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Quantitative analysis of trimethylsilyl derivative of hydroxyurea in plasma by gas chromatography–mass spectrometry

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Abstract

Hydroxyurea is an antitumor drug widely used in the treatment of sickle cell disease. The drug has been analyzed in biological fluids by a number of high-performance liquid chromatography (HPLC) methods. This paper describes a fast and highly reliable capillary gas chromatography–mass spectrometry (GC–MS) procedure that was developed for the detection and quantitation of hydroxyurea in plasma. The compound and its labeled internal standard were liquid extracted from plasma and derivatized with BSTFA before analysis. The detection limit of the assay was 0.078 μg/ml and the limit of quantitation was 0.313 μg/ml with linearity up to 500 μg/ml. Intra-day variation, as coefficient of variation (C.V., %) over the selected concentration range, was 0.3–8.7% and inter-day variation was 0.4–9.6%.

Keywords: Hydroxyurea; Sickle cell disease; Liquid extraction; Quantitation; Derivatization; Gas chromatography–mass spectrometry

1. Introduction

An analog of urea, hydroxyurea, is a structurally simple compound that was first synthesized in 1869 [1]. Due to its antineoplastic activity, it has been used in the treatment of myeloproliferative disorders and other malignancies [2–5]. Hydroxyurea has also been shown to have antiviral effect by inhibiting immunodeficiency virus-type 1 (HIV-1) replication [6–8]. In the treatment of patients with sickle cell anemia, the compound has demonstrated positive outcomes. It stimulated hemoglobin (Hb)F synthesis as well as nitric oxide and guanosine 3′,5′ cyclic monophosphate (cGMP) production [9–11] and increased mean cell volume (MCV) and F reticulocytes [12–15]. A decrease in hemolysis [13], red blood cell (RBC) deformability [12], and painful episodes in addition to a possible prevention of recurrent stroke, was also noted [15]. Thus, in the clinical evaluation of hydroxyurea treatments, a rapid and highly reliable analytical procedure for the measurement of hydroxyurea is fundamental.

A few published analytical methods have been applied to the detection and quantitation of hydroxyurea in biological fluids. Generally, these techniques have not demonstrated the needed speed, sensitivity, and specificity and often require many steps and large volumes of samples and materials. These methods include, colorimetric techniques, high-performance liquid chromatography (HPLC), or HPLC combined with colorimetry or electrochemical detection.

Fabricius and Rajewsky [16] described a colorimetric technique in the quantative analysis of hydroxyurea in blood samples (1 ml) from human subjects, rats, and mice as well as tissue (1 gm) from rat tumor, liver, and embryos. The range of the assay, however, was not very wide, from 0.4 to 4.0 μg/ml of hydroxyurea. Regrettably, preparation of the samples prior to their measurements required much time, many steps and chemicals. Utilizing also a colorimetric technique, a modified micro-urea nitrogen method with the Technicon Analyser, Davidson and Winter [17] determined the levels of hydroxyurea in 2 ml of human serum and urine samples that were first pretreated with urease to destroy urea. However, hydroxyurea itself was slowly degraded by the enzyme. In addition to urea, citrulline, allantoin, and *N*-carboylaspartate were interfering compounds. Unfortunately, blank serum samples reacted with maximal values of about 4 mg/l of hydroxyurea. The assay was able to measure hydroxyurea concentrations as low as 1–2 mg/l. Pluscec and Yuan [18] described an HPLC procedure for the analysis of

