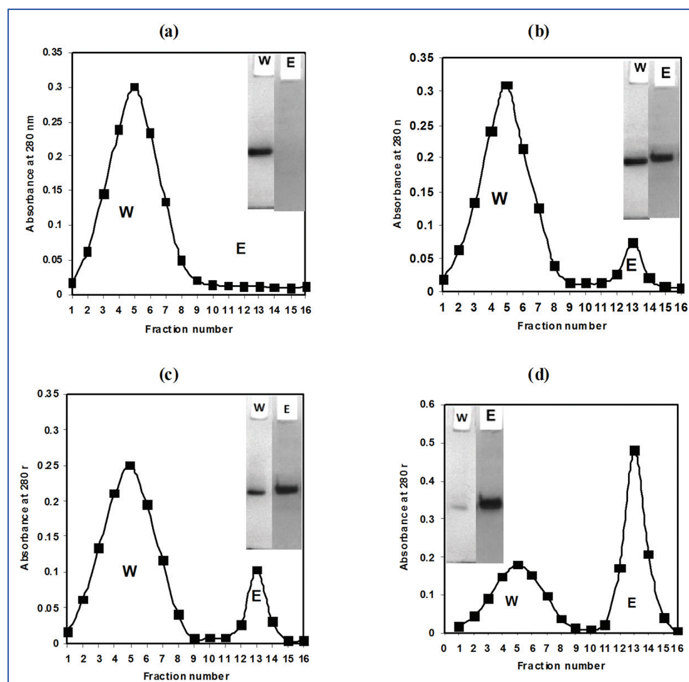
In the previous work, authors have reported that, HSA incubated with increasing concentration of glucose had different content of the Amadori-albumin. Highest amount of the amadori albumin was found in the HSA sample incubated with 50 mM glucose as determined by NBT assay [27]. Native and Amadori-albumin were characterised by various biophysical techniques. Glycation induced structural changes in HSA were evaluated by UV and fluorescence. Native albumin gave a characteristic peak at 280 nm whereas Amadori-albumin showed hyperchromicity (58.4%). Fluorescence Intensity (FI) was found to be significantly decreased in Amadori-albumin as compared to native albumin. Amadori-albumin showed 43.2% reduction in the FI.

Thiobarbituric acid assay for Hydroxymethyl Furfural (HMF): Thiobarbituric acid assay, which evaluates the quantity of ketoamine attached to protein, confirmed the production of Amadori-albumin [21]. HSA modified with 12.5 mM, 25 mM, and 50 mM glucose, yielded HMF 2.8, 4.7, and 8.9 nmol/mg, respectively [Table/Fig-1]. Under similar conditions HSA incubated without glucose, had almost non existent content of HMF. Furthermore, reduction with Sodium borohydride ($NaBH_4$) resulted in a considerable decrease in HMF concentration, which was determined to be 1.4, 2.2, and 3.1 nmol/mg, respectively [Table/Fig-1].



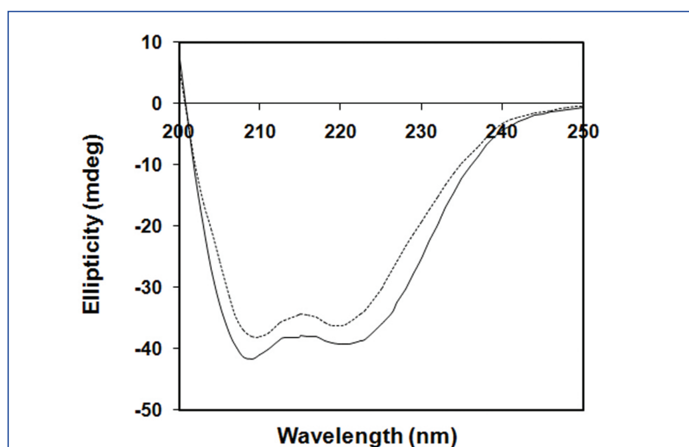
[Table/Fig-1]: Quantitative estimation of hydroxymethylfurfural in HSA modified with 12.5 mM, 25 mM and 50 mM glucose before (■) and after (░) $NaBH_4$ reduction.

