Chemical and Functional Analysis of Hydroxyurea Oral Solutions

Matthew M. Heeney, MD, Matthew R. Whorton, BSc,† Thad A. Howard, MS,† Christina A. Johnson, PharmD,‡ and Russell E. Ware, MD, PhD†*

Abstract: The primary hypothesis of the upcoming NIH-sponsored phase III infant hydroxyurea (BABY HUG) trial is that hydroxyurea can prevent chronic organ damage in infants with sickle cell anemia. Since hydroxyurea is currently commercially available only in capsules, a liquid formulation of hydroxyurea is needed for young patients. Hydroxyurea oral solutions were prepared by dissolving the contents of the capsules in water (room temperature or mildly heated) with vigorous stirring, filtering excipients, and adding flavored syrup to a final concentration of 100 mg/mL. Chemical stability was determined by measuring the hydroxyurea concentration using a standardized analytical colorimetric analysis, while functional stability was determined by measuring the inhibition of phytohemagglutinin-induced T lymphocyte proliferation. Hydroxyurea oral solutions prepared using room-temperature water had statistically equivalent spectrophotometric concentration and inhibition of T-lymphocyte proliferation for 3 to 6 months. Mild heating of the water to facilitate dissolution of the hydroxyurea capsule contents resulted in a reduced concentration and inhibitory activity of the preparations. Hydroxyurea oral solutions (100 mg/mL) prepared and maintained at room temperature have chemical and functional stability for several months. Hydroxyurea oral solutions prepared and dispensed monthly are suitable for use in the upcoming infant BABY HUG trial.

Key Words: children, hydroxyurea, oral solutions, sickle cell anemia

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Hydroxyurea has become an accepted therapeutic option for many patients with sickle cell anemia (SCA). For adults, hydroxyurea has dose-related hematological efficacy and an acceptable short-term toxicity profile.[1] Clinical efficacy has also been proven; in a double-blinded placebo-controlled randomized trial involving adults severely affected with SCA, hydroxyurea significantly reduced the number of painful vaso-occlusive events, blood transfusions, episodes of acute chest syndrome, and hospitalizations.[2] Most recently, long-term hydroxyurea use has been shown to decrease mortality in adults with SCA.[3] For children, hydroxyurea has a similar toxicity profile, with only mild, transient, and reversible myelosuppression.[4,5] The multicenter phase I/II safety trial of hydroxyurea therapy for school-aged children severely affected with SCA (HUG KIDS) showed significant increases in hemoglobin concentration, mean corpuscular volume, and the percentage of both fetal hemoglobin and F cells.[6] Subsequently, hydroxyurea has been shown to aid the growth and development of children with SCA[7] and to help prevent stroke recurrence in children with previous cerebrovascular accident.[8]

Despite this documented laboratory and clinical efficacy for persons with SCA, there are important unresolved issues regarding hydroxyurea in children, particularly with regard to the appropriate age to initiate therapy. In one small group of preschool children, hydroxyurea was well tolerated, with both hematologic and clinical efficacy.[9] For infants with SCA, a prospective multicenter open-label phase I/II pilot trial (HUSOFT) demonstrated hematological benefits with only modest toxicity,[10] suggesting that hydroxyurea might be considered for very young patients before the onset of acute and chronic organ damage. An upcoming NIH-sponsored phase III double-blinded, placebo-controlled randomized clinical trial (BABY HUG) will test formally the hypothesis that hydroxyurea can prevent chronic organ damage in infants with SCA.[11]

A liquid formulation of hydroxyurea is needed for young patients who cannot swallow the commercially available capsule formulations of hydroxyurea. In HUSOFT, an oral solution was prepared in flavored syrup at a final concentration of 100 mg/mL, and refilled every 4 weeks for the duration of the study.[10] That solution was prepared according to a recipe used for in-house neuroblastoma protocols at St. Jude Children's Research Hospital, and was believed to be both stable and functional for at least 1 month at room temperature and 3 months under refrigeration (W. Crum, PharmD, unpublished

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From the *Division of Pediatric Hematology/Oncology, Children's Hospital Boston, Boston, Massachusetts; †Division of Pediatric Hematology/Oncology, Department of Pediatrics, and ‡Department of Pharmacy, Duke University Medical Center, Durham, North Carolina.

