Journal of Clinical Laboratory Analysis 11:208–213 (1997)

Hydroxyurea-Induced Denaturation of Normal and Sickle Cell Hemoglobins in Vitro

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Use of hydroxyurea (HU) to treat sickle cell disease is usually associated with increments in fetal hemoglobin (Hb F) production; however, in vitro studies show that HU may also induce hemoglobin denaturation. Whole blood samples from Hb AA, Hb AS, and Hb SS patients were treated in vitro with 100, 150, 200, 250, and 300 μg/mL HU, incubated at 30°C for up to 12 days, and analyzed by high-performance liquid chromatography (HPLC). Hb AA levels show decrements of 91 to 14% with 100 μg/mL and 89 to 4% with 150 μg/mL after 12 days; 86 to 2% with 200 μg/mL after 10 days; 86 to 8% with 250 and 300 μg/mL after 8 days. Similar treatment and incubation times for Hb AS whole blood demonstrate that HU equally degrades the A and S components of Hb AS. A comparable approach for Hb SS whole blood samples, using a 300 μg/mL HU treatment, showed a hemoglobin denaturing pattern that went from 93% to 1% after 12 days. Globin chain analysis of these samples by reverse-phase HPLC showed that the denaturing effects occur mostly on the β-globin chain. J. Clin. Lab. Anal. 11:208–213, 1997. © 1997 Wiley-Liss, Inc.

Key words: high-performance liquid chromatography; hemoglobin variants; hydroxyurea

INTRODUCTION

The development of new therapies for sickle cell disease treatment was based on two observations: the first, homozygous sickle cell disease individuals of Arabic origin have little clinical complications due to the disease, a fact which was correlated by the persistence of high levels of fetal hemoglobin, Hb F (1); and second, to the work by Watson (2) which indicated that red cells of newborns with sickle cell anemia did not sickle, suggesting that the inhibition of sickling was due to the presence of Hb F. Since these early observations, the objective has been to find ways to increase Hb F production with the intent of reducing morbidity and mortality in individuals with sickle cell disease. Many investigators were elated when results of 5-azacytidine administered to anemic baboons selectively increased levels of Hb F in these animals (3). However, use of 5-azacytidine was discouraged when the potential of carcinogenesis became evident in laboratory animal studies (4–6). Since this initial study, many agents have been tested in an effort to increase Hb F levels and/or to attempt to interfere with the polymerization process of deoxygenated Hb SS (7–15). Studies currently performed in humans with sickle cell anemia (16), Hb EE, β-thalassemia, and unstable Hb variants (17) are demonstrating that hydroxyurea (HU) is an inducer of Hb F production, and posttreatment evaluations are indicating that patients treated with this drug for periods up to 5 years have not developed serious toxicity, cancer, or leukemia conditions (18).

Due to the unusual and promising results obtained with HU, investigations to determine its effect on complete cell blood count, erythrocyte deformation, and rheological properties (19,20) are being conducted. In January 1995, The National Heart Lung and Blood Institute (NHLBI) sent a clinical alert to recommend the use of HU for treatment of sickle cell individuals who suffer severe clinical manifestations. In spite of these important clinical advances (21,22), the mechanism of how HU acts is still under study (23). HU may be the most promising drug suggested thus far as a treatment for patients with sickle cell anemia (24–27). However, its long-term safety and efficacy are still to be ascertained (28). Screening for abnormal hemoglobins is usually done by alkaline, acid, or isoelectric focusing electrophoresis (IEF). High-performance liquid chromatogra-

Contract grant sponsor: NIH; Contract grant number P60-HL38737; Contract grant number GM 08198.

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Received 14 November 1996; Accepted 22 November 1996

phy (HPLC) is being adapted by some state laboratories as a method for hemoglobinopathy screening. Previous work done at our laboratory during hemoglobin screening of 17 normal adults undergoing medication for other ailments showed that their mean percentage of Hb A had diminished by 5 points when compared to the normal adult hemoglobin values previously reported for HPLC (29,30). It appeared that the drugs used were influencing the percentage levels of Hb A. This observation prompted the investigation of the effect of HU on hemoglobin types through an in vitro assay procedure.

