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Heterogeneity of hemoglobin A_{1d}: assessment and partial characterization of two new minor hemoglobins, A_{1d3a} and A_{1d3b}, increased in uremic and diabetic patients, respectively

E. Bissé[a,*], P. Huaman-Guillen[a], P. Hörth[b], A. Busse-Grawitz[a], M. Lizama[a], A. Krämer-Guth[c], W. Haehnel[b], H. Wieland[a]

[a]*Department of Clinical Chemistry, University Hospital, Albert-Ludwigs-Universität, Hugstetter Str. 55, D-79016 Freiburg, Germany*
[b]*Institut für Biologie II der Universität Freiburg, Freiburg, Germany*
[c]*Department of Internal Medicine, University Hospital, Albert-Ludwigs-Universität, Hugstetter Str. 55, D-79016 Freiburg, Germany*

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Abstract

We have separated and quantified two new minor hemoglobins named HbA_{1d3a} and HbA_{1d3b}. The level of HbA_{1d3a} was significantly higher in uremic than in non-uremic patients (3.00±0.50% vs. 1.28±0.26% of total hemoglobin). It correlated well with carbamylated hemoglobin ($r=0.80$, $n=81$, $p<0.002$) and with plasma urea concentration ($r=0.78$, $n=81$, $p<0.002$). These data and the electrospray ionization mass spectrometric analysis provide strong evidence that HbA_{1d3a} is an α-chain modified by carbamylation. The HbA_{1d3b} level in diabetic patients was found to be 1.6-fold that in non-diabetic subjects (3.00±0.49 vs. 1.90±0.33). This was attributed to HbA_{1d3} modified by glycation. Indeed HbA_{1d3b} correlated significantly with HbA_{1c} ($r=0.71$, $p<0.002$) and with serum glucose level ($r=0.62$, $p<0.002$). These two new minor hemoglobins may serve as complements for the objective assessment of averaged long-term uremia and glycemia in uremic and diabetic patients.

Keywords: Hemoglobin A_{1d3a}; Hemoglobin A_{1d3b}

1. Introduction

Several minor hemoglobin species (HbA_{1a-e}) in human red cell hemolysate are post-translational modifications of HbA_0. HbA_{1c} has attracted considerable interest because its level in patients with diabetes mellitus [1–4] is found to be 2- to 3-fold higher than that in normal subjects. Accordingly, it has been extensively studied and characterized [5]. HbA_{1d3} is the most abundant minor hemoglobin next only to HbA_{1c}. It constitutes about 3.5% of the total hemoglobin in normal subjects. Abraham and coworkers [6] were the first to demonstrate that HbA_{1d3} has a modified α-chain and has a decreased oxygen affinity in the presence of 2,3-diphosphoglycerate. This indicated that HbA_{1d3} is functionally different from HbA_{1c}. These workers found that this minor hemoglobin contains significantly less ketoamine than HbA_{1c}. The clinical significance of HbA_{1d3}, however, has not yet been demonstrated. Recently, we [7] reported a linear increase of HbA_{1d3} with increasing urea serum level in uremic patients. We

*Corresponding author.

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found that the HbA_{1d3} level in subjects with normal renal function was 3.46±0.43%. Uremic patients showed a mean value of 5.68±1.22%. The increase of this hemoglobin fraction could not be detected by affinity chromatography based on the affinity of *m*-aminophenyl boronic acid for the *cis*-diol groups present in the glucose portion of hemoglobin. Therefore, we suggested that the increase of HbA_{1d3} was related mainly to carbamylation of hemoglobin by an urea-derived reactant. We have also shown that HbA_{1d3} consists of two components. The separation of these components was not reproducible by the chromatographic conditions we used [8]. The aim of the present study was to develop a chromatographic method which achieves a reproducible and accurate separation of HbA_{1d3} in two peaks, i.e HbA_{1d3a} and HbA_{1d3b}. In addition, HbA_{1d3a} was isolated and partially characterized by combining high-performance liquid chromatography (HPLC) and electrospray ionization mass spectrometry (ESI-MS). The levels of these components are compared with the levels of carbamylated hemoglobin (CarHb) in normal subjects and uremic patients. CarHb was quantified by measuring the valine hydantoin (VH) formed by acid hydrolysis of hemoglobin. The method used was similar to the procedure of Kwan et al. [9] with some modifications.