Boronate affinity chromatography: To authenticate Amadori formation, native and glycated-HSA samples were run through a boronate column. In case of native HSA, no significant binding was found. Through fractionation, the amount of native HSA loaded emerged in wash (W). Glycated-HSA samples, on the other hand, revealed a progressive decrease in the unbound fraction and a systemic increase in the boronate retained fraction [Table/Fig-2a-d]. Amadori formation was clearly visible on Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of the wash through and eluted fraction (E). Amadori HSA was further characterised using HSA treated with 50 mM glucose.



[Table/Fig-2]: Boronate affinity chromatography of (a) native HSA (b) HSA+ 12.5 mM glucose (c) HSA+ 25 mM glu and (d) HSA+ 50 mM glu. Inset: SDS-PAGE of non glycated (W) and glycated (E) HSA. Binding buffer of pH 8.8 was used to collect wash through fractions; Elution buffer of pH 3.4 was used to collect bound fractions.

Circular Dichroism (CD) analysis of native and glycated-HSA: Circular dichroism was used to assess early glycation-induced secondary alterations in HSA. In the far-UV range, the amide chromophores of the proteins' peptide bonds show ellipticity (200-250 nm). Negative bands between 222 nm and 208 nm are produced by proteins with high alpha-helix content. Proteins with pure beta-sheets, on the other hand, show negative bands between 216 nm and 175 nm. The far-UV CD profile of native HSA and its 50 mM glucose modified equivalent is shown in [Table/Fig-3]. The native and glycated-HSA samples have essentially identical far UV CD profiles. However, both native and glycated-HSA samples, on the other hand, showed two minima, one at 208 nm and the other at



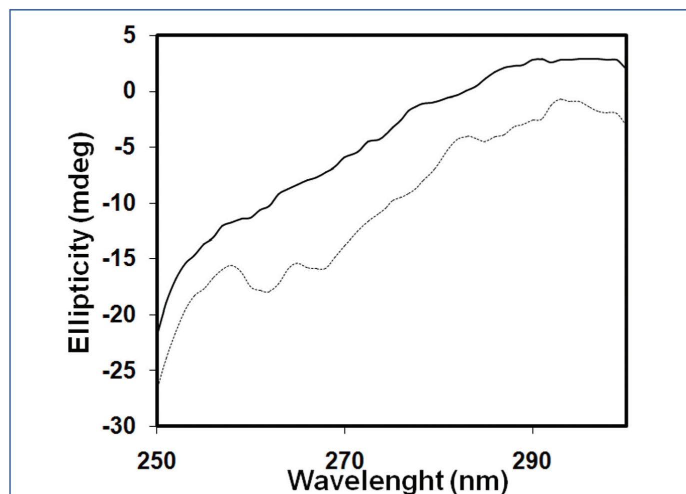
[Table/Fig-3]: Far UV CD profile of native (---) and 50 mM glucose modified-HSA glycated (——).

222 nm, indicating the presence of α-helical content. Native HSA had a 58%-helix, while glycated-HSA had a 65%-helix. HSA's increased alpha-helical content after glycation shows conformational changes. [Table/Fig-4] summarises the MRE and other parameters.

Parameters	Native HSA	Glycated-HSA
Mean residue ellipticity (deg cm^2 mol^{-1})		
222 nm	16848.4	19050.9
262 nm	2042.17	1167.37
268 nm	1803.3	826.68
Alpha helix	58%	65%
β-sheet	8%	5%
Random coil	34%	30%

[Table/Fig-4]: Circular Dichroitic characteristics of native and glycated-human serum albumin.

