CLIN. CHEM. 36/4, 607–610 (1990)

Determination of Carbamylated Hemoglobin by High-Performance Liquid Chromatography

Jonathan T. C. Kwan,[1,3] Edwin C. Carr,[2] Michael R. Bending,[1] and Jeffrey L. Barron[2]

We have developed an HPLC method for measuring carbamylated hemoglobin (CarHb), based on the quantification of valine hydantoin formed from the released NH_2-teminal carbamyl valine residue after acid hydrolysis of hemoglobin. In uremia, CarHb is produced by nonenzymatic post-translational modification of the terminal amino group of hemoglobin monomers by isocyanic acid, derived from the spontaneous dissociation of urea. We measured CarHb in 25 nonuremic control subjects, 24 nonuremic diabetic subjects, and 30 patients with stable chronic renal failure. There was no significant difference between the controls and diabetic patients, their mean (SD) CarHb values being 41 (11.5) and 38 (10.8) μg of carbamyl valine per gram of hemoglobin (μgCV/gHb), respectively. Mean (SD) CarHb values in the uremic patients were much greater, 164 (87.7) μgCV/gHb. There was significant correlation between the concentrations of CarHb and plasma urea in the uremic subjects. Thus CarHb provides a urea-derived index of chronic uremia.

Additional Keyphrases: *uremia · diabetes · chromatography, reversed-phase · carbamyl valine*

The need for monitoring cyanate therapy in patients with sickle cell anemia has led to the development of methods for quantifying carbamylated hemoglobin (CarHb) (*1*).[4] Carbamylation of the NH_2-terminal valine residue of hemoglobin S with isocyanic acid, the reactive form of cyanate in aqueous solution, prevents the gelling of deoxygenated hemoglobin and the sickling of the erythrocytes (*2*) and lengthens the survival of erythrocytes (*3*). Like glycation, carbamylation of hemoglobin is a nonenzymatic post-translational chemical process. In patients with uremia, CarHb is formed from the reaction of isocyanic acid, derived from the spontaneous dissociation of urea in plasma, and the NH_2-terminal valine residue of hemoglobin monomers (Figure 1) (*4*).

In an earlier study, the concentrations of carbamylated hemoglobin S were quantified by amino acid analysis of an alkaline hydrolysate of valine hydantoin, derived from CarHb (*3*). This method involved time-consuming ion-exchange and evaporation procedures and was therefore unsuitable for routine clinical use.

A gas–liquid chromatographic (GLC) method for measuring valine hydantoin released by acid hydrolysis of globin extracts of whole blood involved a labor-intensive globin-purification step, so that only about 40 samples could be processed in one week (*1*). Using this method, Flückiger et al. (*4*) demonstrated increased concentrations of CarHb in uremic patients. They suggested that, in analogy with glycated Hb in diabetic patients, CarHb might give a time-integrated, urea-derived index of chronic uremia.