2. Experimental

2.1. Subjects

The study-group consisted of 31 normal volunteers and 50 uremic patients. Control subjects were diabetes-free and had normal renal function. The basic criterion qualifying the patient for the study was a diagnosis of renal failure. The patients exhibited a broad range in mean serum urea levels in the preceding four weeks. The mean values of serum urea and creatinine in the preceding two weeks were 26.0±8.9 mmol/l and 709.0±392.5 μmol/l, respectively.

2.2. Sample preparation

Blood samples were collected in tubes containing EDTA as anticoagulant. Red cells were isolated by centrifugation and washed three times with an equal volume of isotonic saline (150 mmol/l NaCl). For measuring HbA_{1d3} the samples were prepared as previously reported [8]. Samples for quantifying CarHb were prepared according to the method of Kwan et al. [9] with the following modifications. Fresh hemolysates of washed packed cells were prepared using the freeze–thaw method and centrifugation (3000 *g*, 15 min) to separate red cell stroma. The hemoglobin concentration was adjusted to 100 g/l with distilled water. To split off VH, 500 μl of hemolysate were incubated in 1 ml hydrochloric acid (11 mol/l) and 1 ml acetic acid (17 mol/l) at 110°C for 2 h. After adding 10 μg of internal standard (carbamyl norvaline) the extraction was performed with 5 ml ethyl acetate using a home made 8-ml separating funnel. The ethyl acetate (4.5 ml) was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 500 μl of mobile phase and 40 μl were used for the chromatography.

2.3. Analytical methods

2.3.1. Hemoglobin separation and quantitation by HPLC

Hemoglobins were measured by cation-exchange chromatography using a PolyCATA column (200× 9.4 mm I.D.). The method was essentially the same as previously reported [8]. A sample of 350 μg Hb in red cell lysate was injected onto the column. For the characterization of HbA_{1d3a} and HbA_{1d3b} the respective fractions were collected and concentrated using membrane filtration.

2.3.2. HPLC for measuring carbamylated Hb

The high-performance liquid chromatograph was equipped with an autosampler, a spectrophotometric detector and a computing integrator as previously described [10]. The analytical column consisted of stainless-steel (125×3 mm I.D.) filled with 5 μm C_{18} Multospher material (Ziemer, Mannheim, Germany). The column was protected by a guard column (46×4 mm I.D.) packed with Cyclobond I resin (ICT, Frankfurt, Germany). The method was calibrated using carbamyl valine (CV) and carbamyl norvaline (CNV) as previously reported [9]. A sample volume of 40 μl was injected. VH and CNV were chromatographed at room temperature by isocratic elution at a

flow-rate of 0.7 ml/min. The mobile phase was 3% aqueous acetonitrile which was acidified to pH 4 with acetic acid.

2.4. Calculation and statistical analysis

The carHb level was expressed as μg carbamyl valine per g hemoglobin (μg CV/g Hb). The results are given as mean±standard deviation. Linear regression analysis was used for data correlation.

2.5. Chain separation

The globin chains present in isolated HbA_{1d3a} were separated by reversed-phase HPLC as previously reported [10].

2.6. Molecular mass analysis

Molecular masses of globin chains from isolated HbA_{1d3a} were determined by electrospray ionisation mass spectrometry (ESI-MS) combined with microbore HPLC. The MS–MS mass spectrometer (TSQ700; Finnigan MAT) was equipped with an electrospray interface and controlled by a DEC 5000 computer. A sample volume of 5 μl (5 μg HbA_{1d3a}) was injected into a 100×0.8 mm I.D. column (LC Packings) with Vydac C4, 300 Å material directly connected by a silica capillary to the electrospray interface. The chromatography was carried out with a flow-rate of 20 μl/min provided by a syringe-based HPLC-pump (140B; Applied Biosystems). The procedure makes use of water–acetonitrile–trifluoroacetic acid (TFA) developers (A: 0.1% TFA in water, B: 80% acetonitrile, 0.1% TFA). Globin chains were separated using an isocratic elution (47% B) for 5 min followed by a linear gradient to 52% B in 35 min. The mass M is calculated from a series of peaks at m/z observed for the protein molecule associated with a range of n protons, $M=[(m/z)-1]\cdot n$.

