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Determination of hydroxyurea in plasma and peritoneal fluid by high-performance liquid chromatography using electrochemical detection

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Abstract

A sensitive method has been developed for the determination of hydroxyurea in plasma and peritoneal fluid using reversed-phase high-performance liquid chromatography (HPLC) with electrochemical detection. Plasma or peritoneal fluid samples were treated with acetonitrile to precipitate proteins then injected to the HPLC. A C_{18} analytical column was used to separate hydroxyurea from interfering substances in the biological matrix. The mobile phase, consisting of 0.2 *M* sodium perchlorate–methanol (95:5, v/v) adjusted to pH 5.0, was delivered isocratically at a flow-rate of 1 ml/min and hydroxyurea was detected using a glassy-carbon electrode operating at an applied potential of +800 mV. Hydroxyurea eluted with a retention time of 3 min. The cycle time for analysis is short and the assay precision is acceptable (C.V. plasma=1.4–3.9%, C.V. peritoneal fluid=2.1–9.7%). The method has been validated and is linear from 25 to 400 ng/ml in plasma and 5 to 30 ng/ml in peritoneal fluid. The method has been shown to be applicable for pharmacokinetic studies.

Keywords: Hydroxyurea

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1. Introduction

Hydroxyurea is chemically a relatively simple compound that was first synthesized in 1869 [1]. Its use in the palliative treatment of leukemia and malignant melanoma was first reported in 1962 by Nagel et al. [2]. Hydroxyurea has been used in the treatment of melanoma, carcinoma of the ovary and resistant chronic myelocytic leukemia [3]. Several trials of hydroxyurea in combination with other chemotherapeutic agents are currently ongoing. The precise mechanism of action of hydroxyurea is not clearly understood. It has been suggested that hydroxyurea is an inhibitor of ribonucleotide reductase [4] which selectively inhibits DNA synthesis by depleting the cellular deoxyribonucleotide pool [5]. Since hydroxyurea is a cell specific cytotoxic agent, it has been suggested that in order to achieve effective inhibition of DNA synthesis, it should be administered in a dosing regimen that will ensure adequate tissue levels covering at least one to two cell cycle periods.

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There are few published methods for the determination of hydroxyurea in biological specimens. The colorimetric methods reported by Davidson and Winter [6] and Fabricius and Rajewsky [7] lack sufficient sensitivity and selectivity for pharmacokinetic studies. The HPLC method of Pluscec and Yuan [8] lack sensitivity and is not applicable for pharmacokinetic studies. More recently, the method of Harvard et al. [9] using electrochemical detection is the most sensitive method reported for the determination of hydroxyurea in plasma, with a reported limit of detection of 0.02 mmol/l.

Preclinical studies suggest that hydroxyurea may enhance the cytotoxic activity of the fluoropyrimidines [10]. This enhancement can be explained by lower levels of deoxyuridine monophosphate (dUMP) pools achieved by hydroxyurea leading to reduction in competition of FdUMP for thymidylate synthase (TS). Moran et al. [11] have shown an advantage of adding hydroxyurea to FUdR in the treatment of L1210 mouse leukemia. Combination therapy consisting of intraperitoneal (i.p.) floxuridine (FUdR, 5-fluoro-2′-deoxyuridine) and intravenous (i.v.) hydroxyurea with leucovorin protection is currently being investigated in patients with disseminated intra-abdominal malignancies.

Since we were interested in the pharmacokinetics of drugs used in this combination therapy, it was necessary to be able to determine hydroxyurea concentrations in both plasma and peritoneal fluid. The concentration of hydroxyurea in peritoneal fluid relative to plasma was expected to be relatively low. Previously published methods were not sufficiently selective and sensitive for the monitoring of hydroxyurea levels in biological fluids. Hence, it was necessary to develop a method that could be used in pharmacokinetic studies of hydroxyurea in plasma and peritoneal fluid.

This report describes the development and validation of a method for determination of hydroxyurea in plasma and peritoneal fluid. Sample preparation and the mobile phase were modified from that previously reported by Harvard et al. [9]. This resulted in substantial improvement in sensitivity from 1500 ng/ml (0.02 mmol/l) to 5 ng/ml (peritoneal fluid). The method is simple and reproducible with acceptable precision and accuracy.

2. Experimental

2.1. Reagents and materials

Hydroxyurea, sodium perchlorate and perchloric acid were purchased from Aldrich (Milwaukee, WI, USA). Human plasma was purchased from Sigma (St. Louis, MO, USA). Methanol and acetonitrile (HPLC grade) were purchased from EM Science (Gibbstown, NJ, USA).

