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Standardization method for measurement of hydroxyurea by Ultra High Efficiency Liquid Chromatography in plasma of patients with sickle cell disease

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Sickle cell anemia (SCA) is a recessively inherited disease characterized by chronic hemolytic anemia, chronic inflammation, and acute episodes of hemolysis. Hydroxyurea (HU) is widely used to increase the levels of fetal hemoglobin (HbF). The objective of this study was to standardize and validate a method for the quantification of HU in human plasma by using ultra high performance liquid chromatography (UPLC) in order to determine the plasma HU levels in adult patients with SCA who had been treated with HU. We used an analytical reverse phase column (Nucleosil C18) with a mobile phase consisting of acetonitrile/water (16.7/83.3). The retention times of HU, urea, and methylurea were 6.7, 7.7, and 11.4 min, respectively. All parameters of the validation process were defined. To determine the precision and accuracy of quality controls, HU in plasma was used at concentrations of 100, 740, and 1600 µM, with methylurea as the internal standard. Linearity was assessed in the range of 50-1600 µM HU in plasma, obtaining a correlation coefficient of 0.99. The method was accurate and precise and can be used for the quantitative determination of HU for therapeutic monitoring of patients with SCA treated with HU.

Uniterms: Sickle cell anemia/therapeutic monitoring. Hydroxyurea/quantification in human plasma. Ultra High Efficiency Liquid Chromatography/quantitative analysis.

A anemia falciforme (AF) é uma doença hereditária recessiva caracterizada por anemia hemolítica crônica, inflamação crônica e episódios agudos de hemólise. Hidroxiureia (HU) é amplamente utilizada para aumentar os níveis de hemoglobina fetal (Hb F). O objetivo consiste em padronizar e validar um método para a quantificação de HU no plasma humano utilizando Cromatografia Líquida de Ultra Alta Eficiência (UPLC), a fim de determinar os níveis de HU em pacientes adultos com AF, tratados com HU. Utilizou-se coluna analítica de fase inversa (Nucleosil C18), fase móvel constituída por acetonitrila/água (16,7/83,3). Os tempos de retenção da HU, uréia e metiluréia foram respectivamente de 6,7, 7,7 e 11,4 minutos. Definiram-se todos os parâmetros do processo de validação. Para determinar a precisão e exatidão dos controles de qualidade utilizaram-se concentrações de 100, 740 e 1600 mM de HU no plasma, empregando como padrão interno a metiluréia. A linearidade foi avaliada no intervalo de 50-1600 mM de HU no plasma, obtendo-se coeficiente de correlação de 0,99. O método foi considerado exato e preciso e pode ser realizado com o propósito de determinação quantitativa de HU para monitorização terapêutica de pacientes com AF tratados com esse fármaco.

Unitermos: Anemia falciforme/monitorização terapêutica. Hidroxiuréia/quantificação em plasma humano. Cromatografia Líquida de Ultra Alta Eficiência/análise quantitativa.

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INTRODUCTION

Hydroxyurea (HU), a structural analogue of urea, inhibits the activity of ribonucleotide reductase. It has been used clinically since the 1960s, primarily in the treatment of Chronic Myeloid Leukemia, acting as a cytotoxic, mutagenic, and recombinogenic antineoplastic agent with specificity to the S phase of the cell cycle (preventing cell division) and with specific action on ribonucleotide reductase (interfering with the conversion of ribonucleotides to deoxyribonucleotides) (Bachir *et al.*, 2007; Ballas, 2012). Since 1998, HU became part of the therapeutic program for patients with sickle cell anemia (SCA), and was the first drug with proven clinical efficacy in the treatment of this disease. HU is commercially available (Hydréia®) as a 500 mg tablet and the prescribed dosage ranges from 15 - 25 mg/kg /day. Response to HU treatment is variable, since the therapeutic response is associated with the clinical severity of the factors modulating SCA, including the concentration of fetal hemoglobin (HbF) in groups with haplotype beta globin, co-association with alpha-thalassemia, and the presence of polymorphisms for XmnI, BCL11A, and HBS1L-MYB (Cokic *et al.*, 2008). Moreover, the variability of the response may be associated with variations in drug metabolism (Cançado *et al.*, 2009).