3. Results

The method used for measuring CarHb is precise and highly reproducible. The limit of detection for VH was 0.1 μg CV/g Hb. The recovery of CV (0–10 μg) added to a hemolysate was 98% ($n=10$). The within-run imprecision did not exceed 3% and the

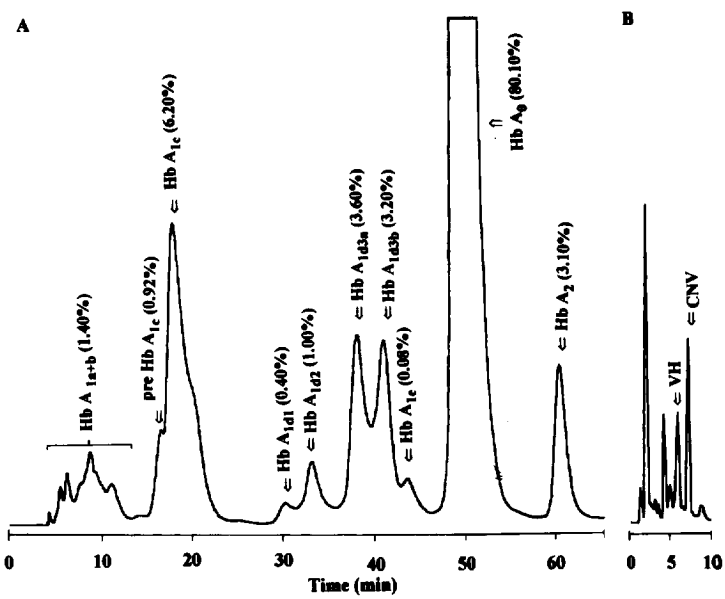

Fig. 1. (A) Separation of hemoglobin components present in red cell lysate from a uremic diabetic patient. (B) Reversed-phase separation of VH present in red cell lysate after acid hydrolysis of hemoglobin. The level of VH was 156.2 μg CV/g Hb. CNV (10 μg) was used as internal standard.

day-to-day variation was 5%. Fig. 1 depicts typical elution profiles of VH present in red cell hemolysate of a uremic patient. The separation of minor hemoglobins in the hemolysate of the same subject is also illustrated in Fig. 1. HbA_{1d3a} and HbA_{1d3b} eluted at 39.6 and 41.2 min respectively. We have studied hemolysates from 31 normal controls and 50 uremic patients. The uremic patients included 10 diabetic and 40 non-diabetic subjects. The HbA_{1d3a} level was significantly higher (3.00±0.51%) in the uremic samples than in healthy controls (1.28±0.26%). The levels of HbA_{1c} (3.97±0.39%) and HbA_{1d3b} (2.00±0.45%) were comparable in healthy controls and in non-diabetic uremic patients. The mean values of HbA_{1d3b} in diabetic and non-diabetic uremic patients were significantly different (3.00±0.49% vs. 1.90±0.43%). For the whole study, carHb (y) and HbA_{1d3a} (x) showed a significant correlation with a coefficient of correlation of $r=0.80$, the regression line being $y=0.01x+1.23$ ($n=81$, $p<0.001$) (Fig. 2). Both CarHb and HbA_{1d3a} correlated well with plasma urea concentration (Fig. 3 and Fig. 4) and allowed a good discrimination between normal controls and uremic patients. The mean levels of CarHb in uremic patients were 117±36 μg CV/g Hb. The normal controls had concentrations of CarHb of 21.7±7.7 μg CV/g Hb. There was also a significant correlation ($r=0.62$) between these components and

