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Development and Validation of a Liquid Chromatography Method with Electrochemical Detection for Hydroxyurea Quantification in Human Plasma and Aqueous Solutions

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Abstract

Hydroxyurea is the unique drug having demonstrated a significant efficacy in sickle cell disease treatment. We developed a liquid chromatography method with electrochemical detection for hydroxyurea analysis in plasma and aqueous solutions. Analytical goals included an analytical range from 2 to 50 mg/L, a total imprecision lower than 15% and a total error lower than 30%. After protein precipitation with acetonitrile, the separation was performed on a C18 Atlantis T3 column and eluted with sodium acetate 25 mM, acetonitrile 2.5%, pH 6.5. Thioacetamide was used as internal standard. The method was linear for drug concentrations ranging from 0.5 to 50 mg/L and recovery was comprised between 100 and 120%. The intra-day precision was lower than 6.0% and between-day precision was lower than 11%. The detection limit was 0.18 and 0.63 mg/L for aqueous solution and plasma, respectively and the quantification limit was 1.0 and 1.2 mg/L for aqueous solution and plasma, respectively. No interference from urea was observed. The liquid chromatography method developed can be used for pharmacokinetic studies in plasma and other biological samples such as saliva or urine. It requires low sample volumes and a simple pre-treatment and it allows a direct measure of non-derivatized hydroxyurea.

Keywords: Hydroxyurea; Hydroxycarbamide; Thioacetamide; Liquid chromatography; Electrochemical detection; Sickle cell disease

Introduction

Hydroxycarbamide, better known as hydroxyurea, is a cytostatic agent used in the treatment of myeloproliferative disorders such as essential thrombocythemia and myelofibrosis [1,2]. It is also the most active drug in sickle cell disease, a genetic disorder due to the mutation of the 6th codon of the β-globin gene, leading to the synthesis of an abnormal hemoglobin (Hb S); sickle cell disease is characterized by chronic hemolytic anaemia and vaso-occlusive crises [3]. The main beneficial effect of hydroxyurea is to increase cellular levels of fetal hemoglobin (Hb F), which reduces Hb S polymerization [4]. Others mechanisms were demonstrated or suggested such as reduced expression of adhesion molecules, increased nitric oxide production, cation transport changes and myelosuppressive effects [5,6].

Despite hydroxyurea therapy has shown clinical improvement for sicke cell patients [7-9], differences in response i.e., the increase in Hb F levels, are observed and accurate predictors of hydroxyurea efficacy do not currently exist [10]. When hydroxyurea is administrated orally at a dose of 20 mg/kg, plasma concentrations normally reach a peak within 2 h (T_{max}) with a mean maximal concentration of 26 mg/L (C_{max}). Nevertheless, two phenotypes have recently been described: a "fast" (T_{max} of 15 or 30 min) and a "slow" one (T_{max} of 60 or 120 min) [10]. Pharmacokinetics studies are therefore required to explain these differences and to establish prediction factor.

Several methods have been developed to quantify hydroxyurea in biological samples: colorimetric techniques [10], liquid chromatography coupled with UV spectrophotometry [11,12], GC-MS [13,14], reversed phase liquid chromatography coupled with mass spectrometry [15]. Colorimetric methods require sample volumes of 250-500 µL and are insensitive. GC-MS needs a preliminary derivatization of hydroxyurea.

Here we describe the development and validation of a simple liquid chromatography method with electrochemical detection to quantify hydroxyurea in aqueous solutions and plasma.

Material and Methods

Chemicals and pre-analytical treatment

A stock solution of hydroxyurea (Sigma Aldrich, Steinheim, Germany) at 1000 mg/L was prepared and kept at -20°C. Aqueous and plasma standards were prepared by dilution of the stock solution in distilled water or human plasma pool.

