Research Journal of Chemical Sciences ISSN 2231-606X
Vol. **3(12),** 6-11, December **(2013)** *Res. J. Chem. Sci.*

Novel Equation for Correction of Glycated Hemoglobin and Calculation of Carbamylated Hemoglobin in Diabetic Uremic Patients

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Available online at: www.isca.in, www.isca.me
Received 29th October 2013, revised 27th November 2013, accepted 2nd December 2013

Abstract

Carbamylated hemoglobin, as a measure of uremic control, has not gained wide acceptance like glycated hemoglobin as a measure of diabetic control, probably due to its difficulty of estimation. Furthermore, earlier studies have shown that hemoglobin A1c concentrations, measured by cation-exchange based methods, were higher in uremic patients. Our aim was to find a mathematical equation for correction of hemoglobin A1c and easy estimation of hemoglobin carbamylation in uremic patients. We performed in vitro glycation and carbamylation of hemoglobin. Hemoglobin A1c was estimated using cation-exchange based assay method, while carbamylated hemoglobin was estimated by high performance liquid chromatography. The results of in vitro glycation of hemoglobin were compared with those of uremic and diabetic uremic patients. We found that urea in the incubation medium increased the hemoglobin A1c value in the in vitro experiments. Moreover, uremia increased its levels in uremic patients compared to healthy subjects and in diabetic uremic patients compared to diabetic non-uremic patients. We extracted a mathematical equation for the correction of hemoglobin A1c and easy estimation of hemoglobin carbamylation in samples with high urea levels. Studying the difference between hemoglobin A1c measured by cation-exchange based methods and affinity based methods against carbamylated hemoglobin estimated by high performance liquid chromatography and performing further studies on a large cohort of patients will be necessary to confirm these findings and to establish the definite mathematical equation for correction of hemoglobin A1c and determination of the extent of hemoglobin carbamylation in uremic patients.

Keywords: carbamylation, glycation, HbA1c, hemoglobin, HPLC

Introduction

Diabetic nephropathy is a major complication of diabetes mellitus[1]. The high blood glucose concentration in patients with diabetes mellitus leads to abnormally high incidence of protein glycation[2,3]. Non-enzymatic carbamylation of hemoglobin is another type of post-translational modification, caused by blood urea[4,5]. Since both carbamylation and glycation reactions involve the free amino groups of the protein, especially the terminal valine residues in the case of hemoglobin, the possible interference of the two reactions should be considered[6,7]. Glycated hemoglobin (hemoglobin A1c, HbA1c) is accepted as a measure of diabetes control[8]. Carbamylated hemoglobin is similarly suggested to indicate uremic control[9]. However, carbamylated hemoglobin has not gained wide acceptance like glycated hemoglobin in the clinical laboratory field, probably due to its difficulty of estimation. Our aim was to extract a mathematical equation for correction of hemoglobin A1c and to find a way of easy estimation of carbamylated hemoglobin applicable to uremic patients. We addressed this problem through the investigation of the mutual interference of glycation and carbamylation of hemoglobin.

Material and Methods

This study has complied with the principles laid down in the Declaration of Helsinki, adopted by the 18th World Medical Assembly, Helsinki, Finland, June 1964, and recently amended at the 59th World Medical Assembly, Seoul, Korea, October 2008. The participants provided signed informed consent for participation in the study as required. In vitro glycation and carbamylation of hemoglobin of a normal (non-diabetic, non-uremic) person was conducted by incubating washed red blood cells (RBCs) with varying concentrations of glucose and urea at 37°C for five days. Also, the study included sixty patients having either diabetes mellitus (diabetic non-uremic group, n=20) or chronic renal failure (uremic group, n=40). The patients of the latter group were further subdivided into non-diabetic patients (non-diabetic uremic group, n=20) and diabetic patients (diabetic uremic group, n=20). Twenty age and sex matched adults were collected and acted as controls. Hemoglobin A1c was estimated using glycohemoglobin determination kit (Stanbio Laboratory, Boerne, Texas, USA). This method depends on binding of non-altered hemoglobin to a cation exchange resin, thus separating glycated hemoglobin, while carbamylated hemoglobin was estimated by high performance liquid chromatography (HPLC). All chemicals used in this study were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

Blood sample collection and processing: For each experiment 5 mL of venous blood sample was withdrawn under complete

aseptic condition and divided into two portions as follows: 3 mL of blood was placed in an EDTA containing tube for determination of hemoglobin using Coulter B66 (Miami, Florida, USA) and measurement of carbamylated hemoglobin and glycated hemoglobin. The remaining 2 mL of blood was placed in a tube without additives and left at room temperature for 30-60 minutes for spontaneous clotting then serum was separated by centrifugation at 3000 xg for 10 minutes. Serum was used in determination of glucose and urea levels using Cobas C 111 Roche Diagnostics GmbH, Mannheim, Germany. Six replicates of each experiment were conducted for the in vitro experiments. For HPLC, whole blood was washed with isotonic saline, then centrifugation was done at 3000 xg for 10-15 minutes and the supernatant was discarded. The pellet containing RBCs was dissolved in isotonic saline and mixed well to obtain a hemoglobin concentration 15 g/dL.

