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# DETERMINATION OF 5-FLUOROURACIL IN PLASMA AND WHOLE BLOOD BY ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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## SUMMARY

5-Fluorouracil in blood or plasma was determined by extraction and column liquid chromatography. Acetonitrile was added to blood or plasma and the mixture was stirred and centrifuged. Zinc sulfate addition was followed by stirring and centrifuging. The acetonitrile was salted out with ammonium sulfate, and an aliquot was evaporated with nitrogen. The residue was dissolved in mobile phase and chromatographed. The stationary phase was a styrene–divinylbenzene copolymer, and the mobile phase was 10 m*M* tetrabutylammonium hydroxide–methanol (74:26). 5-Fluorouracil was detected by UV absorption at 266 nm. Time of the assay was less than 30 min. The detection limit was 10 ng/ml and the relative standard deviation was 4 to 10% depending on the concentration.

## INTRODUCTION

5-Fluorouracil (Fig. 1) is a chemotherapeutic drug that is used widely, especially for solid malignancies. Several high-performance liquid chromatographic (HPLC) methods have been developed for the determination of 5-fluorouracil in biological samples such as plasma [1–15]. However, these methods suffer from two problems: one in the pre-chromatographic treatment and one in the chromatography itself.

Biological samples must be treated prior to chromatography to remove constituents whose peaks would interfere with that of 5-fluorouracil and constituents that shorten column life. Extractions that are used are inefficient because of unfavorable partitioning into the extracting organic layer. Thus, a large volume of organic solvent is required, and this volume lengthens the time required for evaporation of the solvent. The second problem is the poor retention of 5-fluorouracil in reversed-phase chromatography. 5-Fluorouracil can have a $k'$ less than 1 when

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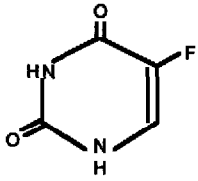


Fig. 1. Structure of 5-fluorouracil.

buffered water is the mobile phase, and adding organic solvents lowers it. If some samples have interfering peaks, the 5-fluorouracil peak cannot be moved to longer retention times by increasing the water content of the mobile phase because it is virtually 100% water already.

We wish to report an HPLC method for determining 5-fluorouracil in blood and plasma that solves these two problems. The extraction is performed relatively efficiently with a water-soluble solvent, acetonitrile, that has been salted out. The chromatography is carried out at a high pH where ion pairing is used to control $k'$.

## EXPERIMENTAL

### *Apparatus*

The chromatograph consisted of a Waters M-6000 pump (Millipore, Waters Chromatography Division, MA, U.S.A.), a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 injector with a 200-$\mu$l loop, a 10 $\mu$m, 10 cm$\times$4.6 mm Hamilton PRP 1 (Hamilton, Reno, NV, U.S.A.) slurry packed column, a Kratos Spectroflow 773 variable-wavelength detector (ABI Analytical Kratos Division, Ramsey, NJ, U.S.A.), and a Houston (Austin, TX, U.S.A.) TM 4500 Microscribe recorder. A 1-ml gas tight Hamilton syringe with a 0.45-$\mu$m Rainin (Woburn, MA, U.S.A.) Nylon 66 membrane filter was used to filter extracted samples. Extraction solvents were evaporated with nitrogen from a Multivap 113 (Organomation Assoc., South Berlin, MA, U.S.A.). The mobile phase was 10 m*M* tetrabutylammonium hydroxide–methanol (74:26, v/v). The mobile phase had a nominal pH of 11.0. The flow-rate was 1.0 ml/min. The detector wavelength was 266 nm, and its sensitivity was varied from 0.002 to 0.50 a.u.f.s.

### *Reagents*

5-Fluorouracil and tetrabutylammonium hydroxide were obtained from Aldrich (Milwaukee, WI, U.S.A.). Acetonitrile, methanol, ethanol, 1-propanol, 2-propanol, and 2-methyl-1-propanol were obtained from Aldrich or Burdick and Jackson Labs. (Muskegon, MI, U.S.A.). Salts were obtained from Fisher (Fair Lawn, NJ, U.S.A.) or Alpha Products (Danvers, MA, U.S.A.). Distilled water was further purified with a Sybron Barnstead (Boston, MA, U.S.A.) system.

