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Short communication

Plasma hydroxyurea determined by gas chromatography–mass spectrometry

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ABSTRACT

Hydroxyurea treatment is efficiently used to ameliorate the clinical course of patients affected with sickle cell disease. To understand the patient's wide variation in the clinical response to that drug and monitor its plasma levels, a new method was developed and validated. Fifty μL plasmatic samples containing hydroxyurea are added with internal standard, deproteinized, evaporated to dryness, silanized, and analyzed by gas chromatography–mass spectrometry, which operates in the selected ion mode after electron impact fragmentation. Linearity was found to extend to at least 100 mg/L. Over a 1–25 mg/L concentration range, coefficients of variation for intra-day and inter-day precision are 5.3% and 7.7%, respectively. Plasma blank-samples reveal endogenous hydroxyurea at a level ≤0.2 mg/L. The performances of the method, which is fast and simple, encounter the analytical goals needed for evaluation of hydroxyurea treatment and for pharmacokinetic studies.

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1. Introduction

Hydroxycarbamide (hydroxyurea, HU), the hydroxylated analogue of urea, is a potent and specific inhibitor of DNA synthesis acting on ribonucleotide reductase [1]. HU has been therefore introduced as myelosuppresive agent in the treatment of myeloproliferative disorders such as polycythemia vera, essential thrombocytosis, and chronic myelogenous leukaemia [2–6].

HU is also the only approved drug for treatment of sickle cell disease (SCD) [7]. It has demonstrated an efficacy in preventing recurrences of vaso-occlusive episodes, acute chest syndromes [8], primary stroke prevention [9] and in reducing transfusional needs in patients severely affected with SCD [8]. However, the estimated proportion of patients who do not benefit from HU treatment has been claimed to be 10–50% [10]. This can be explained by a poor compliance with the treatment and/or by pharmacokinetic variability. HU is administered once a day or, for children, once every 2 or 3 days. While HU half-life is short (ca. 3 h), the course of plasma levels is pulsating and presents trough level.

For these reasons, the demand is high for the development of a quantitative and sensible method suitable for plasma HU monitoring and pharmacokinetics studies. The latter requires methods sensitive enough to measure trough levels and/or to be applicable to small paediatric samples. Several analytical methods have been applied to the quantification of HU in physiological fluids. Spectrophotometric measurements of derivatized HU with picryl chloride [11] or of diazotated nitrites after HU oxidation with iodine [12,13] lack specificity and sensitivity and need a great quantity of blood. A more widespread determination of HU is by high-performance liquid chromatography (HPLC) methods. At the exception of the method of Iyamu et al. [14] who used ion-exclusion, HU is chromatographied on a reversed phase column. The drug is detected underivatized by ultraviolet detection [14] or by electrochemical detection [15–17]. Liquid chromatography of the HU diacetyl derivative has also been described [18,19]. Ultraviolet and colorimetric HPLC methods need 200-500 μL sample volume, and are not sensitive enough to suitably delineate HU pharmacokinetics. HPLC methods with electrochemical detection are far more sensitive, but lack specificity. Indeed, the HU peak is difficult to separate from the solvent peak as well as from other very-soluble compounds that could also be electrochemically active. Moreover, this mode of detection is skill-demanding and needs a close maintenance for guarantee detector stability and good performances.

Until now, gas chromatography–mass spectrometry (GC–MS) was only applied by James et al. [20]. Claimed analytical performances are promising but, in spite of extensive trials, this method does not provide, according to our tests, useful results by reason of a very low extraction yield. Dalton et al. [10] had recourse to tandem MS for analyzing urine samples. This method, still reserved to

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specialized laboratories, offers many capabilities and could be the future method of choice.

A new GC–MS method for the measurement of plasma HU, including a novel approach for the sample preparation, is here proposed.