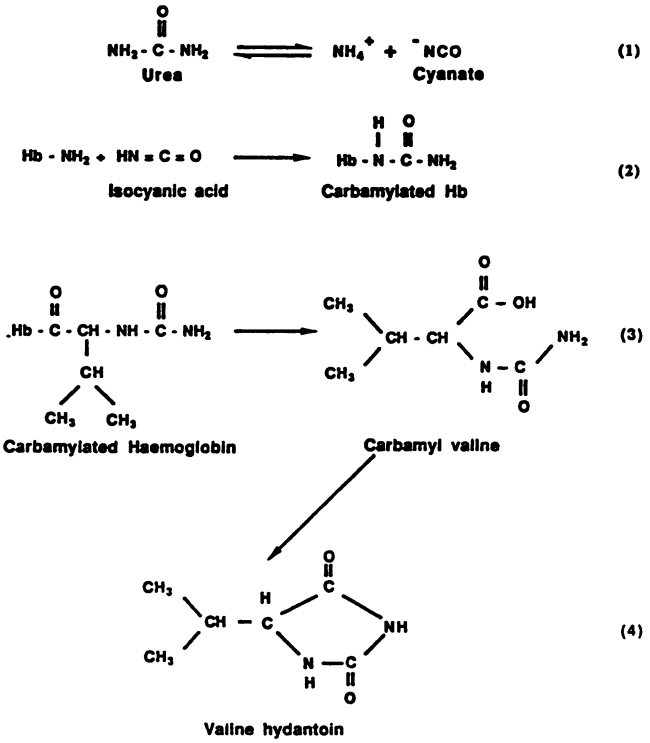

Fig. 1. Schematic representation of hemoglobin carbamylation

1) Urea spontaneously dissociates to form ammonium and cyanate ions. 2) Terminal amino group of hemoglobin reacts with isocyanic acid to form a carbamyl derivative. 3) Hydrolysis of carbamylated hemoglobin releases the NH_2-terminal carbamyl valine residue. 4) Carbamyl valine spontaneously transforms to valine hydantoin in acid

More recently, Smith et al. (*5, 6*), using a slightly modified, but no less time-consuming, GLC method, reported the values of CarHb in normal, diabetic, and uremic patients receiving different modes of treatment. They also suggested the possibility that protein carbamylation could have a pathophysiological role in the mechanism of uremic toxicity.

Here we describe a rapid, precise, and reproducible HPLC method for measuring CarHb, based on the release of carbamyl valine from the NH_2-terminals of α and β chains of Hb by acid hydrolysis and the subsequent quantification of the spontaneously produced valine hydantoin.

Materials and Methods

Apparatus

Our chromatographic system consisted of a ConstaMetric 3000 HPLC pump and a SpectroMonitor 3100 UV detector (Milton Roy, Stone, U.K.). A 4.6 mm × 250 mm 5-μm particle size octadecyl silica-bonded reversed-phase column (Apex II) was maintained at 45 °C in a dry heat-block equipped with a Rheodyne 7125 valve (Jones Chromatog-

[1] South West Thames Regional Renal Unit, and [2] Department of Clinical Chemistry, St. Helier Hospital, Carshalton, Surrey SM5 1AA, U.K.

[3] Current address and address for correspondence: Department of Nephrology, The London Hospital, Whitechapel, London E1 1BB, U.K.

[4] Nonstandard abbreviations: Hb, hemoglobin; CarHb, carbamylated hemoglobin; CV, carbamyl valine; and GLC, gas–liquid chromatography(ic).

Received November 29, 1989; accepted January 11, 1990.

raphy, Hengoed, Wales, U.K.). The chromatograms were recorded with an SP 4290 Integrator (Spectra-Physics, Hemel Hempstead, U.K.). A Dri-Block DB3 dry heat-block (Techne, Duxford, U.K.) with adjustable temperature up to 110 °C and a detachable SC-3 Sample Concentrator (Techne) were used for both hydrolysis and evaporation of the solvent extracts.

Reagents

Analar-grade concentrated HCl (11 mol/L), acetic acid (17 mol/L), NaOH (10 mol/L), anhydrous sodium bicarbonate, HPLC-grade water, ethyl acetate, and acetonitrile were obtained from BDH (Poole, U.K.). *N*-Carbamyl-D,L-valine (32 mg/L) and *N*-carbamyl-D,L-norvaline (96 mg/L) (Sigma Chemicals Ltd., Poole, U.K.) in HPLC-grade water were used as standard and internal standard, respectively.

Samples

We analyzed 2-mL EDTA-treated and 5-mL heparinized blood samples, collected by venepuncture into Vacutainer Tubes (B-D Vacutainer Systems, Oxford, U.K.). Hb was measured in the EDTA sample with an H6000 Cell Counter (Technicon Instruments, Basingstoke, U.K.); urea and creatinine were estimated in an aliquot of the heparinized plasma sample by standard procedures with an Astra 8 analyzer (Beckman Instruments, High Wycombe, U.K.).

