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Determination of 5-fluorouracil in human plasma by a simple and sensitive reversed-phase HPLC method[1]

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Abstract

A simple and sensitive reversed-phase HPLC method with UV detection was developed and validated for the quantitation of 5-fluorouracil (5-FU) in human plasma. After acidification and salting out, 5-FU was extracted into ethyl acetate and back-extracted into a basic buffer. The extract was adjusted to neutral pH before being injected onto the HPLC column. 5-FU was separated from the matrix components on a YMC ODS-AQ column at 40°C using an aqueous mobile phase of 10 mM potassium phosphate at pH 5.5. A linear gradient of 0–25% methanol wash eluted late peaks, maintained column performance, and increased column stability. The run time was 20 min. The linear range was 25–300 ng ml^{-1} ($r^2 > 0.999$). The limit of quantitation was 25 ng ml^{-1}, with a signal-to-noise ratio of 23:1. Interday precision and accuracy of quality control samples were 6.2–8.4% relative standard deviation and −0.1–+1.9% relative error.

Keywords: 5-Fluorouracil; Nucleotide analog; Reversed-phase HPLC

1. Introduction

5-Fluorouracil (5-FU), a pyrimidine analog of the nucleotide uracil (Fig. 1), is an anti-neoplastic agent used in the treatment of malignant tumors of several organs, particularly the liver, colon, breast and stomach [1–3]. Because 5-FU is subjected to very rapid liver catabolism with short $t_{1/2}$, high doses are usually given which results in toxicity. Despite its toxicity, it remains a commonly used agent for neoplastic chemotherapy because of its low cost and availability, as well as the lack of other more effective but less toxic agents [4].

Recent 5-FU therapeutics have been tested by administering low doses targeted at the localized area of the tumor. The pharmacokinetics for typical therapeutic doses of 5-FU are well documented [1–4], but many of the previously described methods lack sensitivity or ruggedness

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for the quantitation of 5-FU administered at low doses. Because of the short $t_{1/2}$ of 5-FU, a sensitive method with a limit of quantitation (LOQ) of < 0.10 μg ml^{-1} will be needed to describe the elimination phase in the pharmacokinetic profile.

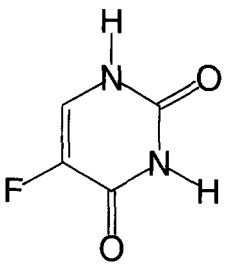
A gas chromatographic–mass spectrometric method for 5-FU has been described recently [5]; however, it required derivatization and extensive sample processing. A gas chromatographic method using nitrogen–phosphorus detection was reported with a LOQ of 1 μg ml^{-1} [6], which was not adequate. Several reversed-phase HPLC methods reported better sensitivity for 5-FU without derivatization. However, they were not sensitive enough for the required LOQ of 0.1 μg ml^{-1} [7–9]. Barberi-Heyob et al. [10] reported an HPLC method with a detection limit of 0.1 μg ml^{-1} at a signal-to-noise ratio of three. However, the k' value was approximately 1.0 which produced a retention time of 1.12 min for 5-FU. This did not leave much room for resolution from potential interference peaks of endogenous compounds. The present authors attempted literature methods using mobile phases of high aqueous content [10,11]. With mobile phases of 2–5% methanol the analyte was poorly retained on the C_{18} stationary phase and not resolved from interferences. When mobile phases of 100% aqueous content were used, column efficiency and performance could not be maintained for an analytical run of more than 25–30 injections. When a linear gradient of 25% methanol was used to rinse the column and elute the late peaks, the column performance was reproducible and very stable.

In this paper a sensitive, accurate, robust and specific assay for 5-FU by reversed-phase HPLC is described which has been used in the assay of clinical samples.