2. Experimental

2.1. Chemicals and reagents

HU and methoxyurea (MU) were obtained from Sigma Chemical Co. (St Louis, USA). *N*,*O*-bis(trimethylsilyl)trifluoroacetamide/ trimethylchlorosilane (100:1, v/v) (BSTFA/TMCS) was purchased from Pierce Company (Rockford, IL, USA). All the other chemicals were of analytical grade and were obtained from Merck (Darmstadt, Germany).

2.2. Gas chromatography–mass spectrometry

Analyses were performed on a Hewlett-Packard HP5890A series II gas-chromatograph coupled with a HP 5972 mass-selective detector. A phenyl (5%)-méthyl (95%) polysiloxane (HP-5-MS) capillary column 30 m × 0.32 mm (i.d.) × 0.25 μm (film thickness) was used. Helium as carrier gas was set at a linear velocity of 40 cm/s. A Gerstel Cooled Injection System® was tuned to the splitless mode, with an initial temperature of 150 °C, increased to 270 °C at a 2 °C/s rate.

The oven temperature program was initial temperature 80 °C for 1 min, rate rise up to 136 °C at 12 °C/min, then to 270 °C at 35 °C/min rate, this final temperature being maintained for 2.5 min (total chromatographic run: 12.0 min). GC–MS interphase temperature was 250 °C.

The mass spectrometer was operated after electron-impact at 70 eV in the selected ion mode (SIM). Monitored ions were *m*/*z* 277 (quantification ion) and 292 (confirmation ion) for HU and *m*/*z* 234 (quantification ion) and 219 (confirmation ion) for MU. Dwell time was 100 ms.

MU was used as internal standard (IS) and the peak area ratio HU/MU was applied to determine the HU concentrations.

2.3. Preparation of standard solutions

Stock solutions of HU and MU were prepared by dissolving the appropriate amount of substance in distilled water at 1000 and 200 mg/L, respectively. These stock solutions were aliquoted and stored at −20 °C.

For validation study, plasma HU working solutions were prepared by appropriate dilution of the HU stock solution in a normal plasma pool in order to reach the following concentrations: 0.2, 0.4, 0.6, 0.8, 1, 2.5, 5, 10, 15, 20, 25, 50, 75 and 100 mg/L. These solutions were aliquoted and stored at −20 °C. Another 10 mg/L HU working solution was used as calibrator for plasma sample analysis. A 10-mg/L MU working solution was prepared by diluting the MU stock solution in distilled water; this solution was stable for at least 1 week at +4 °C.

2.4. Sample treatment

In a 5-mL centrifuge tube, 100 μL of MU working solution were added to 50 μL of patient's plasma sample, HU working solution, or calibrator, followed by 1000 μL of hexane/ethanol (1/1, v/v) for protein precipitation. Each analysis batch contained the 10 mg/L HU working solution as calibrator. The mixtures were vortex-mixed for 1 min and centrifuged at 2800 × *g* at room temperature for 10 min. The lower organic/aqueous phase was carefully taken-up, transferred to a 1.5-mL glass vial and evaporated to dryness at 40 °C under a stream of nitrogen. The residue was added with 100 μL of dichloromethane and re-evaporated at 40 °C in order to ensure full dryness. A 100 μL volume of BSTFA/TMCS:pyridine (100:20, v/v) was added to the vial, which is capped and incubated at 60 °C for 30 min. A 1-μL volume was injected into the GC–MS.

Table 1
Accuracy and repeatability estimated for HU determination in plasma. Five duplicate analyses were performed per level.

Added level	Mean found level ± standard deviation (mg/L)
1.0	0.96 ± 0.083
2.5	2.56 ± 0.262
5.0	5.14 ± 0.285
10.0	10.46 ± 0.772
15.0	14.96 ± 0.571
20.0	20.52 ± 0.621
25.0	25.02 ± 1.048

3. Results

MU and HU appeared on the chromatogram as quite symmetrical peaks, with retention time under the specified conditions of 4.15 and 5.15 min, respectively (Fig. 1A).

