New isocratic high-performance liquid chromatographic procedure to assay the anti-sickling compound hydroxyurea in plasma with ultraviolet detection

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Abstract

A new procedure using high-performance liquid chromatography (HPLC) with ultraviolet detection to assay hydroxyurea (HU) levels in plasma has been developed. The drug was isolated from plasma by a direct deproteinization process with sulfosalicylic acid. Following neutralization of the acidic supernatant, an aliquot was loaded onto an Aminex HPX-72S column (300×7.8 mm). Chromatography was performed at $55°C$ using a mobile phase consisting of acetonitrile–0.025 M ammonium sulfate buffer (pH 8.5) including 0.1% triethylamine, 0.01 M sodium sulfate, and 5 mM sodium heptane sulfonate. The UV absorbance of effluent was monitored at 214 nm. A flow-rate of 0.8 ml/min was used for analyzing HU in both human and mouse plasma. Under these conditions, the drug eluted at 12.6 min. The assay possessed linearity up to 425 µg/ml, with a lower limit of quantitation of 3.32 ± 0.0004 µg/ml (mean$\pm$S.D., $n=10$). Intra-day and inter-day coefficients of variation were less than 8.5% and 8.7% respectively. Absolute differences were less than 7.4%. The method has been employed in clinical studies and the sensitivity of the assay was shown to be adequate for characterizing the plasma pharmacokinetics of HU in mice. In conclusion, the procedure described herein could be ideally suited for therapeutic monitoring of hydroxyurea.   © 1998 Elsevier Science B.V.

Keywords: Sickle cell disease; Antisickling agent; Hydroxyurea

1. Introduction

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The compound, hydroxyurea (Hydrea), first synthesized by Dresler and Stein in 1869, has been used in cancer chemotherapy [1]. Hydroxyurea (HU) current clinical usefulness is primarily limited to control of mature cell numbers in the chronic phases of some myeloproliferative syndromes and the rapid reduction of blast cell counts in patients with acute

leukemia during the early stages of treatment. Recent studies have also revealed the implication of hydroxyurea as an inhibitor of human immunodeficiency virus-type1 replication [2,3]. Because this antineoplastic compound has also shown efficacy in reducing vasoocclusive episodes and hemolysis, it has become important in the management of sickle cell disease. Studies in human subjects indicate a profound increase in the number of reticulocytes containing fetal hemoglobin (Hb F), thereby inhibiting polymerization of sickle hemoglobin (Hb S); since Hb F has been found to have a 'sparing effect' on Hb S polymerization [4]. In view of the usefulness of HU in sickle cell disease treatment and management, The National Heart, Lung and Blood Institute (NHLBI) in January 1995, recommended the use of HU for treatment of sickle cell individuals to reduce the severe clinical manifestations observed among this group of patients.

In order to delineate the pharmacokinetics of HU and to ensure its potential use for therapeutic drug monitoring, we found it necessary to develop a method for its accurate quantitation in blood plasma after interperitoneal and oral administrations in laboratory animals and humans respectively. Although some accomplishments have been made earlier in the development of an analytical method for the determination of hydroxyurea in biological fluids, these authors adopted the colometric method of analysis which lacks sensitivity [5,6]. The only available high-performance liquid chromatographic method, employs an ultraviolet detector used to assess the purity of hydroxyurea in pharmaceutical formulations [7]. However, this method is unsuitable for measurement of HU in biological fluids. Although Havard et al. has published a report on the use of HPLC method in the analysis of hydroxyurea in blood plasma, they utilized an electrochemical detector for analysis which is expensive and of less universal use as the UV detector [8]. Generally, ultraviolet (UV) detectors are favored because of their versatility, reliability, sensitivity and relatively lower cost. Based on the above findings, we have developed an alternative method of analysis using ultraviolet detector and ion-exclusion column. As described herein, the procedure involves isocratic ion-exclusion HPLC with UV detection following isolation of the drug by simple deproteinization with

an ion-chelating organic acid. The assay has been shown to be specific for HU, has been thoroughly validated and proven to be reliable, through extensive application during preclinical pharmacokinetic studies and in therapeutic monitoring of HU in sickle cell patients.