Procedures

Sample preparation. We pipetted 0.5 mL of heparinized blood into a 10-mL plastic-topped plain glass tube (LIP Equipment Ltd., Shipley, U.K.), washed this with 10 mL of isotonic (9 g/L) sodium chloride, centrifuged it at 3000 × *g* for 10 min, and discarded the supernate. The washed samples were stored at −20 °C until required for analysis. Alternatively, if there was any question of hemolysis, we stored a 0.5-mL aliquot of the heparinized blood sample without washing and used it when required.

Hydrolysis and extraction. We hydrolyzed the 0.5-mL sample by adding 1 mL each of 11 mol/L HCl and 17 mol/L acetic acid and heating for 2 h at 110 °C. We cooled the hydrolysate in cold water and then added 2 mL of 10 mol/L NaOH to it, bringing the pH up to about 4, followed by 100 μL of the internal standard solution. After adding 5 mL of ethyl acetate to the hydrolysate and shaking for 1 min, we centrifuged the sample at 5000 × *g* for 10 min. We took 4.5 mL of the supernate to shake with 2 mL of 1 mol/L $NaHCO_3$ for 2 min, and then centrifuged it at 3000 × *g* for 5 min. We evaporated the solvent from 4 mL of this supernate under a stream of air at 70 °C. The resulting extracts were stable when stored desiccated.

Chromatography and assays. We reconstituted each extract with 0.5 mL of mobile phase: HPLC-grade water containing 60 mL of HPLC-grade acetonitrile and 1 mL of 17 mol/L acetic acid per liter (pH about 4). The sample volume injected was 50 μL, the pump speed 1.5 mL/min, the detection wavelength 210 nm, and the sensitivity 0.05 *A* full-scale. Each assay run included two standards (100 and 200 μL of 32 mg/L carbamyl valine) and two quality-control specimens.

Stability study. Aliquots (0.5 mL) of both washed and unwashed whole-blood samples from one subject were left at room temperature for 0, 1, 2, 4, 7, 10, and 14 days. The samples were then stored at −20 °C and were later assayed in the same run.

Analytical variables. The optimal pH for extraction was determined by extracting valine hydantoin and carbamyl norvaline from hydrolysates in the pH range of 2 to 6. The analytical recoveries of both valine hydantoin and carbamyl norvaline were determined by comparison with results of the unextracted solutions. The linearity of the method was checked against concentrations of valine hydantoin and carbamyl norvaline from 0 to 14 mg/L. Possible interference from plasma constituents was tested by assaying plasma samples from five different subjects.

Calculation of results. CarHb, expressed as micrograms of carbamyl valine per gram of hemoglobin (μgCV/gHb), was calculated from the following formula:

$$\text{CarHb} = \frac{a \times d \times 3.2 \times 100}{b \times c \times 0.5 \times \text{Hb}}$$

where a is the peak height of the sample, b is the peak height of internal standard in the sample injection, c is the peak height of the 100-μL (3.2 μg) carbamyl valine standard, d is the peak height of the internal standard in the standard injection, 0.5 mL is the whole-blood sample size, and Hb is the hemoglobin concentration (g/100 mL) in the sample. CarHb could also be expressed as % carbamyl Hb in the sample of Hb, according to this formula:

$$\%\ \text{carbamyl Hb} = \mu\text{gCV/gHb} \times 0.04$$

Results

Figure 2 shows specimen chromatograms of the standards (*a*), washed sample (*b*), and unwashed whole-blood sample (*c*). The retention time of valine hydantoin is around 5 min and that of the internal standard, carbamyl norvaline, is around 6 min. In most of the patients' specimens, no significant peaks were detected at the retention time of carbamyl valine (3.6 min), thus showing total conversion of carbamyl valine to valine hydantoin. The presence of several early- and late-eluting peaks in the whole-blood sample did not significantly interfere with the quantification. However, the use of unwashed whole blood considerably increased column fouling.

