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Short communication

Colorimetric determination of hydroxyurea in human serum using high-performance liquid chromatography

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Abstract

A high-performance liquid chromatography assay for hydroxyurea in human serum was developed based on a commercial colorimetric assay kit for urea (Sigma Diagnostics). Serum (0.5 ml), spiked with methylurea as an internal standard, was treated with 70% perchloric acid. Supernatant (0.2 ml) was combined with 0.7 ml of BUN acid reagent and 0.6 ml of BUN color reagent. The resulting colored reactant (100 μl) was analyzed on a 300×3.9 mm Bondclone 10 C_{18} column coupled with a UV–Vis detector, at 449 nm. The mobile phase was 13% acetonitrile in water. Retention times of colored derivatives of hydroxyurea and methylurea were 6.5 and 12.2 min, respectively. The log–log calibration curve was linear from 0.0065 to 1.31 m*M*. Average accuracy was 99.9±4.0% and the intra- and inter-day error of assay did not exceed 11%. © 1998 Elsevier Science B.V.

Keywords: Hydroxyurea

1. Introduction

Hydroxyurea (HU) is used in the treatment of cancer [1], sickle cell anemia [2] and infection with the human immunodeficiency virus (HIV) [3]. The pharmacokinetics of the drug have been determined in patients with cancer and sickle cell anemia using a colorimetric assay developed by Fabricius and Rajewski in 1971 [4]. By modern standards, the method lacks sensitivity (0.033 m*M*) and selectivity for pharmacokinetic studies [5]. Recently, three high-performance liquid chromatography (HPLC) assays with electrochemical detection of HU have been published [5–7].

The method of Harvard et al. [6] permitted the determination of plasma HU concentrations from 0.02 to 0.35 m*M*, but the drug peak was not adequately separated from the solvent peak. Nevertheless, Villani et al. [7] adapted this assay to assess the pharmacokinetics of HU in patients with HIV. The limit of detection was reported to be 0.0006 m*M*, with linearity between 0.0013 and 0.26 m*M*. Unfortunately, few details of the assay as performed by these authors are included in the report. It is not clear if the drug had the same retention time as that described by Harvard et al. [6] or why the lower limit of detection is orders of magnitude less than

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that of the earlier paper. More recently, Pujari et al. [5] presented an additional method for assaying HU in plasma and peritoneal fluid using an electrochemical detector. For plasma, the assay was linear between 0.0003 and 0.01 m*M* (25 to 400 ng/ml). According to Villani et al. [7], steady-state serum levels of HU in HIV patients, receiving 500 mg of HU twice daily, ranged from 0.008 ± 0.003 to 0.135 ± 0.060 m*M*.

There is increasing interest in using HU as a component of drug combinations in several disease states [5]. At present, the UV–Vis detector is a much more common component of HPLC systems than the electrochemical detector. It is therefore desirable to have available an HU assay with sufficient sensitivity (and selectivity) that can be used on the majority of HPLC systems. In this paper, we describe a novel approach that combines a commercially available colorimetric assay for blood urea with HPLC separation to achieve a simple and effective assay of HU in human serum.

2. Experimental

2.1. Chemicals and reagents

Hydroxyurea, methylurea (MU), Urea Nitrogen diagnostic kit 535-A, perchloric acid and acetonitrile (HPLC grade) were purchased from Sigma (St. Louis, MO, USA). Blank human serum was acquired from ICN Pharmaceuticals (Costa Mesa, CA, USA).

2.2. Preparation of standards

Standard aqueous solutions of HU, ranging from 0.131 to 13.1 m*M*, were prepared. All solutions were stored in a refrigerator. Aqueous solutions are stable under these conditions [5]. Calibration curves for HU in human serum were made by adding standard solutions to blank human serum, yielding HU concentrations of 0.0065, 0.0131, 0.0263, 0.0657, 0.105, 0.131, 0.262, 0.526, 0.788, 1.05 and 1.31 m*M*.

2.3. Extraction procedure

Serum samples (500 μl), 20 μl of 70% perchloric acid and 20 μl of MU (15.5 m*M*) were added to polypropylene tubes. The contents of the tubes were mixed and centrifuged for 5 min at 12 000 *g*. After centrifugation, 0.2 ml of supernatant was mixed with 0.7 ml of BUN acid reagent (No. 535-3) and 0.6 ml of BUN color reagent (No. 535-5) in each polypropylene tube. The tubes were then placed in boiling water to allow the colored complexes of HU and MU to form. After 10 min in boiling water, the tubes were cooled in iced water and 25–100 μl of colored solution was removed for analysis.

