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Carbamylated hemoglobin and carbamylated plasma protein in hemodialyzed patients

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Carbamylated hemoglobin and carbamylated plasma protein in hemodialyzed patients. The carbamylation reaction *in vivo* involves the nonenzymatic, covalent attachment of isocyanic acid, the spontaneous dissociation product of urea, to proteins. Carbamylated proteins have been proposed as markers of uremia and indicators of uremic control. However, the utility of measuring carbamylated proteins has not been investigated adequately. Therefore, this study was done to determine the relationship between the carbamylation of long-lived protein (hemoglobin) with that of short-lived proteins (plasma proteins) in hemodialyzed patients. Significantly higher carbamylated hemoglobin (CHb; 157 ± 40 μg valine hydantoin/g Hb) and carbamylated protein (CTP; 0.117 ± 0.011 absorbance/mg protein) concentrations were found in hemodialyzed patients ($N = 13$) as compared to normal individuals ($N = 9$, 53 ± 20 μg valine hydantoin/g Hb and 0.08 ± 0.01 absorbance/mg protein, respectively). A high correlation was found between CHb and CTP concentrations ($r = 0.87$, $P < 0.0001$), demonstrating a strong relationship between these two different half-lived proteins. A six-month longitudinal study of seven hemodialyzed patients showed that the between subject correlations were significant for CHb versus CTP as well as CHb versus pre-dialysis urea. Correlations were not significant for CTP versus pre-dialysis urea or Kt/V, nor CHb versus Kt/V. Carbamylated hemoglobin fluctuated the most over this time period (30.1% ± 20.2%), pre-dialysis urea and CTP varied less (18.3% ± 13.4% and 14.9% ± 7.5%, respectively), and Kt/V varied the least (6.3% ± 3.3%). Within subject correlations were not significant between any two tests. It is unclear whether the lack of correlations found is real or a function of the small sample size. However, these data do show that CHb and CTP are positively associated and reflect the degree of urea exposure in the blood, but their usefulness for patients on maintenance hemodialysis is not clear.

Increases in blood urea levels have always been associated with a loss of kidney function. Recently, a new approach has been taken for its measurement that has broadened the scope of its use. Urea decomposes to give cyanate and ammonium ions [1]. Cyanate, in its unprotonated form, reacts with unpaired electrons through a nucleophilic reaction [2, 3]. *In vivo*, the most stable reactions are with the electrons of the free amino groups (α and ε), and sulfhydryl groups. Carbamylation studies done *in vitro* [4, 5] have demonstrated that carbamylation causes changes in conformation, decreased or inhibition of enzyme activity, binding changes (to receptors and drugs), and physically causes an increase in anodicity on various types of proteins.

Elevated urea levels therefore not only indicate a loss of kidney function, but may also serve as a source for potential pathophysiological consequences. Several reports have inferred the association of carbamylation with symptom or loss of function such as cataracts [6], neuropathies [7], nausea, drowsiness, memory loss [4, 8], atherosclerosis [9, 10], and drug binding defects [12–14]. In fact, the presence of carbamylated amino acids [15], membranes [16, 17], lipoproteins [9], plasma proteins [12, 14, 18–20], and hemoglobin [11, 21–27] have been found in patients with renal failure. However, no study has yet shown by direct measurement that symptoms seen in patients with uremia are a result of carbamylation.

Nonetheless, there has been ample progress in assessing carbamylated proteins as markers of uremia [3, 11, 15, 16, 18, 19, 21–31]. This concept is analogous to that of glycated proteins in diabetes [32]. When glucose (or cyanate or other potential compound) makes a stable attachment to a protein, it takes on the half-life of that protein. In the assessment of diabetics, it is these glucose-protein adducts that reveal the past dynamics of blood glucose levels, a time-integrated marker. Glucose or urea measurements alone are subject to day-to-day dietary and metabolic influences, thereby reflecting transient time periods. The most common marker proteins measured are hemoglobin, which is a long-term indicator (2 months), and albumin or total plasma protein, which are short-term indicators (2 to 3 weeks) [33].

Carbamylated hemoglobin has been the major carbamylated protein studied owing to initial studies that sought to use cyanate as a therapy for sickle cell anemia [34]. Flückiger et al were the first to identify carbamylated hemoglobin in uremic patients and furthermore correlated HbA_1 with time-averaged urea concentration [28]. Oimomi et al [29] then investigated the possibility of using HbA_1 as an indicator of control in patients with renal failure. A time-course relationship between urea and HbA_1 demonstrated a correlation with HbA_1 and urea concentrations measured one to two weeks earlier. In another study, CHb in renal transplant patients reflected the degree of uremia two to three weeks earlier [27].