Reprints: Matthew M. Heeney, MD, Children's Hospital Boston, 300 Longwood Avenue, Boston, MA 02115-5724 (e-mail: matthew.heeney@childrens.harvard.edu).

observations). The hematological and clinical benefits observed in HUSOFT indeed suggested that a liquid hydroxyurea solution was both chemically stable and functional.[10] However, no specific guidelines were provided regarding the need to refrigerate the oral solution, or the appropriate quantity that patients could use before requiring a fresh formulation.

A liquid hydroxyurea formulation, similar to that used in the HUSOFT study, is proposed for the phase III BABY HUG trial. To document the stability of this product, we tested prepared solutions of hydroxyurea over time using specific assays for both chemical composition and functional activity.

MATERIALS AND METHODS

Preparation and Storage of Hydroxyurea Oral Solutions

Hydroxyurea oral solutions (100 mg/mL) were prepared by hand (i.e., extemporaneously) using commercially available 500-mg capsules (Hydrea, Bristol-Myers Squibb, Princeton, NJ). The contents of the capsules were mixed with a sufficient amount of room-temperature sterile water to achieve a concentration of 200 mg/mL. The solutions were vigorously stirred for several hours using a magnetic stirrer and then filtered to remove insoluble excipients. Flavored syrup (Syrpalta without color; HUMCO; Texarkana, TX) was added to the filtered solutions in sufficient quantity produce a final concentration of 100 mg/mL. To assess the effect of heat on the chemical stability of hydroxyurea, additional solutions were prepared using mildly heated water (41°C) to facilitate dissolution. The resulting solutions were stored at room temperature in amber plastic bottles (Kerr Group Inc., Lancaster, PA).

Chemical Detection of Hydroxyurea

Quantitative determination of hydroxyurea concentration was performed using a modification of the analytical colorimetric method described by Fabricius and Rajewsky.[12] Briefly, upon oxidation of hydroxyurea by iodine, nitrite is formed, which diazotizes sulfanilic acid. After reduction of excess iodine with sodium thiosulfate, the diazotized sulfanilic acid is coupled with N-(1-naphptyl)-ethylenediamine dihydrochloride to generate a colored product that can be measured spectrophotometrically.[12]

At each experimental time point, a standard curve was established using freshly prepared aqueous hydroxyurea samples of known concentration. This was accomplished by dissolving the contents of a single 500 mg hydroxyurea capsule in 66 mL ddH_2O to create a 1.0 mmol/L stock solution. Serial dilutions ranging from 10 to 500 μmol/L were then used to generate the standard curve. For chemical analysis of the 100 mg/mL oral solutions, prepared either with room-temperature or mildly heated water, aliquots were diluted in ddH_2O to 100 μmol/L and measured in triplicate using the colorimetric method at various time points from 0 to 9 months after preparation. Relative units of optical density were measured at 540 nm using a Spectronic 601 UV/Visible spectrophotometer (Milton Roy, Rochester, NY).

Functional Activity of Hydroxyurea

Hydroxyurea produces a temporary dose-dependent inhibition in the in vitro proliferation of T lymphocytes. Peripheral blood mononuclear cells isolated from normal human donors were stimulated in culture with phytohemagglutinin (PHA), as previously described.[13] Cultured cells were harvested after 72 hours of in vitro PHA stimulation, and then tritiated thymidine uptake was quantitated. For functional analysis of hydroxyurea oral solutions over time, aliquots of the original 100 mg/mL solutions were diluted in ddH_2O to 200 μmol/L and measured in triplicate using this assay.