2. Experimental

2.1. Instrumentation

The HPLC system comprised a model 712 autosampler, model 484 detector, and dual model 510 pumps directed by a model 680 gradient controller (Waters, Milford, MA). The injection volume was 120 μl with detection at 266 nm. Both pumps were run at flow rate of 1.20 ml min^{-1}, with one pump for mobile phase A, potassium phosphate (pH 5.5; 0.01 M), and the other for mobile phase B, methanol:potassium phosphate (pH 5.5; 0.01 M) (50:50, v/v). The elution program was: mobile phase A for 5 min, mobile phase B 0%–50% over 1 min, and maintained at 50% for 3 min. Initial conditions were restored by decreasing mobile phase B to 0% over 1 min, and the column equilibrated with 100% mobile phase A for 10 min. The injection cycle was 20 min. The separation was performed by a YMC ODS-AQ C_{18} analytical column (5 μm particle size, 250 mm × 4.6 mm i.d., YMC Co., Ltd., Kyoto,



5-Fluorouracil

Fig. 1. Structure of 5-fluorouracil.

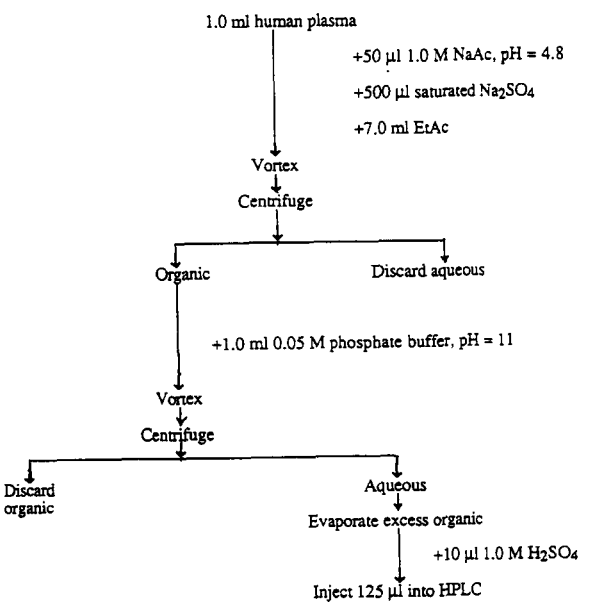


Fig. 2. Extraction flow diagram.

Table 1
Extraction recovery[a] of 5-FU from human plasma (Unextracted matrix-free solutions and extracted standards of 25, 100, and 300 ng ml^{-1} 5-FU in quadruplicate were injected onto the HPLC column. The mean peak height counts of the unextracted samples were 693 (2.4% RSD), 2889 (1.8% RSD), and 8443 (0.9% RSD) for 25, 100, and 300 ng ml^{-1} 5-FU respectively. The % recovery of each extraction was calculated based on the mean peak height counts at each level)

5-FU (ng ml^{-1})					
25		100		300	
Peak Ht.	%Rec.	Peak Ht.	%Rec	Peak Ht.	%Rec
316	45.6	1368	47.4	4042	47.9
328	47.3	1327	45.9	3795	45.0
329	47.5	1385	47.9	3958	46.9
320	46.2	1356	46.9	4009	47.5
Mean	46.7		47.0		46.8
%RSD	1.9		1.8		2.7

[a] Overall % recovery = 46.8.

Japan) at 40°C using a CH-30 column heater and TC-50 controller (Eppendorf North America, Madison, WI). Two separate HPLC systems were used during validation to assure method ruggedness. The second system consisted of autosampler, pumps, and detector from either a different manufacturer or a different model from the first system. The performance was comparable to the first system. Five different analytical columns were tested between the two systems and the chromatographic performance was similar.

2.2. Materials

All chemicals used were of analytical grade or HPLC grade. The organic solvents were from Fisher (Fair Lawn, NJ), with the exception of ethanol which was purchased from Quantum (Rolling Meadows, IL). Inorganic chemicals were purchased from Fisher and Mallinckrodt (Paris, KY). Deionized water was Nanopure® Barnstead and was filtered before use. Ethyl acetate was saturated with water by horizontal shaking in a separatory funnel for at least 1 h, and the bottom layer was drained to waste. Control human heparinized plasma was purchased from Nashville Biological (Cincinnati, OH), or drawn in-house. 5-FU was purchased from Sigma Chemical Company (St. Louis, MO). Potential 5-FU metabolites 5-fluoro-2-deoxyuridine monophosphate, 5-fluoro-2-deoxyuridine, and 5-fluorouridine were kindly supplied by Matrix Pharmaceuticals (Menlo Park, CA). Mobile phase was vacuum-filtered through 0.45 μm nylon membranes from Gelman Sciences (Ann Arbor, MI).