In vitro carbamylation of hemoglobin: In vitro carbamylation was conducted according to the method of[10] with some modifications. Briefly, previously prepared washed RBCs were incubated with different concentrations of urea at fixed hemoglobin concentration 7.5 g/dL and urea concentrations 5, 10, 20, 40, 60 and 80 mmol/L. Then, prepared samples were incubated at 37˚C for 5 days prior to the assay.

Carbamylated hemoglobin was measured by HPLC method based on quantification of valine hydantoin that spontaneously formed from the released NH2-terminal carbamyl valine residue after acid hydrolysis of hemoglobin according to a previously described method[11]. Briefly, 250 µL of previously incubated sample was hydrolyzed by adding 0.5 mL of 11 mol/L HCl and 0.5 mL of 17 mol/L glacial acetic acid then heating was done for 2 hours at 100˚C. The hydrolysate was cooled in cold water and then 1 mL of 10 mol/L NaOH and 2.5 mL of ethyl acetate were added to the hydrolysate followed by good mixing and centrifugation of the sample at 5000 xg for 10 minutes. 2.25 mL of the supernatant was taken and added to 1 mL of 1 mol/L NaHCO3 then shaking was done for 2 minutes followed by centrifugation at 3000 xg for 5 minutes. 2 mL of the supernatant was evaporated and the resulting extract was reconstituted with 250 µL of the mobile phase: HPLC-grade water containing 60 mL of HPLC-grade acetonitrile and 1 mL of 17 mol/L acetic acid per liter (pH=4). Each assay run included 250 µL of phenol (210 mg/L) added to the reconstituted extract as a reference because of its miscibility in the reconstituted extract.

The chromatographic system was Hewlett Packard HPLC (Waldbronn, Germany) equipped with a 4.6 × 200 mm, 5 µm particle size octadecyl silica reversed-phase column. The sample volume injected was 20 µL, the pump speed was 1.55 mL/minute and the detection wavelength was 210 nm.

Valine hydantoin was prepared by incubation of valine (0.1 mol/L) with different concentrations of urea (5, 10, 20, 40, 60 and 80 mmol/L). The mixtures were incubated at 37˚C for 5 days. Peak of valine hydantoin was identified and compared with that of urea alone and valine alone to determine retention time of valine hydantoin.

Carbamylated hemoglobin was expressed as percent of the ratio between peak height of valine hydantoin and that of phenol.

In vitro glycation of hemoglobin: In vitro glycation was conducted according to the method of [12.] Briefly, previously prepared washed RBCs were mixed with different concentrations of glucose in 0.2 M phosphate buffer, pH 7.4 at fixed hemoglobin concentration 7.5 g/dL and glucose concentrations 5, 10, 20 and 40 mmol/L per sample. The prepared samples were incubated at 37˚C for 5 days prior to the assay then glycated hemoglobin was measured by glycohemoglobin kit according to the manufacturer's instructions and the results were expressed as HbA1c (%).

The same protocol was done with addition of urea to a final concentration 80 mmol/L per sample to evaluate the impact of glycation on carbamylation and to a final concentration 40 and 80 mmol/L per sample to evaluate the impact of carbamylation on glycation.

Statistical analysis: The analysis was done using the Statistical Package for the Social Sciences (SPSS software version 19). Data are expressed as mean±standard deviation (SD). ANOVA with post-hoc Tukey's test was applied to compare the means in case of parametric data. $p<0.05$ was considered significant and $p<0.01$ was considered highly significant.

Results and Discussion

In this study, normal non diabetic and non uremic blood was used (mean±SD of hemoglobin, urea and random blood glucose were 15.1±0.99 g/dL, 5±0.23 mmol/L and 99±3.54 mg/dL respectively and HbA1c was 4.75 %. Peaks of valine hydantoin and phenol (210 mg/L) have been identified at 1.478 and 1.917 minutes respectively.