Statistics

Descriptive statistical calculations were performed using the Primer of Biostatistics (McGraw-Hill, New York, NY) software package.

RESULTS

Chemical Stability of Hydroxyurea Oral Solutions

Standard curves produced by the colorimetric assay were linear at hydroxyurea concentrations between 20 and 200 μmol/L (see Fig. 1A, $r^2 = 0.979$, $P < 0.001$). Accordingly, the chemical stability of each hydroxyurea oral solution was assessed using aliquots diluted to 100 μmol/L. Hydroxyurea solutions prepared using room-temperature water had excellent chemical stability over a 6- to 9-month observation period (see Fig. 1B). There was only a 5% loss in chemical activity over the first 3 months ($P > 0.05$) and no additional loss out to 9 months of room-temperature storage. However, the use of mildly heated water (41°C) to aid in the initial dissolution of hydroxyurea resulted in an immediate 40% loss in chemical activity compared with the preparation dissolved in room-temperature water ($P < 0.001$). This decreased chemical activity was then stable over time (see Fig. 1B).

Functional Stability of Hydroxyurea Oral Solutions

The functional activity of hydroxyurea solutions was measured by the inhibition of PHA-stimulated T lymphocyte proliferation. A dose-dependent effect was noted, with approximately 50% inhibition of proliferation observed with a freshly prepared 200 μmol/L hydroxyurea solution (see Fig. 2A). Accordingly, this dose was selected to analyze the functional activity of hydroxyurea solutions over time. Similar to the results for chemical stability, hydroxyurea solutions prepared in room-temperature water had less than 5% loss of