3.1. Validation of plasma HU determination

Estimations for the precision were conducted on plasma working solutions. Seven of them (levels 1, 2.5, 5, 10, 15, 20 and 25 mg/L) were analyzed in duplicate on 5 different days (Table 1). Treatment of data by two-way analysis of variance gave an intra-day coefficient of variation of 5.3% and an inter-day precision of 7.7%. Within-day coefficients of variation were also indicatively estimated outside this main level range by duplicate or replicate measurements. For low concentrations, duplicates of the plasma working solutions 0.2, 0.4, 0.6, 0.8 and 1.0 mg/L gave an overall CV% of 5.7%; overall repeatability for high concentrations, estimated on the plasma working solutions 50, 75 and 100 mg/L each analyzed four times, was 4.0%.

The analytical response, i.e. area ratio HU/MU as a function of spiked levels in the working solutions, indicates a very good linearity of the signal over the whole tested range which expands from 0 to at least 100 mg/L. As shown in Table 1, accuracy of the found levels as a function of concentration added to plasma (working solution) is also very satisfactory. Indeed, the regression line recovered levels vs. added levels had the following values: slope ± standard deviation 1.0063 ± 0.0144, intercept ± standard deviation 0.088 ± 0.202 mg/L, standard error of the estimate 1.018 mg/L (seven levels analyzed in duplicates on 5 different days).

Limit of detection estimated as the level corresponding to a signal/noise ratio of 3 in water is very low, i.e. 0.002 mg/L. However, analysis of plasma samples quoted the presence of *m*/*z* 277 and 292 signals at the retention time of HU, revealing the very likely presence of endogenous HU. Measured corresponding levels, determined on eight different drug-free plasmas, are 0.03–0.2 mg/L.

An analytical consequence of this observation is the irrelevance of estimating practical limit of detection or of quantification in plasma if they are close to or below the endogenous HU level. Our method permitting levels lower than 0.2 mg/L to be determined in water, we can assume that in practice a ca. 0.5 mg/L is the lowest level that can be measured with confidence as iatrogenic HU in plasma.

