Full Length Research Paper

A colorimetric method for measurement of carbamylated haemoglobin in patients with chronic kidney disease using a spectrophotometer

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Abstract

Haemoglobin carbamylation occurs when isocyanate; a spontaneous breakdown product of urea reacts with the terminal valine residues of the α and β polypeptide chains of haemoglobin (Hb). Hb carbamylation depends on the duration of exposure to high levels of urea. Carbamylated haemoglobin (CHB) is thus elevated in renal failure. The aim of this study was to develop a colorimetric method for the measurement of CHB in patients with renal failure. Twenty patients with chronic kidney disease (CKD) and twenty persons with normal renal function were recruited in the study to determine their blood levels of CHB. CHB was measured using a colorimetric method. Mean CHB levels were significantly higher in the CKD patients (107.5±16.2) than in the normal controls (65±11.9); CHB expressed as microgram valine hydantoin per gram Hb (µgVH/gHb). There was significant correlation between CHB and urea, creatinine and Hb concentration, r=0.832, r=0.566 and r=-0.733 respectively. Following multiple regression analysis, urea was the only predictor of CHB. At a CHB cut off level of 100µgVH/gHb, the sensitivity and specificity of this test to identify persons with CKD from those with normal renal function was 95% and 80% respectively. CHB levels can be determined with reasonable accuracy using a colorimetric method.

Keywords: Carbamylated haemoglobin, colorimetry, chronic kidney disease.

INTRODUCTION

Carbamylation is a chemical reaction between the amino (NH$_2$) group of an amino acid and isocyanate ion derived from the spontaneous breakdown product of urea. In solution, urea is in equilibrium with cyanate ion. Carbamylation of proteins can occur when the terminal amino acid attached to the protein interact with the more reactive isocyanate ion than the cyanate ion with which it is in equilibrium. Carbamylation of haemoglobin is the most studied carbamylation reaction of the N-terminal valine residues of the alpha and beta chains of haemoglobin with isocyanate ion (Fluckiger et al., 1981).

Studies on haemoglobin carbamylation were initiated following the use of cyanate therapy in sickle cell anae-

mia. Cyanate therapy was considered for treatment of sickle cell anaemia because carbamylation of the amino acid group of the terminal valine residue of haemoglobin S with isocyanate prevented gelling of deoxygenated haemoglobin and hence the sickling of the erythrocytes. Thus, methods of quantifying carbamylated haemoglobin were developed for its use as a measure of cyanate toxicity (Gillette et al., 1971).

Several methods have been used to quantify carbamylated haemoglobin (CHB). Manning et al (Manning et al., 1973) used ion exchange and evaporation technique followed by gas chromatographic amino acid analysis of an alkaline hydrolysis of valine hydantoin released to quantify carbamylated haemoglobin S. Gas liquid chromatographic (GLC) method was used in other studies to measure valine hydantoin released after acid hydrolysis of haemoglobin extracted from whole blood (Smith et al., 1988). Recently,

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high performance liquid chromatography (HPLC) has been used to quantify CHB based on the release of carbamyl valine from the N-amino acid terminals of the alpha and beta chains of haemoglobin after acid hydrolysis (Kwan et al., 1990; Davenport et al., 1993). Davenport et al (Kwan et al., 1990 and Kwan et al (Kwan et al., 1992) measured CHB in patients with renal failure using the HPLC method. In this paper the Rosen method (Rosen, 1957) for measurement of CHB was adapted for a modified colorimetric analysis of CHB and to access its suitability for identifying patients with renal failure.

MATERIALS AND METHODS

Patients

Twenty consecutive patients with chronic kidney disease who presented at the department of medicine, University of Benin Teaching Hospital, Benin City and twenty age matched controls with normal renal function were recruited for the study. Approval was obtained from the hospital ethics committee and informed consent was obtained from the patients and control subjects. Patients who received blood transfusion in the preceding three months, those who were already on haemodialysis treatment and those with diabetes mellitus were excluded from the study. Persons with normal serum urea and creatinine, normal blood pressure and urinalysis were recruited as controls. The patients were subjected to clinical examination. The diagnosis of chronic kidney disease was made based on facial and /or leg swelling, oliguria, general malaise, pruritus all lasting for more than three months with contracted kidneys on ultra sound examination, elevated urea greater than 85 mg/dl (14.2 mmol/L) and serum creatinine of at least 3mg/dl (265µmol/L)

Blood samples

Blood samples (10ml) were collected from all patients and control subjects and distributed into plastic tubes containing lithium heparin and potassium EDTA (ethylene diamine tetra acetate) for plasma urea and creatinine estimation respectively. All haematological estimations were done according to standard practice (Dacie and Lewis, 1991). Plasma creatinine was determined using the alkaline picric acid Randox kit method (from Randox laboratories Ltd, Crumlin, UK) based on Jaffe's reaction (Randox kit lot № II9ICR). Plasma urea was estimated using the urease- hypochlorite Berthelot method (Randox kit, lot № 2414 UR).