2.2. Instrumentation

The HPLC system consisted of two LKB Model 2150 solvent delivery pumps, LKB Model 2152 gradient controller and a Rheodyne manual injector. A pressure stabilizer valve (Altech, Deerfield, IL, USA) was connected in series between the pump and the injector (improves stability of the electrochemical detector due to its extreme sensitivity to fluctuations in mobile phase pressure and flow resulting in base line drift and negative peaks). An Econosil C_{18} analytical column, 250×4.6 mm, 10 μm particle size) was purchased from Altech. The electrochemical detector consisted of a LC-4C amperometric detector, a CC-4 cell system consisting of a glassy-carbon dual electrode cell and an Ag/AgCl reference cell (Bioanalytical Systems, West Lafayette, IN, USA). The electrode was connected in single electrode mode with an applied potential of +800 mV against the Ag/AgCl reference cell. The filter setting was 0.10 HZ. The auxiliary block and cell were cleaned with water–methanol. Chromatograms were recorded on a Soltec 1243 chart recorder. The recorder, detector, cell and HPLC pump shared a common ground.

2.3. Mobile phase

The mobile phase consisted of 0.2 *M* sodium perchlorate–methanol (95:5, v/v) adjusted to pH 5.0 using perchloric acid. The solution was filtered through a Millipore 0.4 μm nylon filter and degassed by sonication for 20 min. Finally, the warm (40°C) mobile phase was purged with helium gas for 15 min then cooled to room temperature prior to use.

2.4. Preparation of standard solutions

A stock solution of hydroxyurea was prepared by dissolving 1.0 mg of hydroxyurea in 1.0 ml of methanol. The stock solution was stored at −20°C until ready for use. Other standards were prepared by making serial dilutions of this standard. For preparation of the standard curve, plasma or peritoneal fluid was spiked with appropriate volumes of hydroxyurea stock solution. The concentrations used for the preparation of the standard curve in plasma were 25, 50, 100, 200, 300 and 400 ng/ml. For peritoneal fluid, the concentrations used were 2, 5, 10, 20 and 30 ng/ml.

2.5. Plasma sample preparation

To 10 μl of plasma sample or plasma standard containing hydroxyurea, was added 100 μl of acetonitrile to precipitate the plasma proteins. The mixture was vortexed for 1 min then centrifuged at 5000 *g* for 5 min. The liquid phase was transferred to a second vial and evaporated to dryness using a stream of nitrogen gas. The dried sample was reconstituted with 1 ml of mobile phase and 5 μl was injected into the HPLC system.

2.6. Peritoneal fluid sample preparation

Peritoneal fluid samples were prepared similarly to that used for plasma samples. To 25 μl of peritoneal fluid was added 100 μl of acetonitrile. The mixture was vortexed for 1 min then centrifuged at 5000 *g* for 5 min. The liquid phase was transferred to a second tube and evaporated to dryness under a stream of nitrogen gas. The dried sample was reconstituted in 25 μl of mobile phase and 5 μl of the solution was injected into the HPLC system.

2.7. Stability of hydroxyurea in plasma

The stability of hydroxyurea in plasma was determined at room temperature over a 4 day period. Hydroxyurea in methanol (100 μl of a 0.1 mg/ml solution in methanol) was transferred to a 5 ml glass tube. The methanol was evaporated to dryness under a stream of nitrogen gas. Plasma (2 ml) was added and the mixture vortexed. Serial plasma samples (20 μl) were removed at various time intervals over a 72-h period and assayed for hydroxyurea.

2.8. Stability of hydroxyurea in peritoneal fluid

The stability of hydroxyurea was also determined in peritoneal fluid at room temperature over a 5-day period. Hydroxyurea (100 μl of a 0.01 mg/ml solution in methanol) was transferred to a 10-ml glass tube. The methanol was evaporated to dryness under a stream of nitrogen. Peritoneal fluid (5 ml) was added and the mixture vortexed. At various time intervals, serial samples (100 μl) were removed over a 144-h period and assayed for hydroxyurea.

3. Results and discussion

Initially, a modification of the HPLC method of Pluscec and Yuan [8] using UV detection was attempted. This method was developed for the determination of hydroxyurea in pharmaceutical formulations. The UV spectrum of hydroxyurea in methanol showed a very weak absorbance at 210 nm ($\epsilon=251$). During the course of this study, various column/mobile phase combinations, including ion-pair chromatography, were attempted. Hydroxyurea did not bind strongly to most columns tested making it difficult to obtain adequate chromatographic separation. However, separation of hydroxyurea from matrix interference was obtained using a C_{18} reversed-phase column.