### *Procedure*

Stock solution A was prepared by dissolving 50.0 mg of 5-fluorouracil in water and diluting to 100 ml in a volumetric flask. Stock solution B was prepared by diluting stock solution A 100-fold. Enough solution B was combined with enough

blood, plasma, or water to make total volumes of 1.0 ml with a concentration range of 0–800 ng/ml. Enough solution A was combined with enough blood, plasma, or water to make total volumes of 1.0 ml with a concentration range 1.0–1.6 μg/ml. In the case of blood, the cells were lysed prior to making working solutions by freezing the blood and then thawing it.

To 1.0 ml of a working solution were added 2.0 ml of acetonitrile. The resulting mixture was vortex stirred and centrifuged to separate proteins. The supernatant was decanted, and 50 mg of zinc sulfate were added to it. The mixture was vortex stirred and centrifuged. The supernatant was decanted, and more than enough ammonium sulfate was added to saturate the solution. After again vortexing and centrifuging 1.8 ml of the upper salted out acetonitrile layer was removed. The acetonitrile was evaporated with nitrogen in a 90°C water bath. The residue was dissolved in 200 μl of mobile phase. The solution was filtered through a 0.45-μm membrane filter. A 5-μl aliquot of the solution was injected into the chromatograph.

*Calculations*

Calibration curves were obtained by plotting peak heights in cm as the $y$-axis and concentration in ng/ml of working solutions of blood, plasma, or water as the $x$-axis. Regression analysis gave the best values for $m$ and $b$ in the equation: $y = mx + b$.

The blood or plasma of patients being treated with 5-fluorouracil was determined by the extraction and chromatographic procedures described above. Patients' extracts were interspersed with extracts from calibration working solutions. From the measured peak height from the patient's sample, the concentration of 5-fluorouracil was calculated with the use of the above linear equation.

*Recovery*

Recovery experiments were conducted in the following manner. A patient's plasma concentration was determined. An amount of 5-fluorouracil in a small volume was then added to the plasma to double the drug's concentration. The spiked sample was redetermined. The difference between the first and second determination was divided by the added concentration to give the data shown in Table IV.

## RESULTS

Fig. 2a shows a chromatogram obtained from a whole blood extract containing 5-fluorouracil. Comparison with Fig. 2b where the sample contained no 5-fluorouracil shows that the method produced a well resolved 5-fluorouracil peak. Fig. 3a shows a chromatogram obtained from an extract of plasma containing 5-fluorouracil, and Fig. 3b shows a chromatogram from a plasma extract where no 5-fluorouracil was present. Again, the chromatograms show no peaks interfering with the peak of interest. However, nearby peaks prevented the use of internal standards with retention times near that of 5-fluorouracil. 5-Chloro-, 5-bromo-,

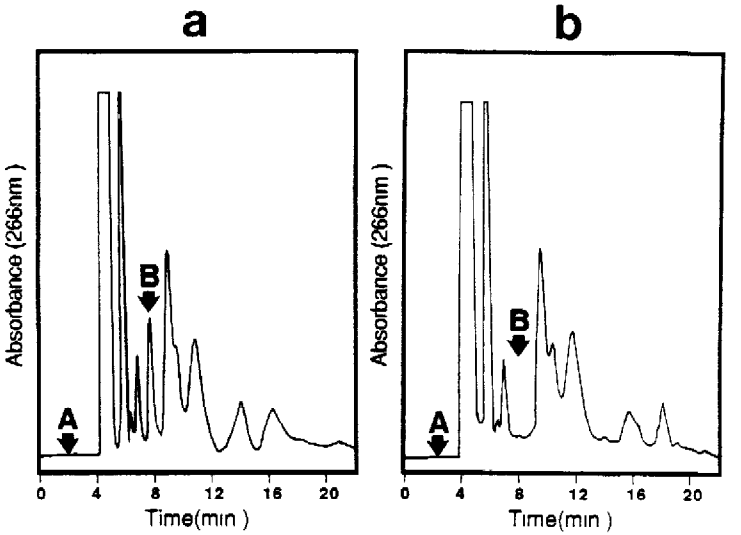


Fig. 2. Chromatograms of an extract of: (a) patient's whole blood containing 5-fluorouracil and (b) whole blood containing no 5-fluorouracil. Detector 0.005 a.u.f.s. Peaks: A, injection point; B, 5-fluorouracil.