2. Experimental

2.1. Reagents and chemicals

Hydroxyurea, potassium phosphate (monobasic), sodium heptane sulfonate, triethylamine and HPLC grade acetonitrile, were all purchased from Sigma (St. Louis, MO, USA). Sulfosalicylic acid, EDTA and HPLC grade methanol were from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure ammonium sulfate was obtained from J.T. Baker (Phillipsburg, NJ USA). Anhydrous sodium sulfate was purchased from Mallinckrodt, (Paris, KY, USA). Mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA). HPLC-grade deionized water was purchased from Aldrich Chemical Company (Milwaukee, WI, USA). A 5-ml Heparin-containing VACUTAINER was purchased from Becton Dickinson VACUTAINER Systems, (F-ranklin Lakes NJ, USA).

2.2. Instruments

The HPLC system was a Waters 600E multisolvent delivery system controller, consisting of a Model 700 satellite WISP sample processor, Model 484 tunable absorbance UV detector, and a Model TCM temperature controller (Waters Chromatography Division, Millipore, Milford, MA, USA), a BIO-RAD Aminex HPX-72S ion exclusion analytical column, (BIO-RAD Laboratories, Hercules, CA, USA). Samples filtration was achieved with a 10-ml syringe (Becton Dickinson, Rutherford, NJ, USA) attached to a Nalgene disposable filter (Nalgene, Rochester, NY, USA). Microspin centrifuge was purchased from Sorvall instrument (Dupont, Atlanta, GA, USA). The chromatogram was recorded with NEC PowerMate 1 advanced personal computer (NEC Information systems, Boxborough, MA, USA)

with an AMDEK 12″ monitor (AMDEK, Elkgrove Village, Ill, USA).

2.3. Chromatographic conditions

The mobile phase contained 0.025 mol/l ammonium sulfate, 0.005 mol/l sodium heptane sulfonate, 2.5% acetonitrile, 0.1% triethylamine, and 0.01 sodium sulfate. The flow-rate was fixed at 0.8 ml/min (column pressure approximately 4×10^6 Pa), The column temperature was maintained at 55°C and the eluent was monitored at 214 nm. Hydroxyurea eluted at 12.6 min with other extraneous materials from plasma without coelution. Analysis was complete in 30 min.

2.4. Stock and working solutions

A stock solution of hydroxyurea was prepared by dissolving 15 mg in 10 ml of deionized water. This solution was used to prepare working standard solutions for different concentrations between 11.25 μg/ml and 426 μg/ml. The 1 mg/ml solution was diluted 1:50 with deionized water to prepare working standard solutions of 3.32 μg/ml and 5.75 μg/ml. All standard and stock solutions were stored in reagent bottles at 4°C and periodically checked against freshly prepared standards.

2.5. Preparation of plasma standards and deproteinization procedure

Approximately 500 μl of whole blood from ICR mice and humans were collected into a 1.5-ml heparinized Eppendorf tube (in accordance to the institutional guidelines for the care and use of animals and the institutional Review Board for research in humans) and centrifuged at 2500*g* for 5 min. The plasma supernatant was pipetted into a separate Eppendorf tube and stored at −20°C until ready for use. Working stock solutions of hydroxyurea (100 μl) were added to 200 μl of heparinized blank plasma and mixed thoroughly resulting in a concentration range of 3.32 μg/ml to 425 μg/ml standards. Samples for accuracy, precision, and stability determination were prepared in the same manner. For clinical and pharmacokinetic studies

samples, 100 μl of deionized water was added to the 200 μl of plasma in Eppendorf tubes (no hydroxyurea standard was added). To deproteinize the plasma, 400 μl of 10% sulfosalicylic acid (SSA) were added to the standards or study samples which were then vortex-mixed for 30 s followed by centrifugation at 5000*g* for 10 min. The supernatant was collected and neutralized with 15–20 μl of 10 mol/l sodium hydroxide. Following a further centrifugation at 5000 *g* for 5 min, the supernatant was filtered and transferred to micro-vials and 80 μl were injected onto the HPLC column for analysis. The results were calculated by interpolation after correction for dilution.