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the drug in pharmaceutical formulations and in raw material. The authors achieved good separation of hydroxyurea which had a retention time of about 6 min. The analysis, though, was extended because the internal standard, uracil, eluted at about 13 min. Unfortunately, the analytical method was not designed for measuring hydroxyurea in biological samples. In contrast, the HPLC method of Iyamu et al. [19] measured hydroxyurea in human and mouse plasma. The retention time of hydroxyurea was 12.6 min. The chromatograms depicted the presence of eluted endogenous components whose peaks were sharper and bigger than that of the hydroxyurea peak. The assay had a range from 3.32 to 425 μg/ml. Manouilov et al. [20], on the other hand, combined colorimetry with HPLC to determine the concentrations of hydroxyurea in human serum. A volume of 500 μl of sample was used. The method produced good separation of the drug peak which had a retention time of 6.5 min. However, the assay was prolonged since the internal standard, methylurea, eluted at 12.2 min. The range of the assay was from 0.5 to 100 μg/ml. Gwilt et al. [8] also reported the use of a technique of combined HPLC and colorimetry in their pharmacokinetic assessment of hydroxyurea in plasma and cerebrospinal fluid (CSF) of HIV-infected subjects. Absent the chromatograms from the report of the assay as performed by the authors, it is not known how well the hydroxyurea peak was separated. Nonetheless, the standard curve of the assay was reported to be linear in the 0.5–20 mg/l range. Hydroxyurea was determined to have a retention time of 7.9 min and methylurea, the internal standard, 17.2 min. A more sensitive method was developed by Pujari et al. [21] to measure hydroxyurea in human plasma and peritoneal fluid. The authors used HPLC in conjunction with electrochemical detection. Very little sample (10 μl plasma and 25 μl peritoneal fluid, respectively) was used in the assay and the chromatograms indicated good peak separation of the drug. Although the retention time of hydroxyurea was just over 3 min, the plasma sample produced a matrix interference peak at about 4.5 min. The assay was linear from 25 to 400 ng/ml for plasma and 2 to 30 ng/ml for peritoneal fluid. Similarly, Havard et al. [22] coupled HPLC with electrochemical detection to measure hydroxyurea in plasma. The authors also discovered that the plasma generated a matrix interference peak. That peak eluted about 2 min after the hydroxyurea peak which had a retention time of about 1 min. The assay was linear to 0.35 mM with a limit of detection of 0.02 mM.

This paper describes a novel approach to the measurement of human plasma hydroxyurea by GC–MS using BSTFA as a derivatizing agent and labeled urea as the internal standard.

2. Experimental

2.1. Chemicals and reagents

Hydroxyurea was obtained from Sigma (St. Louis, MO, USA). Urea was purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Isotopically labeled urea (^{13}C, 99%; $^{15}N_2$, >98%), the internal standard, was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA, USA). Acetonitrile, gas chromatography (GC) grade, was purchased from EM Science Company (Gibbstown, NJ, USA). *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce Company (Rockford, IL, USA). Toxi-Tubes A were purchased from ANSYS Diagnostics Inc. (Lake Forest, CA, USA).

2.2. Instrumentation

A Hewlett-Packard model HP 5890A gas chromatograph equipped with a mass selective detector (MSD), model 5970B, an HP 7673A autoinjector, and an HP 310 computer (Wilmington, DE, USA) was used for the analysis. Extraction of hydroxyurea was done by a process of liquid–liquid extraction coupled to centrifugation. A Brinkman Sample Concentrator, model SC/27R (Westbury, NY, USA), was used to concentrate the drug. This instrument is a variable temperature aluminum heating block designed for the efficient concentration of drug samples.

2.3. Chromatographic conditions

An HP-1 fused-silica capillary column (12 m × 0.20 mm I.D. × 0.33 μm film thickness, and consisting of cross-linked methyl silicone gum phase) was used. Perfluorotributylamine (PFTBA) was used to autotune the system daily. The multiplier was set at the autotune voltage. The carrier gas (helium, 99.995% purity) flow rate was 0.59 ml/min at a linear velocity of 31.25 cm/s. The gas chromatograph was run with the splitless valve on for 0.75 min and an initial column temperature of 90 °C which was increased by a rate of 50 °C/min up to 280 °C. The analytical run was allowed to go periodically beyond 5 min held at 280 °C to eliminate the possibility of artifacts. The temperatures of the injection port and transfer line were 275 °C and 280 °C, respectively. Operation of the MSD was in the electron-impact (EI) mode at 70 eV with a solvent delay of 1.3 min and a dwell time of 25 ms. Data were first acquired in the scanning mode in order to obtain full-scan EI mass spectra of the compounds for the selection of the appropriate ions for the selected ion monitoring (SIM) method. Quantitation ions using SIM were *m*/*z* 277 and 292 for hydroxyurea and *m*/*z* 192 for labeled urea. Ion chromatograms of each of the quantitation ions were analyzed after each run. A peak area ratio of hydroxyurea vs. the internal standard was used to determine the concentrations of hydroxyurea. Integration parameters for the threshold and peak width were set so as to optimize the detection of the peaks at the calibration retention time. These were 0.02 amu for the peak width and 9 for the threshold.