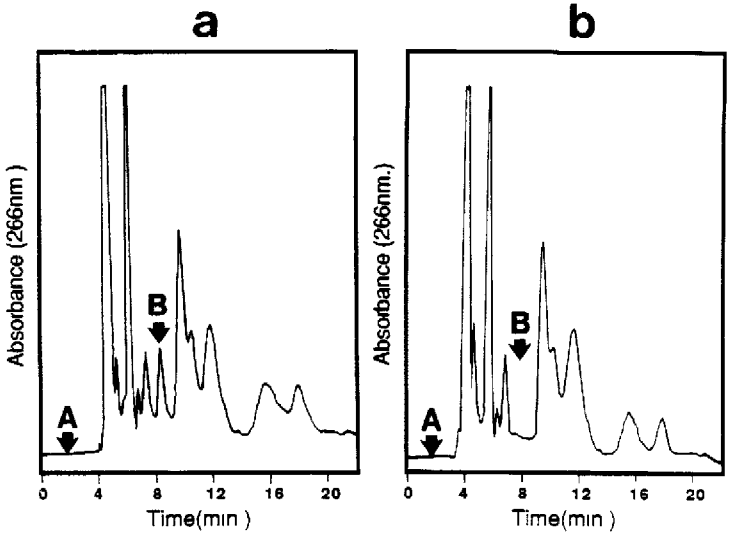


Fig. 3. Chromatograms of an extract of (a) patient's plasma containing 5-fluorouracil and (b) plasma containing no 5-fluorouracil. Detector: 0.005 a.u.f.s. Peaks: A, injection point; B, 5-fluorouracil.

and 5-iodouracil were investigated, but they could not be well resolved from peaks of blood and plasma constituents.

Calibration curves of peak height versus concentration were used instead of an internal standard. Data from the curves are shown in Table I. The slopes of curves for these media are the same at the 99% confidence level. The intercepts are very nearly zero, and therefore, any one of these fluids can be used for calibration for

TABLE I

LINEAR REGRESSION ANALYSES OF CALIBRATION CURVES

$r = 0.998$ to 0.999.

| Curve | Fluid extracted | Slope (mean ± S.D.) | Intercept (mean ± S.D.) | Identity of slopes ($t_{calc}$*) |
|---|---|---|---|---|
| A | Blood | 0.0249 ± 0.0020 | 0.066 ± 1.7 | A and B (0.0674) |
| B | Plasma | 0.0250 ± 0.0015 | 0.028 ± 1.3 | B and C (0.0106) |
| C | Water | 0.0249 ± 0.0015 | 0.098 ± 1.4 | A and C (0.0571) |
| D | No extraction** | 0.0404 ± 0.0025 | 0.126 ± 2.0 | |

*$t_{table} = 2.98$, 99% confidence.
**Standards dissolved in mobile phase.

patient blood or plasma samples. In most cases, water would be the most convenient to obtain.

Table I also shows a calibration curve obtained when standards were chromatographed without extraction. The ratio of slopes for curves from extracted samples to the slope for the curve from direct chromatography gives the efficiency of pre-chromatographic treatment. The efficiency was 62%, and this value is comparable to what has been reported by others, 50–68% [2,13,14]. However, the volume of extracting solvent was significantly smaller in the procedure reported here. The time required for evaporation was therefore much less.

Table II shows the reproducibility of the method over a range of concentrations

TABLE II

REPRODUCIBILITY OF THE METHOD OVER A RANGE OF 5-FLUOROURACIL CONCENTRATIONS

Fluid spiked with 5-fluorouracil at the concentrations shown, $n = 8$.

| Fluid | Concentration (ng/ml) | S.D. (ng/ml) | R.S D. (%) |
|---|---|---|---|
| Blood | 50.0 | 5.2 | 10 |
| Blood | 400 | 30 | 7.5 |
| Blood | 800 | 38 | 4.8 |
| Blood | 1600 | 61 | 3.8 |
| Plasma | 100 | 7.2 | 7.2 |
| Plasma | 600 | 33 | 5.5 |
| Plasma | 1300 | 54 | 4 2 |
| Plasma | 1600 | 56 | 3.5 |
| Water | 50.0 | 4.8 | 9.6 |
| Water | 200 | 13 | 6.5 |
| Water | 1000 | 46 | 4.6 |
| Water | 1600 | 56 | 3.5 |