Extraction buffer pH. Extraction for both valine hydantoin and carbamyl norvaline was optimal within pH 3–5.

Precision. The within-run CVs for CarHb concentrations of 100 and 328 μgCV/gHb were 7% (n = 16, SD = 7 μgCV/gHb) and 5% (n = 22, SD = 16 μgCV/gHb), respectively. The corresponding between-run CVs were 8% (n = 17, mean = 102, SD = 8.2 μgCV/gHb) and 6% (n = 24, mean = 326, SD = 20.3 μgCV/gHb), respectively.

Analytical recovery. The mean analytical recoveries of valine hydantoin and carbamyl norvaline were 68.8% (n = 4) and 64.0% (n = 4), respectively.

Linearity, detection limit, and interference. The results of the method varied linearly with concentrations of valine hydantoin and carbamyl norvaline in the range of 0 to 14 mg/L, the full range of CarHb concentrations likely to be encountered in clinical practice. The lowest detection limit was determined to be 1 μgCV/gHb, but this could vary by altering the recorder sensitivity or injection volume. No interference in the chromatograms by plasma constituents was apparent.

Sample storage. Storage of heparinized whole-blood samples or washed cells for 14 days at 21 °C did not significantly alter the concentrations of CarHb.

Clinical studies. Carbamylated hemoglobin was mea-

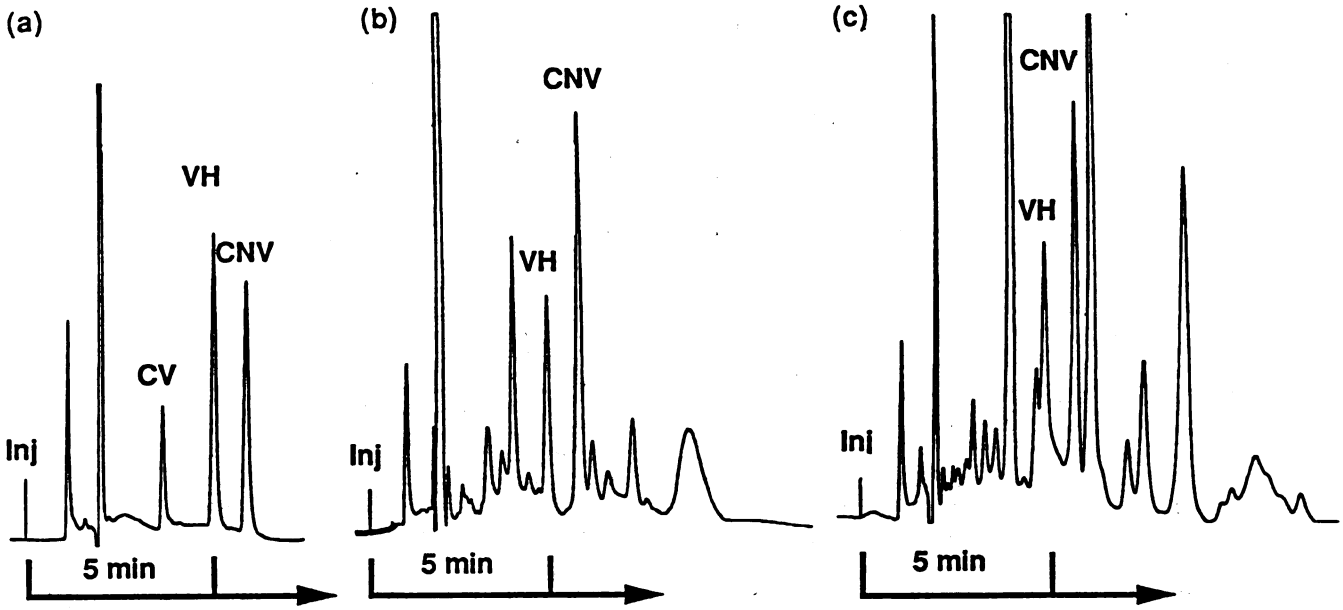

Fig. 2. HPLC tracings of (*a*) standards with carbamyl valine added to the final extract, (*b*) washed blood sample, and (*c*) unwashed whole-blood sample