MATERIALS AND METHODS

Whole blood samples were collected in EDTA tubes. Nine male adult African American (18–35 years of age) patients, previously screened for Hb SS, Hb AS, and normal Hb AA by cellulose acetate and IEF, were the subjects of this study. For each hemoglobin type assayed, blood from three subjects were used for the duration of the study. In accordance with IRB regulations, informed consent from each patient was obtained. For the in vitro studies, 1 mL whole blood samples from each of the nine selected patients were deposited into 1.5 mL Eppendorff tubes (three for each hemoglobin type, Hb AA, Hb AS, and Hb SS). Whole blood samples from the same patient were treated with 0, 100, 150, 200, 250, and 300 μg/mL HU and placed in an oscillating incubator at 30°C. Every 2 days for up to 12 days, a 50 μL aliquot of each incubating blood sample was collected and eluted with a hemolysate reagent (Helena Laboratories, Beaumont, TX). Ten microliter hemolysate solutions were transferred to H-style, Nalgene vials for hemoglobin variant analysis by HPLC using a Poly Cat A column (The Nest Group, Southboro, MA). The HPLC set up consisted of a Waters 600 E multisolvent delivery system with an automatic sample processor (Waters, Milford, MA). The gradient conditions employed were as previously reported (29,30). At the end of each run, qualitative and quantitative peak integrations for each hemoglobin were obtained.

Hemoglobin chain separation of hemolysates was performed by reverse HPLC using a Vydac column (The Nest Group). The gradient system used was made of mobile phase A, which contained 60% acetonitrile (HPLC grade) and 1 mL/L trifluoroacetic acid (TFA), and mobile phase B, which was composed of 20% acetonitrile and 1 mL/L (TFA). The gradient conditions were: mobile phase A 45, 65, 100, 35, 45% at 0, 20, 25, 40, 50, and 60 minutes, respectively. The operating conditions included a flow rate of 0.8 mL/min, a wave length of 220 nm, and a 60-minute elution time per sample. At the end of each HPLC run, qualitative and quantitative peak integration results expressed in percentages were automatically recorded.

RESULTS

Hemoglobin Analysis

Table 1 shows the Hb AA mean percentages (B-100) of blood samples collected from three patients. Analysis of whole blood samples treated in vitro with HU concentrations ranging from 0 to 300 μg/mL for up to 12 days was performed by HPLC. The aliquots were taken from blood coming from the three patients with the same hemoglobin type. These percentages are also tridimensionally shown in Figure 1 to illustrate the denaturing patterns of Hb AA at various HU concentrations and exposure times. The mean percentage values show Hb AA losses from 91 to 14% and 89 to 4% when treated with 100 and 150 μg/mL after 12 days; while 200 μg/mL HU reduced the percentage to 2% in 10 days. Similarly, levels of 250 and 300 μg/mL HU showed a reduction of Hb AA to 8% after 8 days. A similar approach was followed with the Hb AS and Hb SS samples, and the results are expressed in Figures 2 and 3.

Figure 2 represents comparative tridimensional degrading patterns for components A and S of Hb AS when exposed to 100, 150, 200, 250, and 300 μg/mL HU concentrations for a period of up to 12 days. The component A denaturing pattern observed here is comparable to one observed for Hb AA (Fig. 1) after the same treatment and analysis. Figure 4 shows a typical HPLC chromatogram for an Hb AS control (HU untreated sample) and the denatured profiles of A and S of the Hb AS sample treated with 200 μg/mL HU after 8 days.

Figure 3 shows the base 100 percentage changes of Hb SS untreated control and treated with 300 μg/mL HU. After a 12-day incubation period at 30°C, the hemoglobin degradation trends of HU-treated samples fluctuated from 93% at

TABLE 1. HPLC Hemoglobin Determination of In Vitro HU-Treated Hb AA Whole Blood Samples[a]

Days	Control (HU)	HU treatment (μg/mL) 100	150	200	250	300
1	100	90	89	86	86	85
2	95	88	82	79	73	71
4	94	82	73	53	40	41
6	91	65	41	23	17	15
8	90	46	24	11	8	8
10	89	31	9	2	0	0
12	88	14	4	0	0	0

[a]Hemoglobin results are expressed in adjusted mean B-100 percentage values corresponding to three patients per each day tested. Fifty microliter aliquots from the same patient were used to perform HPLC hemoglobin analysis every 2 days for up to 12 days for each HU concentration.