The efficacy of HU treatment for SCA is generally attributed to its ability to enhance HbF, reducing the intra-erythrocytic polymerization of HbS in conditions of deoxygenation. However, HU also promotes a reduction in the number of neutrophils, reduced expression of erythrocyte adhesion molecules, and increased synthesis and bioavailability of nitric oxide by the activation of guanylyl cyclase and subsequent increase in cyclic GMP (Friedrisch *et al.*, 2008; Gladwin *et al.*, 2002).

Besides increasing the concentration of HbF, these mechanisms confer benefits such as reduced hemolysis, decreased adhesion of erythrocytes, leukocytes, and platelets to the vascular endothelium, and vasodilatation, contributing to a reduction in vaso-occlusive and inflammatory complications (Lettre *et al.*, 2008).

Despite its proven clinical efficacy, many aspects of the role of HU in patients with SCA remain unclear, such as optimal therapeutic dose, mechanism of drug action, the intense variability in therapeutic response, and cytotoxicity and genotoxicity. The carcinogenic potential of HU is not well established; some studies associate HU with an increased risk of leukemia in patients with myeloproliferative disorders (Manouilov, McGuire, Gwilt, 1998; Silva, Shimauti, 2006).

In order to establish a standard treatment regimen based on the pharmacokinetics of HU in patients with SCA, it is necessary to develop and validate a method for the quantification of HU in human plasma. This method was developed based on the methods described by Manouilov *et al.* (1998) and Bachir *et al.* (2007). We performed modifications in the sample preparation, using a smaller volume of plasma. For the colorimetric reaction, no commercial kits were used, while an Ultra High Performance Liquid Chromatography with diode detector (UV) was employed as it affords a higher resolution of the chromatograms analyzed. These modifications in the method de Manouilov (1998) and Bachir *et al.* (2007) resulted in a method with increased sensitivity, lower quantification limit, and reduced time of analysis; nevertheless, its validation is necessary (Manouilov, McGuire, Gwilt, 1998; Bachir *et al.*, 2007).

MATERIAL AND METHODS

Chemicals

Hydroxyurea (>98%; 04/04/2013), methylurea (97%; 04/04/2013) (internal standart), diacetyl monoxime ($C_4H_7NO_2$), tiosemicarbazide (CH_5N_3S) and ferric chloride ($FeCl_3$) were purchased from SIGMA® (St. Louis, MO, USA). Sodium chloride (NaCl) from LABSYNTH® (SP, Brazil), sulfuric acid from Cromato Produtos Químicos Ltda (SP, Brazil), orthophosporic acid from TEDIA® (RJ, Brazil), trichloroacetic acid and acetonitrile were purchased from VETEC® (RJ, Brazil). Plasma standards were obtained from Hemocenter of the State of Ceará, Brazil.

Solutions

A stock solution of hydroxyurea was prepared in acetonitrile/water (1:1) at a concentration of 10,000 μM, which was dissolved in acetonitrile/water (1:1) to obtain concentrations of 8000, 6000, 3200, 2000, 1000, 500, and 250 μM. The standard solutions were stored in a 4 °C, and after comparison with the test solutions prepared freshly observed stable for 20 days. The methylurea solution used as internal standard was prepared at a concentration of 15.5 mM.

The colorimetric solution (A) was prepared by combining 0.2 g thiosemicarbazide, 0.9 g sodium chloride, and 0.1 g diacetyl monoxime in distilled water qsp 100 mL. The acid solution (B) was prepared by adding 6 mL sulfuric acid (H_2SO_4) and 1 mL phosphoric acid to 80 mL of distilled water, dissolving 0.01 g ferric chloride in this solution, and filling to 100 mL with distilled water.