The low molar absorptivity of hydroxyurea at 210 nm made UV detection unsuitable for high assay sensitivity. However, high sensitivity could be obtained using an electrochemical detector. Because the mobile phase was modified from previously published methods, a voltammogram (Fig. 1) for hydroxyurea was run. No detector current was observed at applied potentials below 400 mV. However, as the applied potential was increased to greater than 400 mV, increases in detector current were observed which began to plateau at voltages above 900 mV. A working electrode potential of +800 mV relative to the Ag/AgCl reference electrode was selected for the

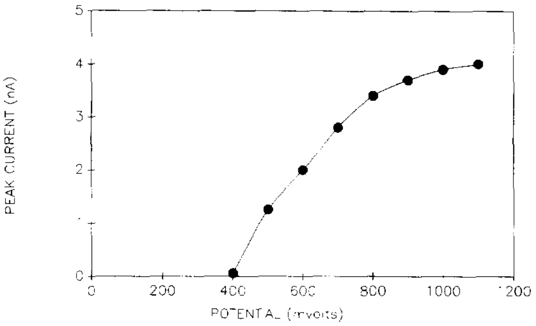

Fig. 1. Voltammogram of hydroxyurea in mobile phase. Injection of 5 μl of a 100 ng/ml solution.

detector, which is consistent with the potential used by Harvard et al. [9]. At this potential, matrix interference from either plasma or peritoneal fluid was not observed. This was likely due to other substances present in the matrix not being subject to oxidation–reduction reactions under the experimental conditions. Using a different mobile phase but with the identical glassy-carbon electrode, Harvard et al. [9] also showed that peak height tends to stabilize above +800 mV. Higher voltages were not used in order to minimize possible matrix interference and to prolong the reliability and longevity of the glassy-carbon electrode.

Typical HPLC spectra of hydroxyurea in plasma and peritoneal fluid using electrochemical detection are shown in Fig. 2. At a mobile phase flow-rate of 1 ml/min, hydroxyurea has a retention time of approximately 3 min with a matrix interference peak from plasma eluting in about 4.5 min. The use of higher mobile phase flow-rates resulted in sharper peaks and in a longer duration of life of the glassy-carbon electrode before cleaning and polishing were required. The order of elution of hydroxyurea and the matrix interference peak was not effected by changes in either pH or ionic strength of the mobile phase. This observation was also made by Harvard et al. [9] using a different buffer system. No matrix interference peak was seen with peritoneal fluid.

3.1. Determination of standard curve and assay validation

Six-point standard curves were prepared for hydroxyurea in both plasma and peritoneal fluid by plotting peak height versus hydroxyurea concentration. The use of peak areas was not reliable since occasional transient negative spikes and large negative currents were observed. This was consistent with previous reports [9] that experienced similar difficulties using the electrochemical detector.

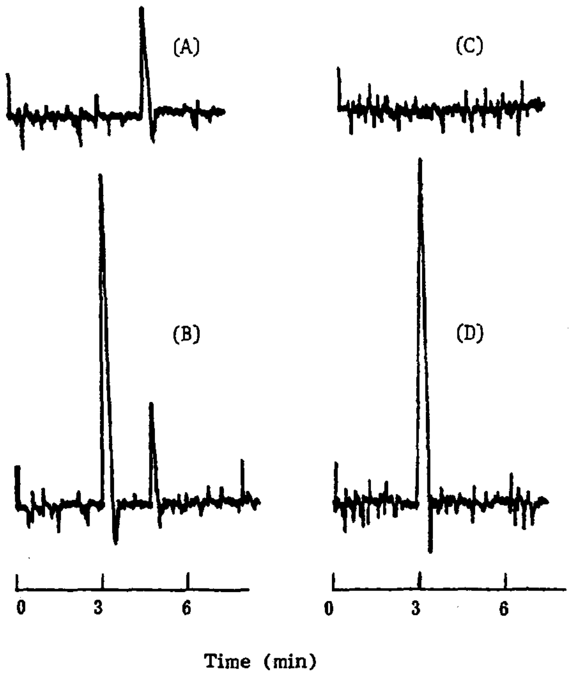

Fig. 2. Chromatogram of hydroxyurea in plasma and peritoneal fluid. (A) Plasma blank; (B) plasma spiked with hydroxyurea, 100 ng/ml; (C) peritoneal fluid blank; (D) peritoneal fluid spiked with hydroxyurea, 10 ng/ml.