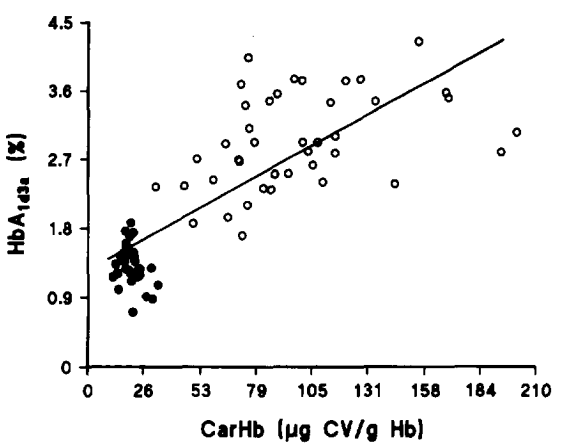

Fig. 2. Relationship between HbA_{1d3a} (y) levels and CarHb (x) values in 31 normal controls (solid circle) and 50 uremic patients (open circle). Standard errors of intercept (SEI) and slope (SES) of the regression line ($y=0.015x+1.240$, $r=0.795$, $p<0.001$) were 0.114 and 0.001, respectively.

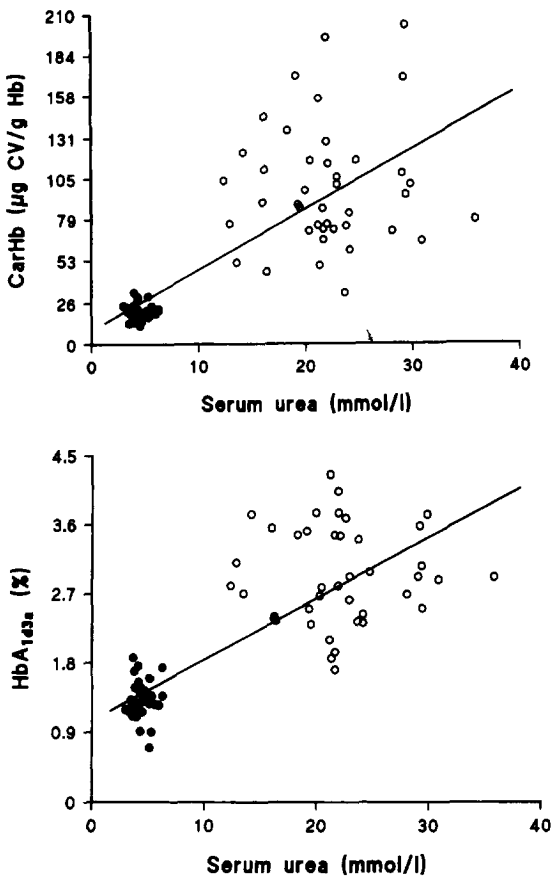

Fig. 3. Top: Correlation between serum urea level (x) and CarbHb values (y) in 31 normal (solid circle) subjects and 50 uremic patients (open circle). SEI and SES of the regression ($y=3.760x+10.230$, $r=0.730$, $p<0.001$) were 7.790 and 0.090, respectively. Bottom: Correlation between serum urea levels and the percentages of HbA_{1d3a} (y). The regression line was $y=0.08x+1.11$ ($r=0.780$, $p<0.001$). SEI and SES were 0.130 and 0.001, respectively.

serum creatinine levels. HbA_{1c} correlates significantly with HbA_{1d3b} ($r=0.71$, $p<0.05$) but not with CarHb ($r=0.49$) and HbA_{1d3a}.

We examined the formation of carbamylated and glycated hemoglobin as previously reported [7]. Incubation of washed erythrocytes with cyanate (20 mmol/l) resulted in an increase of HbA_{1d3a} and induced a new fraction eluting just after HbA_{1c} (data not shown). Incubation with glucose did not affect HbA_{1d3a}, but caused a significant rise of $preHbA_{1c}$ and HbA_{1d3b}. Reversed-phase chromatographic separations of globins obtained from the isolated HbA_{1d3a}