In vitro carbamylation: The percentage of the ratio of peak height of valine hydantoin obtained from incubation of washed RBCs with different concentrations of urea to the peak height of phenol (210 mg/L) was determined and compared to that of washed RBCs not incubated with urea. We found that the level of urea affects the extent of carbamylation of hemoglobin and there was linear increase in carbamylated hemoglobin level with increase urea after subtracting (0) value representing the in vivo carbamylation, table (1) and figures (1 and 2). To evaluate the impact of in vitro glycation on carbamylation, valine hydantoin (%) obtained from each assay was determined. We detected a decrease in carbamylated hemoglobin with increase glucose concentration at a fixed level of urea (80 mmol/L) (table (2).

In vitro glycation: There was a steady, statistical highly significant, increase of hemoglobin glycation with increasing glucose concentration in the incubation medium ($p<0.01$) (figure (3).

To evaluate the impact of carbamylation on glycation, washed RBCs were incubated with urea (40 and 80 mmol/L) and different concentrations of glucose. We found that HbA1c (%) was affected by the level of urea, since it increased with increase urea concentration. By comparing samples having the same concentration of glucose, HbA1c (%) was higher in the sample containing higher urea concentration (table (3).

Comparison of glycated hemoglobin between study groups: Glycated hemoglobin levels were higher in non-diabetic uremic group compared to control group (5.5±0.39 versus 5±0.24) and in diabetic uremic group compared to diabetic non-uremic group (9.76±1.19 versus 9.16±1.22) (table (4).

Correction of glycated hemoglobin and calculation of carbamylated hemoglobin: Presence of urea led to a significant increase of the glycated hemoglobin value in a urea concentration-dependent manner, figure (4). Furthermore, uremia increased glycated hemoglobin independent of diabetes. Dividing the difference between mean glycated hemoglobin of non-diabetic uremic and control groups or between diabetic uremic and diabetic non-uremic groups by the mean urea level of the corresponding uremic group resulted in the value of carbamylated hemoglobin per unit blood urea concentration. Based on the curves of figure (4) and the results of tables (3 and 4), the following equations were extracted for correction of glycated hemoglobin and calculation of carbamylated hemoglobin:

Corrected glycated hemoglobin (HbA1c %) =
estimated glycated hemoglobin (HbA1c %) – (X/50).
Where X= blood urea level in mmol/L.

Carbamylated hemoglobin (%) = estimated glycated hemoglobin (HbA1c %) - corrected glycated hemoglobin (HbA1c %).

Table-1
Comparison of valine hydantoin (%) obtained by incubation of washed RBCs with different concentrations of urea (mmol/L)

Urea concentration (mmol/L)	Mean valine hydantoin (%)	SD	Minimum	Maximum	p*
0	2.90	0.33	2.53	3.36	<0.01
5	3.94	0.50	3.40	4.64	
10	8.50	1.03	7.19	9.81	
20	15.69	2.36	11.68	18.31	
40	34.75	2.47	31.39	37.91	
60	52.95	2.33	50.03	55.59	
80	71.03	5.11	61.88	75.87	

*p<0.01 is highly significant.

Table-2
Comparison of valine hydantoin (%) obtained by incubation of washed RBCs with 80 mmol/L urea and different concentrations of glucose (mmol/L)

Glucose concentration (mmol/L)	Mean valine hydantoin (%)	SD	Minimum	Maximum	p*
0	71.03	5.11	61.88	75.87	<0.01
5	58.25	3.07	53.92	61.20	
10	48.66	1.89	46.14	51.13	
20	43.80	1.28	42.27	45.67	
40	38.45	2.43	35.44	42.15	

*p<0.01 is highly significant.

Table-3
Comparison of glycated hemoglobin (%) obtained by incubation of washed RBCs with different concentrations of glucose (mmol/L) and urea (40 and 80 mmol/L) for five days

Glucose concentration (mmol/L)	no urea		urea (40 mmol/L)		urea (80 mmol/L)	
	Mean HbA1c (%)	SD	Mean HbA1c (%)	SD	Mean HbA1c (%)	SD
0	4.95	0.201	5.75	0.052	6.55	0.08
5	5.33	0.161	6.13	0.043	6.93	0.194
10	5.68	0.191	6.48	0.042	7.28	0.043
20	6.4	0.202	7.2	0.245	8.0	0.072
40	7.14	0.215	7.94	0.024	8.74	0.201
	*p<0.01		*p<0.01		*p<0.01	

*p<0.01 is highly significant.