Evaluation of carbamylated hemoglobin in patients with various degrees of renal function has shown that its measurement has potential clinical value (Table 1) [11, 21–27, 31]. There are significant differences between CHb in control groups [22, 24, 31] compared to patients with chronic renal failure [21, 22, 24, 29, 31], patients on continuous ambulatory dialysis [22, 24], hemodialysis

Key words: carbamylated hemoglobin and plasma protein, hemodialysis, urea, proteins and urea, measuring carbamylated proteins.

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Table 1. Carbamylated hemoglobin (CHb) values in normal individuals and patients with renal failure

	CHb[a] *μg CVAL/g Hb*	Patients *N*	Reference
Normal			
Normal renal function	25 (2)	27	[35]
	34 (20–63)	31	[36]
	26 (2)	20	[22]
	28 (n/a)	20	[25]
	41 (12)	25	[21]
	37 (7)	(n/a)	[27]
	37 (7)	30	[24]
	96 (24)	13	[37]
	38 (12)	33	[24]
Diabetic			
IDDM	38 (11)	24	[21]
IDDM and NIDDM	38 (11)	29	[24]
Primary renal disease	29 (2)	22	[22]
Nonsymptomatic Prerenal azotemia	166 (88)	16	[37]
Renal failure			
Acute renal failure			
<10 day	29 (27–35)	11	[25]
	33.7 (24.6–55.8)	b	[36]
>10 days	72 (60–83)	9	[25]
	68.7 (36.0–93.9)	b	[36]
Acute on chronic renal failure	42 (31–67)	22	[24]
Chronic renal failure	137 (10)	40	[22]
	164 (88)	30	[21]
	186 (92)	43	[24]
	148 (122–210)	24	[24]
	115 (34.6–286.5)	53	[36]
Continuous ambulatory peritoneal dialysis			
	127 (8)	24	[22]
	143 (32)	18	[24]
Hemodialysis			
	100 (5)	31	[22]
	131 (42)	27	[24]
	106.5 (38.2)	55	[35]
Adequate dialysis, *adequate PN*[c]	142 (29)	11	[23]
Inadequate dialysis, *adequate PN*	197 (30)	6	[23]
Adequate dialysis, *inadequate PN*	105 (28)	3	[23]
Transplant			
	43 (4)	16	[22]
	76 (33)	16	[24]
Transplant (renal failure)	87 (14)	14	[22]

[a] Data are mean ± SEM [22], median and interquartile ranges [25], median and range [36], mean ± standard deviation [21, 23, 24, 27, 35, 37]
[b] <10 days and >10 days = 37 patients
[c] Protein nutrition

[22, 24, 28, 31], and patients with failed transplants [22]. No differences are seen between CHb in control groups and patients with primary renal disease [22], successful transplants [22, 24], or diabetic patients without renal failure [21, 24, 29]. However, CHb has been found to be capable of differentiating acute from chronic renal impairment [25]. The positive predictive value for this test was 80% with a sensitivity of 100% when the ratio of CHb/creatinine was set at 0.2.

Furthermore, there are significant correlations between carbamylated hemoglobin values compared to urea, creatinine, and time-averaged urea concentration (TAC) measurements [11, 21–24, 27–29], but not with bicarbonate and phosphate values [31].

Table 2. Carbamylated protein values in normal individuals and patients with renal failure

Protein	Normal *μg HC/g protein*[a]	Uremic *μg HC/g protein*	Reference
Total protein			
Carbamyl groups	ND	1247 (284)	[12]
Homocitrulline[a]	~208	~473	[19]
Albumin			
Carbamyl groups	ND	938 (378)	[38]
Homocitrulline[b]	219 (164–329)	737 (55–903)	[14]

For comparison purposes carbamyl groups were assumed to be homocitrulline residues.
Conversion factors used were 69,000 g/mol for HSA and 189 g/mol for homocitrulline.
Values in parentheses are SD's or ranges. ND is not detected.
* μg homocitrulline/g protein.
[a] Estimated from a graph
[b] Calculated from molar ratio's.

Considerably less information is available on the relationship of other carbamylated compounds in renal patients. However, carbamylated plasma protein (CTP) [12, 14, 18, 19], leukocytes [16], erythrocytes, and amino acids [15] in uremic individuals have all been found to be higher compared to normal individuals. Table 2 lists the carbamylated protein values in normal individuals and patients with renal failure who have been reported in the literature.