Standard curves were determined from four separate runs in both plasma and peritoneal fluid. For plasma, the standard curve was linear from 25 to 400 ng/ml ($y=0.34x+0.03$, $r^2=0.999$), using the 10 nA detector range. For peritoneal fluid, the standard curve was linear from 2 to 30 ng/ml ($y=0.32x+0.04$, $r^2=0.999$) using a detector range of 1 nA. The method was validated by determining the within-run and between-run precision at five separate concentrations. A mid-point external standard was injected every fifth to sixth sample to determine the performance of the electrochemical cell. Between-run precision was obtained by running sample solutions on five different days using identical concentrations prepared from the same stock.

3.2. Stability

The results of a stability study of hydroxyurea in plasma and peritoneal fluid stored at room temperature indicated that approximately 90% of the original hydroxyurea concentration remained after 72 h. It was previously reported that after deproteination of the plasma, hydroxyurea was stable for at least 40 days [9]. All patient specimens were stored at −70°C prior to analysis.

3.3. Extraction and recovery

The use of solid-phase extraction for hydroxyurea from plasma was not successful because of the poor binding properties of hydroxyurea to solid-phase extraction columns. Solvent extraction using ethyl acetate gave poor recovery and showed substantial interference using either UV and electrochemical detection. Ethyl acetate could not be used with the electrochemical detector due to loss of sensitivity of the glassy-carbon electrode after only two injections.

Hydroxyurea recovery from plasma and peritoneal fluid was determined at different concentrations. Each sample concentration was analyzed in triplicate and the peak height was compared to a sample of identical concentration prepared in the mobile phase. The mean recovery for various concentrations in plasma and peritoneal fluid was 89% and 94%, respectively. Recovery results for plasma and peritoneal fluid are shown in Table 1.

Table 1
Recovery of hydroxyurea from plasma and peritoneal fluid

Concentration (ng/ml)[a]	% Recovery following protein precipitation (Mean±S.D.) (%)
Plasma	
25	101.0±0.6
50	82.9±4.4
100	85.9±7.0
200	89.9±3.4
300	85.4±0.8
Mean recovery	89.0±7.2
Peritoneal fluid	
5	91.1±1.9
10	96.3±1.5
20	73.6±0.6
30	95.2±3.3
Mean recovery	94.1±2.3

[a] $n=3$ at each concentration.

3.4. Assay validation in plasma and peritoneal fluid

The between-run and within-run precisions in plasma were determined at five different concentrations, 25, 50, 100, 200 and 500 ng/ml, reflecting typical low, medium and high plasma concentrations. The between-run means±standard deviations were determined from five separate assays run on five separate days at each concentration. The within-run means were determined from assaying five different concentrations on five separate assay runs. The results the validation studies in plasma are shown in Table 2. Over the concentration range studied (25–500 ng/ml), the between-run coefficients of variation (C.V.) varied from 1.4–3.9% with an error of 0.9–4.0%. The coefficients of variation for the within-run validation were 0.3–10.4% with an error ranging from 0.1–5.6%.

The between-run and within-run precision in peritoneal fluid was important because lower hydroxyurea concentrations were anticipated requiring greater sensitivity of the electrochemical detector. Five different concentrations were used (2.0, 5.0, 10.0, 20.0, 30.0 ng/ml) reflecting typical low, medium and high peritoneal fluid concentrations. The results of the assay validation in peritoneal fluid are shown in Table 3. For the between-run validation, samples were assayed on five separate days. Coefficients of variation for the assays at each concentration ranged from 1.6–9.7% with an error from 0.3–23.0%.

The results of the within-run validation show C.V.s ranging from 1.1–5.6% with an error of 0.5–26.5%. The results of the within-run and between-run validation indicate that the method is not sufficiently precise for a peritoneal fluid concentration of 2 ng/ml and that the minimum quantifiable concentration in peritoneal fluid is between 2 and 5 ng/ml. Stability of the detector was achieved by allowing the cell to stabilize overnight at an appropriate working potential with perfusion of the cell by the mobile phase. Mobile phase must be prepared using high grade reagents and high purity deionized, glass