2.4. Chromatography

The HPLC system used for analysis included a LC-10A solvent delivery system, a SCL-10A system controller, a SIL-10AXL autoinjector, a SPD-6AV spectrophotometric detector and a SR501 chromatopac integrator, all from Shimadzu (Columbia, MD, USA). Separation of HU and MU in serum was performed on a 300×3.9 mm Bondclone 10 C_{18} column (Phenomenex, Torrance, CA, USA). The mobile phase comprised 13% acetonitrile in water. The flow-rate was 1.7 ml/min and detection was at 449 nm. The integrator was connected with the 10 mV full scale recorder terminal of the SPD-6AV.

2.5. Quantitation

Calibration plots of HU in human serum were constructed by regression analysis, without weighting, of the logarithm of peak-area ratio (HU/MU) versus the logarithm of the HU serum concentration.

2.6. Precision, accuracy and sensitivity

The intra-day precision of the assay in serum was estimated by assaying five samples at each concentration level, i.e. 0.0263, 0.105, 0.26, 0.78 and 1.05 m*M*, on the first day. For the inter-day assay precision, samples with the same concentrations were analyzed on five separate days. Precision is defined as the relative standard deviation (R.S.D.). Accuracy was calculated by comparing measured HU concentrations to the known values and was expressed as a percentage of a nominal value.

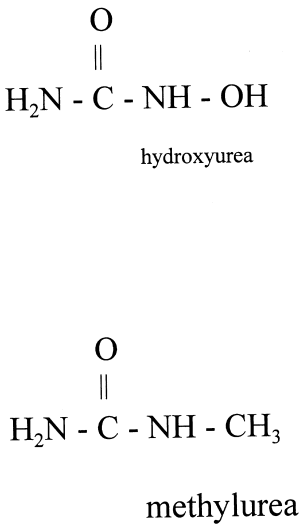

Fig. 1. Structural formulae of urea analogs.

3. Results and discussion

An HPLC assay for hydroxyurea in human serum with methylurea, as the internal standard (Fig. 1), was developed based on a commercial colorimetric assay kit for urea (Sigma Diagnostics).

The absorbance spectra of HU and MU products of the colored reaction have a broad band in the visible range, with maximum absorbances at 449 and 485 nm, respectively. Therefore, the spectrophotometric detector was set at 449 nm to achieve maximum sensitivity for HU.

Chromatograms of blank human serum (A) and human serum spiked with HU and MU (B) are shown in Fig. 2. The retention times for HU and MU were 6.5 and 12.2 min, respectively, and no interference with endogenous substances occurred. The large peak eluting just after HU represents serum urea. The colored products of both HU and MU were unstable and, within 6 h, the absorbance declined to 72–75% of the initial value. However, the rates of decay of the products were similar, so that the ratio of the areas under peaks of HU and MU remained constant. HU is stable in plasma stored at room temperature, 4°C or −20°C without deproteinization for up 48 h after collection and for at least 40 days after being deproteinized [6].

Eleven-point standard curves for HU in human serum were prepared by plotting the peak area ratio