Measurement of carbamylated protein could therefore provide the clinical information needed as an indicator of uremic control. It could be used to monitor the deterioration of renal function, to assess compliance to dietary therapy, and to evaluate the adequacy of dialysis treatment [23]. The benefit of using carbamylated proteins as markers is that they can be used as alternatives to TAC measurements, particularly in dialyzed patients. The currently accepted method to quantify dialysis therapy and define its adequacy is through a mathematical description of urea balance called urea kinetic modeling (UKM) [39]. The calculation of UKM parameters requires collection of numerous pieces of data, including accurately timed urea measurements, and a computer to solve the simultaneous equations. In comparison, measurement of carbamylated protein requires only a single measurement on one blood sample that could be performed easily in a clinical laboratory.

However, more clinical studies are needed to fully establish the usefulness of carbamylated protein measurement in dialyzed patients, particularly in assessing adequacy of dialysis. Therefore, a six-month longitudinal study was undertaken to evaluate the changes in carbamylated hemoglobin, carbamylated protein, urea, and dialysis dose (Kt/V, a UKM derived parameter) in seven thrice weekly hemodialyzed patients. Furthermore, the relationship between CTP and CHb concentrations in normal individuals and hemodialyzed patients were assessed.

METHODS

Chemicals

N-Carbamyl-DL-valine (CVAL), N-carbamyl-DL-norvaline (CNVal), albumin (~99%), and 4-aminodiphenylamine (semidine) were purchased from Sigma Chemical Co. (St. Louis, MO,

USA). High pressure liquid chromotomographic (HPLC) grade acetonitrile and acetic acid, diacetyl monoxime, and sulphuric acid were purchased from BDH Inc. (Toronto, ON, Canada). 2-Aminobenzoic acid was from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA).

Patients

Seven stable outpatient hemodialyzed patients who were dialyzed thrice weekly in the Renal Dialysis Unit at the Hotel Dieu-Grace Hospitals (Grace site) were evaluated in the longitudinal study (selection based on availability of blood samples for six consecutive months). All patients had been on maintenance hemodialysis for more than one year and had not received any blood transfusions, nor were they on erythropoetin during the course of the study. Approval for the study was given by both the University of Windsor Ethics Committee and the Salvation Army Grace Hospital (now Hotel Dieu-Grace Hospitals), Windsor, Ontario, Canada.

For the cross-sectional study, a random selection of hemodialyzed patients ($N = 13$) and outpatients ($N = 9$) with normal urea and glucose, were chosen for the uremic group and normal group, respectively.

Blood samples

Ethylenediaminetetraacetic acid (EDTA) whole blood and serum were drawn during regular dialysis treatments and the routine tests performed on them. The whole blood samples were further processed for determination of CHb and CTP. These samples were stored overnight at 4°C routinely and up to two weeks in some cases. Although carbamylation does not cease at low temperatures [40], no significant changes in CHb values were found during this time period. This is in agreement with Kwan et al [21] and reflective of *in vitro* urea decomposition studies [41]. Only slight hemolysis (1 to 2%) was seen in a few samples. Red cells were separated from the plasma by centrifugation, washed three times with phosphate buffered saline (PBS) and resuspended in an equal volume of PBS. The plasma and the washed red blood cells were then used immediately or frozen at −20°C until required.

Measurements

Blood tests. Serum samples were analyzed for urea, creatinine, phosphate, total protein (TP), albumin, calcium (total and ionized), potassium, sodium, chloride, carbon dioxide, glucose, alkaline phosphatase, γ-glutamyl transferase, and aspartate aminotransferase (Ektachem 700 or 700XR; Eastman Kodak Co., Rochester, NY, USA). Hemoglobin measurements were made using a total hemoglobin test kit (Sigma Diagnostics Canada, Mississauga, ON, Canada). Low concentrations of protein were measured manually using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada), with human serum albumin as standard.

Preparation of carbamylated albumin. Human serum albumin (99%) was carbamylated by incubation with 100 mM sodium cyanate in 0.1 M phosphate buffer, pH 7.4 at room temperature. The incubation time varied depending on the extent of carbamylation required. Unreacted cyanate was removed from the protein by gel filtration (Bio-Gel P-6G). The extent of carbamylation was estimated with the trinitrobenzene-sulfonic acid assay [42]. Cyanate removal was detected by reaction with 2-aminobenzoic acid [43].