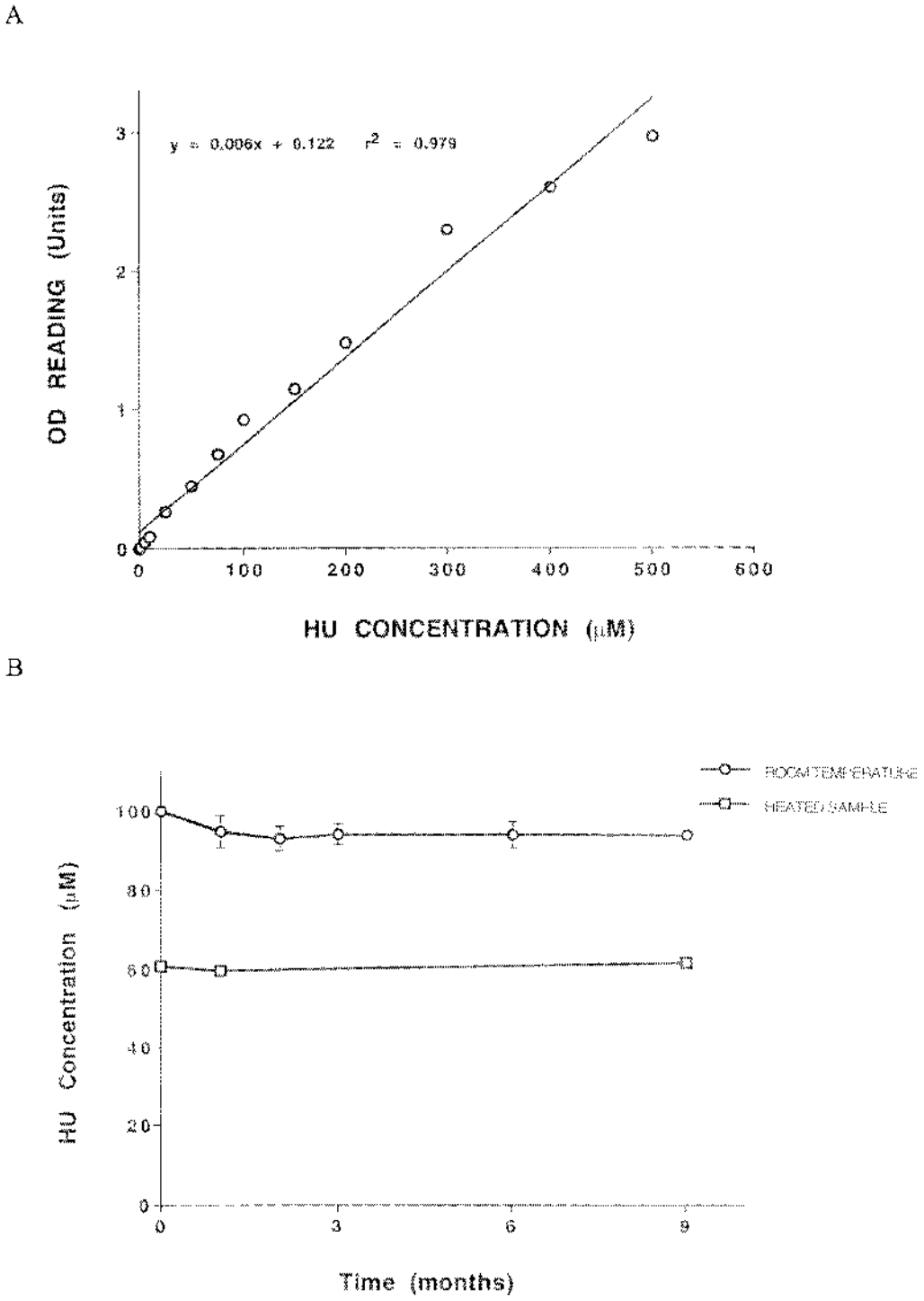

FIGURE 1. Chemical stability of hydroxyurea (HU) oral solutions. (A) Representative standard curve for analysis of hydroxyurea concentration using the colorimetric method of Fabricius and Rajewsky,[12] with a resultant linear curve, $r^2 = 0.979$, $P < 0.001$. (B) Stability of 100 mg/mL hydroxyurea solutions prepared at room temperature (circles), with <5% loss of chemical activity over 6 months. Mild heating of the water to facilitate dissolution of the hydroxyurea (squares) reduced activity to 60% of baseline activity, $P < 0.001$. All assays were performed in triplicate, and the results (mean ± 1 standard deviation) reflect three to eight independent experiments at each time point.