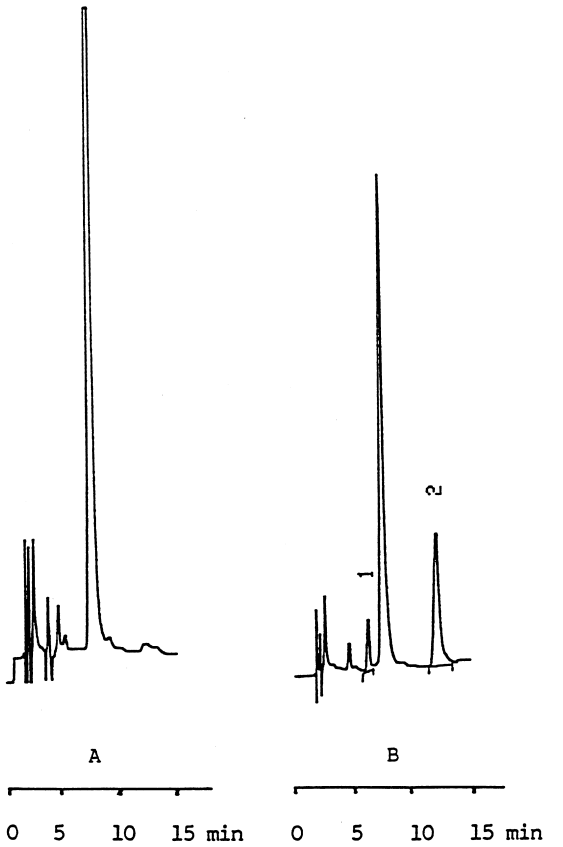

Fig. 2. Chromatograms of (A) blank human serum, and (B) of serum spiked with HU (1) at a concentration of 0.065 m*M* and internal standard MU (2) at a concentration of 0.62 m*M* (integrator set at width=20 s, attenuation=32 mV, speed=1.6 mm/min).

(HU/MU) versus standard HU concentrations. Concentrations as low as 0.0065 m*M* were detected and quantified. The log-linearity of response with respect to concentration was tested with standard concentrations ranging from 0.0065 m*M* (0.5 μg/ml) to 1.31 m*M* (100.0 μg/ml). Fig. 3 shows the HU/MU area ratio relationship for the above-mentioned concentration range for analyses performed on five different days.

Accuracy and inter-day precision for the lowest, medium and highest range of concentrations were satisfactory (Table 1). A maximum of 15% inaccuracy and imprecision was chosen as the criterion for the minimum quantifiable concentration. The lowest analyzed concentration (0.0065 m*M*) satisfies this

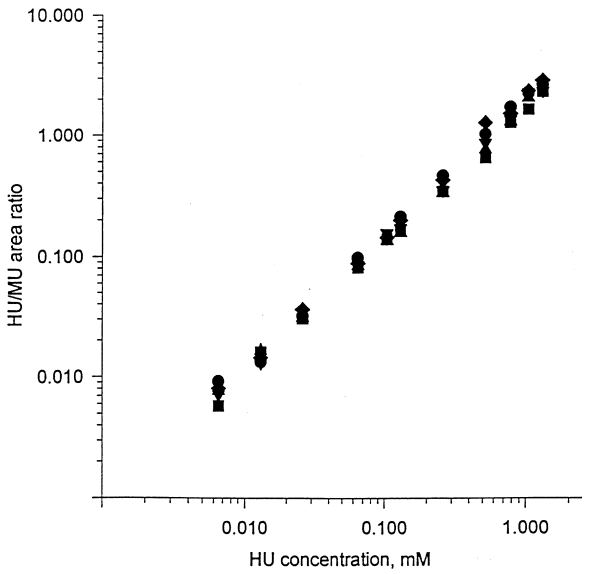

Fig. 3. Response of area ratio HU/MU at eleven concentrations of HU on five different days.

condition. This concentration is lower than the limit of detection reported by Harvard et al. [6] for HPLC with electrochemical detection and by Fabricius and Rajewsky [2] using the colorimetric method. The range of linearity of this assay (0.0065–1.315 m*M*) is significantly greater than that reported by Harvard et al. [6] (0.02–0.35 m*M*) or Pujari et al. [5] (0.0003–0.01 m*M*).

According to Villani et al. [7], concentrations in HIV patients taking 500 mg twice daily range from 0.01 to 0.13 m*M*. The present assay can therefore be used to measure HU in this patient population as well as in patients with cancer and sickle cell anemia, who generally are administered higher doses of HU.

The method has been used to characterize HU binding in plasma and serum and to assess HU concentrations in whole blood over a range from 0.1 to 1.3 m*M*. The current assay is applicable to patients with normal BUN values. Its extension to patients with significant renal failure and, hence, elevated BUN levels, may require an increase in the volume of the reagents in the commercial urea kit.

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Table 1
Accuracy and precision of assay for hydroxyurea in human serum

Nominal concentrations (m*M*)	Measured concentrations (mean±SD)	Accuracy	Precision	
		Inter-day (%)	Inter-day (%)	Intra-day (%)
0.0065	0.0068±0.0006	105	8.74	
0.013	0.013±0.0015	100	11.3	
0.026	0.026±0.0014	100	5.4	5.6
0.065	0.064±0.0014	98	2.3	
0.105	0.103±0.0062	98	6.0	3.4
0.131	0.124±0.0060	95	4.8	
0.262	0.242±0.0130	92	5.4	0.6
0.526	0.534±0.0511	102	9.6	
0.789	0.791±0.0277	100	3.5	0.9
1.052	1.122±0.0927	107	8.3	2.3
1.315	1.344±0.0953	102	7.1	