2.6. Calibration and calculations

The calibration graph was obtained by plotting the peak area of hydroxyurea in plasma standards, after correction for dilution, against the concentration (μg/ml). The best fit was obtained using the linear regression that was best described by the equation: $y = ax + b$, without inclusion of the origin, to determine the slope, *y*-intercept and correlation coefficient of the best-fit line. Analytes of unknown concentrations in pharmacokinetic plasma as well as plasma in clinical specimens were calculated by interpolation. All plasma samples and standard solution concentrations are expressed as free base of hydroxyurea.

2.7. Mouse pharmacokinetic study

Male ICR mice weighing 30–42 g were treated by intraperitoneal injection with hydroxyurea (200 mg/kg) dissolved in physiological saline solution (0.9% sodium chloride). At seven time intervals ranging from 3 min to 75 min after dosing, groups of three animals were anesthetized with ether and bled (500 μl) from the retro-orbital sinus using heparinized capillary tubes into Eppendorf heparin-coated microcentrifuge tubes (Curtin Matheson Scientific, Jessup, MD, USA). The plasma was separated from the red blood cells by centrifugation at 2500 *g* for 5 min, kept frozen at −80°C, and processed for analysis within 48 h.

3. Results

3.1. Selectivity

A typical liquid chromatogram of drug-free mouse plasma and mouse plasma spiked with HU as determined by UV detection at 214 nm is shown in Fig. 1A and B. Fig. 1C represents the chromatogram of a 12-min postdose plasma sample collected from a mouse treated with a single 200 mg/kg interperitoneal administration of hydroxyurea. The chromatograms of drug-free human plasma and HU-spiked human plasma are shown in Fig. 2A and C respectively, and the chromatogram of a 45-min postdose oral administration of HU in sickle cell patient (10 mg/kg) in Fig. 2C. Comparison of the chromatograms demonstrates the absence of endogenous components eluting at the retention time of HU (t_R 12.6 min) and the drug was well resolved.
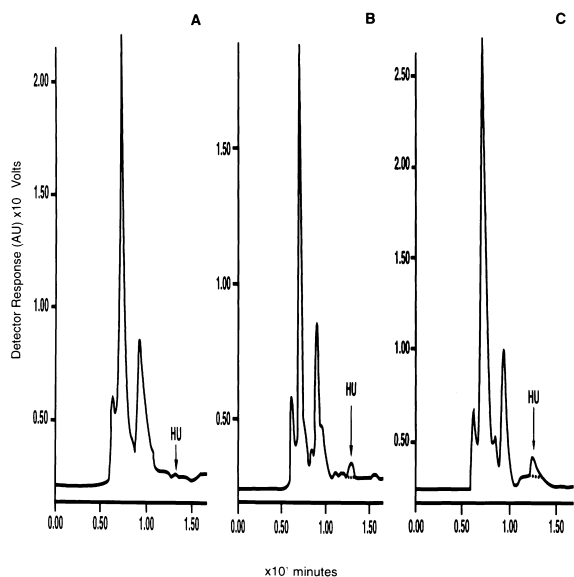

Fig. 2. Chromatographic analysis of deproteinized human plasma without hydroxyurea (A), plasma spiked with 53.0 μg/ml of hydroxyurea (B), and (C) plasma from patient receiving hydroxyurea (oral dose: 10 mg/kg once daily, plasma 18.3 μg/ml). The separation was performed as described in Section 2.3, at a flow-rate of 0.8 ml/min. The arrow indicates the retention time of hydroxyurea. Sensitivity was set at 0.005 a.u.f.s.

3.2. Linearity

Calibration curves for extracts from plasma was determined and the graph was linear to 425 μg/ml. Standard curves obtained from different calibration lines performed on different days were ($y = 0.7717x + 0.99914$, $r^2 = 0.998 \pm 0.001$, $n = 10$), slope $= 0.7717 \pm 0.275$ and intercept $= 0.99914 \pm 0.0665$ with the limit of quantitation of 3.32 μg/ml. Above 425 μg/ml, the calibration graph was nonlinear. For both mouse and humans, 0.2 ml of plasma was extracted per sample. A typical signal-to-noise ratio at the lowest concentration level was greater than 3.