Chromatographic conditions

The equipment Ultra High Performance Liquid Chromatography (UPLC) consists of a pumping system with diode array detector (PDA) (Accela® Thermo Scientific) and data acquisition system ChromQuest 5.0 (Thermo Scientific®). Compounds were separated by reverse-phase chromatography on analytical column NUCLEOSIL C18, 250_4.6 column (5 µm particle size) from Phenomenex® and pre-column C18 (4x30 mm). The mobile phase used was acetonitrile/water (16.7 / 83.3) flow of 1 mL/min, wavelength 440 nm.

Sample preparation

In a 10 mL tube, 400 µL aliquots of plasma from patients treated with hydroxyurea were combined with 80 µL of 15.5 mM methylurea (internal standard) and 800 µL of 20% trichloroacetic acid to precipitate proteins. After vortexing (30 s) and centrifugation (3500 rpm for 10 min), 1000 µL of supernatant were transferred to another tube, and 1000 µL of solution A (colorimetric) and 1000 µL of solution B (acid reagent) were added. The mixture was heated in a water bath to 100 °C for 10 min and then cooled to 4 °C for 10 min. A 25 µL sample of the reaction product was subjected to chromatographic analysis.

Calibration curve

To construct the calibration curve (performed in triplicate), 400 µL aliquots of human plasma blanks (those not treated with hydroxyurea) were enriched with 100 µL of an appropriate concentration of the working standard solution to obtain hydroxyurea concentrations of 50, 100 200, 400, 740, 1200, and 1600 µM in plasma; these concentrations were chosen based on the expected plasma levels of HU and comprised seven data points, including the quantification limit. The internal standard (80 µL, 15.5 mM) was added to each solution, which was then subjected to the process of sample preparation and chromatographic analysis as described above.

The linear regression equations and correlation coefficients were obtained by dividing the peak area of hydroxyurea by the area of the internal standard (hydroxyurea/IS), plotted as a function of their concentrations. The acceptance criteria for calibration curves included a linear correlation coefficient greater than or equal to 0.99, a deviation less than or equal to 20% compared to the nominal concentration for the lower limit of quantification, and a standard deviation less than or equal to 15% over nominal concentration for the other

concentrations of the calibration curves.

Validation

The validation process was performed in October 2012 in accordance with Resolution RDC no 27 of 17/05/2012, the Ministry of Health – National Health Surveillance Agency, which is also in accordance with the Guidance for Industry – Bioanalytical Method Validation, 2013. (Brazil, 2012; FDA, 2013)

Limit of quantification

The limit of quantification was defined as the lowest concentration quantified with precision and accuracy with relative standard deviation (RSD) and coefficient of variation (CV%) ± 20%. Samples of human plasma were enriched with hydroxyurea to yield concentrations as low as 50 µM. The samples were analyzed in quintuplicate.

Linearity

Linearity was assessed at the concentrations used in the calibration curve, by gradually increasing the concentration to 1600 µM hydroxyurea in plasma. After. we determined the linear regression equations and correlation coefficients.

Selectivity

The interference from endogenous components of plasma was assessed by the analysis of plasma aliquots obtained from six different individuals who were not treated with hydroxyurea.

Precision and accuracy

To determine precision and accuracy, quality controls were used at concentrations of 100, 740, and 1600 µM of hydroxyurea in plasma.

To evaluate precision within assays, 5 aliquots were analyzed for each control on a single day, or across a single calibration curve. To evaluate the precision between assays, aliquots were analyzed in quintuplicate, with quality controls on three consecutive days. Precision was expressed as coefficient of variation (CV%), assuming no values of ± 15% (fifteen percent).