Measurement of protein carbamylation. The determination of protein carbamylation was based on the method of Hunninghake and Grisolia [44]. Plasma samples were first passed through a Sephadex G-25 column (1 × 8.5 cm) to remove urea and other potential interfering small molecules. Briefly, into a 10-ml borosilicate test tube the following was added in order: 1 ml sample or control (albumin or carbamylated albumin), 0.5 ml diacetylmonoxime (3% wt/vol), 0.5 ml H_2SO_4, and 0.5 ml semidine (0.2% wt/vol). The mixture was vortexed and heated for 10 minutes at 90°C. The mixture was then allowed to cool for 5 to 10 minutes (temperature 30° to 60°C). Then, 1 ml of a 0.001 M $FeCl_3$-H_2SO_4 solution was added and mixed thoroughly. The mixture was cooled to room temperature and absorbances read within one hour at 525 nm. The extent of protein carbamylation was expressed in relative units (absorbance/mg protein) since no appropriate standard is available for this method. This assay was found to be linear with amount of carbamylated albumin (CHSA) tested. Carbamylated albumin (74%) was used as the positive control.

In vitro preparation of valine hydantoin. Valine hydantoin (VH) standard was prepared by incubating carbamyl valine (13.5 mg) with 11.6 M HCl (5 ml) for eight hours in a dri-bath set at 120°C and then left overnight at room temperature [45]. The solution was rotoevaporated until only a few drops of yellow liquid remained. The tube was then left under a vacuum overnight to remove the last traces of liquid. The resulting residue was slightly yellowish and had a melting point of 145°C. The residue was redissolved in approximately 3 ml of water and filtered through a 2-μm Acrodisc LC13 polyvinylidene fluoride (PVDF) syringe filter (Pall Gelman Sciences, Inc., Ann Arbor, MI, USA). The filtrate was then left to recrystallize overnight on a watch glass.

Preparation of valine hydantoin from hemoglobin. The preparation of valine hydantoin from hemoglobin was based on the method by Manning et al [45]. A 0.5 ml aliquot of red blood cells was placed into a heavy walled 15 ml screw top test tube. To this was added 5 ml of 2% (wt/vol) HCl in acetone (4°C). The mixture was vortexed, centrifuged for three minutes at 3000 × g and the supernatant discarded. The pellet was washed three times with 5 ml of acetone to remove all the heme. The off-white pellet was then washed with 5 ml of diethyl ether, centrifuged and heated in warm water (*ca.* 45°C) until dry. The pellet was then suspended in 0.5 ml of 50% glacial acetic acid and 0.5 ml of concentrated HCl. The tube was capped with a glass marble and the mixture heated for one hour at 100°C in a dri-bath. The globin is subsequently hydrolyzed. Carbamyl valine residues are released which then spontaneously cyclizes to form valine hydantoins. The hydrolyzate is cooled immediately in an ice bath. To this were added 0.8 ml of 10 N NaOH and 0.5 ml of saturated NaCl and the resulting suspension mixed well. The pH at this point should be between 4 to 5 for optimal extraction. Then 100 μl of the internal standard, 100 mg/ml carbamyl norvaline, was added. The valine hydantoin and carbamyl norvaline are then extracted by adding 5 ml of ethyl acetate and vortexing for one minute. The ethyl acetate supernatant (4 ml) was transferred to a clean 10-ml borosilicate tube and evaporated to dryness under a stream of air in a dri-bath (70°C). The residue was then placed over NaOH pellets in a vacuum dessicator overnight to remove the last traces of acid.

Measurement of valine hydantoin. The method of Kwan et al [21] was adapted for the measurement of valine hydantoin. The mobile

phase (6% acetonitrile) was prepared by first adjusting approximately 900 ml of Type I water (Milli-Q water system; Millipore Corp., Milford, MA, USA) to pH 4.0 with 0.5 M acetic acid, adding 60 ml acetonitrile and diluting to a final volume of 1 liter. The prepared extract was resuspended with 0.5 ml of the mobile phase, filtered through a 2-μm PVDF filter syringe, and 50 μl were autoinjected into a Shimadzu HPLC system (SCL-10A; Shimadzu Corporation, Kyoto, Japan) fitted with a heated (45°C) Apex II reversed-phase column (Jones Chromatography Ltd., Lakewood, CO, USA). The absorbance was read at 210 nm. Each run included two standards, 50 μl of valine hydantoin (10 μg/ml) and 50 μl of carbamyl norvaline (10 μg/ml).

Calculation of hemoglobin carbamylation. Carbamylated hemoglobin, expressed as μg valine hydantoin/g hemoglobin (μg VH/g Hb) was calculated according to the following formula:

$$\text{CHb} = (\text{A} \times 0.5\ \mu\text{g/B} \times \text{B/C} \times 20)/\text{g Hb}$$

where A is the peak height of valine hydantoin (VH) in the sample, B is the peak height of the 50 μl (0.5 μg) CNVal (external standard), and C is the peak height of CNVal in sample (internal standard). The ratio 0.5 μg/B converts the peak height of the sample VH to μg VH, since the absorbance of VH is the same as CNVal at 210 nm. The ratio of B/C is a correction factor for loss during extraction and the value 20 adjusts for the volume injected (50 μl) compared to the total volume (1 ml) prepared from 0.5 ml of washed red blood cells. The amount of Hb in the sample is calculated by multiplying the concentration of Hb (g/liter) in the sample by the sample volume processed (0.5 ml).