CV = carbamyl valine, VH = valine hydantoin, and CNV = carbamyl norvaline

sured in 25 nondiabetic subjects (12 men, 13 women) with normal renal function (controls), 24 insulin-dependent diabetic patients (12 men, 12 women) with normal renal function, and 30 patients (18 men, 12 women) with stable chronic renal failure who were not on renal replacement therapy (Table 1). In the control subjects the concentrations of CarHb ranged from 17 to 59 μgCV/gHb (0.7% to 2.4% carbamyl Hb). The diabetic patients had concentrations similar to the normal subjects, 20 to 63 μgCV/gHb (0.8% to 2.5% carbamyl Hb); however, the concentrations of CarHb in uremic patients were much higher. Figure 3 shows the correlation of CarHb with plasma urea and creatinine in the uremic patients.

Discussion

The purpose of this study was to develop a rapid, practical, specific, and precise method for measuring CarHb. To the best of our knowledge, this is the first HPLC method described for this. A trained laboratory scientist who is familiar with HPLC techniques can easily handle 20 samples in the course of one working day, a throughput unlikely to be attained by the previously described GLC methods.

It is not surprising that the concentrations of CarHb in the controls and diabetic patients are similar, because carbamylation depends on the presence of cyanate derived from urea in plasma. The plasma urea was not significantly different in both of these groups of patients. CarHb causes

Table 1. Age, Plasma Urea, Plasma Creatinine, and Carbamylated Hemoglobin in Three Groups of Subjects (Mean ± SD)

	Subjects[a]		
	Controls	IDDM	CRF
No. of patients	25	24	30
Age, years	58 ± 13.0	36 ± 10.3	55 ± 15.6
Plasma urea, mmol/L	5.0 ± 1.6	5.3 ± 1.5	20 ± 7.6
Plasma creatinine, μmol/L	78 ± 14	80 ± 15	400 ± 175
Carbamylated hemoglobin, μgCV/gHb	41 ± 11.5	38 ± 10.8[b]	164 ± 87.7[c]

[a] IDDM, insulin-dependent diabetic patients with normal renal function; CRF, patients with stable chronic renal failure.

[b] Not significantly different from controls ($P = 0.284$, Student's *t*-test).

[c] Significantly different from controls ($P < 0.0001$, Student's *t*-test)