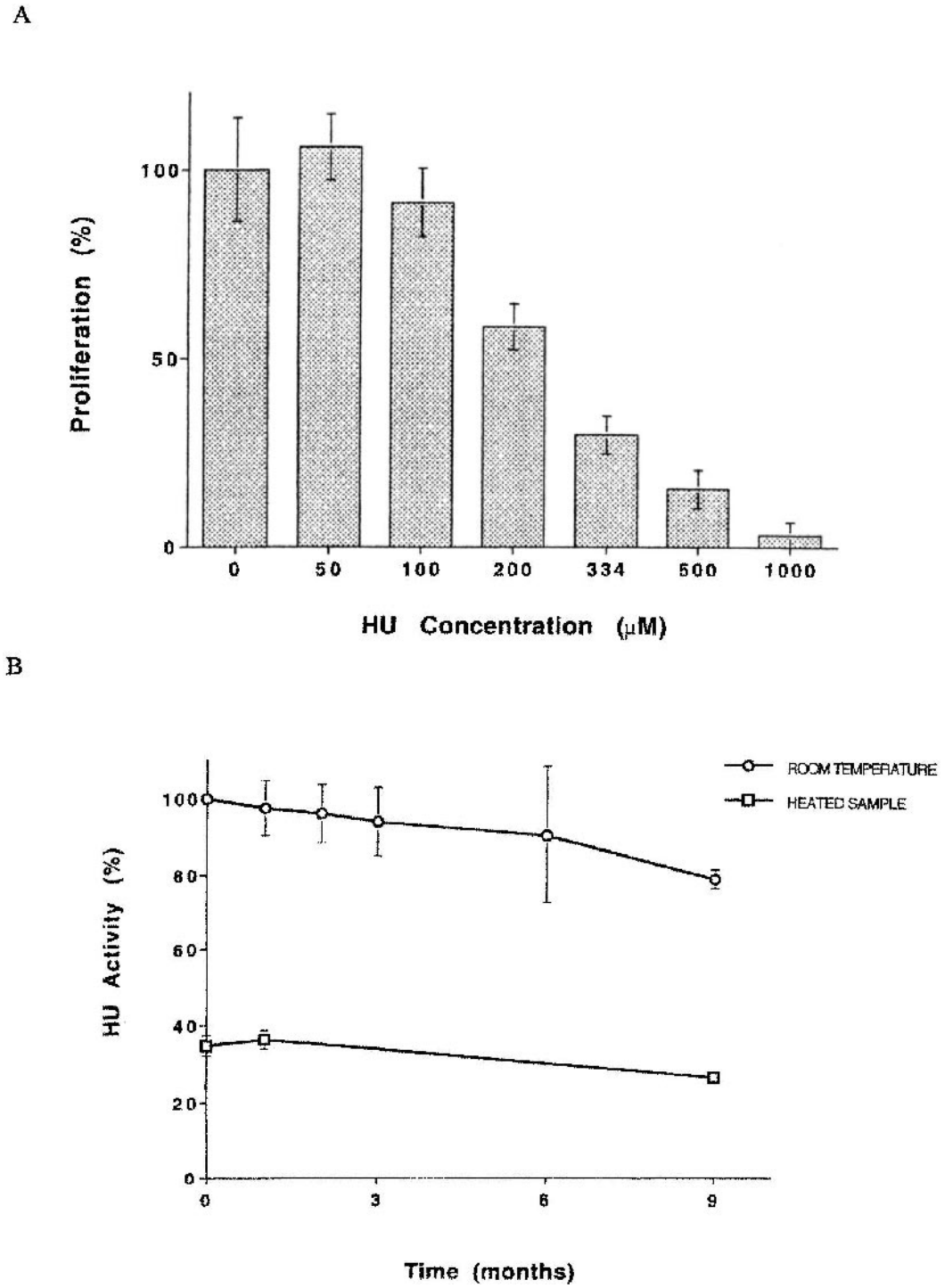

FIGURE 2. Functional activity of hydroxyurea (HU) solutions. (A) Dose-dependent inhibition of PHA-stimulated T lymphocyte proliferation, with about 50% inhibition observed with 200 μmol/L hydroxyurea. (B) Stability of 100 mg/mL hydroxyurea solutions prepared at room temperature (circles), with <10% loss of functional activity over 6 months. Mild heating of the water to facilitate dissolution of the hydroxyurea (squares) reduced its functional activity to 30% to 40% of baseline, $P < 0.001$. All assays were performed in triplicate, and the results (mean ± 1 standard deviation) reflect four to six independent experiments at each time point.

activity after the first 3 months ($P > 0.05$), which became significant ($P < 0.001$) only for measurements at 9 months (see Fig. 2B). Samples prepared using mildly heated water (41°C) to dissolve the hydroxyurea had a greater reduction in activity. There was less than 40% functional activity initially ($P < 0.001$) that was then relatively stable over time (see Fig. 2B).