The within-run coefficient of variation for the standards was 1.7% for both VH and CNVal. Between-run coefficient of variances for the standards VH and CNVal were 2.7% and 3.6%, respectively. Variation between samples was calculated from three patient samples prepared in duplicate and run in the same batch. The within-run difference was 10.2% for VH and 2.5% for CNVal.

Calculation of dialysis dose (Kt/V). Dialysis dose (Kt/V), was calculated using a preprogramed calculator (Fresenius-Brent, Toronto, ON, Canada) incorporating the variable volume single-pool model developed by Gotch and Sargent [46].

Statistical analysis

Simple correlation calculations were done by using Microcal Origin (Microcal Software Inc., Northhampton, MA, USA). Multiple regression analyses were performed using SAS (SAS Institute Inc., Cary, NC, USA), and weighted correlation coefficients were calculated using BMDP (BMDP Statistical Software Inc., Los Angelas, CA, USA).

RESULTS

Carbamylated hemoglobin and carbamylated protein in normal individuals and hemodialyzed patients

Figure 1 shows the relationship between carbamylated hemoglobin and carbamylated total protein in normal ($N = 9$) and uremic patients ($N = 13$). The overall correlation of carbamylated hemoglobin with carbamylated total protein was highly significant ($P < 0.0001$) with a correlation of 0.87 (0.01). Individually, however, the normal group shows no association between CHb and CTP values ($r = 0.07$, $P = 0.85$), and the uremic group shows a fair association ($r = 0.61$, $P = 0.026$). Furthermore, a significant

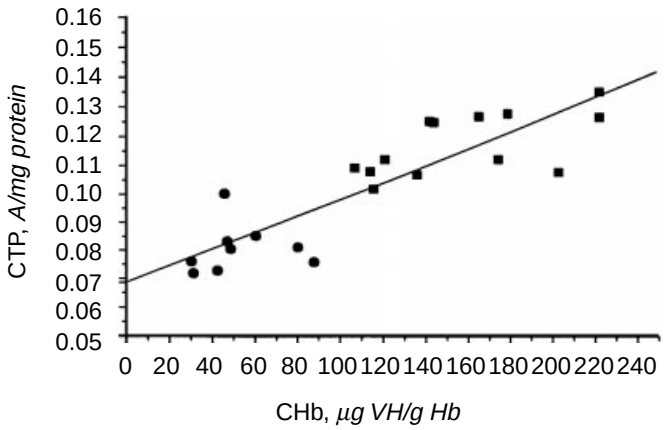

Fig. 1. Correlation between carbamylated plasma protein (CTP) and carbamylated hemoglobin (CHb) in normal and uremic subjects. Relationship between carbamylated hemoglobin and carbamylated total protein in 9 normal (●) and 13 uremic (■) patients. The regression line was $y = 2.9E^{-4}x + 0.069$ ($r = 0.87$, SD = 0.01, $P < 0.0001$). Duplicate determinations were done for each sample.

difference was found between the normal and uremic groups with carbamylated hemoglobin ($P < 0.0001$) and carbamylated total protein ($P < 0.0001$). The mean value for carbamylated hemoglobin in normal individuals was 53 (± 20) μg VH/g Hb, and in uremic patients it was 157 (± 40) μg VH/g Hb. The mean value for carbamylated total protein in normal individuals was 0.08 (± 0.009) A/mg protein, and in uremic patients it was 0.117 (± 0.011) A/mg protein.

The relationship between carbamylated hemoglobin and carbamylated total protein also shows that the y-intercept crosses at 0.069 (absorbance/mg protein). This is a very high y-intercept considering that the highest y-value is only about twice that value. This may suggest that carbamylated total protein can be detected sooner than carbamylated hemoglobin.

Longitudinal study: Correlation with uremic markers

The results of the longitudinal study comparing urea (pre- and post-dialysis), Kt/V, carbamylated protein and carbamylated hemoglobin in seven patients are shown in Figure 2. There did not appear to be any clear relationships, or discernable patterns between any sets of curves that were consistent among all patients.