Table-4
Comparison of urea (mmol/L) and HbA1c (%) between study groups

Groups	Group 1 (Control)	Group 2 (Non-diabetic uremic group)	Group 3 (Diabetic non-uremic group)	Group 4 (Diabetic uremic group)
Urea (mmol/L)	4.99±0.11	25±0.85$^{*\$\dagger}$	5±0.13$^{\#\dagger}$	29.9±1.19$^{*\#\$}$
HbA1c (%)	5±0.24	5.5±0.39$^{\$\dagger}$	9.16±1.22$^{*\#}$	9.76±1.19$^{*\#}$

The results were expressed as mean±SD. Highly significant difference at $p<0.01$. *$p<0.01$ versus group 1, $^{\#}p<0.01$ versus group 2, $^{\$}p<0.01$ versus group 3 and $^{\dagger}p<0.01$ versus group 4.

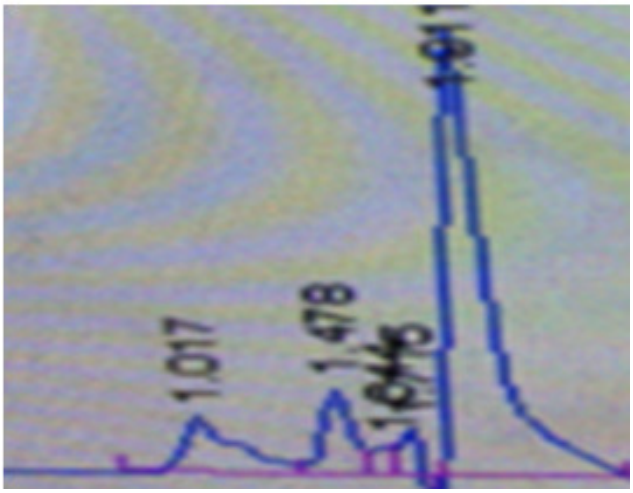

Figure-1
Representative HPLC tracing of valine hydantoin obtained by incubated washed RBCs sample with urea followed by addition of phenol (210 mg/L) before injection. Peak of valine hydantoin was at retention time 1.478 minute, peak of phenol was at retention time 1.917 minute, other peaks were insignificant

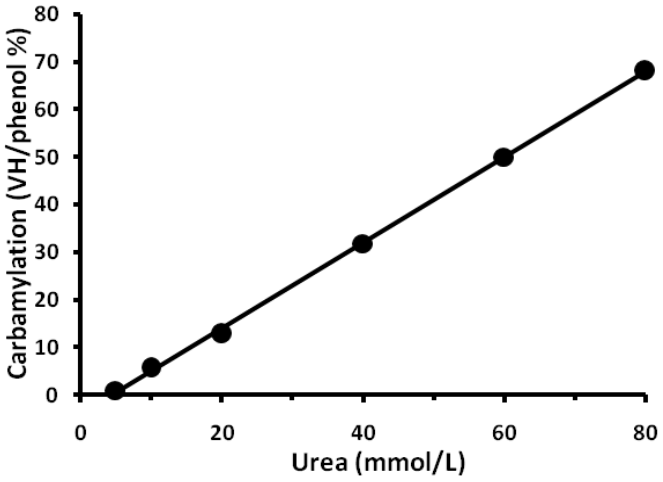

Figure-2
Mean valine hydantoin (%) obtained by incubation of washed RBCs with different concentrations of urea (mmol/L) after subtracting 0 value

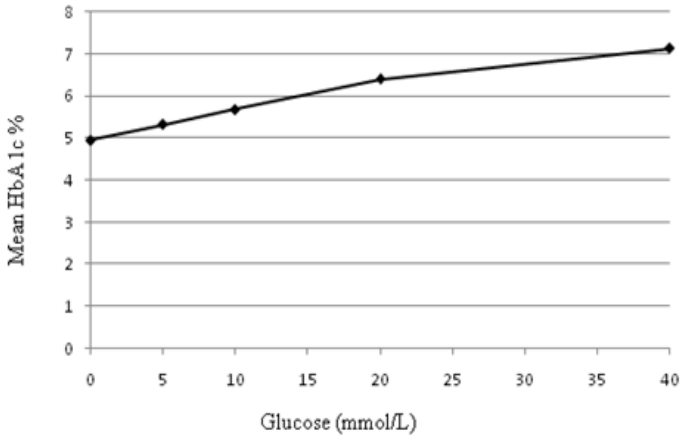

Figure-3
Hemoglobin A1c (%) after incubation of red blood cells with various concentrations of glucose (mmol/L) for five days