Accuracy within and between assays was also evaluated using the experimental results from the precision evaluations. The accuracy was expressed through the Relative Standard Error (RSE), not including values

outside the range of ±15% (fifteen percent) of the nominal value.

$$RSE = \frac{\text{Average concentration experimental} - \text{nominal value}}{\text{nominal value}} \times 100$$

Clinical and experimental protocol

We analyzed 22 adult patients (5 men and 17 women, aged between 20 and 40 years) with clinical and laboratory diagnoses of SCA, confirmed by molecular biology, with a baseline treatment with hydroxyurea for over a year, accompanied at the outpatient clinic of a university hospital in Fortaleza, Ceará, Brazil. Baseline determination was based on the criteria described by Ballas (2012): absence of painful episodes and/or concurrent illnesses such as infections or inflammation in the four weeks preceding the study; no hospital admissions in the 2-3 days prior to study; and absence of blood transfusion in the four months preceding the study. The exclusion criteria included an absence of molecular diagnosis of SCA, pregnancy, presence of renal or liver failure, or smoking (Ballas, 2012).

Informed consent was obtained from all patients (or from the responsible person) prior to enrollment into the study, and the study protocol was submitted to and approved by the Ceará Federal University Ethics Committee under protocol N° 110.10.10.

Venous blood was collected in containers containing the anticoagulant heparin, 2.5 h after drug intake. Plasma samples for chromatographic analysis were obtained by centrifugation of the blood samples at 4000 rpm for 10 min and were stored at -80 °C until the time of analysis.

Plasma concentrations of hydroxyurea in the patients tested was calculated using the calibration curves.