3.3. Reproducibility or precision

Intra-day and inter-day reproducibility of the assay were determined in human plasma samples in the concentration range 5.75 μg/ml to 136 μg/ml. To determine intra-day reproducibility, samples were

Fig. 1. Representative liquid chromatograms of 80-μl injections of deproteinized mouse plasma. (A) Hydroxyurea-free plasma; (B) plasma spiked with 23.0 μg/ml hydroxyurea; (C) plasma from mouse 12 min after intraperitoneal administration of HU (dose: 200 mg/kg; plasma: 26.42 μg/ml). Samples were prepared for analysis and separated as described under Sections 2.5 and 2.7. The arrow indicates the retention time of hydroxyurea. Sensitivity: 0.005 a.u.f.s.
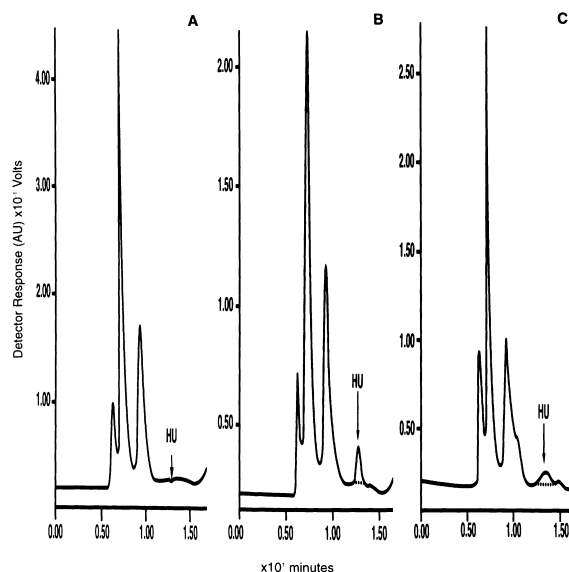
Table 1
Precision of hydroxyurea determination from human plasma

Added concentration (μg/ml)	Intra-assay precision ($n=5$)		Inter-assay precision ($n=6$)	
	Found concentration (mean±S.D.) μg/ml	C.V. (%)	Found concentration (mean±S.D.) μg/ml	C.V. (%)
136.00	139.86±9.81	7.0	139.24±10.61	7.6
46.00	44.37±3.80	8.5	45.02±3.29	7.3
23.00	23.03±0.76	3.3	22.59±1.10	4.8
11.50	11.06±0.54	4.8	10.99±0.96	8.7
5.75	5.68±0.29	5.2	5.62±0.47	8.5

spiked in five replicates at each concentration and analyzed on the same day. Inter-day reproducibility of the assay was assessed by analyzing the spiked samples at each concentration (fresh samples were prepared daily) on six consecutive days. The results are summarized in Table 1. The coefficients of variation (C.V., %) for intra-day and inter-day assay precision results ranged from 3.3 to 8.5% and 4.8 to 8.7%, respectively.

3.4. Accuracy

The accuracy of the method was evaluated as percent error [(mean of measured-mean of added)/mean of added]×100 at five different HU concentrations in plasma samples. Spiked samples were prepared at least in triplicate at each concentration in the range of 13.25 μg/ml to 212 μg/ml. The results are summarized in Table 2. The absolute difference between the spiked and found concentrations of hydroxyurea ranged from 0.90 to 7.4%.

3.5. Extraction recovery

The extraction recovery of hydroxyurea following deproteinization of mice and human plasma was determined by comparing the peak areas of HU from extracted standards to those from the unextracted standards at the same concentrations within the validated range of 3.32 to 425 μg/ml. Five replicates at each concentration were analyzed and the recovery was in the range of 92 and 104%.