Table 2
Between-run and within-run precision for hydroxyurea in plasma

Sample No.	Hydroxyurea plasma concentrations (ng/ml)				
	25	50	100	200	500
Between-run precision					
1	26.7	50.2	103.5	199.2	506.6
2	25.6	49.8	102.6	197.6	505.3
3	25.1	53.3	106.5	208.3	520.0
4	26.4	54.4	99.4	203.5	518.8
5	26.2	50.8	98.3	199.9	507.9
Mean±S.D.	26.0±0.64	51.7±2.0	102.1±3.3	201.7±4.3	511.5±7.2
C.V. (%)	2.5	3.9	3.2	2.1	1.4
Error (%)	4.0	3.4	2.1	0.9	2.3
Within-run precision					
1	25.1	50.7	104.0	197.4	499.7
2	25.1	51.7	103.3	202.1	501.2
3	25.4	50.1	103.3	197.7	498.1
4	25.1	53.2	103.3	195.8	498.1
5	31.3	51.7	106.5	194.2	501.2
Mean ± S.D.	26.4±2.7	51.5±1.2	104.1±1.4	197.4±3.0	499.7±1.6
C.V. (%)	10.4	2.3	1.3	1.5	0.3
Error (%)	5.6	3.0	4.1	1.3	0.1

Table 3
Between-run and within-run precision for hydroxyurea in peritoneal fluid

Sample No.	Hydroxyurea peritoneal fluid concentrations (ng/ml)				
	2	5	10	20	30
Between-run precision					
1	2.41	5.13	10.2	19.9	30.1
2	2.42	4.23	9.9	20.6	29.6
3	2.45	4.29	10.4	20.3	30.9
4	2.83	5.04	10.4	19.5	29.8
5	2.17	4.65	10.8	20.2	29.8
Mean ± S.D.	2.46±0.24	4.67±0.41	10.3±0.33	20.1±0.42	30.1±0.49
C.V. (%)	9.7	8.8	3.2	2.1	1.6
Error (%)	23.0	6.6	3.0	0.5	0.3
Within-run precision					
1	2.56	4.67	10.7	20.5	30.7
2	2.41	4.52	10.0	19.9	30.1
3	2.71	5.13	10.2	19.6	30.7
4	2.41	4.83	10.4	19.9	30.1
5	2.56	5.13	10.5	19.6	30.1
Mean±S.D.	2.53±0.13	4.86±0.27	10.4±0.27	19.9±0.37	30.3±0.33
C.V. (%)	5.0	5.6	2.6	1.8	1.1
Error (%)	26.5	2.8	4.0	0.5	1.0

distilled water. Impurities in the mobile phase reduce sensitivity and life of the electrode. The stabilization of the detector can also be achieved by increasing the filter value. This generally results in reduction in baseline noise, but the assay sensitivity is adversely affected. No interfering peaks from other substances were observed using the reported experimental conditions.

Plasma and peritoneal fluid samples were collected from 25 patients in a phase I clinical trial of i.v. hydroxyurea and i.p. 5-fluoro-2′-deoxyuridine (FUdR). Patients were administered a combination consisting of 3 gm of FUdR infused i.p. and escalating doses of hydroxyurea infused i.v. over a 72-h period. Serial blood and peritoneal fluids samples were obtained over 24 h and were assayed for hydroxyurea. The mean hydroxyurea plasma and peritoneal fluid concentrations over a 24-h period in 15 patients following a dose of 2.0 gm/m^2/day is shown in Fig. 3. Note that the peritoneal fluid concentration achieved is only 1/1000 of the plasma concentration.

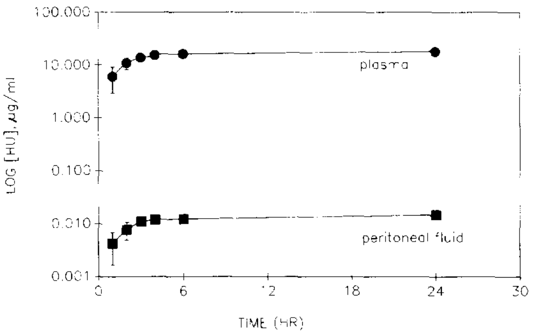

Fig. 3. Plot of mean hydroxyurea plasma (●) and peritoneal fluid (■) concentrations versus time following administration of hydroxyurea as a continuous i.v. infusion at a dose of 2.0 gm/m^2/day ($n=15$). Error bars indicate one standard deviation. Absence of an error bars indicates that the standard deviation is smaller than the data point symbol.

The method has been shown to be sensitive and reproducible. It has been demonstrated to be applicable for determination of hydroxyurea in plasma and peritoneal fluid in pharmacokinetic studies.

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