2.3. Preparation of solutions

The 5-FU reference standard was dried in an oven at 80°C, under vacuum, for 4 h before weighing. Primary stocks of approximately 100 $\mu g\ ml^{-1}$ for standards and quality control samples (QCs) were prepared from separate weighings. Spiking standards were diluted with ethanol from 10 $\mu g\ ml^{-1}$ substock to concentrations from 0.25 to 3.0 $\mu g\ ml^{-1}$. After 1.0 ml portions of the spiking standards were aliquoted into microcentrifuge tubes, they were stored at 4°C. The spiking standards were stable for at least 15 days. Working standards were prepared by drying 100 μl of the appropriate spiking standard in 16 mm × 100 mm polypropylene screw-cap tubes (Sarstedt Inc., Newton, NC) under nitrogen, and reconstituting with 1.0 ml of control human heparinized plasma. The QC substock of 10 $\mu g\ ml^{-1}$ was diluted with plasma to produce concentrations of 75, 100, and 225 ng ml^{-1}. QCs were aliquoted in 2.25 ml volumes into polypropylene tubes and were stored at −20°C with the clinical samples until assayed. Another group of QCs was set aside and stored separately at −20°C for long term storage stability tests.

2.4. Sample processing procedures

The flow of sample processing is shown in Fig. 2. 1 ml aliquots of QCs or analytical plasma samples were added to 16 mm × 100 mm polypropylene screw-cap tubes for processing with a single set of working standards. Each plasma sample was adjusted to pH 6.0 by adding 50 μl of sodium acetate buffer (pH 4.8; 1.0 M). A saturated sodium sulfate solution (200 g l^{-1}), 250 μl,

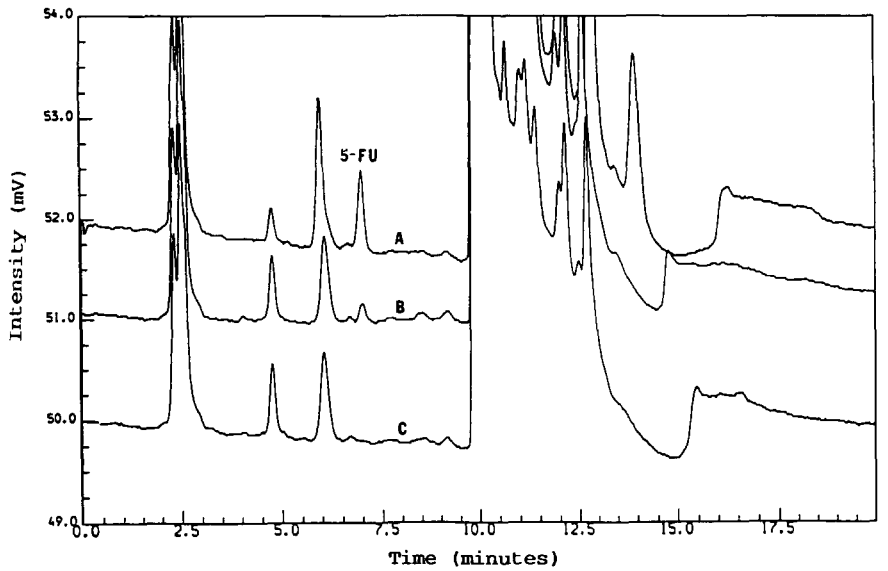


Fig. 3. Chromatograms of 5-FU in plasma extracts: (A) 100 ng ml^{-1} QC; (B) 25 ng ml^{-1} standard (LOQ); (C) blank control plasma.