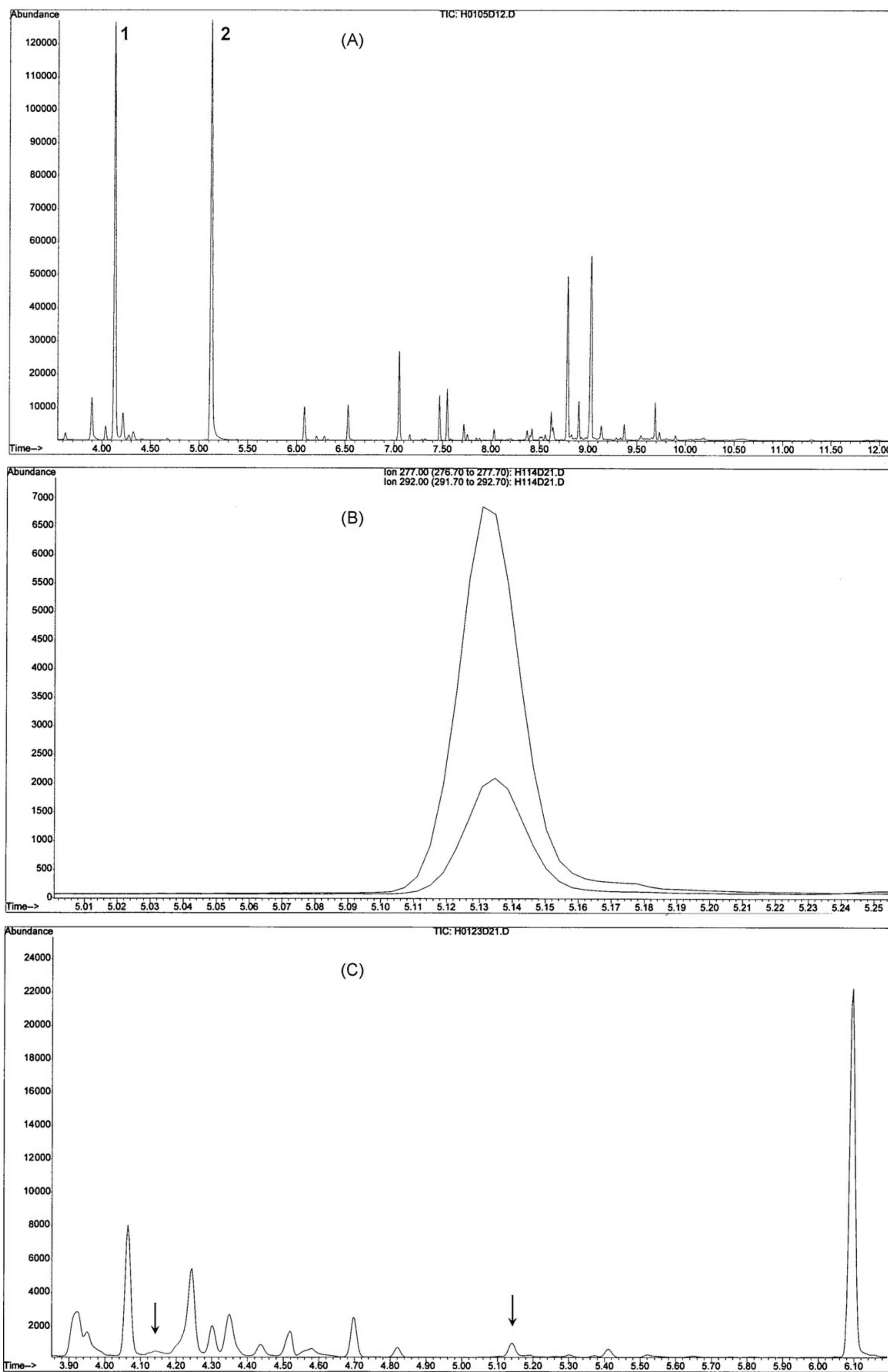

Fig. 1. (A) Total ion chromatogram (obtained in the selected ion mode) of a HU treated patient's plasma, level 9.8 mg/L. Peak 1: MU, peak 2: HU. Time scale is in min. (B) Part of the extracted ion chromatogram of the 0.4 mg HU/L plasma HU working solution (HU peak). Larger curve represents m/z 277 and the lowest curve m/z 292. Note the expanded abundance scale. (C) Part of a total ion chromatogram (obtained in the selected ion mode) of a plasma blank. Arrows indicate retention times of MU and HU. Note the expanded abundance scale.

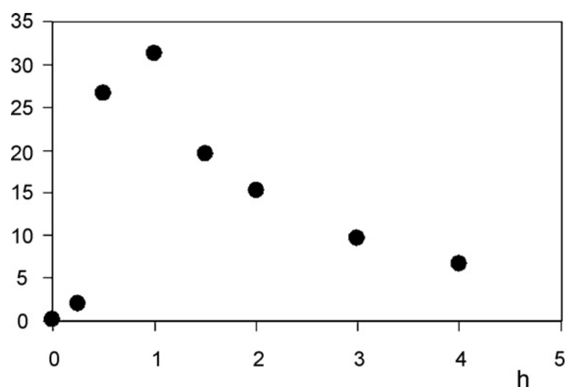

Fig. 2. Example of pharmacokinetics in a 9-year-old patient administered with 500 mg HU. Each point represents HU plasma levels (mg/L) during the 4-h period following administration.

3.2. Application to patients' plasma

A 9-year-old female patient[1] weighing 22.3 kg was orally administrated with 500 mg HU. The plasma concentrations observed during the 4-h period after administration is plotted in Fig. 2. The chromatogram of the sampling at time to +3 h is shown in Fig. 1A.