RESULTS

Analysis of hydroxyurea in plasma

Figures 1 and 2 show the chromatographic analysis of a plasma blank enriched with solutions of hydroxyurea and methylurea (internal standard) and plasma from a patient with sickle cell disease being treated with hydroxyurea (1000 mg/day), respectively. It was observed that the retention times of hydroxyurea, urea, and methylurea were 6.763 min, 7.778 min, and 11.472 min, respectively.
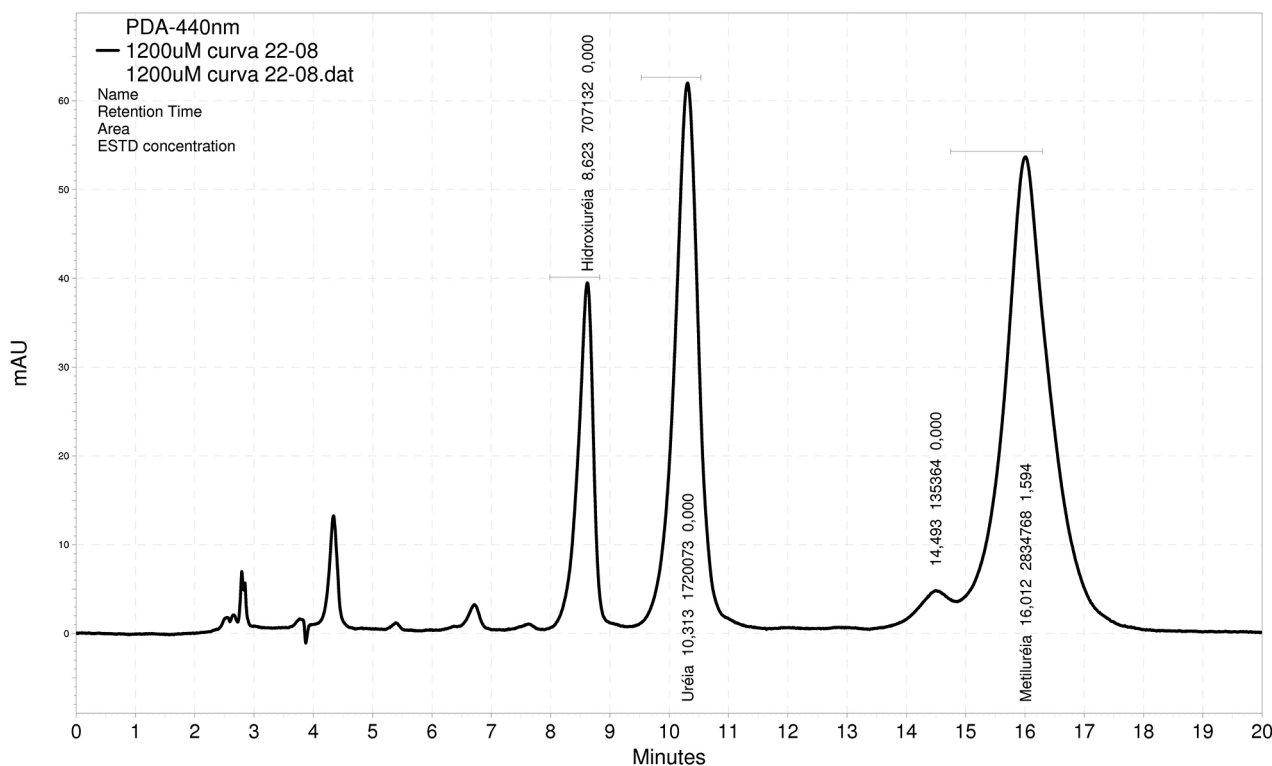
FIGURE 1 - Chromatogram of plasma enriched with 1200 µM hydroxyurea and 15.5 mM methylurea (internal standard).
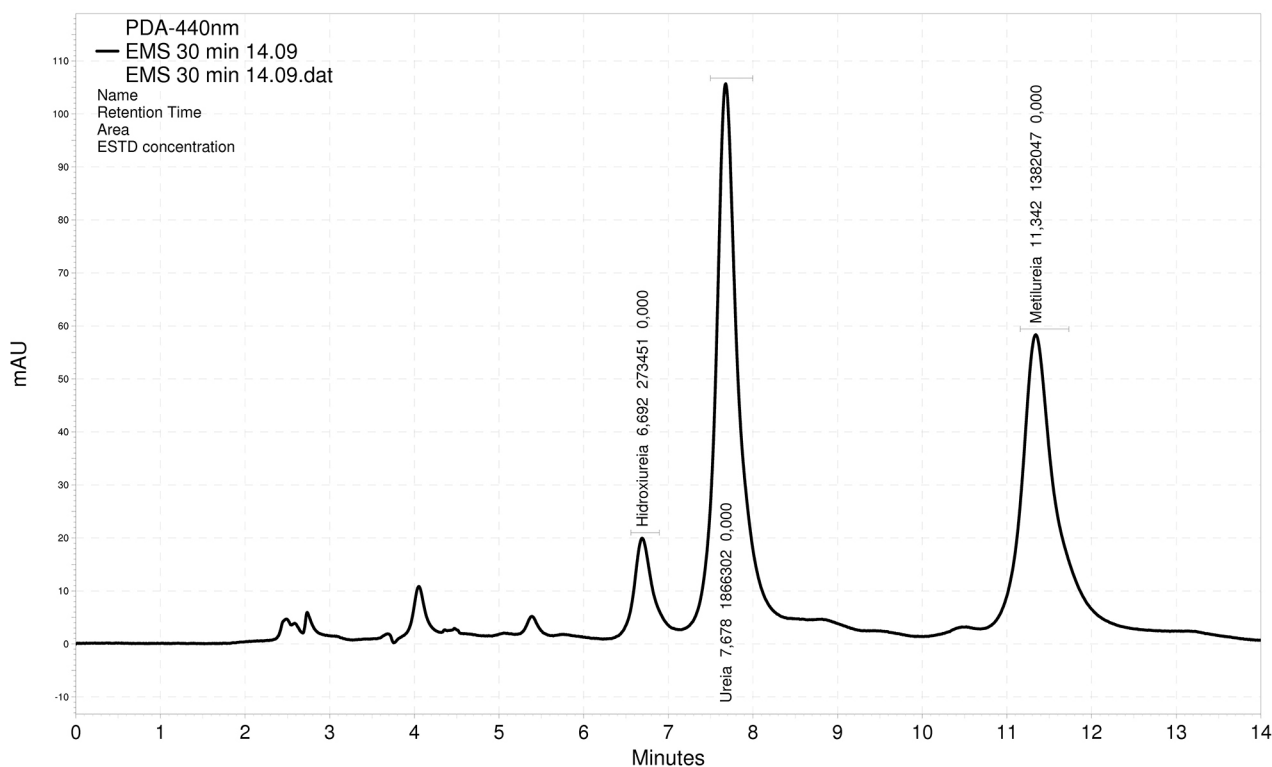
FIGURE 2 - Chromatogram of plasma from patients undergoing SCA therapy with hydroxyurea 1000 mg/day enriched with 15.5 mM methylurea (internal standard).