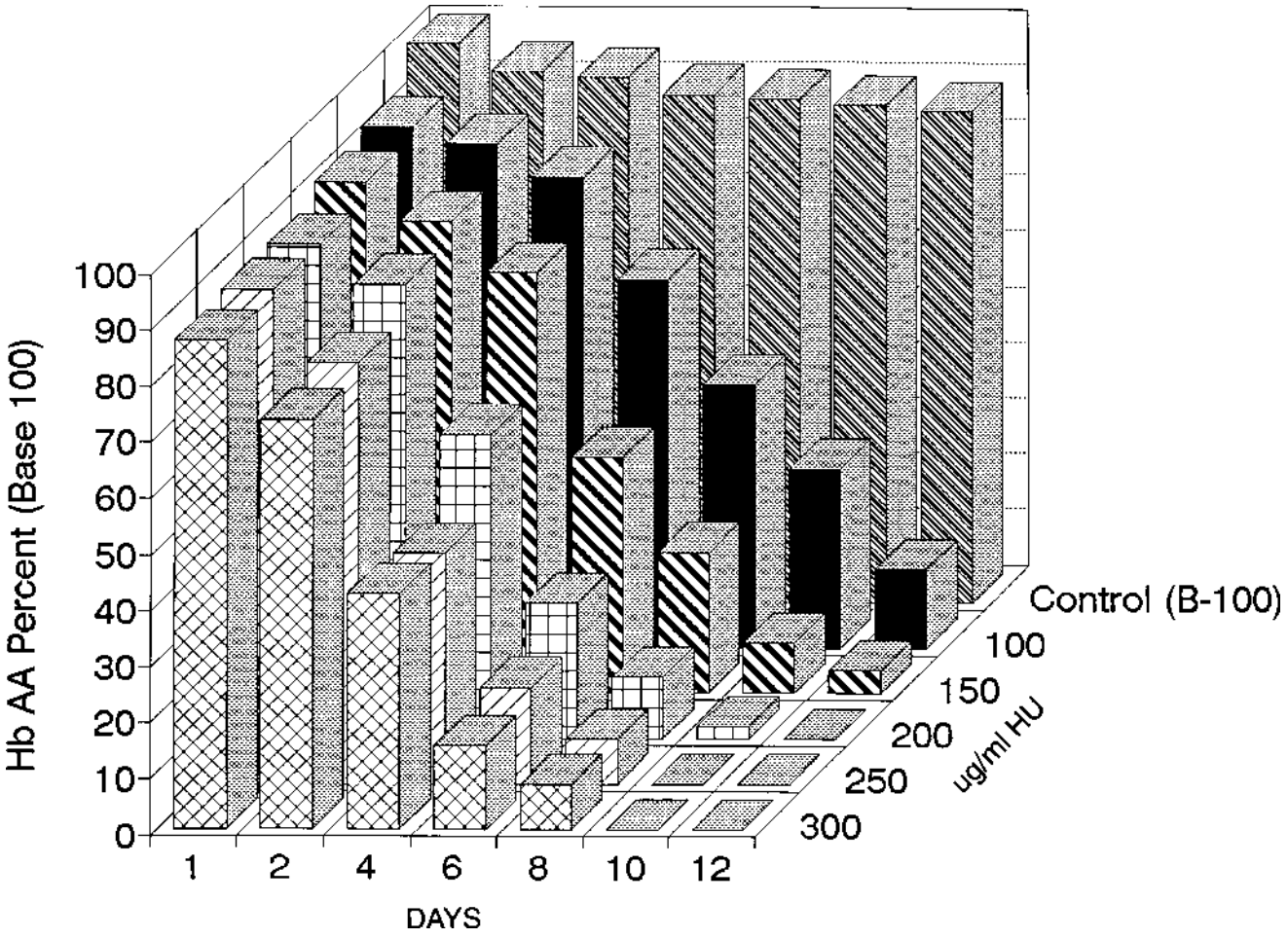

Fig. 1. Comparative HPLC (base 100) percentage trends of Hb AA whole blood samples incubated at 30°C, treated with various levels of HU, and recorded up to 12 days.

