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Short Communication

Determination by high-performance liquid chromatography of hydroxyurea in human plasma

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ABSTRACT

An assay using reversed-phase high-performance liquid chromatography with electrochemical detection was developed to measure hydroxyurea in plasma at concentrations suitable for pharmacokinetic studies. The sample preparation is simple, the analysis rapid and assays can be batched. The between-run precision is excellent (coefficient of variation = 2.8–4.5%) and the limit of detection is 0.02 mmol/l. Preliminary studies have shown that the method is suitable for pharmacokinetic studies.

INTRODUCTION

Hydroxyurea, a hydroxylated analogue of urea, is currently used as an antineoplastic drug in the treatment of leukaemia [1]. Clinical trials have demonstrated that when hydroxyurea is administered simultaneously with radiotherapy, it enhances its efficacy [2,3], by arresting the dividing cell at the G1-S interface, when the cell is most radiosensitive [4].

There is a need for a simple, sensitive method for assaying hydroxyurea to delineate its pharmacokinetics in humans. Davidson and Winter [5] and Fabricius and Rajewsky [6] have published colorimetric assays which lack sensitivity. The only available high-performance liquid chromatographic (HPLC) method describes an assay used to assess the purity of hydroxyurea in pharmaceutical formulations [7]. However, this method is unsuitable for measurement of hydroxyurea in plasma, due to interference from plasma con-

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stituents. The method described in this article involves deproteinization, separation on a reversed-phase HPLC column and measurement using an electrochemical detector. Hydroxyurea is measured in undiluted plasma at concentrations between 0.02 and 0.35 mmol/l with higher concentrations being measured after dilution in mobile phase.

EXPERIMENTAL

Reagents

Hydroxyurea was purchased from Sigma (St. Louis, MO, USA), anhydrous sodium acetate, AR, from Mallinckrodt (St. Louis, MO, USA), glacial acetic acid and trichloroacetic acid (TCA), AR, from BDH Australia (Kilsyth, Australia) and tetrabutylammonium hydroxide (TBAH), 40% (w/w) solution in water, from Aldrich (Milwaukee, WI, USA).

Chromatography

The HPLC system consisted of a Waters 6000A pump, Waters 710B autosampler (Waters Chromatography Division, Millipore, Milford, MA, USA), a Phase-II ODS 3-μm, 100 mm × 3.2 mm I.D. column (Bioanalytical Systems, West Lafayette, IN, USA), preceded by a 0.5-μm Rainin inline solvent assembly (Rainin, Woburn, MA, USA). The electrochemical detector was a BAS LC-4B (Bioanalytical Systems) with a glassy carbon electrode set at a potential of +800 mV *versus* an Ag/AgCl electrode. Chromatograms were recorded with a Waters 730 data module (Waters).

The mobile phase was 0.05 mol/l sodium acetate containing 5 mmol/l TBAH, adjusted to pH 6.75 ± 0.02 with 0.05 mol/l acetic acid. With a flow-rate of 0.5 ml/min (column pressure approximately 11 MPa), hydroxyurea eluted at 1.1 min, followed by various plasma constituents between 2.3 and 4.0 min. Analysis was complete in 5 min.

Plasma preparation

Plasma was deproteinized with two volumes of 5% (w/v) TCA, vortexed-mixed for 30 s and centrifuged at 7000 *g* for 5 min. A 5-μl aliquot of the supernatant was injected onto the column.

Standards and quality control samples

A stock solution of hydroxyurea was prepared at a concentration of 600 mmol/l in mobile phase and stored at −20°C. Working standards were prepared by spiking drug-free plasma with diluted stock, deproteinizing and storing the supernatant at 4°C. Aliquots (5 μl) were injected onto the column.

Quality control stocks were prepared similarly, but by staff not involved in the preparation of standards.

Calibration and calculations

A seven-point calibration graph was obtained by plotting the peak height of hydroxyurea in spiked plasma standards against the concentration (mmol/l). Automatic calculation using an integrator was unreliable due to the proximity of hydroxyurea to a negative peak (see Fig. 2). For this reason, peak height was calculated manually. The concentration of hydroxyurea in an unknown was read from this graph. Samples with concentrations above the linear range (0.35 mmol/l) were diluted with mobile phase and reanalysed. The regression equation was calculated from the calibration graph.

Precision and recovery

To determine the within-run precision, ten aliquots of plasma spiked with hydroxyurea to concentrations of 0.06, 0.12 and 0.24 mmol/l were deproteinized and analysed in a single working day. Between-run precision was assessed for each concentration, by analysing on ten consecutive days, ten aliquots of plasma which had been spiked and deproteinized on the first day.

