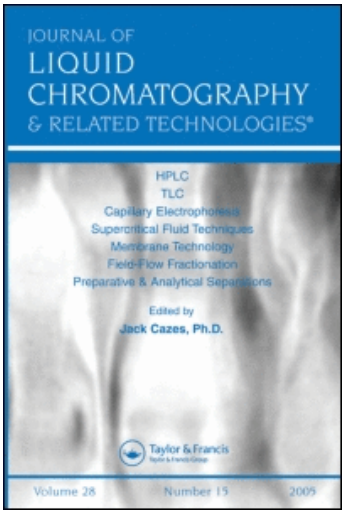


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# An HPLC Method for Measuring 5-Fluorouracil in Plasma

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# AN HPLC METHOD FOR MEASURING 5-FLUOROURACIL IN PLASMA

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## ABSTRACT

5-Fluorouracil in plasma was determined by extraction with methyl isobutyl ketone, evaporation of the ketone, and reverse phase high performance liquid chromatography of the evaporation residue. With UV detection at 280 nm the lower limit of detection is 10.0 ng/ml and interfering peaks eliminated. The method is highly reproducible.

## INTRODUCTION

5-Fluorouracil (5-FU) is a frequently used chemotherapeutic agent in many cancer treatment protocols. Despite frequent use and twenty years of opportunity to study this drug, the optimal dose and method of administration are uncertain. Part of this uncertainty is due to its rapid clearance ($T_{\frac{1}{2}}$ is 5-15 minutes) from the body which decreases the time cancer cells are exposed to the drug. Blood level measurements and therefore pharmacokinetic studies have been difficult to obtain and are highly variable since very small amounts of drug are present. Recent use of a continuous intravascular infusion of this drug has necessitated the development of a sensitive and accurate method for

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analyzing 5-FU in nanogram per milliliter concentrations in plasma samples. The method described here has a lower limit of detection of 10 ng/ml and is reproducible and accurate. No interfering peaks in the chromatogram have been detected.

## MATERIALS AND METHODS

### Instrumentation

A Waters Associates (Milford, Mass.) HPLC system was used consisting of a model WISP 710B autoinjector, a model 6000M solvent delivery system, a model 440 wavelength detector set at 280 nm and .005 aufs, a model 720 data module, a column heater set at 39°C, and a Waters model 730 system controller.

The column (Alltech) was 25 cm reverse phase $C_{18}$, 10 μ particle size. A 3 cm pellicular $C_{18}$ guard column was also used. The mobile phase was .05M $NaH_2PO_4$ buffer to which 3.2 g/l of tetrabutylammonium hydroxide (Aldrich) was added for a pH of 6.2. The column and guard column were protected from microrganisms and fine particles with a 2 micron inline filter. Flow rate was 1 ml/min (600 psi av.) for the first 10 minutes then increased to 3 ml/min (1200 psi av.) for the next 25 minutes. The retention time for 5-FU averaged 5.40 minutes, $k' = 0.80$. Column life averaged 120 injections.

### Calibrations

Calibration curves were prepared by diluting a stock solution of 5.0 μg/ml 5-FU (Aldrich Chemical Co., Milwaukee, Wisconsin) from 12 ng/ml to 100 ng/ml in water. The stock solution was also added to pooled plasma to achieve final concentrations of 25 ng/ml and carried through the entire procedure to produce another linear calibration curve with intercepts at or near zero. Linear water and pooled plasma calibration curves were obtained for each set of patient plasma samples analyzed. The ratio of the slope of the plasma calibration curve to the slope of the water calibration curve gave the efficiency of the extraction.

**Procedure**

Patient blood samples were drawn from a peripheral vein into a 7 ml Vacutainer tube (Becton-Dickinson) containing sodium heparin. This was centrifuged at 3500 RPM for 5 minutes, and the plasma was removed and frozen until the analysis was performed.

Methyl isobutyl ketone (5 ml) and 6N HCl (2 drops) were added to 1 ml plasma, vortexed for 30 seconds and centrifuged at 3500 RPM for 5 minutes. One further extraction of the plasma with methyl isobutyl ketone (5 ml) was performed and the organic phase combined and evaporated at 40-45°C under a stream of nitrogen. The residue was dissolved in 200 µl of mobile phase, centrifuged at 3500 RPM for 5 minutes and the supernatant transferred to 1.5 ml polypropylene microfuge tubes which were centrifuged at 12,000 RPM for one hour at 5°C. 25 µl of this supernatant was injected into the HPLC apparatus.



## RESULTS

A typical chromatogram of plasma alone and with 95 ng/ml of 5-FU added is shown in Figure 1. No interfering peaks have been identified in a large number of patients with varying disease processes while most were receiving multiple other drugs.

The relationship of peak height to amount of 5-FU injected in water and extracted from plasma is shown in Figure 2. The relationship is linear over the range $6 \times 10^{-10}$ gm/ml to $2 \times 10^{-5}$ gm/ml with typical correlation coefficients of 0.999 for 5-FU in water and 0.997 for 5-FU in plasma. The extraction efficiency of the procedure averaged 49.3% $\pm$4.8 (SD) over a period of several weeks. Five separate extractions of the same plasma sample on one day gave an average value 70.3 $\pm$ 2.45 (SD) ng/ml 5-FU for a coefficient of variation of 3.5%. Ninety-six injections of the same water standard gave an average peak height of 551.9 $\pm$ 16.6 mm for a coeffieient of variation of 3.0%.