Analytical Procedure for Carbamylated Haemoglobin

I. Reagents 0.1M sodium cyanide solution

II. Acetate buffer (PH 5.3 - 5.4). prepared from 3.6g sodium acetate dissolved in 200 ml distilled water and glacial acetic acid (66.5ml) and made up to 1 litre of distilled water.

III. Acetate - cyanide reagent made from 2ml NaCN solution and 100ml acetate buffer.

IV. Ninhydrin Reagent (indanetrione hydrate) made from 3g Ninhydrin dissolved in 100ml organic solvent (95% v/v n-butanol and 5% v/v glacial acetic acid).

V. Dilute isopropanol prepared from (1 vol Isopropanol + 1 vol distilled water)

VI. Valine stock solution. Dissolve 0.5855g valine in 1 litre distilled water, equivalent to 585.5µg/ml.

VII. Valine standard solutions: low standard (LS) 29.28µg/ml and high standard (HS) equivalent to 58.55 µg/ml.

Principle of the Method

Carbamylated haemoglobin released after haemolysis of the red cells is hydrolysed using hydrochloric acid- glacial acetic acid reagent. The N-terminal valine residues of the α and β chains of haemoglobin are released as carbamyl valine. Carbamyl valine undergoes cyclization to form valine hydantoin which is then extracted with an organic solvent. The amino acid valine released from the hydantoin using acetate-cyanide solution reacts with ninhydrin reagent to produce a colour which is measured spectrophotometrically at 570nm (Milton Roy Spectronic 2OD Spectrophotometer, Linden Avenue,New York USA).

Sample preparation

Haemoglobin concentration of blood samples in each of the EDTA bottles was estimated. Whole blood was washed three times with physiological saline and diluted 1 in 100 (0.1 ml blood with 9.9 ml distilled water) to obtain a haemoglobin haemolysate. This dilution gives approximately 0.09gHb to 0.15gHb range. To 1.0 ml of the haemolysate was added 0.5ml acid mixture (10ml conc. Hydrochloric acid / 1.0 ml glacial acetic acid) and incubated at 37°c for 30 minutes.

n-Butanol- acetic acid mixture (1.0ml) was added to the haemolysate to extract the valine hydantoin formed. To each test tube marked blank, test and standard was added 1.0 ml distilled water, 1.0ml extracted valine hydantoin and 1.0ml each of the valine standards (LS and HS) respectively. Acetate cyanide solution (0.5ml) was then added to all the tubes followed by 0.5ml ninhydrin solution and tubes placed in boiling distilled water for 20 minutes. The dilute isopropanol (0.5ml) was immediately added to all the tubes. The content of the tubes were allowed to cool to room temperature and then read spectrophotometrically at 570nm using 1 cm cuvette after setting to zero with the blank reagent.

Table 1. Comparison of mean biochemical parameters of CKD and Control subjects.

Parameter	Normal range	Control	CKD	p value
Age (years)		39.4±13.3	38.7±13.3	>0.05
BMI (Kg/m^2)	18.5-24.9	26.3±3.6	23.4±3.9	>0.05
Hb conc (g/dl)	11.5-15.5	13.3±1.5	8.6±2.3	<0.05
creatinine (mg/dl)	0.5-1.5	0.8±0.2	10.3±7.7	<0.05
urea (mg/dl)	15-40	25.7±6.6	179.5±57.4	<0.05
CHB (µgVH/gHb)		65±11.9	107.5±16.2	<0.05

P values less than 0.05 is taken as significant

The concentration of valine was calculated using the absorbance readings obtained. The results were expressed as valine hydantoin equivalent of carbamylated haemoglobin.

$$CHB (µgVH/gHb) =$$
$$\frac{\text{Absorbance of test}}{\text{Absorbance of std (HS)}} \times 58.55 \times \text{Hb conc/ml}$$

$$OR = \frac{\text{Absorbance of test}}{\text{Absorbance of std(LS)}} \times 29.28 \times \text{Hb conc/ml}$$

The haemoglobin level was cross-checked by a PCV estimation by a standard microhaematocrit method; the precision and reproducibility of the carbamylated haemoglobin, urea and creatinine were monitored using commercial quality control with known values (Wellcomtrol, Welcome Reagents Ltd) and a pooled extracted valine hydantoin samples of the controls (mean 70 µg VH/gHb; range 65-80 µg VH/ gHb). The inter assay coefficient of variation for 20 determinations each for urea, creatinine and carbamylated haemoglobin was 4.6% 4.8% and 10.0% respectively. The sensitivity of the CHB test in correctly identifying true positives as such that at a cutoff point of 100 µg VH/gHb was 100%; while specificity at the same cut off of CHB was95% while the positive predictive value i.e. proportion of test positives that are truly positive was 67%.