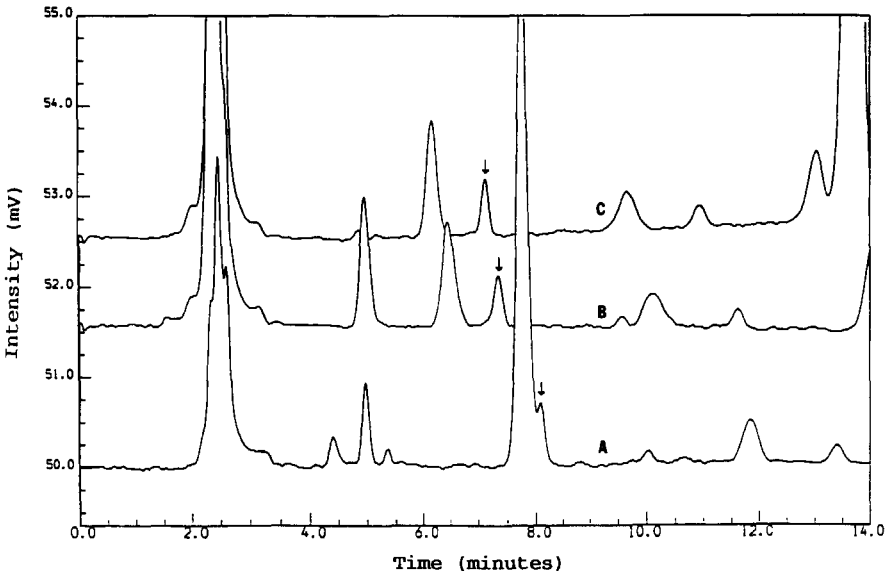


Fig. 4. Effect of column temperature on resolution of 5-FU (↓) from matrix peaks; (A) 25°C; (B) 35°C; (C) 40°C.

was added and mixed by vortexing. After adding 7.0 ml of water-saturated ethyl acetate, the tubes were capped and vortexed vigorously for 90 s. After centrifuging for 5 min, 6.0 ml of the organic layer was transferred to a clean polypropylene screw-cap tube containing 1.0 ml of phosphate buffer (pH 11; 0.05 M). The tubes were capped, vortexed and centrifuged to separate the phases. The organic layer was aspirated to waste and the residual solvent flushed from the aqueous portion with nitrogen at room temperature ($\approx$ 10 min). The aqueous sample was adjusted to neutral pH with the addition of 10 μl of sulfuric acid (1.0 M), and 125 μl of the sample was injected onto the HPLC column.

Table 2
Calibration standards interday variation. Data were from five analytical runs

Parameter	Calibration standard (ng ml^{-1})							
	25	40	60	80	100	150	250	300
Mean	25.1	39.4	59.5	80.7	103	149	251	299
%RSD	3.3	1.2	2.5	1.8	4.0	4.7	1.7	2.7
%RE	+0.4	−1.4	−0.9	+0.9	+2.6	0.5	+0.3	−0.4

3. Results and discussion

3.1. Extraction procedure

Methods using solid-phase extraction of unwashed silica [10] as well as combined silica gel solid phase and liquid–liquid extraction [12] were reported. To avoid problems of lot-to-lot variability and sample overloading, a liquid–liquid extraction method was chosen. Initially, a single extraction procedure was attempted. This procedure produced a very dirty sample even after filtration. It caused high back pressure in the analytical column and a very noisy baseline. Endogenous compounds were eluted as late as 90 min, presenting potential interferences for subsequent chromatographic runs. The addition of a back-extraction procedure resulted in a much cleaner sample. The baseline noise was decreased substantially, increasing the sensitivity for 5-FU.

Christophidis et al. [13] back-extracted 5-FU from 2-butanol; ethyl ether (15:85) into a phosphate buffer (pH 11; 0.05M). In the initial extraction step, the plasma was adjusted to pH 6 so that the compound remained unionized (pK_a of 5-FU is 8). A saturated salt solution was used to salt-out the compound because 5-FU is highly protein-bound [14]. This method was tested as well as combinations of other organic solvents. All of them yielded similar results chromatographically and recoveries of less than 50%. It was observed that water-saturated ethyl acetate alone produced the cleanest chromatography and a consistent recovery of 46.8%. Table 1 shows that percent recoveries were 46.7, 47.0, and 46.8 with %RSDs (relative standard deviations) of 1.9, 1.8, and 2.7 for 25, 100, and 300 μg ml^{-1} 5-FU respectively.