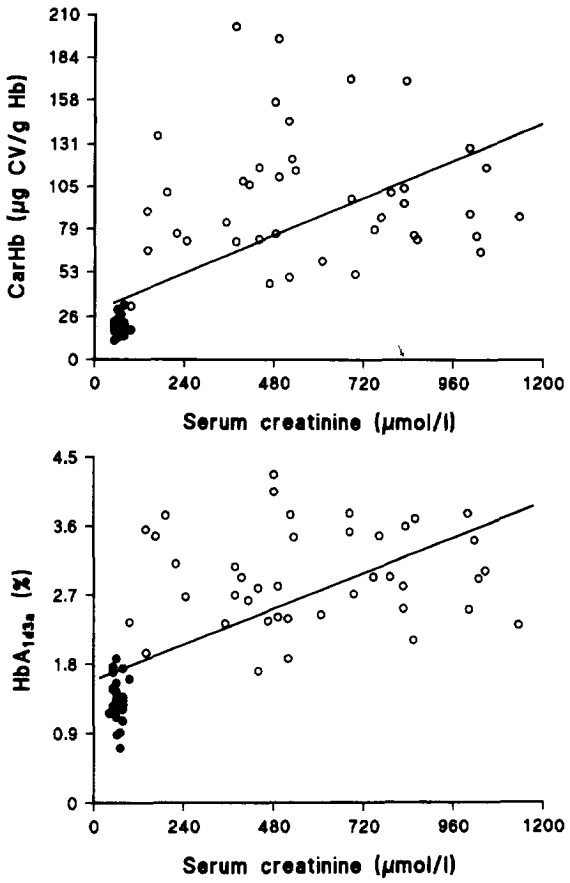

Fig. 4. Top: Relationship between CarHb values (y) and serum creatinine levels (x), $y=0.090x+32.340$ ($r=0.620$, $p<0.004$). SEI and SES were 6.700 and 1.200, respectively. (patients: open circle; controls: solid circle). Bottom: Correlation between serum creatinine levels (x) and the percentages of HbA_{1d3a} (y), $y=0.002x+1.11$ ($r=0.680$; $p<0.004$). SEI and SES were 0.121 and 0.021, respectively.

and that from the whole hemolysate are illustrated in Fig. 5. The analysis of isolated HbA_{1d3a} revealed two α-peaks (Fig. 5, bottom) in different amounts (18.5% and 33.6% of total globin chains, respectively), one eluting at 33.82 min similar to normal α-chain, the other at 37.90 min. Because of its low percentage in whole hemolysate the peak eluting at 37.90 min was difficult to recognize (Fig. 5, top). The ESI-MS analysis of these chains gave mass spectra with several series of multiple charged peaks (Fig. 6, top). The average over the series of spectra is shown as deconvoluted mass spectrum in Fig. 6 (bottom). The β-chain gave an average molecular

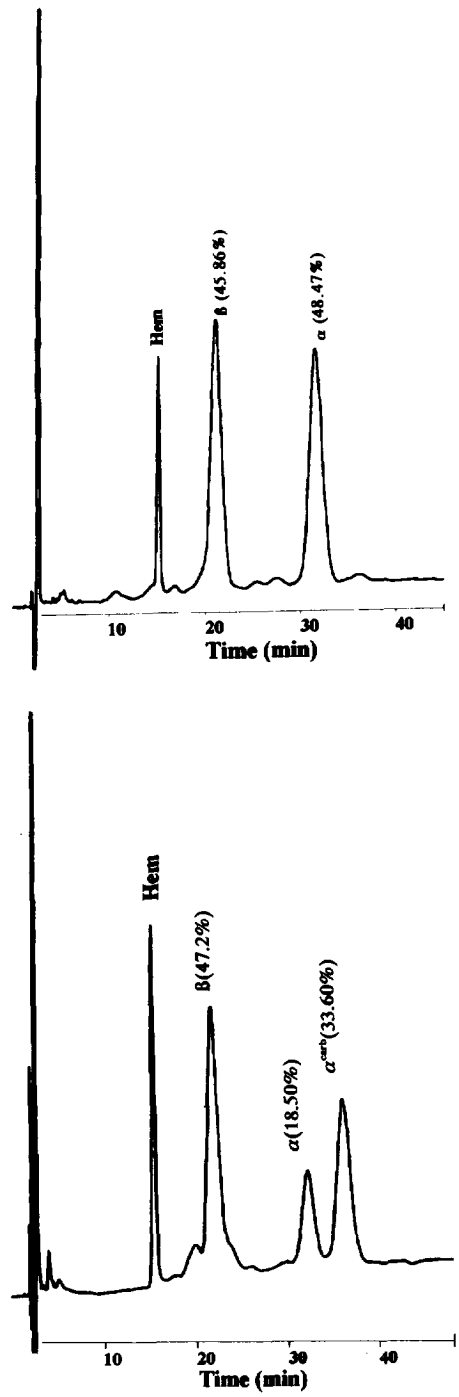

Fig. 5. Reversed-phase chromatographic separations of globin chains from the whole hemolysate (top) and from the isolated HbA_{1d3a} (bottom; α^{carb}=carbamylated α-chain).