Thiourea, methylurea, 2-thiouracil and thioacetamide (Sigma Aldrich, Steinheim, Germany) were tested as internal standard. The solutions were prepared at a final concentration of 100 mg/L containing 3 g/L albumin (Behring Institut, Marburg, Germany).

10 µL of samples or standards were added with 10 µL of internal standard solution and 100 µL acetonitrile (Biosolve, Dieuze, France). After centrifugation at 4000 rpm during 10 minutes, 80 µL of supernatant were recovered and either diluted to 400 µL with mobile phase or dried under nitrogen and reconstituted in 400 µL of mobile phase.

Two mobile phases were tested. The first one was described by Pujari and collaborators [16] and was composed of 0.2 M perchlorate and methanol 95/5 (V/V) (Sigma Aldrich, Steinheim, Germany). The

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second one, reported by Jong and collaborators [17] was composed of 25 mM sodium acetate (Merck, Darmstadt, Germany), 120 mg/L NaCl (VWR International, Leuven, Belgium), 0.1% diethylamine (Sigma Aldrich, Steinheim, Germany) and 2.5 M acetonitrile 2.5% (Biosolve, Dieuze, France), pH 6.5.

Liquid chromatography

The chromatography was performed on a Waters instrument (Waters 717 plus autosampler with a Waters 515 pump) connected to a Waters 2465 electrochemical detector using a carbon working electrode and an Ag/AgCl reference electrode. Elution was carried out on an Atlantis T3 column (5 µm, 250×4.6 mm). Two potentials (560 and 610 mV) were applied to the working electrode. Data were collected at rate of 5 points / second. Five µL of sample were injected and eluted at a flow rate of 0.5 mL/min.

Calibration

Calibration curve was performed correlating HU-enriched plasma or HU aqueous solution concentrations and height ratio between HU and internal standard. Five points are used: 2; 5; 10; 20; 30 and 50 mg/l. Two injections were performed and analyzed. Regression lines were built by linear regression with $1/x^2$ weighting, using EMPOWER2 software.

Method validation

Linearity: Aqueous and plasma standards at different concentrations (0.5, 1, 2, 5, 10, 20, 30, 50, 100 mg/L of hydroxyurea) were analyzed in duplicate. Regression lines corresponding to concentrations ranging from 0.5 to 100 mg/L, 0.5 to 50 mg/L, 0.5 to 30 mg/L, and 1 to 50 mg/L of hydroxyurea were calculated and slopes were compared by the Student t-test. A statistical difference between slopes was considered as a proof of non linearity.

Limit of detection: The mean hydroxyurea concentration corresponding to 10 determinations of hydroxyurea-free plasma or aqueous solutions plus two times the standard deviation was considered as the limit of detection.

Limit of quantification: Plasma and aqueous standards at different concentrations (0.5, 1.0, 1.2, 1.4, 1.6, 1.8, 2.0, 3.0; 4.0 and 5.0 mg/L of hydroxyurea) were analyzed in triplicate. The limit of quantification was the lowest concentration associated with a total error of less than 30%.

Accuracy: The relative recovery of drug added to plasma was calculated at 4 levels in duplicate. The method was considered as accurate at concentrations where the recovery was comprised between 80 and 100%.

Precision: For intra-day precision, aqueous and plasma standards containing 10 mg/L of hydroxyurea, were analyzed 20 times during the same day. For inter-day precision, standards containing 10 mg/L of hydroxyurea were analyzed 20 times on different days over a period of 2 months. Mean and coefficient of variation were calculated.

Specificity: The influence of urea, tested at a concentration of 100 mg/L, was assessed in the regular chromatographic conditions.

Results and Discussion

Optimization of extraction and liquid chromatography conditions

Hydroxyurea is a very hydrophilic compound and solvent extraction from plasma is not possible. Therefore, the samples were treated by protein precipitation with acetonitrile. An additional step of evaporation did not enhance chromatographic results and was not retained for the final protocol.