2.4. Assay procedure

A stock solution of aqueous hydroxyurea solution (1 mg/ml) was prepared fresh each day. Working standard solutions were prepared from this stock solution by making serial dilutions. To a 10-ml test tube, a volume of 200 μl of plasma was spiked with 200 μl of the appropriate concentration of the working hydroxyurea standard solution for the preparation of the standard curve: blank, 0.313, 0.625, 1.25, 2.5, 5, 10, 25, 50, 100, 250 and 500 μg/ml. The internal standard (100 μl,

200 μg/ml) was then added to each sample. Solutions of similar concentrations of urea were also prepared for comparative analysis without ever being added to plasma because of its endogenous presence. The sample was extracted in a Toxi-Lab A tube for 5 min using a rotating shaker. The extraction tube was centrifuged at 2665 × *g* for 10 min. The organic phase was transferred to a concentration glass vial. The specimen was evaporated to dryness at 75 °C under vacuum and reconstituted with 50 μl of acetonitrile. It was then transferred to a 1-ml autosampler vial. A volume of 50 μl of BSTFA was added to the mixture and incubated at 60 °C for 25 min. A volume of 1–2 μl was injected into the GC–MS and analyzed.

3. Results and discussion

3.1. Sample preparation

Samples were prepared as outlined above. Extraction of hydroxyurea and urea was accomplished by using Toxi-Lab A extraction tubes which consist of an organic liquid mixture (1,2-dichloroethane, dichloroethane, heptane, and isopropyl alcohol) and buffered salts. These commercially available tubes facilitate quick and efficient extraction of basic drugs (including hydroxyurea and urea) and neutral drugs for GC–MS analysis [23]. As a result, a recovery of over 95% of hydroxyurea was obtained. This recovery was consistent with either freshly prepared samples containing plasma and standard solutions of hydroxyurea or samples stored for months at 4 °C. The analytical method, therefore, was never adversely affected with time by the possible degrading action of urease on hydroxyurea as reported by Iyamu et al. [19].

GC–MS analyses of derivatized hydroxyurea and urea are shown in Fig. 1a and b, respectively. Analyses occurred in the scan mode. The mass spectra of the derivatized compounds appeared to be very similar to one another. This is to be expected since the only structural difference among the underivatized compounds is the presence of the additional functional group, –OH, found in hydroxyurea. The derivatized analytes, however,