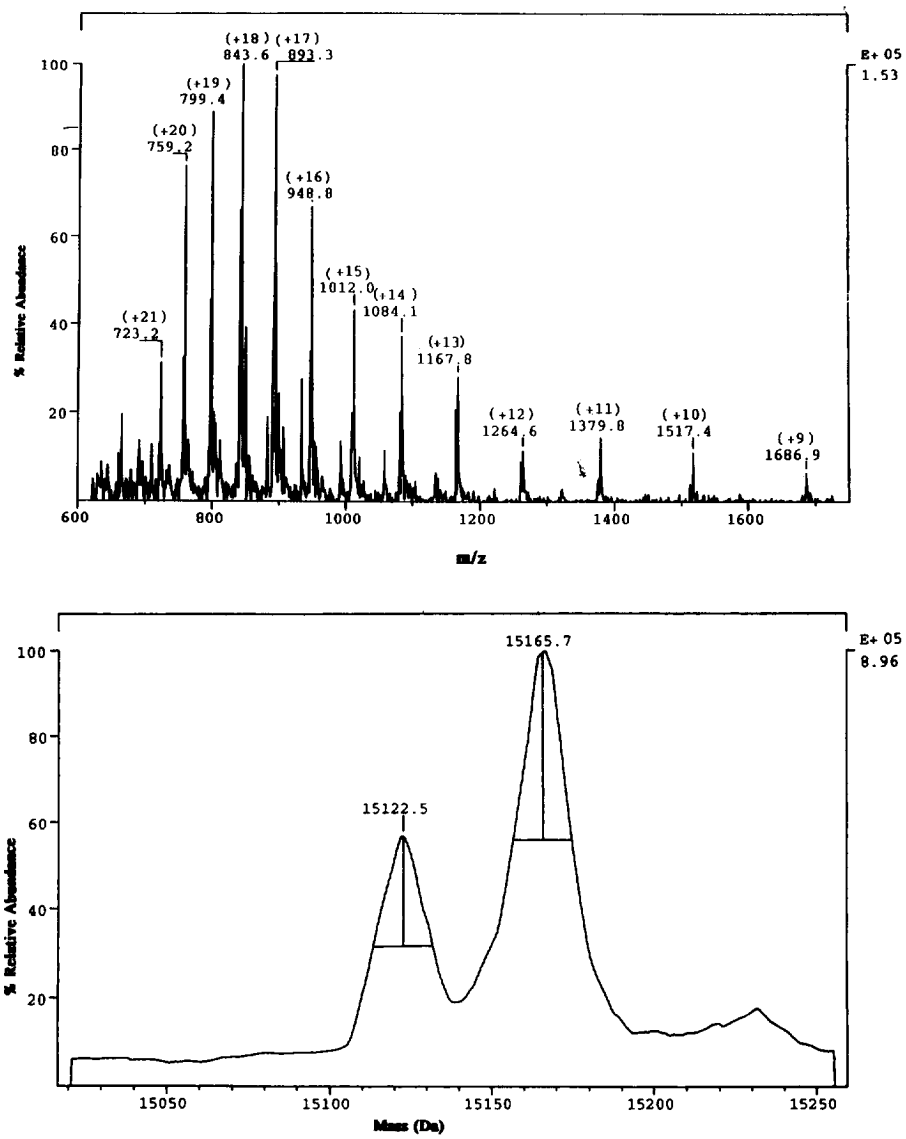

Fig. 6. (Top) Electrospray ionization mass spectrum (top, the peaks are labelled with the m/z values) and transformed mass spectrum (bottom) of α-chain from isolated HbA_{1d3a}. The numbers in parentheses give the number of protons associated with the protein detected at the individual peak. The peaks of the α-chain with an average mass of 15 165.7 are labeled. (Bottom) Profile of the relative abundance of the mass for the averaged peaks after deconvolution. The mass is given for the centroid of the peaks.