Statistical analysis

The statistical package for social sciences (SPSS 16) was used to analyse data. The statistical significance of the difference between the means in the groups was calculated using Student's t-test; a p value of less than 0.05 was considered statistically significant. Pearson's correlation coefficient was used to determine any significant association between CHB, urea and creatinine, multiple linear regression were used to determine the predictors of CHB.

RESULTS

Forty subjects were recruited for the study; twenty pat-

ients with chronic kidney disease (CKD) and twenty persons with normal renal function as controls matched for age and sex. The age range for the study subjects was 19-65 years, twenty males and twenty females.

Table 1 illustrates the mean parameters of the control subjects compared to the CKD patients. Mean serum urea, serum creatinine and CHB were significantly higher in the CKD group compared to the control group. Mean Hb conc and BMI were higher in the control group.

Correlation analysis using Pearson's coefficient showed significant association between CHB and serum urea, serum creatinine and Hb conc among the study subjects see Figure 1 (a),(b),(c) and Table 2

Multiple regression analysis using urea, creatinine and Hb conc as independent factors showed that urea was the only significant predictor of CHB (Table 3). The regression model generated had a good fit, with 70% of the variation in CHB being explained by the model.

DISCUSSION

The purpose of this study was to attempt to measure carbamylated haemoglobin (CHB) by a colorimetric method. Previous research studies on CHB measurement have been carried out using chromatographic methods including high performance liquid chromatography(Manning et al., 1973; Kwan et al., 1990; Davenport et al., 1993; Kwan et al., 1992) but in a resource poor environment, we adapted the Rosen method (Rosen, 1957) for a colorimetric measurement of CHB using spectrophotometric instrumentation.

The mean reference value for CHB in subjects with normal renal function was reported by some workers (Kwan et al., 1992; Davenport et al., 1996) as 28µgVH/gHb (range 26-32) using the HPLC. The mean CHB reference value of 65µgVH/gHb (range 46-84) in control subjects by our colorimetric method is however higher than that reported elsewhere however the inter assay coefficient of variation of 10% by the colorimetric method would indicate good precision and reproducibility for our colorimetric method as a satisfactory analytical method for measurement of CHB.

Elevated plasma urea and creatinine values usually

(a)

$y= 0.24x + 61.58$
$r = 0.832$

(b)

$y= 2.02x + 75.05$
$r= 0.566$

(c)