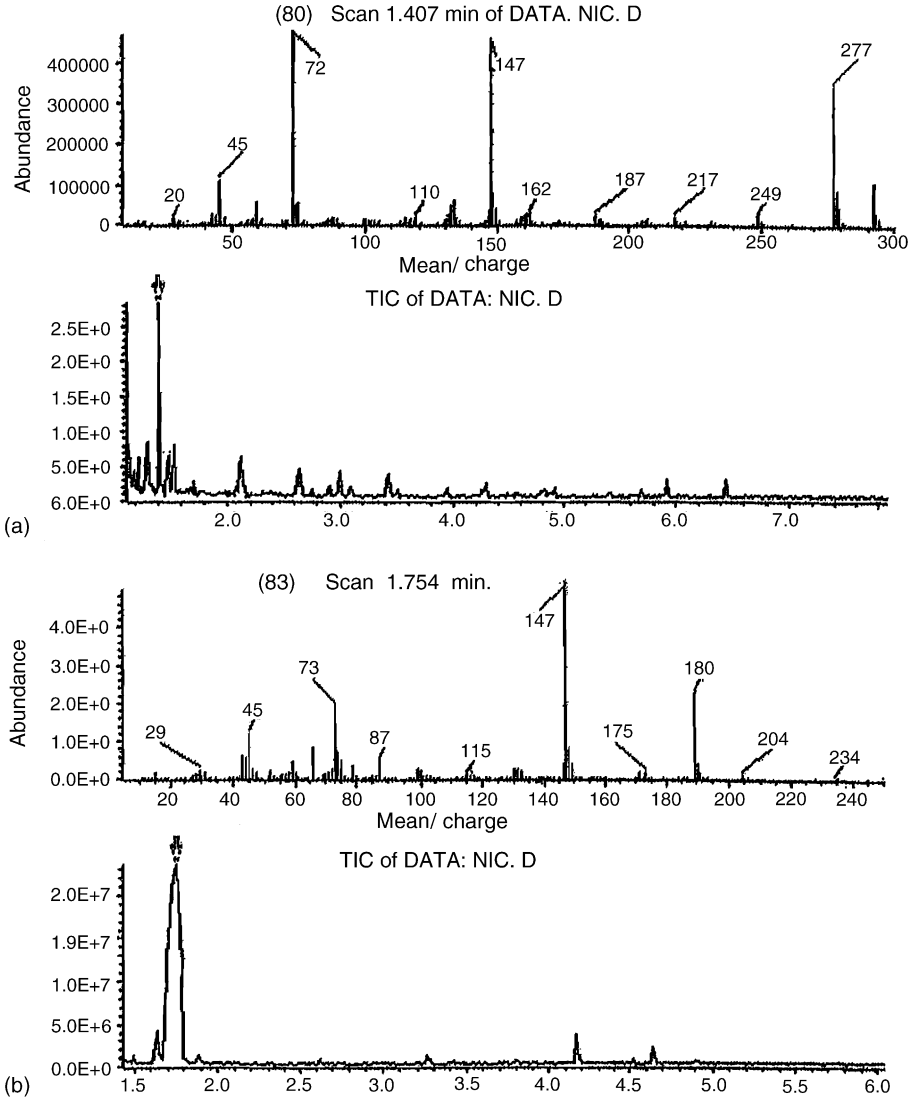

Fig. 1. (a) Total ion chromatogram and mass spectrum of 100 μg/ml of hydroxyurea extracted from spiked plasma and derivatized. Run under scan with 1-μl injection. (b) Total ion chromatogram and mass spectrum of 100 μg/ml of urea extracted from spiked plasma and derivatized. Run under scan with 1-μl injection.

were easily differentiated from one another by the presence of a few distinguishing ion peaks used in their positive identification: *m*/*z* 189 and 204 for urea; *m*/*z* 192 for labeled urea (figure not shown); and *m*/*z* 277 and 292 for hydroxyurea. Under the specified conditions, the typical retention time for hydroxyurea was 1.5 min; urea and labeled urea, eluting slightly sooner, were 1.4 min. The major ion peaks using the scan mode (10–500 amu) were *m*/*z* 73, 147, 277, and 292, for hydroxyurea; *m*/*z* 73, 147, 189, and 204, for urea; and *m*/*z* 192 and 207, for isotopically labeled urea.

3.2. Gas chromatography–mass spectrometry

In the SIM mode, the masses unique to hydroxyurea and urea present in trace levels in the samples, were selected and monitored for quantitative analyses. As a result, selectivity and sensitivity were obtained. The 189 amu ion of urea was 50% of base peak and the 192 amu ion of labeled urea was the base peak (data not shown). The 192 amu ion of the isotopically labeled urea was due to the three additional amu derived from the ^{13}C and $^{15}N_2$ isotopes. Fig. 2a and b, respectively, depict quantitative chromatographic analyses of plasma extracts spiked with hydroxyurea and derivatized. Similarly, drug-free plasma extracts, also derivatized, were analyzed. The typical chromatogram (Fig. 2c) shows the absence of endogenous components eluting at the retention time of either hydroxyurea or urea. The EI mass spectrum of extracted and derivatized plasma hydroxyurea from a sickle cell patient is shown in Fig. 2d. The patient was administered hydroxyurea orally (50 mg/kg) for 3 days.