TABLE III

DISTRIBUTION OF 5-FLUOROURACIL PLASMA LEVELS IN PATIENTS

Drug given by continuous intravascular infusion 5-Fluorouracil infusion fluid concentrations varied.

| Concentration range (ng/ml) | Number of patients |
|---|---|
| 10- 49 | 28 |
| 50- 99 | 46 |
| 100-149 | 24 |
| 150-199 | 17 |
| 200-299 | 6 |
| 300-399 | 9 |

TABLE IV

RECOVERY OF 5-FLUOROURACIL FROM PATIENTS' PLASMA

| Concentration added* (ng/ml) | Concentration found (ng/ml) | Recovery (%) |
|---|---|---|
| 50 | 52 | 104 |
| 77 | 89 | 116 |
| 93 | 86 | 92 |
| 115 | 109 | 95 |
| 176 | 187 | 106 |
| 244 | 252 | 103 |
| 363 | 361 | 99 |
| Average recovery | | 102 |
| S.D. | | 7.3 |

*Equal to the concentration determined in the patient's plasma.

that might be found in patients using 5-fluorouracil. Blood, plasma, and water were the fluids studied. The relative standard deviation (R.S.D.) is about 10% at the low level of 50 ng/ml and falls to about 4% at a high level of 1600 ng/ml. This reproducibility is comparable to that obtained by others [12] using an internal standard, 5.2–8.0% R.S.D. The standard deviations for the slopes of calibration curves in Table I also indicate the reproducibility of the method.

5-Fluorouracil levels in 130 patient samples were determined by the method. The distribution of the levels is shown in Table III. The majority of samples has 5-fluorouracil levels of less than 100 ng/ml. Thus, a method for the determination of 5-fluorouracil in the plasma of patients being treated with the drug must be very sensitive. Table IV shows the results of recovery experiments of 5-fluorouracil spiked into some patients' plasma. The recovery averaged $102 \pm 7\%$. The detection limit of the method was 10 ng/ml at a singal-to-noise ratio of 3.

## DISCUSSION

The method reported herein extracts the very water-soluble 5-fluorouracil from blood or plasma with a water-soluble solvent, acetonitrile. In order to obtain two

phases for extraction, the acetonitrile was separated from the blood or plasma by saturating with ammonium sulfate. The combination of acetonitrile and ammonium sulfate was very effective in precipitating proteins in addition to producing extraction.

For salting out acetonitrile, sodium chloride and potassium dihydrogenphosphate were tried. Each gave an extraction efficiency comparable to that of ammonium sulfate but each failed to remove interfering chromatographic peaks as well as did ammonium sulfate. Methanol, ethanol, 1-propanol, 2-propanol, and 2-methyl-1-propanol were investigated as extracting solvents. None was as effective in precipitating proteins as acetonitrile, and they produced chromatograms with huge interfering peaks.

The $pK_a$ value for 5-fluorouracil is 8.0 [16]. By using a basic mobile phase, the 5-fluorouracil was converted to an anion for chromatography. Tetrabutylammonium ion was added to the mobile phase to pair with the 5-fluorouracil anion to increase its $k'$ value. At zero concentration of sodium chloride in the mobile phase, the $k'$ of 5-fluorouracil was 4.0, but at 40 m$M$ sodium chloride $k'$ was 2.8. Thus, retention of the ion pair could be varied adjusting the sodium chloride concentration. This retention alteration could be useful in determining 5-fluorouracil in patient's blood or plasma when an interfering peak is present.

A styrene–divinylbenzene polymeric stationary phase was chosen because it is stable to high pH mobile phases as compared to silica-based reversed-phase stationary phases. Methanol, ethanol, 2-propanol and acetonitrile were investigated as mobile phase components with water. Ethanol and 2-propanol gave poor resolution of the 5-fluorouracil peak from nearby peaks. Methanol and acetonitrile were equally effective. Methanol was chosen because it is less expensive. The percentage of methanol in the mobile phase was a compromise between sensitivity and resolution: at higher levels of methanol, sensitivity was greater but resolution was poor because of a lower $k'$ value and lower plate number; at lower levels of methanol, resolution was very good but sensitivity fell off.

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