$y = 154.32 - 6.21x$
$r = -0.733$
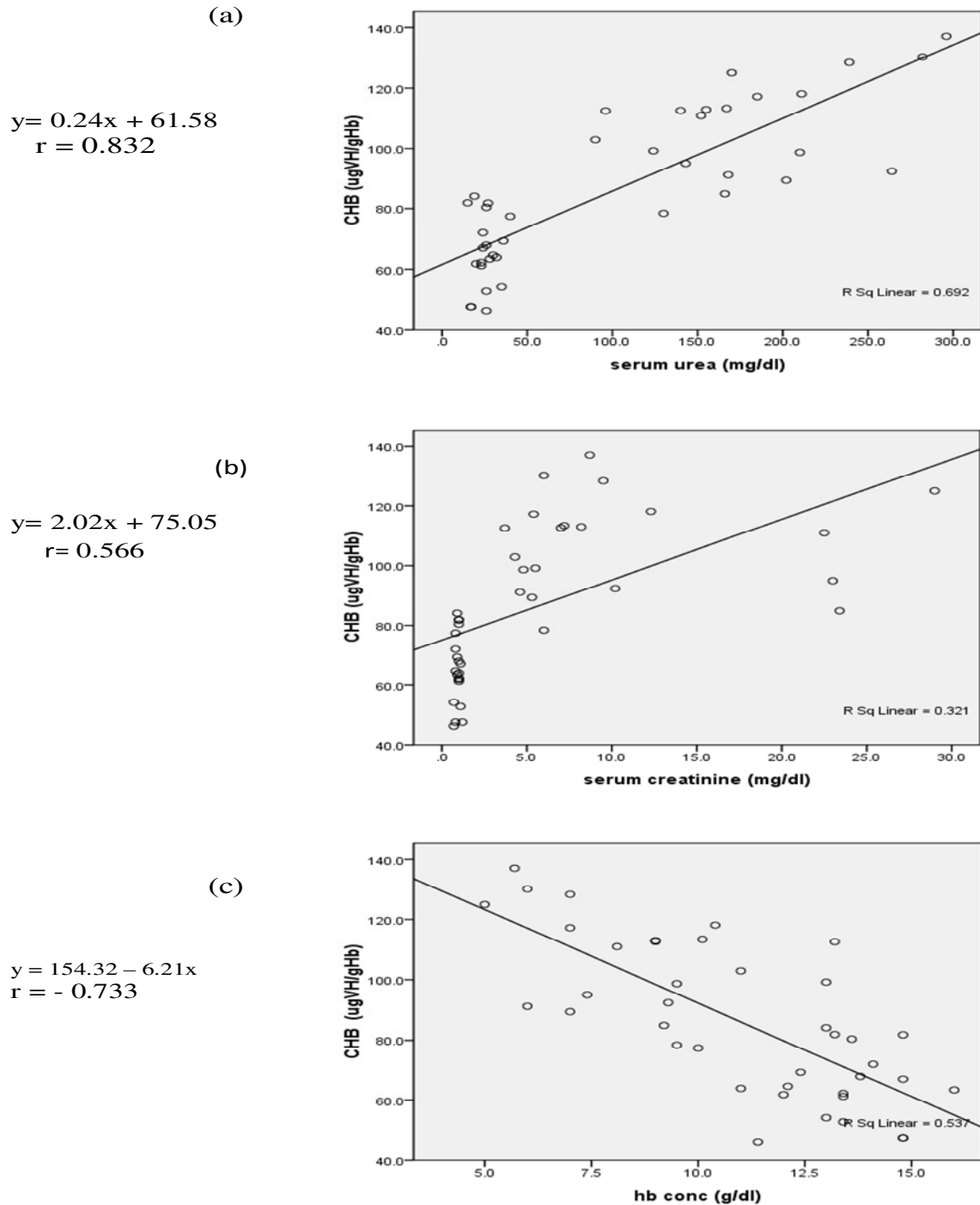
Figure 1. Scatter graphs showing univariate correlation between CHB and (a) urea (b) creatinine (c) Hb conc.

Table 2. Correlation between CHB and other Parameters.

Parameters	Pearson coefficient	P value
Age	-0.156	>0.05
BMI	-0.083	> 0.05
Serum Urea	0.832	<0.05
Serum Creatinine	0.566	<0.05
Hb Conc	-0.733	<0.05

Table 3. Multiple regression model coefficients for CHB.

Model	B coefficient	standard error	t statistic	p value
(Constant)	68.642	21.370	3.212	<0.05
Urea	0.208	0.490	4.289	<0.05
Creatinine	0.395	0.427	0.925	>0.05
Hb conc	0.530	1.509	-0.351	>0.05

Table 4. Percentage of study groups with CHB values below different cut-off values.

CHB cut-offs (μgVH/gHb)	control group (n=20)	CKD group (n= 20)
< 60	25% (5)	0
61 – 80	80% (16)	5% (1)
81 – 100	100% (20)	40% (8)
>101	0	100%(20)

*Absolute number of patients in brackets

associated with patients in renal failure were significantly higher in the control subjects so also were the values of CHB in patients with renal failure (Table 1). There was significant correlation between CHB and serum urea. Plasma urea had the strongest association with CHB (r=0.832) and urea was the only predictor of CHB following multiple regression analysis. This agrees with findings in previous studies (Kwan et al., 1992).

The association between CHB and haemoglobin concentration was negative (r=-0.733). The mean haemoglobin concentration of the patients was 8.6g/dl. Since only available haemoglobin can be carbamylated, anaemia may have an influence on CHB levels in patients with advanced CKD. Previous workers on CHB did not consider the effect of anaemia but in our environment, patients in renal failure present late to the nephrologist and often with anaemia. Also, facilities to treat anaemia in renal failure patients like erythropoietin therapy are usually unaffordable to these patients. There was significant correlation between CHB and Hb conc (r= 0.733) and variation of a regression model using Hb conc as an independent factor was explained 53% but after considering the effect of urea and creatinine, Hb conc was not a predictor of CHB. We also found creatinine not to be a significant predictor of CHB as in previous works (Frazao et al., 1995).

As a diagnostic test for patients with chronic kidney disease, our finding was that, at a CHB cut off value of 100μgVH/gHb, the sensitivity was 95%% and specificity 80% (Table 4) as calculated (Marshall, 1989).

We conclude that CHB can be determined with reasonable degree of accuracy and reliability using a colorimetric method. The CHB values obtained by this method are reliable enough to be used as a diagnostic test to identify those in renal failure. The colorimetric method is cost effective in terms of the instrumentation used and in terms of the non special manpower training requirement.

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