The ion chromatograms of derivatized urea and labeled urea indicated that silylation, an analytical derivatization reaction, allowed only two trimethylsilyl (TMS) groups of BSTFA (the derivatizating agent) to be coupled to one molecule of urea or labeled urea. This was likely accomplished by the replacement of two labile hydrogen atoms, one each from the two nitrogen atoms of each compound. The reaction resulted in the formation of the unfragmented neutral derivatized urea or labeled urea (parent peak ion) having a mass of *m*/*z* 204 or 207, respectively. Subsequently, fragmentation of each TMS derivative elicited strong 189 and 192 amu ions, respectively. Additionally, each showed

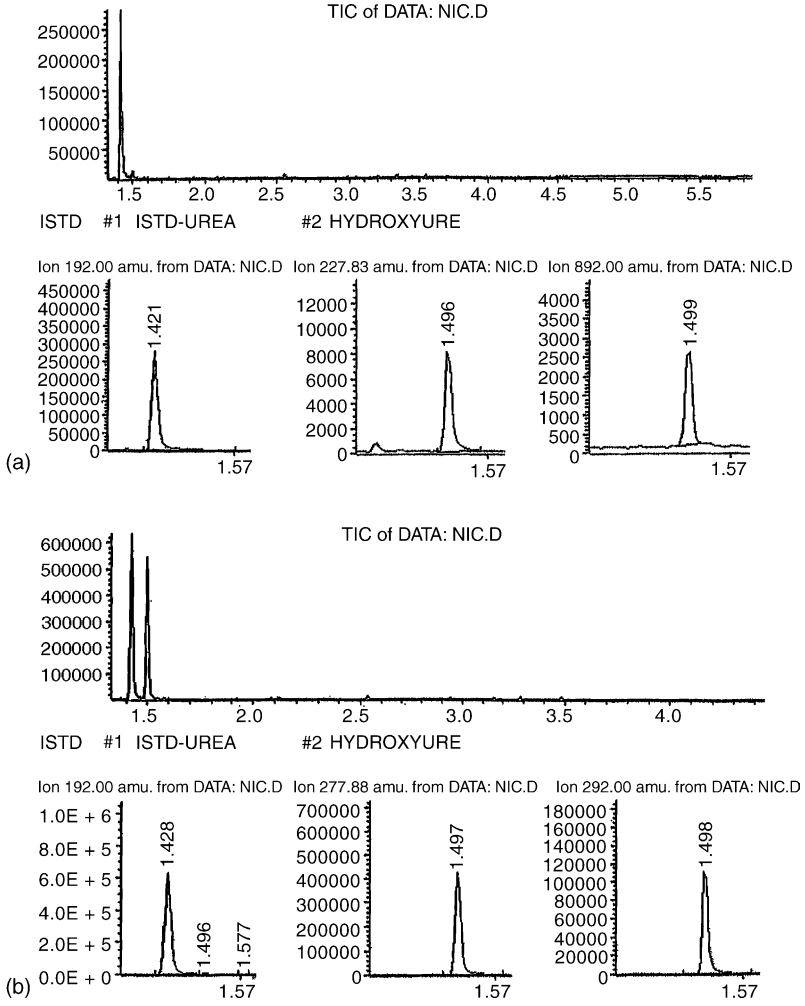

Fig. 2. (a) Total ion chromatogram of 0.313 μg/ml of hydroxyurea and 100 μg/ml of labeled urea extracted from spiked plasma and derivatized. Run under SIM with 1-μl injection. (b) Total ion chromatogram of 100 μg/ml of hydroxyurea and 100 μg/ml of labeled urea extracted from spiked plasma and derivatized. Run under SIM with 1-μl injection. (c) Chromatogram of hydroxyurea-free plasma. Run under SIM with 1-μl injection. (d) Total ion chromatogram of 1.36 μg/ml of hydroxyurea and 100 μg/ml of labeled urea extracted from plasma of sickle cell patient (oral dosage: 50 mg/kg for 3 days) and derivatized. Run under SIM with 1-μl injection.