Calibration Curve

The Figure 3 shows the calibration curve in the range of 50-1600 mM of hydroxyurea in human plasma. The correlation coefficient was 0.9908.
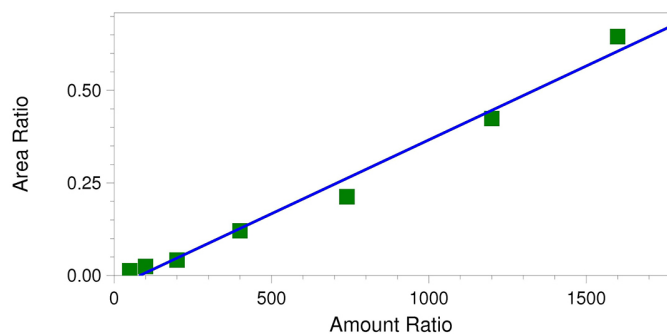

FIGURE 3 - Calibration curve of HU in the range of 50-1600 µM hydroxyurea in human plasma.

Validation of method

Table I shows the results of the validation of the analytical method of hydroxyurea in human plasma. The calculated correlation coefficient (r) indicates a linear relationship between the area and the concentration of hydroxyurea. Method was validated according to national guidelines for the validation of analytical and bioanalytical methods (RDC 27/2012) and to the ICH (International Conference on Harmonisation) guidelines for bioanalytical method validation (ANVISA, 2012; International Conference on Harmonisation, 2001).

Analysis of patients

We analyzed 22 adult patients (5 men and 17 women, aged between 20 and 40 years) with clinical and laboratory diagnoses of SCA treated with hydroxyurea for over one year, at doses of 500 mg/day (1 patient), 1000 mg/day (18 patients), and 1500 mg/day (3 patients), and the average HU concentrations in plasma collected from these dosage groups were 98.31 µM, 190.88 µM, and 302.71 µM, respectively.

DISCUSSION

Hydroxyurea therapy in patients with sickle cell

TABLE I - Confidence limits for the method of analysis of hydroxyurea

	Hydroxyurea
Linearity (50-1600 µM)	
Line Equation	y = 0.00029x - 0.00285
R	0.99084
Limit of Quantification (50 µM)	
Precision (CV%, n = 5)	13.45
Accuracy (RSE, n = 5)	4.96
Precision within assays	
(CV%, n = 5)	
100 µM	2.22
740 µM	2.52
1200 µM	4.52
Precision between assays	
(CV%, n = 15)	
100 µM	6.39
740 µM	7.04
1200 µM	10.38
Accuracy within assays	
(RSE%, n=5)	
100 µM	0.80
740 µM	-4.26
1200 µM	-5.94
Accuracy between assays	
(RSE%, n = 15)	
100 µM	-1.35
740 µM	-2.50
1200 µM	3.32

CV = Coefficient of variation [(SD/média)x 100];
r = linear correlation coefficient.
RSE= [(Caverage experimental-C added)/C added]x100