Recovery of hydroxyurea from plasma was investigated by spiking plasma with hydroxyurea to 0.12 and 0.24 mmol/l, deproteinizing and measuring the drug against a calibration curve of standards made in mobile phase, *i.e.* without deproteinization. Six aliquots were analysed at each concentration and the recovery calculated by comparison with the true concentration.

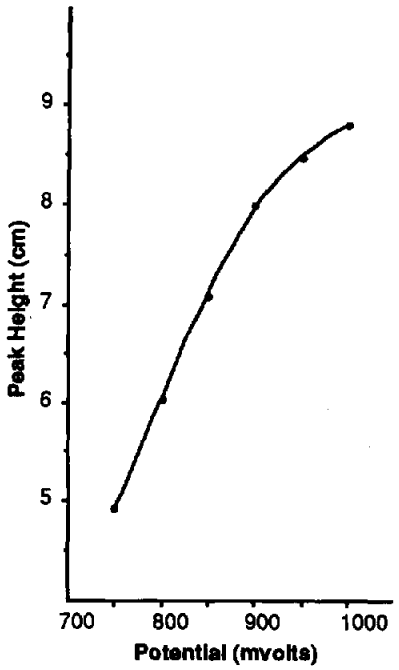

Fig. 1. Voltammogram of hydroxyurea in mobile phase: 5 μl of 0.06 mmol/l.

RESULTS AND DISCUSSION

Separation

The method described by Pluscec and Yuan [7] for determination of hydroxyurea in pharmaceutical preparations uses a C_{18} column, a mobile phase consisting solely of water and measurement at 214 nm with a UV detector. We found that this method did not separate hydroxyurea from other UV-absorbing compounds in plasma. However sufficient specificity was gained by using an electrochemical detector and a different mobile phase. A graph of peak height *versus* potential is shown in Fig. 1. The working electrode potential of +800 mV was chosen as this maximised hydroxyurea's response while minimising interference from other compounds.

Fig. 2 shows typical chromatograms of plasma: (A) from a healthy, drug-free volunteer, (B) from the same volunteer, but spiked with hydroxy-

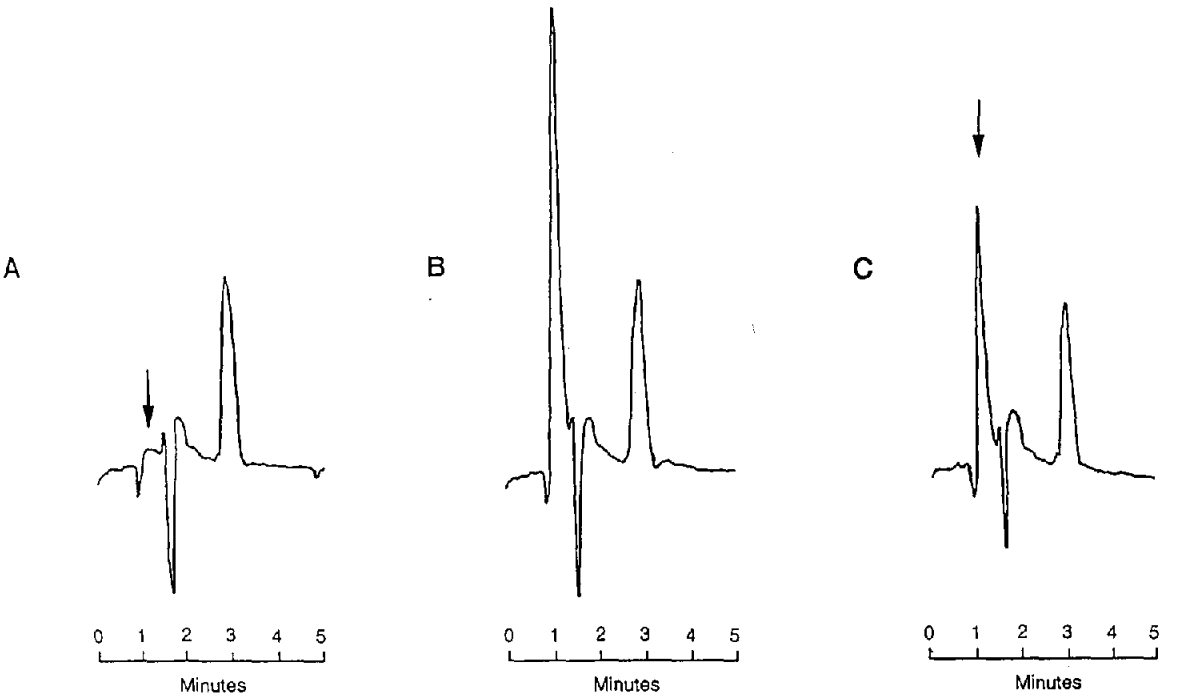

Fig. 2. Chromatograms of 5 μl injections of deproteinized plasma. (A) Hydroxyurea-free plasma; (B) plasma spiked with 0.15 mmol/l hydroxyurea; (C) plasma from a patient receiving hydroxyurea (dose: 1 g twice daily, plasma: 0.09 mmol/l). The arrow indicates the retention time of hydroxyurea.

urea, (C) from a patient receiving hydroxyurea.