Thiourea, methylurea, 2-thiouracil and thioacetamide were tested as internal standard. Only the last one provided the required criteria of stability, detection and separation from hydroxyurea.

A mobile phase containing sodium acetate was selected for the final method, with a detection voltage of 610 mV. A voltage of 560 mV was associated with lower signal stability. Symmetrical hydroxyurea peaks were detected at a retention time of 5.8 minutes. The detector response for thioacetamide was not stable in aqueous solutions but well in plasma. To overcome this problem, solutions were enriched with human albumin at a concentration of 3 g/L and were renewed each day.

Method validation

Hydroxyurea and the internal standard were separated within 12 minutes without interference from endogenous compounds (Figure 1

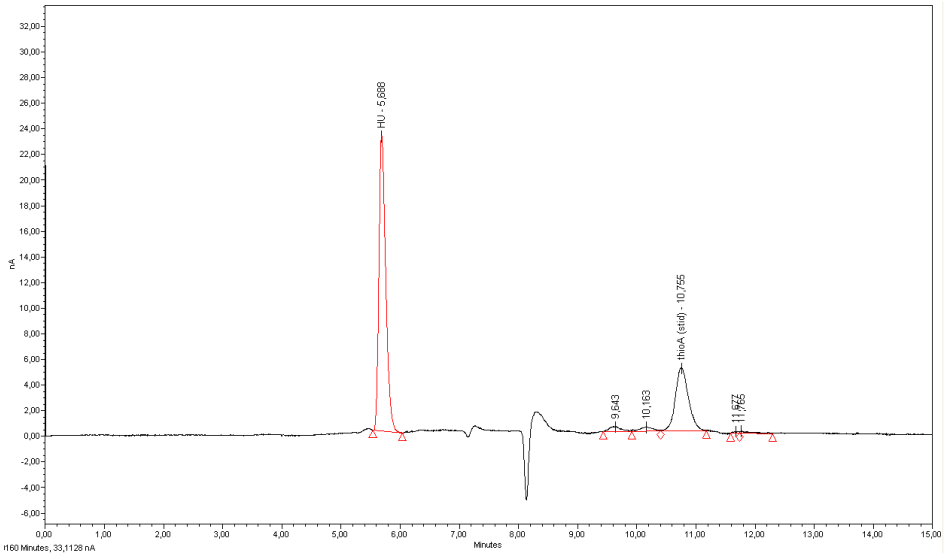

Figure 1: Chromatogram of hydroxyurea aqueous solution (10 mgL) enriched with internal standard.