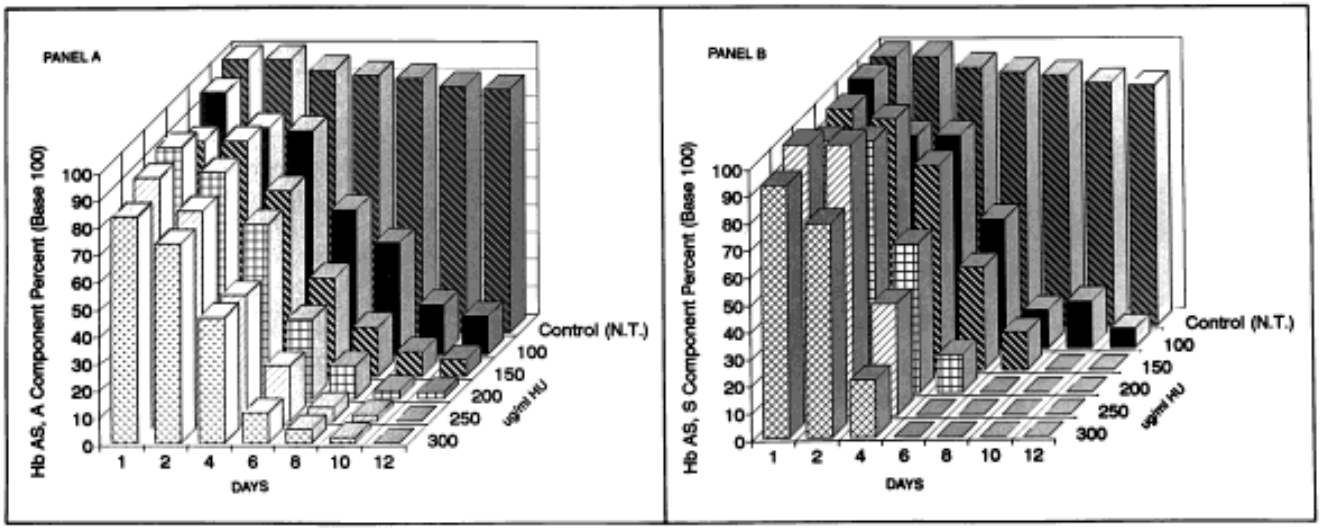

Fig. 2. Comparative HPLC (base 100) percentage trends of Hb AS whole blood samples incubated at 30°C, treated with various levels of HU, and recorded at different time periods. **A:** Tridimensional changing patterns of the A component. **B:** Changes for the S component of Hb AS.