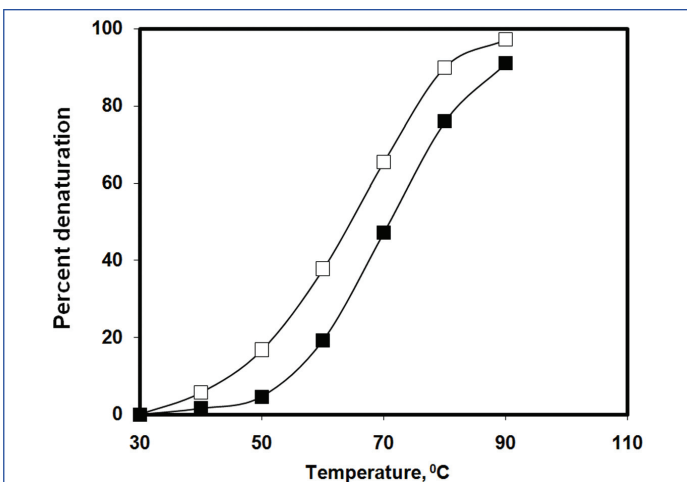
Near-UV CD measurements of native and glycated-HSA: CD measurements in the near-UV region were used to investigate glucose-induced structural alterations in more depth. It should be remembered that peptide backbone orientations influence far-UV CD characteristics, whereas side chains of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) influence CD characteristics in the near-UV CD region (250-300 nm) [28,29], revealing the proteins' tertiary structure. The near-UV CD profile of native and glycated-HSA is shown in [Table/Fig-5]. Two minima were observed in native HSA, one at 262 nm and the other at 268 nm. The 292 nm minimum is typical of disulphide and aromatic chromophores [30]. The reduction of signal at 292 nm, as well as the removal of the 262 nm and 268 nm minima, suggest a significant loss in tertiary structure of glycated-HSA.



[Table/Fig-5]: Near UV CD profile of native (---) and 50 mM glucose modified-HSA glycated (——).

Thermal denaturation of native and glycated-HSA: Heat induced variations in absorbance at 280 nm were used to investigate the effect of glycation on the thermal stability of HSA. The melting temperatures of native and glycated-HSA were determined to be 63˚C and 70˚C, respectively. The Tm value increased by 7°C, indicating that glucose modification has stabilised the structure of HSA. [Table/Fig-6] shows the thermal denaturation profiles of native and glycated-HSA.

HPLC study of native and Amadori-HSA: Standard furosine's HPLC profile [Table/Fig-7a] reveals a well-defined peak with a retention duration of 28.1 minutes. Furosine was not found in native HSA [Table/Fig-7b], which was used as a control. Whereas, Glycated-HSA displayed a peak with a retention period of 28.3 minutes [Table/Fig-7c], which corresponds to furosine's retention duration. It implies that when glucose is incubated with HSA, fructosyl lysine (Amadori adduct) is formed, which is identified as furosine. Furosine, as a result, is a characteristic of early glycation. The furosine peak in $NaBH_4$ decreased glycated-HSA was not visible [Table/Fig-7d]. This



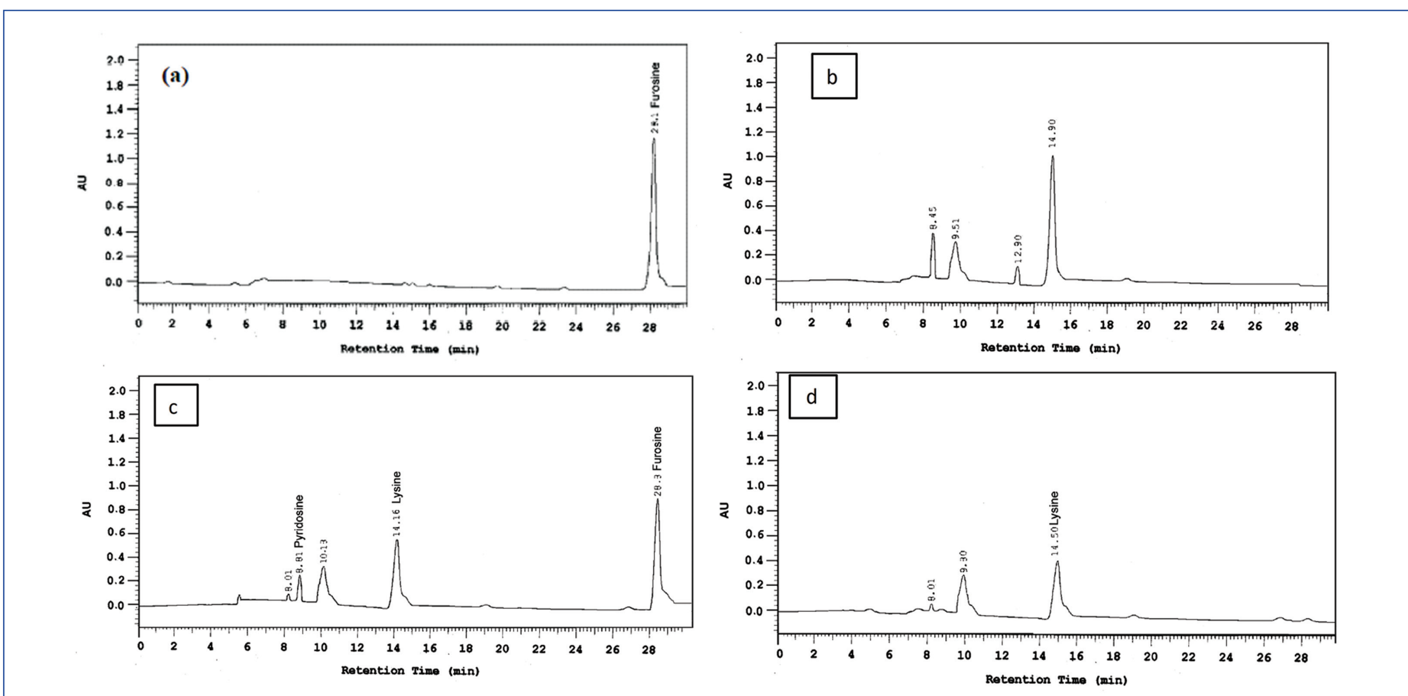
[Table/Fig-6]: Thermal denaturation profile of native (-□-) and 50 mM glucose modified-HSA (-■-).