TABLE 2 - Average concentrations of HU in patients with SCA

Treatment dose (mg/day)	Average Concentration (µM) ±SD	N
500	98,31±0	1
1000	190,88 ± 100,7433	3
1500	302,71± 212,6223	18

anemia (SCA) has a proven clinical and laboratory efficacy. Administered in concentrations of 15 to 25 mg/kg/day, it can induce an increase of 10 to 40% of HbF. However, the variability in therapeutic response is high and may be associated with pharmacogenetic factors that lead to phenotypic characteristics of fast or slow metabolizers. Given this variation, the search for a safe therapeutic use that avoids the cyto- and genotoxicity of

the drug through a standard regimen based on therapeutic monitoring is essential (Steinberg *et al*., 2010; Veale *et al*., 1988). Thus, the quantification method of hydroxyurea in human plasma by UPLC with diode detector (UV) was developed in order to follow up patients treated with this drug. It was developed from the methods of Manouilov *et al*. (1998) and Bachir *et al*. (2007) and is based on the formation of a color reaction between hydroxyurea and solutions A (thiosemicarbazide + sodium chloride + diacetyl monoxime) and B (sulfuric acid + phosphoric acid + ferric chloride) (Manouilov, McGuire, Gwilt *et al*., 1998; Bachir *et al*., 2007).

While developing the method, the optimal wavelength was chosen by performing a "scan" of wavelengths between 300 and 600 nm. A wavelength of 440 nm was identified as having the highest absorption. To choose the best mobile phase, adjustments were made in the proportions of acetonitrile/water, and the analysis time obtained was of 13 min, a reduction of 50% compared to the method of Bachir *et al*. (2007), which used 16.7% acetonitrile (Bachir *et al*., 2007).

The plasma sample was treated by an initial precipitation of proteins (clean-up) using trichloroacetic acid instead of perchloric acid, as described by Bachir *et al*. and Manouilov *et al*., thereby reducing interference by endogenous substances. After this step, the colorimetric reaction was carried out (Manouilov, McGuire, Gwilt, 1998; Bachir *et al*., 2007).

The specificity of the method was confirmed when it was observed that the retention times of hydroxyurea, urea, and methylurea were 6.763 min, 7.778 min, and 11.472 min, respectively, with no interference to methylurea (internal standard) or urea from endogenous substances at high concentrations in the plasma. The calibration curve in the concentration range of 50-1600 µM hydroxyurea in plasma was selected from therapeutic concentrations observed in the dosages of 15 mg/kg/day to 30 mg/kg/day of hydroxyurea.

The calibration curve in the concentration range of 50-1600 µM hydroxyurea in plasma was selected from therapeutic concentrations observed in the dosages of 15 mg/kg/day to 30 mg/kg/day of hydroxyurea.

The method was deemed precise and accurate, using quality controls at low, middle, and high concentrations of 100 µM, 740 µM, and 1200 µM, respectively, presenting a quantification limit, selectivity, precision, and accuracy compatible with application in the analysis of hydroxyurea in patients treated with a multiple dose regimen. Moreover, the method was considered to be linear, since the obtained correlation coefficient was greater than 0.99.

We note that, in the validation process, hydroxyurea,

methylurea standard solution, and solutions A and B used in colorimetric reaction all showed a stability of greater than 20 days. The color reaction product was unstable after 5 h of reaction, with a decrease in absorbance, and plasma samples containing hydroxyurea are unstable for a period longer than six months even when stored at -80 °C.

The mean concentrations obtained 2.5 h after ingestion of hydroxyurea in doses of 500 mg, 1000 mg, and 1500 mg per day were 98.31, 190.88, and 302.71 µM, respectively, compatible with the pharmacokinetics of the drug, whose maximum concentration (Cmax) is 793.75 µM for a dose of 2000 mg/day with maximum time (Tmax) of 1.22 h (Ware *et al*., 2011; Rodriguez *et al*., 1998). However, it should be noted that there are phenotypic differences in drug metabolism; in rapid metabolizers, Cmax is achieved 15 to 30 min after ingestion, whereas in poor metabolizers Cmax is achieved 60 to 120 min after ingestion (Ware *et al*., 2011; Rodriguez *et al*., 1998). Therefore, for therapeutic monitoring of patients treated with HU, the ideal collection time would be 30 min and 1.5 h after drug intake.

The results of this study demonstrate that the method presents highly linear correlation and can be used for quantitative determination of hydroxyurea for therapeutic monitoring in patients with sickle cell anemia.

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