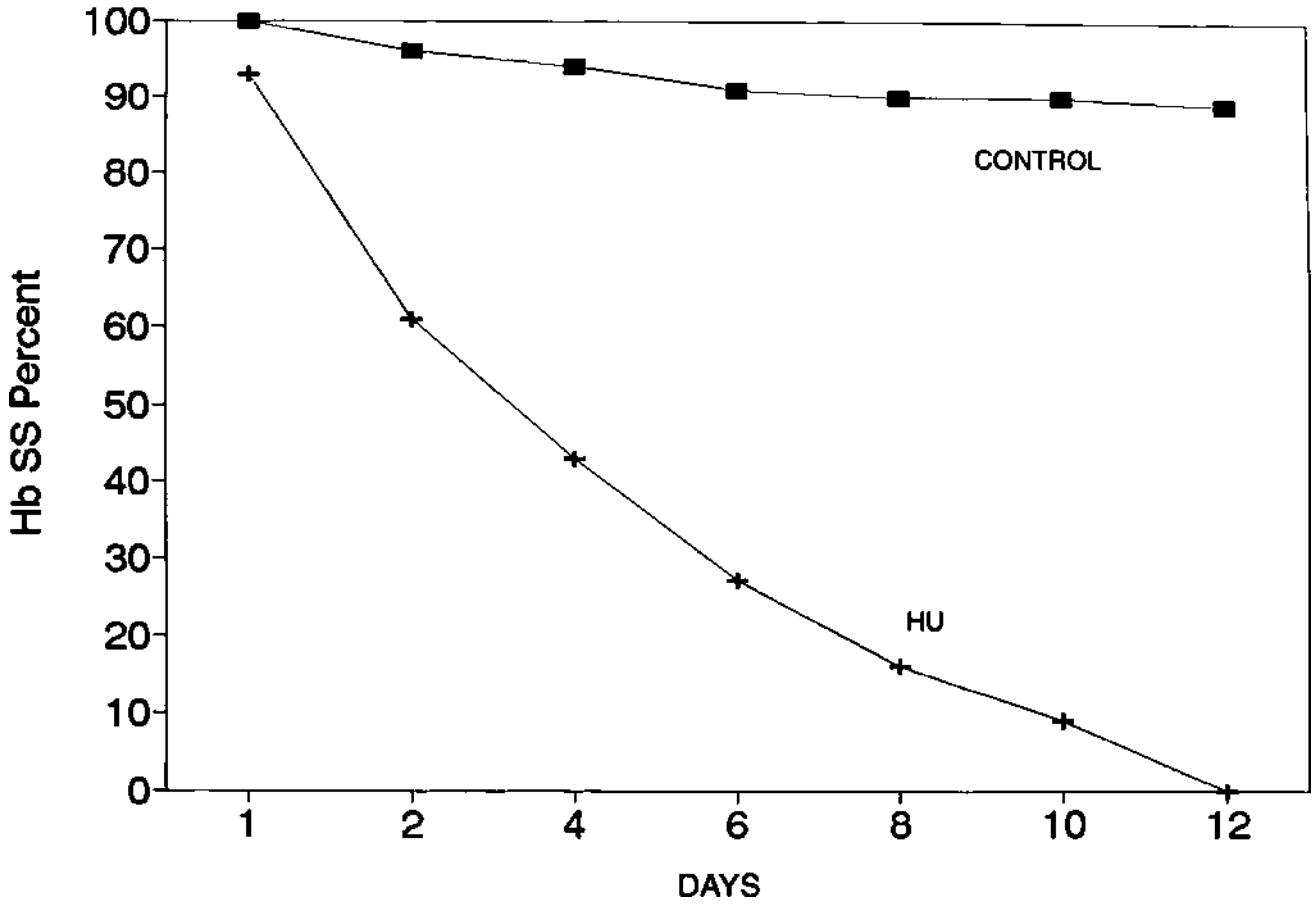

Fig. 3. Comparative (base 100) percentage trends of an Hb SS whole blood control sample and a 300 μg/mL HU-treated sample incubated at 30°C and HPLC analyzed at different time intervals up to 12 days.

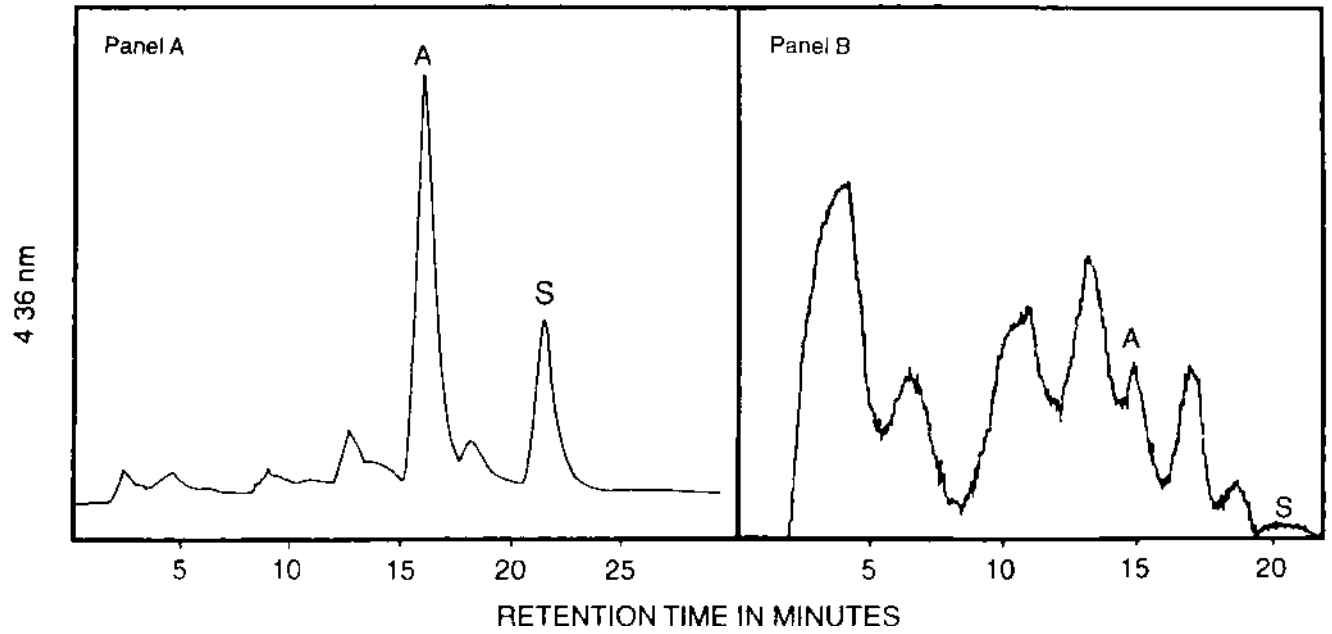

Fig. 4. Representative HPLC chromatograms of an Hb AS whole blood control sample **(A)** and another treated with 200 μg/mL HU **(B)**, after an 8-day incubation period.

day 1 to 1% at day 12; while the hemoglobin profile of the Hb SS whole blood control was practically unchanged.