Because of the very different half-lives of carbamylated protein (3 weeks) and carbamylated hemoglobin (2 months), correlations done at single times are of little value in assessing the relative clinical utility of the two assays. Furthermore, it is difficult to compare parameters that have different measurement scales. To accommodate these obstacles another approach was taken to analyze the data. The data were recalculated in terms of relative percent change over time, that is, the initial time interval was set to 0% change and each subsequent value was expressed relative to the previous value in the time series. The mean and SD were calculated for each test using the mean relative percent value for each patient. Kt/V values varied the least (6.3% ± 3.3%) over this time period, whereas CHb varied nearly five times more (30.1% ± 20.2). Pre-dialysis urea and CTP values had similar relative fluctuations (18.3% ± 13.4% and 14.9% ± 7.5%, respectively),

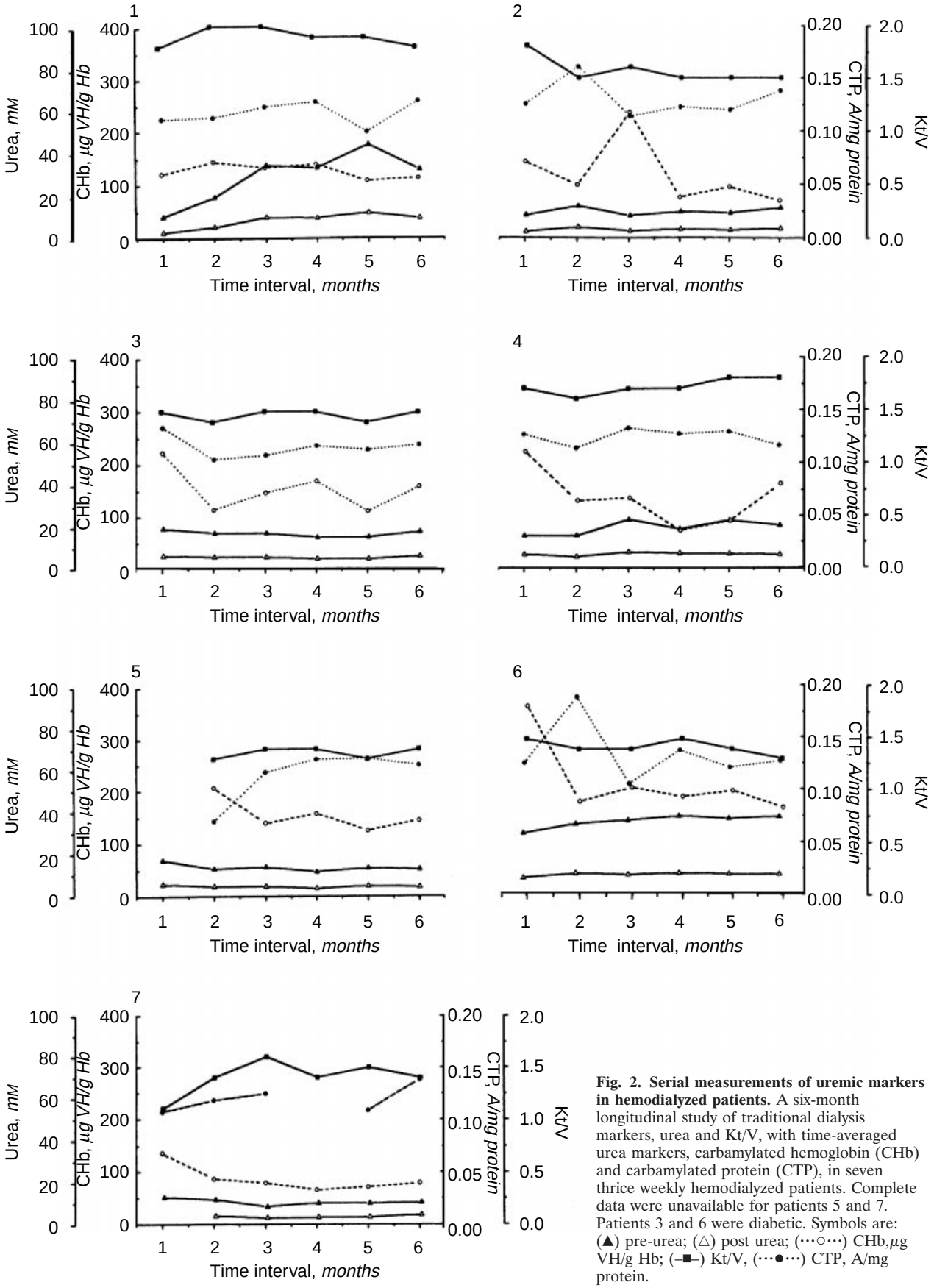

Fig. 2. Serial measurements of uremic markers in hemodialyzed patients. A six-month longitudinal study of traditional dialysis markers, urea and Kt/V, with time-averaged urea markers, carbamylated hemoglobin (CHb) and carbamylated protein (CTP), in seven thrice weekly hemodialyzed patients. Complete data were unavailable for patients 5 and 7. Patients 3 and 6 were diabetic. Symbols are: (▲) pre-urea; (△) post urea; (···○···) CHb,µg VH/g Hb; (–■–) Kt/V, (···●···) CTP, A/mg protein.

between those of Kt/V and carbamylated hemoglobin values. This indicates that CHb is a more sensitive in monitoring urea fluctuations than either pre-dialysis urea, CTP, or the dialysis dose, Kt/V.