4. Discussion

4.1. Sample preparation

HU is a very hydrophilic molecule, practically insoluble in organic solvents. As its extraction from an aqueous or biological matrix is not so workable, the only way to prepare the sample for chromatography is to eliminate most the potentially interfering compounds, mainly proteins and lipids. The approach of James et al. [20] who extracted HU in the organic solvent mixture contained in Toxi-Lab® A tubes, did not produce feasible results in our hands, the extraction yield being less than 1%. Several deproteinizing reagents (perchloric acid 70 g/dL, trichloracetic acid 10 g/dL) and several solvents (ethanol, chloroform, hexane) in different proportions were tested. The hexane/ethanol mixture (1/1, v/v) appeared to be the most efficient as well as providing a handy aqueous phase after centrifugation. This aqueous/organic solution, if well suited for liquid chromatography, cannot be injected in a gas chromatographic column; it must be evaporated to dryness and reconstituted in an adequate medium, in this case the silanizing reagent mixture.

For improvement of precision and accuracy, an original IS was added to all analyzed samples and solutions. Several substances were tested in this aim: thiourea, glycine hydroxamate, *N*-methylurea, and methoxyurea. Only this last candidate encountered criteria of good derivatization reactivity and adequate retention time.

4.2. Gas chromatography–mass spectrometry

Mass spectra acquired in the scan mode (m/z 40–300) allowed to choose the appropriate ions monitored in the SIM mode. The major ion peaks were m/z 73, 147, 277, 292 for HU and m/z 73, 147, 219, 234 for MU. The m/z 292 ion indicates that three trimethylsilyl (TMS) groups took part in the silylation reaction of HU. These groups replace the hydrogen atoms of the amine groups and the hydroxyl moiety of HU. Subsequent fragmentation of the derivatized substance results in a m/z 277 ion. The MU spectrum confirms that silylation allows only two TMS groups to be coupled.

The silanized analyte and the IS can easily be differentiated one from the other and from other extracted compounds on the basis of their retention times and by a positive identification provided by characteristic ions (m/z 277 and 292 for HU, and m/z 234 and 219 for MU) (Fig. 1B). For the SIM mode, fragments m/z 277 and 234 were selected as quantification ions of HU and MU, respectively. As expected after the mode of preparation of the sample, GC–MS of plasma in the scan mode reveals a chromatogram burdened by several peaks of water soluble endogenous substances, among them urea and glucose being predominant. In the SIM mode however, mass spectrometry response shows no significant peak at the region of MU retention time and is particularly flat in the region of HU retention time, as shown on the chromatogram of a plasma blank (Fig. 1C). No interference of urea, which elutes at ca. 4.95 min, is noted.

Use of a novel internal standard, MU, guarantees the analytical precision and accuracy. It can however be assumed that recourse to a stable isotope derivative of HU can contribute to a further improvement of these performances.

4.3. Analysis of blank plasma samples

When analyzing plasma blanks (plasma from subject not taking HU) by our GC–MS method, signals corresponding to a HU low level have been discovered. Endogenous HU was also highlighted in our laboratory by two other methods: HPLC with electrochemical detection (method adapted from Yong et al. [15]) and HPLC after colorimetry (method adapted from Manouilov et al. [18]), methods for which a small peak at the retention time of HU has also been noted. A previous paper suggested the presence of HU in human liver cells and the authors made the supposition that part of urea could be hydroxylated in microsomes [21]. Our discovery of endogenous HU made irrelevant, whatever the analytical method, any estimation of the limit of detection or of quantification in plasma at a level close to or lower than the endogenous HU level (ca. ≤0.2 mg/L),. The very high sensitivity of the method allows the procedure to be applied to lower sample volume if necessary.

5. Conclusion

Thorough validation of a new GC–MS procedure developed for the quantitative determination of plasma HU revealed a sensitive and specific, yet fast and simple method for HU monitoring in a ≤50 μL plasma sample. The sensitivity and specificity of this method also permitted to point out presence of low concentrations of endogenous HU (≤0.2 mg/L). Pharmacokinetic studies, including measurement of trough levels and drug monitoring of HU treated patients are thus fully feasible by this novel analytical approach.

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