Globin Chain Analysis

HPLC analysis for α and β-globin chains from Hb AA, Hb AS, and Hb SS whole blood treated with 300 μg/mL HU and incubated for 10 days is shown in Table 2. The results are expressed in actual percentages. The superscript letters (i.e., "b") indicate the appearance of side peaks and the intensity of the denaturation process. These changes were specifically observed in the β-globin chain. High levels and prolonged exposures to HU may also cause small distortions of the α-chain. The α/β ratio is increased in the Hb SS whole blood sample when the same HU (300 μg/mL) levels are used. High HU levels employed cause the rise of side peaks and almost complete denaturation of the β-globin chain.

DISCUSSION

Hemoglobin denaturation was observed in Hb AA, Hb AS, and Hb SS whole blood samples after HU treatment in vitro. The HPLC procedures utilized have enabled the detection and quantification of these hemoglobin changes. Analysis performed by cellulose acetate, citrate agar, and IEF has also shown the presence of degraded hemoglobin bands (data not shown), but accurate quantitative analysis of these results by densitometric scanning has not been possible. Solubility tests by Sickledex and Sickle Quick solubility kits have been used to identify the presence of Hb S. HPLC techniques have enabled the detection and quantification of the "denaturing patterns," which occur in hemoglobin when whole blood samples are exposed to various levels of HU after incubation for several days. The term "denaturing patterns" used in this work refers to the appearance of other "peaks" along the various regions of the HPLC chromatogram profile. These peaks are most probably denatured or decomposed residues of the hemoglobin being analyzed. This approach has permitted us to establish relationship parameters between the effect of drug concentration and the time of drug exposure. The data obtained with Hb AA, Hb AS, and Hb SS whole blood samples have already demonstrated that there are no significant profile changes with the controls (see background bars in Figs. 1 and 2A,B; and the control trend of Hb SS in Fig. 3), but proportionally significant profile changes occurred when samples were treated with 100, 150, 200, 250, and 300 μg/mL HU and were incubated for short periods of time. The effect of HU (300 μg/mL) on these hemoglobins, Hb AA, Hb AS, and Hb SS demonstrates that degradation of the A component in Hb AA and in Hb AS followed the same decreasing patterns when exposed to different levels of HU; the S component degraded more rapidly (4 days) in Hb AS samples than in the homozygous Hb SS, whose denaturing process was prolonged up to the tenth day.

HPLC reverse-phase analysis of Hb AA, Hb AS, and Hb SS whole blood samples has shown a greater effect of HU on the β-globin chain component. The β-chain denaturing process can be appreciated by the appearance of small side peaks, diminishing peak areas, and irregular configuration of the peak areas. The α-chain is also affected after prolonged exposure to high levels of HU. The increase in the α/β ratio indicates mainly the decrease of the β-globin chain levels specifically with the Hb SS samples after 2 days exposure to 300 μg/mL HU.

In summary, the in vitro studies suggest that:

1. HU may induce the hemoglobin denaturation process of Hb AA, Hb AS, and Hb SS whole blood samples.
2. Hemoglobin degradation is proportional to HU concentration levels and incubation time of exposure employed.
3. HU's specific denaturing effects occur mostly on the hemoglobin β-chain, and more definitely with the Hb SS sample.

Finally, we may conclude that these in vitro results could prove most useful when assessing patient response to HU treatment.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Ms. Cristina Roa and Ms. Bernadine Strode for the preparation of charts, text, and

TABLE 2. Comparative HPLC Globin Chain Percentage Determinations of Hb AA, Hb AS, and Hb SS Whole Blood Samples Treated With 300 μg/mL HU

	Hb AA			Hb AS			Hb SS		
Days	α	β	α/β ratio	α	β	α/β ratio	α	β	α/β ratio
0 (control)[a]	38	35	1.09	38	31	1.23	34	32	1.06
1	35	34[b]	1.03	38	31	1.23	32	22	1.46
2	38	33	1.15	37	31[b]	1.19	43	20[b]	2.15
4	38	23[b]	1.65	34	26[b]	1.31	42	15[b]	2.80
6	40	30[b]	1.33	35	20[b]	1.75	40	9[b]	4.44
8	33	27[b]	1.22	34	34[b]	1.00	39	13[b]	3.00
10	41	22[b]	1.86	38	21[b]	1.81	45	12[b]	3.75

[a]Control = no HU treatment.
[b]Presence of side peaks and/or peak size reduction.

figures, and Mr. Richard Redmon for his assistance in reviewing this manuscript.

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