Correlation calculations (using the relative % change data) did not seem to reveal much more in terms of relationships than did the visual observations of the initial data. No correlations between any two tests were seen in most cases. Furthermore, the correlations that did exist between any two tests were mixed, positive or negative, and in very few cases were these correlations significant ($P < 0.05$). The reason for these apparent ambiguities could be due to the nature of the tests, that is, they do not measure the same thing at the same time, or the lack of a larger sample size (frequency of sampling and number of patients).

Correlations between pre-dialysis urea and CHb, and pre-dialysis urea and CTP on the data sets ($N = 7$) were done for each time period ($N = 6$). There was a much higher association between pre-dialysis urea and CHb ($r = 0.97, 0.86, 0.71, 0.52, 0.51, 0.40$) than with pre-dialysis urea and CTP ($r = 0.48, 0.77, -0.11, 0.66, 0.26, 0.08$).

To achieve greater statistical power, multiple regression analysis was done to statistically answer the question as to whether there is a relationship between any two tests within a patient. The overall correlation coefficient was obtained by removing the variation due to individual subjects [47]. No significant correlation was found with any combination of tests, which is probably a reflection of the variation in the correlations of the individual patients, that is, regression to the mean.

Furthermore, the data were also looked at in terms of between subject correlation. That is, to answer the question whether on average do patients with high (or low) values for test A also tend to have high (or low) values for test B. The weighted correlation coefficients were determined between various tests using the subject means. The significance of the correlation was based on the number of subjects ($N = 7$) and not the number of data pairs ($N = 40$) because of the repeated observations per subject [48]. Significant correlations (at $\alpha = 0.05$, $r = 0.6786$) were found between CHb versus CTP ($P = 0.707$) and CHb versus pre-dialysis urea ($P = 0.671$). The correlation between CTP versus pre-dialysis urea was almost significant ($P = 0.534$), and there was a very poor correlation between Kt/V versus pre-dialysis urea ($P = 0.278$). The other comparisons done were between Kt/V versus CTP or CHb ($P = -0.327$ and -0.387, respectively).

Laboratory tests for the seven hemodialyzed patients showed that all had low hemoglobin concentrations and low hematocrits. Albumin values were within the reference interval and pre-dialysis urea values were above the reference interval. Post-dialysis urea values were all lower than pre-dialysis urea values and in some patients within the reference range. Creatinine concentrations were all well above the reference interval before dialysis and decreased after dialysis, but remained above the reference interval. Glucose was elevated only in the two diabetic patients. Values for potassium, sodium, chloride, carbon dioxide, glucose, calcium (total and ionized), phosphate, alkaline phosphatase, γ-glutamyl transferase, and aspartate aminotransferase were in the reference range for most patients. No relationships were evident in cases where there were elevated levels of an analyte when compared to CHb, CTP or Kt/V values.

DISCUSSION

Carbamylated hemoglobin and carbamylated plasma protein in normal individuals and hemodialyzed patients

The correlation between carbamylated hemoglobin and carbamylated total protein concentrations in normal individuals and uremic subjects (Fig. 1) showed a high correlation ($r = 0.87$). Furthermore, the y-intercept showed a bias in favor of carbamylated total protein. Similarly, in glycation studies the bias favors fructosamine and corroborates other studies that show fructosamine as a shorter term indicator of glycemic control compared to glycated hemoglobin [49].

Since assay methodology could also influence this bias, a general idea for how much carbamylation (number of carbamyl groups independent of site or rate of attachment) occurs on albumin versus hemoglobin was obtained by interpolating data from two representative studies [14, 21]. The amount of carbamylation was assessed by comparing homocitrulline residues for albumin and carbamyl valine residues for hemoglobin. This comparison (molar ratio) showed that there was fivefold more carbamyl groups on albumin [0.08 (0.06 to 0.12)] as compared with hemoglobin (0.016 ± 0.005) in normal individuals. Similarly, there were also more carbamyl groups on albumin [0.027 (0.02 to 0.033)] than on hemoglobin (0.066 ± 0.035) in patients with renal failure (4-fold). Therefore, the potential for carbamylation is greatest for plasma proteins where the concentration of urea and cyanate are the highest.