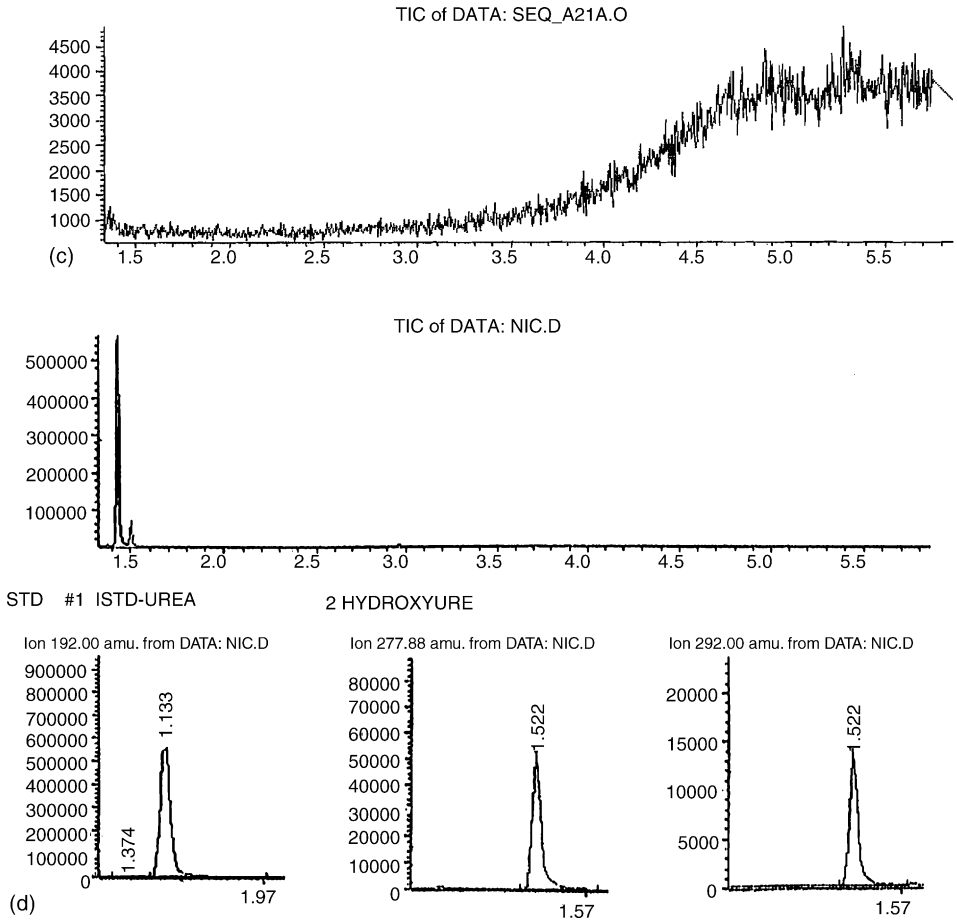

Fig. 2. (*Continued*).

slight 204 and 207 amu responses, respectively, that represented only a small percentage (4%) of base peak.

In the silylation of hydroxyurea, on the other hand, the ion chromatogram suggested that three TMS groups and not two (as in the case of urea or labeled urea) took part in the reaction. The three groups replaced three active hydrogen atoms of the analyte. The third hydrogen atom was derived from the hydroxyl moiety of hydroxyurea. As a result, the unfragmented derivatized hydroxyurea compound gave a mass of 292 amu. Subsequent fragmentation of the substance resulted in a very strong 277 amu ion and a fair 292 amu ion that was used as the confirming ion in the identification and quantitation of hydroxyurea.