3.6. Stability studies

Blank plasma from both human and mouse was spiked with three concentrations of 5.75, 11.5, and 23 μg/ml HU, deproteinized and frozen at $-20°$C over a 7-day period. For daily analyses, a 80-μl sample was injected onto the column. The analytical results showed that HU was unstable in the deproteinizing agent with a consequent loss of 4, 28 and 40% of the initial concentration after 24, 48, and

Table 2
Accuracy and precision of the HPLC method

Added concentration (μg/ml)	Found concentration (mean±S.D.) μg/ml	C.V. (%)	Bias (%)	Recovery (%)
212.00	3	196.22±13.60	6.9–7.4	93
106.00	3	100.08±5.47	5.5–5.5	95
53.00	5	54.91±4.11	7.5+3.6	104
26.50	6	26.24±2.23	8.5–0.9	99
13.25	3	12.87±0.48	3.7–2.8	97

72 h respectively. For this reason, fresh samples and standard solutions were prepared daily for analyses. Further studies revealed that hydroxyurea is stable in plasma stored at room temperature, 4°C, and −20°C without deproteinization for up to 48 h after collection as also observed by Havard et al. [8]. After a 14-day period at −80°C, the HU concentration in plasma without deproteinization reduced considerably to an undetectable level. The reduction of hydroxyurea concentration in plasma samples as observed, may be attributable to the action of urease, since Davidson and Winter reported that urease degrades hydroxyurea [5]. Stock solutions of HU stored at 4°C was however, stable for several months without any apparent degradation.

3.7. Pharmacokinetics of hydroxyurea in ICR mice

The mean plasma concentration versus time curves of hydroxyurea in ICR mice following a single 200 mg/kg interperitoneal dose are depicted in Fig. 3. Hydroxyurea was cleared rapidly from the system circulation following interperitoneal administration of the compound as indicated by a short terminal

half-life of 15.57 min and a high systemic clearance of 1.98 $1\,kg^{-1}\,h^{-1}$. The pharmacokinetics profile of HU in ICR mice also indicated an apparent first order kinetics between 3 and 12 min. Hydroxyurea however, did not distribute well in the body as the volume of distribution was relatively low, 0.6 l/kg. The compound was 100% bioavailable in ICR mice after interperitoneal administration. Maximum plasma concentration of 177.45 μg/ml was reached at 8.25 min.

3.8. Interferences

No possible interfering peaks from plasma constituents were observed when plasma from 10 different subjects were tested. Furthermore, drugs commonly prescribed with hydroxyurea in sickle cell disease management/treatment were also tested for coelution. The result shows that the assay proved to be free of interference from these compounds as summarized in Table 3.

4. Discussion and conclusion

In a previous investigation [8], the authors encountered difficulties in determining the HU level in plasma using a reverse phase HPLC with UV detection system. The use of electrochemical detector with reversed-phase HPLC gave a better resolution and specificity of HU in blood plasma. The initial difficulties encountered by these authors is feasible in that the HU remains positively charged ($pK_a = 10.6$) throughout the pH range (pH 2–8) compatible to most reversed-phase (RP) column. In addition, several organic compounds and other extraneous materials from body fluids exhibit maximum absorption within the same UV region at which HU absorbs maximally (210 to 214 nm) thereby creating a problem of interferences. We found that the addition of an ion-pairing agent (sodium heptane sulfonate) to the mobile phase for sufficient separation of HU from other extraneous materials in plasma did not solve the problem of coelution using a reversed-phase column with a UV detector. Several buffers (acetate, phosphate, and bicarbonate), with different concentrations and variation in pH and ion-pairing agents (tetrabutyl ammonium hydroxide and sodium
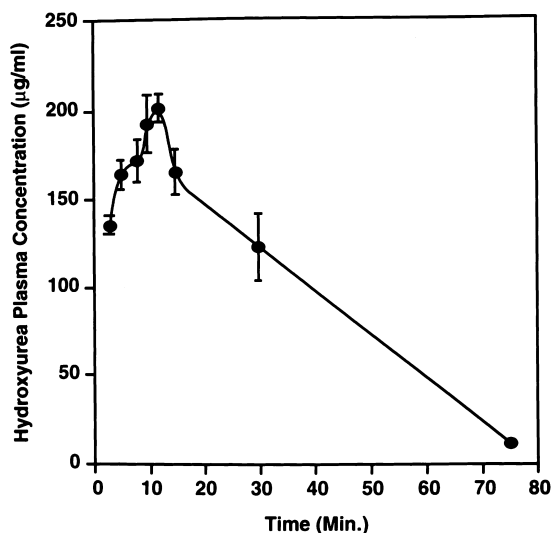

Fig. 3. Plasma concentration–time profile of HU in mice after intraperitoneal administration of 200 mg/kg dose. The plot shows the experimental geometric mean plasma concentrations of three mice per time point and the best-fit line generated by nonlinear regression analysis using Pharmkit software by Johnston and Woollard [12].