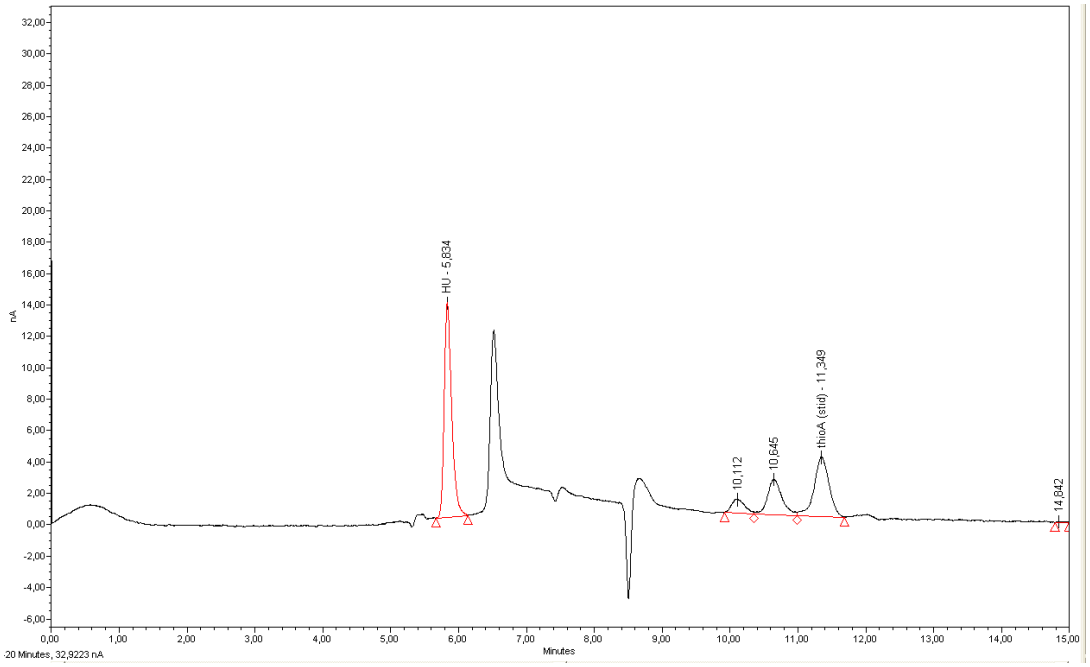

Figure 2: Chromatogram of hydroxyurea plasma solution (10 mgL) enriched with internal standard.

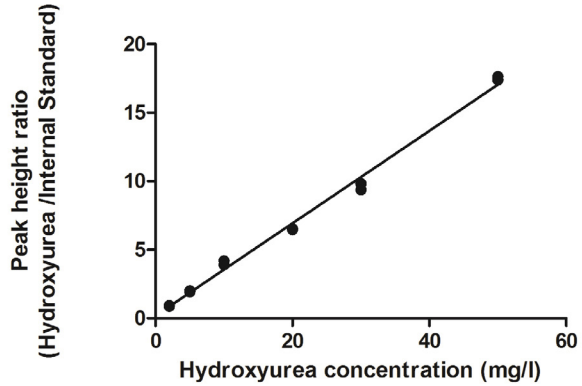

Figure 3: Typical plasma calibration curve.

Theoretical concentration (mg/l)	Calculated concentration (mg/l)	% Recovery
2.0	2.4	118
5.0	6.0	120
20.0	20.0	100
30.0	31.8	106

Table 1: Accuracy and recovery values for concentrations: 2; 5; 20 and 30 mg/l of hydroxyurea standards based in enriched plasma.

and 2). The column efficiency, the resolution and the peak symmetry were satisfactory. A typical calibration line is shown in Figure 3.

The method displayed good analytical performances with a linear range of 0.5-50 mg/L. Accuracy and recovery values for each concentration tested are presented in Table 1. All were lower or equal to 120%. The intra-day precision was 10.3% for aqueous and plasma standards and the inter-day precision was 5.1% and 6.0% for aqueous and plasma standards, respectively.

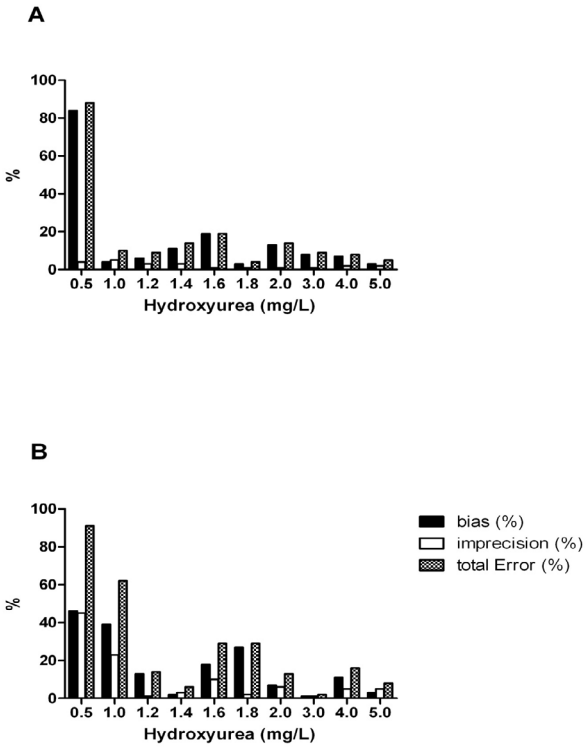

Figure 4: Estimation of the limit of quantification (A) In water (B) In plasma.