indicates that $NaBH_4$ has entirely decreased the Amadori product and that no furosine has formed as a result.

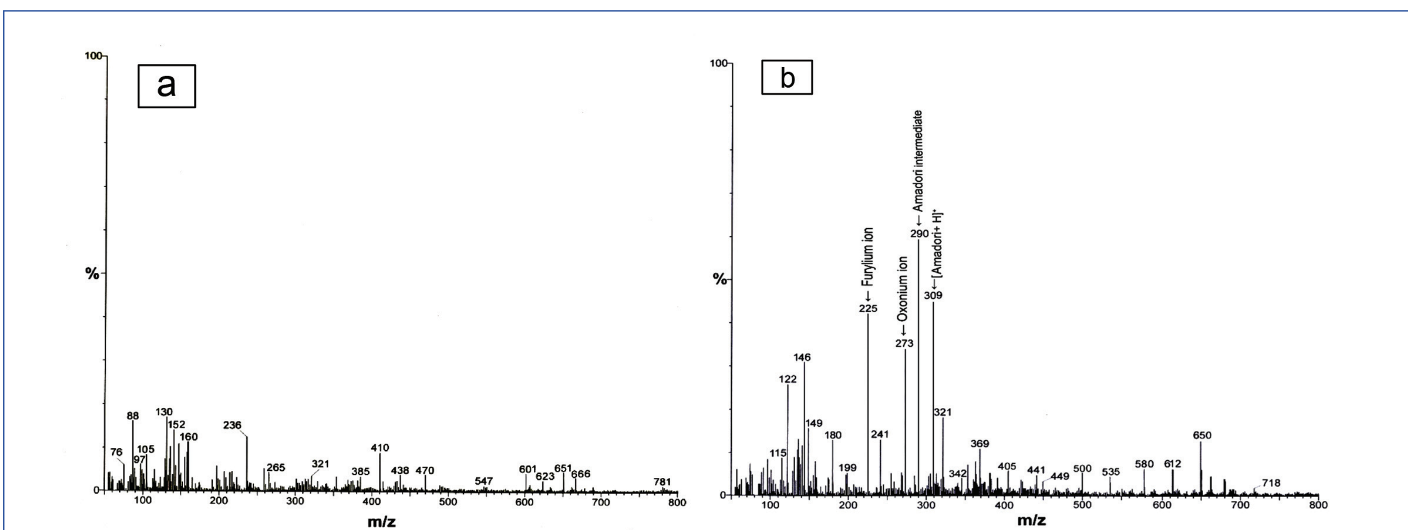
Electrospray Ionisation Mass Spectrometry (ESI-MS) of native and Amadori-HSA: To validate the formation of the Amadori product, hydrolysates of native and glycated-HSA samples were submitted to ESI-MS analysis. The profiles of hydrolysed native and glycated-HSA are shown in [Table/Fig-8 a-b], respectively. The Amadori product (fructosyl lysine) is compatible with the ion at m/z value of 309 (M+H)+, which results from the condensation of lysine residue (Mr 146.16) with glucose (Mr 180) with the loss of a water molecule. The ion with the m/z value of 291 is thought to be the result of a water molecule being lost from the Amadori product. An additional water molecule was lost, resulting in the product ion with m/z of 273. The fragment ion m/z 225 was speculated to form after the removal of a water molecule and the loss of the CHO group from the glucose moiety.