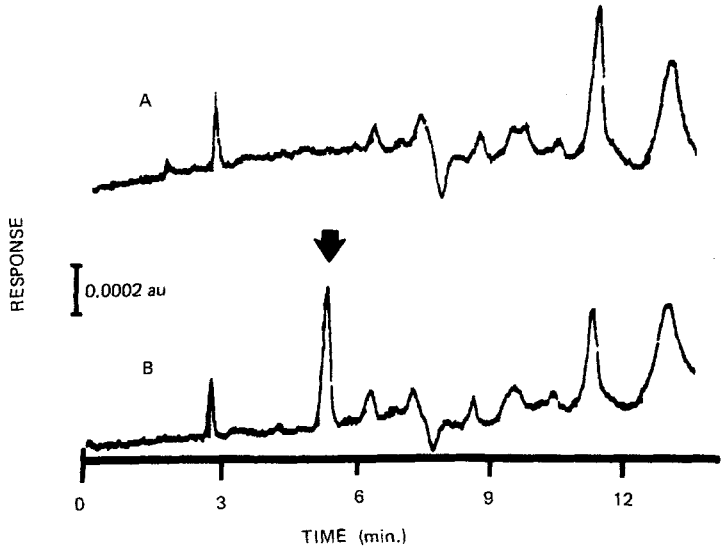


Figure 1A. A typical chromatogram of human plasma from time of injection to 14 min at 0.005 aufs.
B. The same plasma sample to which 95 ng/ml 5-FU (arrow) had been added. Retention time is 5.37 min.



## DISCUSSION

Plasma 5-fluorouracil has been determined by gas (1,2,3,4,5) and liquid chromatography. (6,7) The liquid chromatography methods have required a separate clean-up procedure to attain a sensitivity of 100 ng/ml (7) or require a variable wavelength detector.(6) Our method achieves a lower limit of detection of 10 ng/ml while using the commonly available 280 nm detector.

The methyl isobutyl ketone extraction removes interfering peaks from the chromatogram and concentrates the sample for higher sensitivity. The evaporation step caused a loss of less than 10% of the 5-FU from the original sample, but 40% at the 5-FU was not extracted from the plasma. This loss however, has been consistent between samples and between days, and is measured for each batch of plasma samples processed and is not considered a major

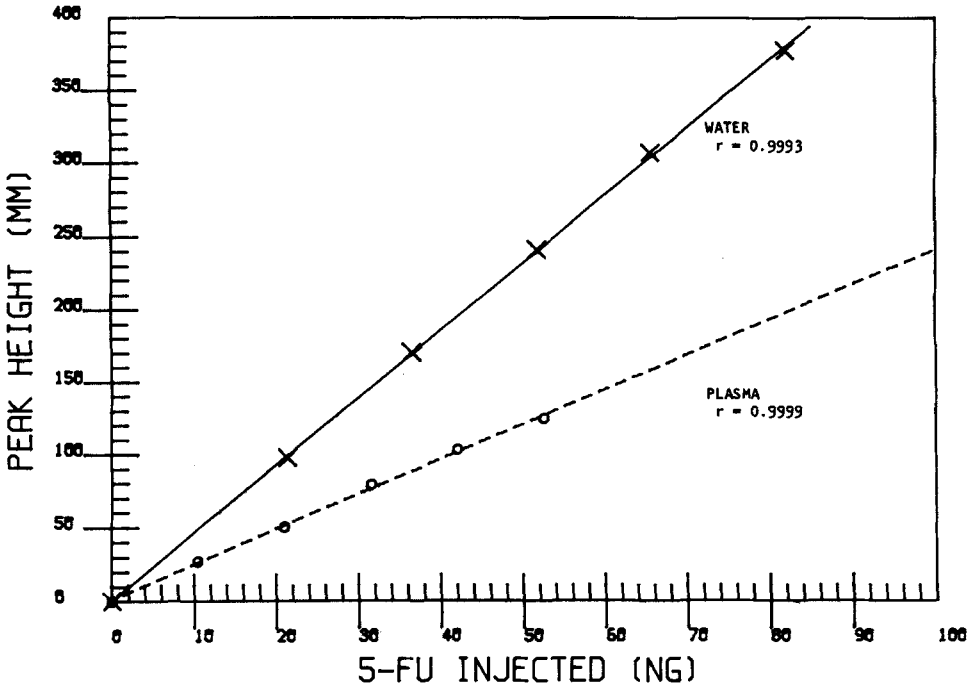


Figure 2. 5-FU was added to water and injected directly into the HPLC. Similar amounts of 5-FU were added to plasma which was then extracted and injected into the HPLC. "r" is the correlation coefficient for the linear regression equations.



disadvantage. Adherence of 5-FU to glass has been reported (1), but this was not detected in a series of experiments conducted by us.

The mobile phase chosen in the HPLC step contains tetra-n-butyl-ammonium ion. Without this ion, resolution of the 5-FU peak from nearby peaks was poor.

When a 254 nm detector was used, 5-FU absorbance was higher than at 280 nm, but resolution from a nearby peak was difficult. At 280 nm the source of this nearby peak had little or no absorbance and, consequently, the interference disappeared.

The method does not employ an internal standard. When 5-bromouracil was used as a standard its retention frequently coincided with one or more

plasma peaks. Therefore, a separate set of plasma standards was used giving a new calibration curve for each series of patient plasma samples analyzed.

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