Longitudinal study

This is the first longitudinal study that has looked at the changes in carbamylated hemoglobin and carbamylated protein in hemodialyzed patients. Parameters of dialysis dose (Kt/V), pre- and post-dialysis urea, CHb and CTP for all seven patients were measured and plotted with respect to the time interval (1 month). Visual observation of these parameters did not show any strong associations or trends (Fig. 2).

Although the correlations between pre-dialysis urea and CHb were high for most time periods, they did not agree and few were significant. The large range in correlation values (0.21, 0.51, 0.52, 0.71, 0.86, 0.98) suggests that there is no consistent relationship between pre-dialysis urea concentration and CHb. This discrepancy is probably due to the small sample size ($N = 7$) or could also be reflecting the large fluctuations in urea generated by dialysis. Studies with larger sample sizes have shown inconsistent relationships as well. Kwan et al [21] showed a correlation of 0.69 ($N = 20$) in hemodialysis patients for pre-dialysis urea versus CHb. However, Smith et al [22], using a similar method, showed a correlation of only 0.44 ($N = 31$) in hemodialysis patients. They also found higher correlations in all other groups studied (control $r = 0.55$, $N = 20$; chronic renal failure $r = 0.75$, $N = 40$; continuous ambulatory peritoneal dialysis $r = 0.55$, $N = 24$; transplant rejection $r = 0.87$, $N = 14$). Another study reported a correlation of 0.84 in a combined group of normal and uremic subjects [19].

The correlations between pre-dialysis urea and CTP among the six time periods were very poor and widespread ($-0.38, -0.11, 0.07, 0.48, 0.66, 0.77$). This variation could also be due to the same factors as discussed above.

In general, the half-lives of carbamylated proteins (CTP) and carbamylated hemoglobin (CHb) can be assumed to be similar to

the half-lives of plasma proteins and hemoglobin (3 weeks and 2 months, respectively). Therefore, detecting a relationship between the CTP and CHb values when measurements are taken only every 30 days is therefore difficult (Fig. 2). If time points had been available weekly, the possibility of detecting a trend may have increased. Also, since the patients studied were in good control already (dialysis regime and diet), very little variation or significant difference in any individual test was seen. Untreated patients or patients on continuous ambulatory peritoneal dialysis may have shown more suggestive results.

This longitudinal study showed no clear relationship between pre-dialysis urea or Kt/V and CTP or CHb measurements. Because hemodialysis patients are maintained on a fairly stable dialysis regime, the utility of measuring carbamylated proteins may be limited. However, they may be of value if concentrations are above a predetermined cutoff value or a pattern emerges over time. Kwan et al [23] reported that CHb concentrations $< 175\ \mu g$ CVal/g Hb indicated adequate dialysis.

The interpretation of carbamylated hemoglobin concentration can be complicated by the anemia in uremia, the shortened life-span of erythrocytes, and transfusions that dilute the erythrocyte mass and the CHb concentration. Measurement of CTP, however, would not be accompanied by these same problems. The plasma protein content is relatively stable in patients with end-stage renal disease, although lower than normal (nutritional effect) [50].

Nonetheless, measurement of carbamylated hemoglobin may reflect a therapeutic time interval that would be appropriate in the clinical management of the uremic outpatient. Carbamylated protein concentration, an index of integrated urea levels over the preceding two weeks, may reflect adequacy of dialysis over too short a time interval to be of much practical use in and outpatient setting. However, in cases of acute renal failure the estimation of carbamylated protein may be more appropriate than carbamylated hemoglobin [25].

In summary, to our knowledge this study has for the first time shown the relationship between CHb and CTP in hemodialyzed patients. It has also found no correlation between these measurements and Kt/V, the currently used, but poorly evaluated [39], indicator of morbidity and mortality in the hemodialyzed patient. Therefore, the data suggest that CHb and CTP are unique indicators of uremia, and their potential value necessitates their examination in a valid outcome study.

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APPENDIX

Abbreviations used in this article are: A/mg protein, absorbance/mg protein; CHb, carbamylated hemoglobin; CHSA, carbamylated human serum albumin; CNVal, N-carbamyl-DL-norvaline; CTP carbamylated total protein; CVal, N-carbamyl-DL-valine; EDTA, ethylenediaminetetraacetic acid; Hb, hemoglobin; Kt/V, dialysis dose; PBS, phosphate buffered saline; TAC, time averaged urea concentration; UKM, urea kinetic modeling; VH, valine hydantoin.

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