DISCUSSION

Increased protein glycation in hyperglycaemia plays a key role in the pathophysiology of diabetic complications. Many proteins in circulation are modified by Amadori in diabetics [31]. Amadori-modified albumin has been found to be an independent and potent trigger of molecular mediators that contribute to diabetes problems, which are alleviated by inhibiting albumin glycation [32,33]. Elevated levels of AGA associate independently with diabetic nephropathy and retinopathy in patients with type 1 diabetes, and positively associate with cardiovascular disease in patients with type 2 diabetes, suggesting that Amadori albumin may serve as a biomarker for diabetic complications.



[Table/Fig-7]: a) HPLC chromatogram of standard furosine, b) native HSA, c) glycated-HSA, d) $NaBH_4$ reduced glycated-HSA.



[Table/Fig-8]: Full scan ESI-MS spectra of (a) acid hydrolyzed native HSA and (b) acid hydrolyzed glycated- HSA.

Reckoning the above detailed facts the present study was performed to characterise Amadori rich glycated HSA. Previously the formation of Amadori-HSA was confirmed by NBT which is a specific and reliable parameter for Amadori products and not for AGEs [34,35]. In the present study, the amount of HMF formed further authenticated the presence of Amadori HSA. Moreover, the extent of Amadori adduct in glycated-HSA samples was also evaluated on boronate affinity matrix. The species retained on the boronate matrix speaks of Amadori product. It may be plausible to think that with increasing concentration of glucose the percentage of the adduct is increased and the formation is also facilitated in a short period of time.

Albumin is a lysine rich protein and it has been shown that specific lysine residues in HSA are involved in non enzymatic glycation in-vivo [36]. Early stages of glycation occurs primarily on the e-amino groups of lysine residues, which might play an important role in altering the secondary structure of the protein [17,37]. Amadori products have a definite effect on protein structure since they are persistent ketoamines [38]. The far-UV CD results of the present study showed that the incubation of HSA with glucose for 7 days caused an increase in alpha helical content in glycated HSA as compared to native HSA. This is in agreement with an earlier report [39]. Furthermore, when glycated HSA was compared to native HSA, the mid-point melting thermal temperature (Tm) was found to be higher in glycated HSA, indicating an increase in alpha helical content, which is consistent with the current study's Far-UV results.

The most specific and crucial biomarker of early glycation is furosine, which is produced during acid hydrolysis of fructosyl-lysine [25,40]. It is known to be a gold standard of early glycation [41]. In the present study, a characteristic peak was obtained in the HPLC profile of glycated HSA similar to the peak found in the HPLC profile of standard furosine suggesting amadori product formation. However, the current study did not repeat the same findings in native HSA.

In a typical non enzymatic glycation reaction, the carbonyl group of reducing sugars (such as glucose) must first react with the free amino groups of a protein to generate Schiff's base, followed by Amadori rearrangement of the intermediate [42]. Previously, LC-MS study was carried out which authenticated the presence of furosine [27]. Furthermore, in this study ESI-MS results also substantiated the presence of Amadori products in the glycated HSA. The ESI-MS data is consistent with the above events, and the ions at m/z 309, 273, and 225, respectively, suggest Amadori product, oxonium ion, and furylium ion.

Limitation(s)

Early glycation induces significant structural changes in HSA which might hamper its functional properties. Prospective studies are needed to determine whether increased plasma levels of early glycated albumin in diabetic patients precede the development of diabetic complications. Furthermore, Amadori-glycated albumin inhibition could be a focus for reducing diabetic vascular problems. However, the limitations of the present study were that the in-vitro experimental conditions for glycation of HSA were not exactly the same like in-vivo.

CONCLUSION(S)

As inferred from the data obtained, authors propose that the alterations induced in albumin by Amadori modification have ramifications for the management of diabetes and its complications. Moreover, evidence has continued to accumulate to the present day that Amadori glycated albumin has clinical relevance and pathophysiologic importance and reducing its formation independent of hyperglycaemia may have therapeutic benefit.

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