Table 3
Drugs studied for possible interferences[a]

Class	Drug	Trade name	t_R (min)	Remark
Penicillins	Penicillin V	Pen-Veek	10.13	−Ve peak
Penicillins	Ampicillin	Several	10.13	−Ve peak
Cardio glycosides	Digoxin	Lanoxin	9.98	−Ve peak
Diuretics	Furosemid	Lasix	10.01	−Ve peak
Chelating agents	Deferoxamine mesylate	Desferal	6.67	+Ve peak
Vitamins	Folic acid	Folic acid	–	no peak
NSAID[b]	Ibuprofen	Motrin	–	no peak

[a] Analyses for interferences were performed by spiking human plasma with equal concentrations of appropriate drug and HU (24 µg/ml) followed by deproteinization, as described in Section 2.5.
[b] Nonsteroidal anti-inflamatory drugs.

octane sulfonate) were investigated. However, no sufficient separation of HU was obtained. The use of an ion-exclusion column with a mobile phase containing ammonium sulfate and sodium heptane sulfonate (see experimental) gave a well-defined peak and a very good recovery behaviour with UV detector. Furthermore, the number of peaks eluting near the solvent front were greatly reduced and the baseline was well reestablished before the analyte emerged.

An assay for HU based upon HPLC with UV detection system was pursued with the objective of developing a method with commonly available chromatographic instrumentation keeping in mind, its specificity, accuracy, and cost effectiveness. The reported method is innovative in that most of the HPLC analyses involving biogenic amines, and basic drugs (including hydroxyurea), in biological fluid utilize electrochemical and fluorimetric detectors [9–11] which require several accessories to the HPLC with accompanied higher cost. Extraction with an immiscible organic acid has been commonly used to isolate basic compounds from plasma prior to chromatographic analysis [8,10]. Similarly, we found that hydroxyurea could also be efficiently extracted from plasma by sulfosalicylic acid with markedly clean liquid chromatograms of plasma extracts. During the course of these studies however, it became apparent that the drug was unstable in the presence of (SSA). This should not be surprising, in that SSA, an ion-chelating agent may have complexed with the positively charged hydroxyurea upon prolonged exposure. This problem was resolved when samples for analyses were freshly prepared daily. As also ob-

served by other investigators, hydroxyurea is unstable in plasma probably due to plasma protein interaction. Furthermore, we observed that plasma specimens could be kept for only 48 h at −20°C before the extent of degradation became significant. During deproteinization procedure, a very good percentage recovery of HU was attained by agitating the deproteinizing agent (SSA) while the plasma sample was being added.

In summary, a procedure based upon isocratic ion-exclusion HPLC has been developed for the determination of HU in plasma. The preparation of the samples involves a simple deproteinization procedure with SSA. Following the neutralization of the sample, 80 µl were injected onto the column and analyzed with UV detection at 214 nm. Several biological samples have been run on a single analytical column without degradation in its performance. The lowest concentration of HU quantified with precision was 3.32 µg/ml. This degree of sensitivity permitted the plasma concentration–time profile of intact drug to be definitively characterized in mice following intraperitoneal administration of 200 mg/kg dose. Furthermore, the specificity of the method for the determination of HU was conclusively established through the analysis of clinical samples acquired from sickle cell disease patients on a low daily oral dose of 10 mg/kg. We also observed no interference from plasma when samples from 10 different subjects were tested. The assay proved to be also free of interference from commonly prescribed drugs. The procedure has been thoroughly validated and shown to be accurate, specific, and easy-to use. The ease of sample preparation coupled

with its cost effectiveness, renders the assay procedure well-suited for preclinical and clinical pharmacokinetic studies as well as routine analysis for therapeutic monitoring of hydroxyurea.

Acknowledgements

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