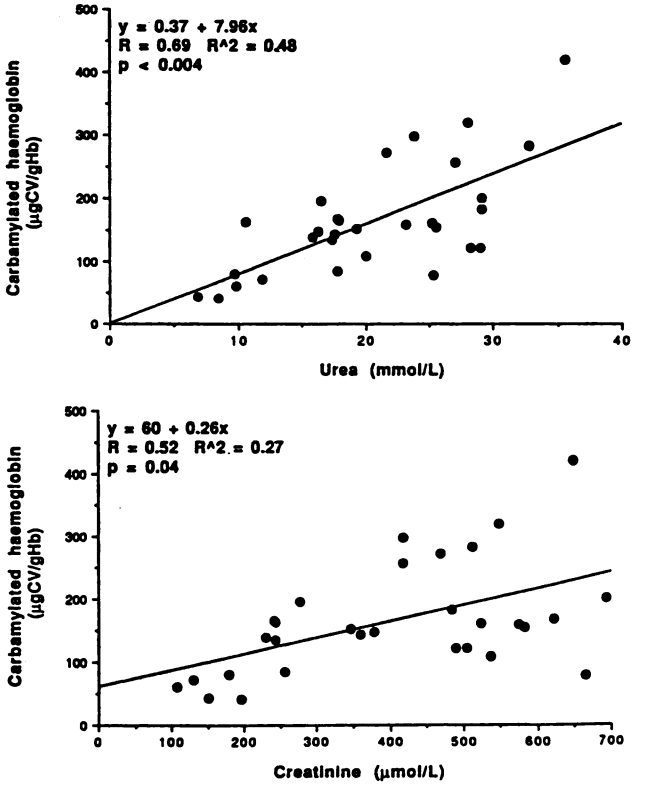

Fig. 3. Correlation of carbamylated hemoglobin with plasma urea (*top*) and plasma creatinine (*bottom*) in 30 patients with stable chronic renal failure

interference with glycated Hb methods that are based on charge separation of Hb species (*4*), which might explain why higher concentrations of glycated Hb were found in uremic patients (*7–9*). In this study diabetic patients had the same range of concentrations of CarHb as that of the controls, indicating no methodological interference from glycated Hb.

Flückiger et al. (*4*) found a strong correlation of CarHb with time-averaged urea concentration in their uremic subjects. This finding was supported by our observation of significant correlation between CarHb and urea in uremic patients with stable renal function (Figure 3). A much weaker correlation, however, was seen between CarHb and plasma creatinine (Figure 4). Therefore, CarHb is more likely to be a urea-derived index of uremia than a good index of renal function.

In previous communications the concentrations of CarHb were expressed as number of carbamyl groups per molecule of hemoglobin (*1*), molar ratio (*4*), and nanograms of valine hydantoin per milligram of globin (*5, 6*). In our method, CarHb is expressed as μgCV/gHb. These methods are therefore not directly comparable. However, the concentrations of CarHb seen in the subjects of Smith et al. (*5, 6*) were of the same order of magnitude as ours. The quantification of CarHb in our method also relies in part on the precision of Hb measurement, but a CV <5% for Hb is achievable by most modern instruments.

The method we described is sensitive, specific, and precise, requiring only minimal sample preparation and standard HPLC equipment. Blood specimens were stable up to 14 days even when stored at room temperature. With this HPLC method, we have commenced longitudinal studies of the kinetics of hemoglobin carbamylation and its relevance to the pathophysiology of uremia.

References

1. Manning JM, Lee CK, Cerami A, Gillette PN. Gas chromatographic determination of the carbamylation of hemoglobin S by cyanate. J Lab Clin Med 1973;81:941–5.
2. Cerami A, Manning JM. Potassium cyanate as an inhibitor of the sickling of red blood cells in vitro. Proc Natl Acad Sci USA 1971;68:1180–3.
3. Gillette P, Manning JM, Cerami A. Increased survival of sickle cell erythrocytes after treatment in vitro with sodium cyanate. Proc Natl Acad Sci USA 1971;68:2791–3.
4. Flückiger R, Harmon W, Meier W, Loo S, Gabbay K. Hemoglobin carbamylation in uremia. N Engl J Med 1981;304:823–7.
5. Smith WGJ, Holden M, Benton M, Brown CB. Carbamylated haemoglobin in chronic renal failure. Clin Chim Acta 1988; 178:297–304.
6. Smith WGJ, Holden M, Benton M, Brown CB. Glycosylated and carbamylated haemoglobin in uraemia. Nephrol Dial Transplant 1989;4:96–100.
7. Stanton KG, Davies R, Richmond J. Glycosylated haemoglobin-A in renal failure [Letter]. Lancet 1978;i:100.
8. de Boer M-J, Miedema K, Casparie AF. Glycosylated haemoglobin in renal failure. Diabetologia 1980;18:437–40.
9. Zawada ET Jr, Virji A. Glycosylated haemoglobin test in diabetic and non-diabetic haemodialysis patients. Dial Transplant 1980;9:360–2.