DISCUSSION

Hydroxyurea is a structurally simple compound, $H_2NCONHOH$, that is a hydroxylated derivative of the symmetrical urea molecule. Although hydroxyurea was first synthesized in 1869,[14] a century passed before it was found to have an antitumor effect.[15] Hydroxyurea is now recognized as an S-phase-specific cytotoxic agent that inhibits the enzyme ribonucleotide reductase and thereby blocks DNA synthesis; further, it has potent activity against a wide variety of neoplasms but has a modest toxicity profile.[16] The drug can be safely administered orally or intravenously, with maximal serum levels detected after 60 to 120 minutes and excretion into the urine.[17]

Analytic measurement of hydroxyurea in biologic tissues and fluids can be performed using a variety of laboratory techniques. Early studies described chemical methods with colorimetric endpoints, which were accurate but somewhat labor intensive.[12,18,19] Newer techniques have been described, including high-performance liquid chromatography (HPLC)[20] and capillary gas chromatography.[21] Each of these techniques has drawbacks; HPLC requires special equipment for column resolution and ultraviolet detection, while gas chromatography is an indirect assay that requires high temperatures and therefore measures the degradation product pyridine. We found the colorimetric technique of Fabricius and Rajewsky[12] to be relatively simple to perform and highly reproducible, with linearity in the standard curve between 20 and 200 μmol/L (see Fig. 1A). The functional activity of hydroxyurea in this clinical setting is not as easily quantified, since the mechanism of action by which hydroxyurea induces fetal hemoglobin has not been fully elucidated. Inhibition of T lymphocyte proliferation is a reasonable marker, however, since it reflects the effects of hydroxyurea on ribonucleotide reductase to block DNA synthesis.

The stability of hydroxyurea in aqueous solution has not been clearly defined. The *Merck Index* states that hydroxyurea is freely soluble in water,[22] and *Martindale* lists hydroxyurea as a hygroscopic compound that is soluble in water but decomposes in the presence of moisture.[23] The *Children's Oncology Group Pharmacology Manual* advises that moisture causes degradation of hydroxyurea and that an aqueous solution should be administered immediately.[24] Furthermore, the 1998 manufacturer's package insert states that hydroxyurea is hydrolyzed by water and that aqueous solutions are stable for only 18 hours at room temperature, with 50% breakdown after 4 days.[25] The only published research on the stability of hydroxyurea solutions used extremely low drug concentrations (10–50 μg/mL) and the indirect capillary gas chromatography technique. Poor stability of hydroxyurea solutions was observed, with a 6% daily loss of compound using a refrigerated product and a 13% daily loss of compound using a heated product.[21]

In contrast to these data, we observed excellent stability of extemporaneously prepared hydroxyurea solutions, both in chemical composition and functional activity, over a period of months (see Figs. 1 and 2). This apparent paradox is possibly explained by the fact that these pharmacological oral solutions of hydroxyurea were prepared at much higher concentrations than those used in previous laboratory analyses. For example, an aqueous solution of hydroxyurea at 100 mg/mL is 1.3 mol/L, which is many orders of magnitude higher than the concentrations of hydroxyurea that are accurately detected by analytic techniques (10–300 μmol/L). Oral hydroxyurea preparations at 100 mg/mL might be predicted to be relatively stable over time, and preservatives or other additives in the flavoring syrup might further prolong the chemical and functional stability. Although our experiments did not include refrigeration of stored liquid preparations, our results indicate that refrigeration is not necessary to provide long-term stability.

Compounding a solution of hydroxyurea for administration to patients incapable of swallowing a capsule is necessary for therapeutic trials involving young children. Hydroxyurea has clinical activity not only for young patients with SCA but also for children with myeloproliferative disorders,[26] nonmalignant hematological conditions,[27] certain brain tumors,[28] and even as adjuvant therapy for those who are HIV positive.[29] Our data indicate that hydroxyurea solutions can be extemporaneously prepared with chemical and functional activity that is well preserved over time. These laboratory data support the hematological and clinical efficacy of liquid hydroxyurea preparations observed in the HUSOFT trial.[10] Based on our data, we recommend that hydroxyurea solutions be prepared in room-temperature water without any initial heating and then stored at room temperature. Such preparations appear to be stable from both a chemical and functional standpoint, and would allow patients to have a convenient 4-week supply for home use. Oral solutions of hydroxyurea refilled monthly should be suitable for use in the upcoming phase III infant BABY HUG trial.

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