mass of 15 862±1.4. The extended range in Fig. 6 (bottom) indicates for the α-chain two molecular masses of 15 122.5±1.9 and 15 165.7±1.8, respectively. These masses can be attributed to a normal and modified α-chain, respectively. This is in agreement with the data obtained by reversed-phase chromatography (Fig. 5). The increase of the mass by 43 Da is consistent with a post-translational modification of the α-chain by carbamylation, converting $-NH_2$ to $-NHCONH_2$. Therefore the peak

eluting at 37.90 min on reversed-phase chromatography was attributed to carbamylated α-chain (α^{Carb}).

4. Discussion

We used reversed-phase HPLC for measuring carbamylated hemoglobin and the method used was similar to that described by Kwan et al. [9]. The major modifications are that we introduce a 3-mm I.D. column and extraction of VH using a separating funnel. These changes allowed analysis of as many as 30 samples per day without difficulty and enhanced the analytical recovery. Previous studies have described HbA_{1d} as being an artifact which results from the disulphide interchange reaction of HbA with oxidized glutathione [11]. However, in our study the incubation of hemolysate with oxidized glutathione affected mostly HbA_{1dx} [6,8] while HbA_{1d3} remained unaffected (data not shown). This observation suggests that HbA_{1d3} is not an artifact. The HbA_{1x} component was described by Castagnola et al. [12,13]. Its elution on Bio-Rex 70 column was similar to that of HbA_{1d}. These workers suggested that HbA_{1x} is different from HbA_{1d} and that it could be glycated at ϵ-amino groups of lysines, or at the N-terminal valine of the α-chain. HbA_{1d3} was separated using a Bio-Rex 70 column and described as a modified α-chain by glycation [6]. Therefore, we believe that HbA_{1x} and HbA_{1d3} could represent the same component. The heterogeneity of this Hb component could not be demonstrated by previous workers [6,12]. The use of a 9.4 mm I.D. PolyCATA column provides a clear and reproducible separation of HbA_{1d3} in two components, HbA_{1d3a} and HbA_{1d3b}. Our experiments involving incubation of red cells with cyanate and glucose revealed that HbA_{1d3a} and HbA_{1d3b} could be carbamylated and glycated, respectively. Indeed, by the analysis of the isolated HbA_{1d3a} on reversed-phase HPLC the α-chain was demonstrated to be heterogenous, consisting of two distinct peaks. The ESI-MS analysis of HbA_{1d3a} showed also a composition of two species representing a normal and a modified α-chain, respectively. The molecular masses determined for normal α- and β-chain agree with those reported previously [14]. The difference of 43 Da between the two masses is consistent with a carbamylation of the α-chain. In addition, the significant relationship (r= 0.8) found between HbA_{1d3a} and CarHb suggests that the modification occurs at N-terminal valine of the α-chain. The elution profile of α-chains (Fig. 5) shows that the modification by carbamylation had induced a significant decrease of the net positive charge. On the basis of our experiments (data not shown) and those of other workers [15] CarHb derives principally from HbA_{1a+b}, HbA_{1c}, and HbA_{1d3} fractions. Our data suggest that among these components HbA_{1d3a} represents the most abundant carbamylated minor hemoglobin. Therefore its measurement would be useful in management of patients with renal failure.

The isolated HbA_{1d3b} fraction was contaminated with trace amounts of HbA_{1e} and therefore not submitted to the ESI-MS analysis. However, the significant relationship between this Hb component, HbA_{1c}, and serum glucose suggests that it is glycated. HbA_{1d3b} may represent the minor hemoglobin postulated to be monoglycated using thiobarbituric acid colorimetric test [6,11].

The influence of an Hb acetaldehyde adduct on HbA_{1d3} has been demonstrated in vitro [16]. This finding is not supported by the physiological levels of HbA_{1d3a} determined in our subjects. Moreover, acetaldehyde protein adducts are known to be easily reversible [17], and therefore it is not expected that long-term changes of HbA_{1d3a} result from Hb acetaldehyde adduct. In addition, none of our patients were known to be alcoholics.

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