The assay we have described is unusual in that hydroxyurea elutes ahead of the plasma constituents when a totally aqueous mobile phase is used. Separation is achieved by retarding the elution of the latter; a more acidic mobile phase elutes the plasma constituents earlier, with consequent decreased resolution of hydroxyurea. Similarly, concentrations other than 5 mmol/l ion-pairing agent and 0.05 mol/l acetate decrease the resolution of hydroxyurea and the plasma constituents. Several buffers (monochloroacetate, acetate and phosphate) and ion-pairing agents (TBAH and sodium octylsulphate) were investigated in various combinations to achieve the optimal separation. A phosphate buffer (0.1 mol/l) containing 5 mmol/l TBAH at pH 6.5 gave a separation equivalent to that achieved using the acetate–TBAH mobile phase. However, use of the mobile phase containing phosphate buffer resulted in a large negative peak close to the retention time of hydroxyurea with all protein-precipitating agents tested (ethanol, acetonitrile, sulphosalicylic acid and TCA). The presence of this large negative peak interfered with accurate measurement of the hydroxyurea peak, making quantitation unreliable. A similar peak seen with the acetate–TBAH mobile phase (see Fig. 2) was smaller, better resolved and allowed reliable quantitation.

Since hydroxyurea elutes earlier than the plasma constituents regardless of the ionic strength and pH of the mobile phase employed, it appears that this separation is influenced by other mechanisms as well as reversed-phase hydrophobicity. Furthermore, during the development of this method, a column from a different batch lot gave a faster elution time for both hydroxyurea and the plasma constituents, suggesting that the separation is sensitive to minor variations in either the loading of C_{18} on the stationary phase or end-capping.

Validation

The calibration graph was linear to 0.35 mmol/l ($y = 0.025x + 0.058$, $r^2 = 1.000$) with a limit of detection of 0.02 mmol/l (signal-to-noise ratio 3:1). Above 0.35 mmol/l, the calibration graph was non-linear. The recovery of hydroxyurea from spiked plasma was 100.78% at 0.12 mmol/l and 105.00% at 0.24 mmol/l. The precision of the method was examined at three concentrations and the results are shown in Table I.

Stability

Hydroxyurea is stable in plasma stored at room temperature, 4°C or −20°C without deproteinization for up to 48 h after collection. However, longer storage at these temperatures resulted in loss of hydroxyurea.

If plasma is deproteinized within 48 h, the hydroxyurea concentration of the supernatant is stable for at least 40 days when stored at room temperature, 4°C or −20°C. We have not elucidated the mechanism whereby deproteinization stabilises the hydroxyurea in plasma, but presumably it involves an interaction between hydroxyurea and plasma proteins. Davidson and Winter [5] have reported that urease degrades hydroxyurea. The degradation rate they have observed, 1 mg/l/h, would account for the decreases we have found.

TABLE I

PRECISION OF HYDROXYUREA DETERMINATION

Spiked concentration (mmol/l)	Within-run precision (n = 10)		Between-run precision (n = 10)	
	Mean concentration found (mmol/l)	C.V. (%)	Mean concentration found (mmol/l)	C.V. (%)
0.060	0.068	2.8	0.067	3.6
0.120	0.126	1.7	0.126	2.8
0.240	0.244	0.9	0.246	4.5

Interferences

Electrochemical detection was chosen because of its sensitivity and specificity.

Hydroxylamine is a possible catabolic breakdown product of hydroxyurea [8] and may be a contaminant of the drug in therapeutic preparations [7]. It is electroactive at +800 mV, elutes at 1.3 min but exhibits only 6% of the detector response of the same concentration of hydroxyurea. Furthermore it is unstable in plasma; after storage at 4°C for three days, a plasma spiked with hydroxylamine hydrochloride contained 10% of the original concentration.

No interfering peaks were seen in plasma from 100 inpatients requiring routine biochemistry or from inpatients and volunteers receiving salicylate, paracetamol, caffeine, valproate, carbamazepine, phenobarbitone, phenytoin, ethosuximide or morphine.

CONCLUSION

Preliminary studies on two patients have been performed to determine the suitability of this method for pharmacokinetic studies. Each patient was given a single oral dose of 5 g hydroxyurea and one week later 5 g intravenously. The hydroxyurea concentration in plasma samples collected from these patients in the 24 h following administration of each dose of the drug ranged from below the limit of detection to 5.5 mmol/l. We conclude that the method described is simple, rapid and suitable for pharmacokinetic studies.

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