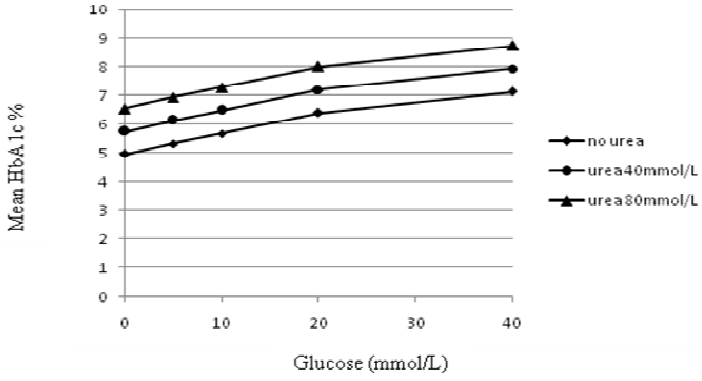

Figure-4
Hemoglobin glycation in absence and presence of urea (40 and 80 mmol/L) for five days

Discussion: Both glycation and carbamylation are post-translational modification of hemoglobin involving the free amino groups, especially the N-terminal valine residues. The interference of the two processes has already been documented[6,13]. We recently re-evaluated this mutual interference through in vitro study done on washed RBCs. Furthermore, we investigated this interference by comparing

HbA1c results in uremic patients being either diabetic or non diabetic aiming for access an easy and accurate assessment way to the extent of carbamylated hemoglobin as index of the degree of uremia and also to find an equation capable of correction of HbA1c values in uremic diabetic patients based on the level of blood urea.

We found that the level of urea affects the extent of carbamylation of hemoglobin measured by HPLC method as there was linear increase in carbamylated hemoglobin level with increase urea concentration by plotting valine hydantoin (%) on the vertical (Y) axis against the urea concentrations in mmol/L on the horizontal axis (X) after subtracting (0) value which represents the in vivo carbamylation induced by almost urea concentration 5 mmol/L. This agrees with previous studies that demonstrated a strong relationship between carbamylated hemoglobin and averaged urea concentration in uremic patients[6,10,12,14].

Re-measuring carbamylated hemoglobin after in vitro glycation showed lower levels compared to those obtained from RBCs samples incubated only with the same urea concentrations. This indicated that in presence of a fixed level of urea, carbamylated hemoglobin decreases with increase glucose concentration which can be explained by that glycation of some hemoglobin free amino groups prevents their participation in carbamylation reaction. This result was in agreement with a previous report[6] which demonstrated that carbamylated hemoglobin was significantly lower in diabetic patients and provided further evidence of decrease carbamylation in samples with abnormally high glucose concentrations through in vitro carbamylation experiment using a fixed urea concentration of 70 mmol/L.

It is not surprising that the measured HbA1c is elevated in presence of high urea concentration when the former is measured by a method depending on the positive charges on non-altered hemoglobin. We were expecting an attenuation of this elevation at high glucose and urea concentrations due to interference of the two reactions: glycation and carbamylation involving the same amino groups. However, the carbamylation of hemoglobin appeared to add to the measured value of HbA1c almost equally at all concentrations of glucose, depending on the urea concentration. This means that carbamylated hemoglobin was already measured by this method and expressed as hemoglobin A1c.

Therefore this study represents another evidence on the mutual interference of glycation and carbamylation of hemoglobin witnessed by the significant decrease of valine hydantoin production with increase glucose concentration and the additive effect of urea that produced carbamylated hemoglobin leading to elevation of glycated hemoglobin levels estimated by this cation-exchange based method.

The impact of carbamylation on glycation was evaluated using two concentrations of urea (40 and 80 mmol/L) to confirm our results and to help establishment of the equation which can correct the results of cation-exchange based assay methods of glycated hemoglobin in blood samples with abnormally high levels of glucose and urea so that the corrected result reflects the actual glycation of hemoglobin and helps in estimation of carbamylated hemoglobin through an easy widely used method. Measuring HbA1c in uremic patients either non diabetic or diabetic showed falsely high values which confirmed the results of our in vitro experiments.

Conclusion

Repetition of these experiments on a large cohort of diabetic patients complicated with uremia and studying the difference between glycated hemoglobin measured by an affinity method, which is expected to exclude carbamylated hemoglobin and cation-exchange based assay methods against carbamylated hemoglobin estimated by HPLC will help in validation of the equation proposed in this study for correction of cation-exchange based assay methods of glycated hemoglobin in patients suffering from diabetes and uremia thus providing a good way for proper planning and management of these patients.

Conflict of interest: The authors have no conflicting interests, including any financial, personal or other relationships with other people or organizations, and are not supported or funded by any company.

Acknowledgment

The authors thank members of the Internal Medicine Department, Faculty of Medicine, Ain Shams University, Cairo, Egypt for providing the patients' clinical data included in the study.

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