The limit of detection was 0.18 and 0.63 mg/L for aqueous and plasma solutions, respectively, and the limit of quantification was 1.0 mg/L for aqueous and 1.2 mg/L for plasma standards, in agreement with the analytical goals (Figure 4).

No interfering peak was observed when the urea solution was

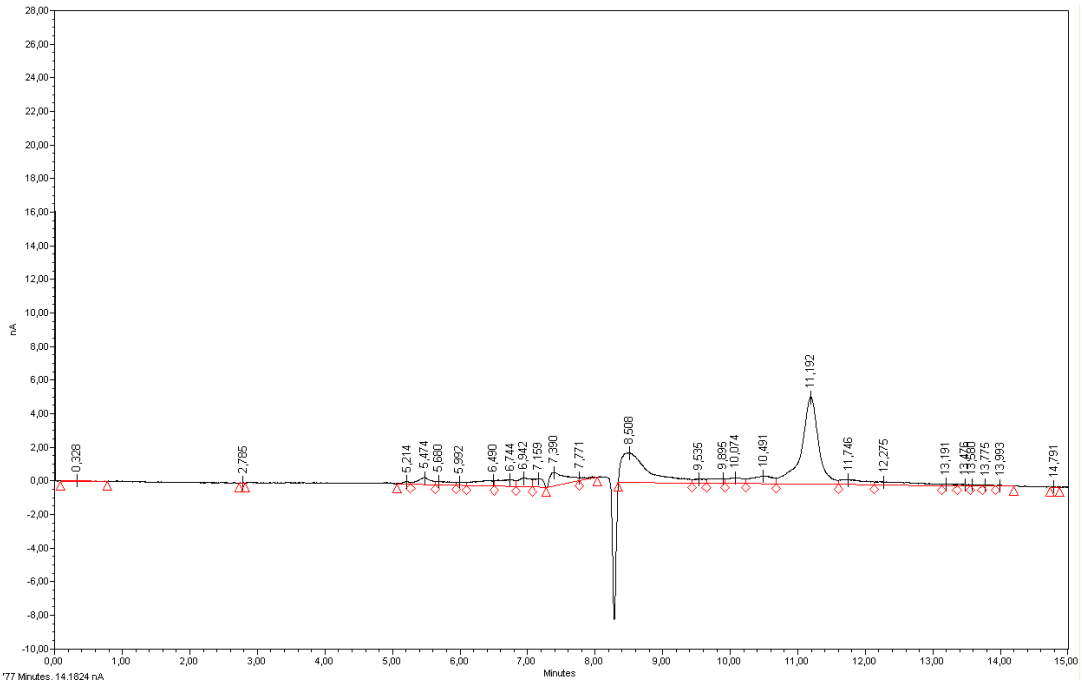

Figure 5: Chromatogram of urea solution (100 mgL).

analysed, reflecting a good specificity regarding this endogenous compound (Figure 5).

These data confirmed that the method was reliable and reproducible for the measurement of all targeted values expected in pharmacokinetic studies, both in plasma and saliva.

Conclusions

The accurate measurement of hydroxyurea in plasma is critical for a better understanding of hydroxyurea response and toxicity and to adapt the patient dose for a personalised treatment with improved clinical benefits. In different situations such as anemia or childhood, the sampling of saliva in place of blood is beneficial. Therefore, a method suitable for saliva analysis is needed too.

In this report, we describe a liquid chromatography method with electrochemical detection that permits the quantification of hydroxyurea in plasma and in aqueous solutions. Moreover, compared to previously published methods, this one presents several advantages like a low sample volume, a simple pretreatment and the direct measure of non-derivatized hydroxyurea.

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