3.3. Standard curve determination

Standard curves were determined for hydroxyurea in plasma from four separate assays over the selected concentration range, 0.313, 0.625, 1.25, 2.5, 5, 10, 25, 50, 100, 250, and 500 μg/ml. The results are shown in Table 1. The graph was linear from 0.313–500 μg/ml ($y = 0.05 + 0.999x$, $r^2 = 1.000$). The limit of detection was 0.078 μg/ml. Concentrations above 500 ng/ml needed to be diluted first before analysis since the calibration graph showed nonlinearity beyond that point. Published data obtained from clinical studies on cancer patients treated with hydroxyurea showed drug serum levels of 12.4–156.8 mg/l [2], 5 mg/l [17], and 0.58–2.47 mM [3], levels that fell within our calibration range. Additionally, results of preliminary studies obtained in our laboratory on three sickle cell patients (two adults and one pediatric) indicated also that plasma hydroxyurea levels fell well within our calibration curve (in the lower part).

Table 1
Results of various concentrations of hydroxyurea in spiked plasma[a]

Concentration added (μg/ml)	Concentration determined (mean ± S.D.) (μg/ml)[b]	C.V. (%)[c]	Recovery (%)
0.313	0.289 ± 0.01	4.50	90
0.625	0.651 ± 0.03	5.33	90
1.25	1.33 ± 0.03	2.15	92
2.5	2.48 ± 0.05	2.02	94
5	5.19 ± 0.23	4.56	97
10	10.31 ± 0.65	6.60	96
25	25.62 ± 0.29	1.17	96
50	49.45 ± 0.38	0.75	96
100	101.10 ± 3.90	3.89	99
250	250.27 ± 0.55	0.22	100
500	500.11 ± 0.47	0.09	102

[a] Determinations were made on four different days at each concentration.
[b] Standard deviation of the mean.
[c] Percent coefficient of the mean.

Table 2
Intra-assay variation and inter-day variation for hydroxyurea in spiked plasma

Concentration added (μg/ml)	Intra-assay variation ($n = 5$)			Inter-assay variation ($n = 5$)		
	Concentration determined (mean ± S.D.) (μg/ml)[a]	C.V. (%)[b]	Bias (%)	Concentration determined (mean ± S.D.) (μg/ml)[a]	C.V. (%)[b]	Bias (%)
0.313	0.316 ± 0.02	6.3	5.4–7.3	0.314 ± 0.03	9.6	8.6–10.5
5	5.24 ± 0.03	6.9	5.4–4.2	5.08 ± 0.27	5.3	7.0–3.8
10	10.33 ± 0.90	8.7	12.2–5.7	10.13 ± 0.33	3.3	4.6–2.0
50	49.88 ± 0.85	1.7	1.5–1.9	49.90 ± 0.76	1.5	1.3–1.7
250	249.94 ± 0.89	0.4	0.3–0.4	249.74 ± 1.21	0.5	0.4–0.6
500	499.66 ± 1.52	0.3	0.2–0.4	499.85 ± 1.85	0.4	0.3–0.4

[a] Standard deviation of the mean.
[b] Percent coefficient of the mean.

3.4. Hydroxyurea recovery

Hydroxyurea recovery was obtained by comparing the results of determined standard concentrations with those of the respective added concentrations. The recovery was in the range of 90–102%, a mean recovery of 95.6%. The results are shown in Table 1.

3.5. Validation of analytical method

Samples for validation studies were prepared as described above. Spiked plasma samples, representative of low, medium, and high concentrations, were assayed at 0.313, 5, 10, 50, 250, and 500 μg/ml. Additionally, a blank plasma sample was always included in the assay. A total of over 25 such blank matrix sources were studied. The intra-day variation was determined from five replicates at each concentration assayed on the same day. Evaluation of the inter-day variation was made on the same concentrations analyzed on five separate days. The average coefficients of variation (C.V., %) over the intra-day and inter-day ranges were 4.05% and 3.43%, respectively. Thus, the data, as shown in Table 2, indicate that the method is quite suitable for quantitation of hydroxyurea levels of plasma samples from 0.313 to 500 μg/ml.

4. Conclusion

In this report, a fast, simple, sensitive and specific GC–MS method is described for the analysis of hydroxyurea in human plasma. Importantly, the assay is negative for endogenous urea, a major metabolic product. In spite of a large number of analytical runs, performance of the capillary column has not been compromised. The assay, therefore, is highly suited for the evaluation of hydroxyurea treatments.

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