Because of the consistent recovery, it was possible to quantitate 5-FU accurately and precisely without the use of an internal standard. Bromouracil, chlorouracil, iodouracil, and thymidine were tried as potential internal standards without success. Bromouracil eluted near an endogenous matrix peak which varied in size from various lots of control plasma. The recovery of chlorouracil was inconsistent between different plasma lots in an analytical run. Iodouracil eluted close to a large matrix peak at 25 min and the separation of iodouracil from the potential metabolite 5-fluoro-2-deoxyuridine was marginal. Thymidine was highly retained on the column and would require gradient elution with a stronger mobile phase, as described by MacMillan et al. [15]. This resulted in a run time of at least 30 min per sample and undesirable chromatography. Another HPLC assay used 5-fluorocytosine as an internal standard on a similar chromatography system [13]. On that system the fluorocytosine eluted before 5-FU and coeluted with an endogenous peak. Similarily, on the current chromatographic system 5-fluorocytosine would elute in front of 5-FU, in a region of multiple endogenous interfering peaks.

3.2. Chromatography

Because 5-FU is a polar, low molecular weight compound, it is poorly retained on a reversed-phase HPLC column. Mobile phases using ion-pair agents were attempted to improve retention. The ion-pair agent tetrabutylammonium phosphate had no effect on the k' value of 5-FU on a reversed-phase C_{18} column eluted with 20 mM phosphate buffer at pH 5.0 [16]. Because the pKa value of 5-FU is ≈ 8, the k' value of 5-FU could

Table 3
QC interday variation. Data were from five analytical runs

Parameter	QC sample (ng ml^{-1})		
	75	100	225
Mean	74.7	99.9	226
%RSD	8.4	6.2	6.5
%RE	−0.4	−0.1	+1.9
N	20	20	20

be changed with a high pH mobile phase. A high pH mobile phase was tested with cetrimonium bromide as ion-pair agent on a polymer column [17]. Retention of 5-FU increased significantly under these conditions. Because of the poor efficiency of the polymer column, the peak shape was too poor to accurately quantify 5-FU at low concentrations. An alumina column gave a much improved peak shape, but the chromatography required a complicated valve-switching system to eliminate late-eluting peaks. It was decided to use a simpler, more reproducible HPLC system.

Chromatograms of extracted blank plasma, and plasma spiked with known amounts of 5-FU are shown in Fig. 3. The endogenous uracil peak was eluted before the compound of interest and did not interfere. Several stationary phases, such as CN and phenyl, were tried according to methods reported in the literature [4,12], but C_{18} gave the best retention of 5-FU and separation from the uracil peak. A column temperature of 40°C was crucial to this separation. At room temperature, the two peaks were poorly separated with $R_s < 0.4$. As the column temperature increased, the interfering peak moved in faster than the 5-FU peak (Fig. 4). Resolution was increased to $R_s > 2.0$ at 40°C. The higher column temperature also served to shorten the run time by decreasing the retention time of late-eluting peaks.

Table 4
QC intraday variation

Parameter	QC sample (ng ml^{-1})		
	75	100	225
Mean	78.0	99.3	204
%RSD	1.4	2.4	3.0
%RE	+4.0	−0.7	−9.6
N	4	4	4

The YMC-AQ column was chosen because its structure is specifically designed for highly aqueous mobile phase. Since the compound was very polar, the amount of organic solvent in the mobile phase must be kept low to provide adequate retention. Very small amounts of organic solvent, such as 2% and 5% methanol, as previously reported [10,11] were attempted, but the compound was eluted in the void volume. Therefore, a 100% aqueous mobile phase was used. The retention time of 5-FU on the 250 mm × 4.6 mm, 5 μm C_{18} column was 6.8–7.3 min.

With the aqueous mobile phase some hydrophobic components in the sample extract accumulated on the column, resulting in a loss of peak shape, late peaks, and increased back pressure. A gradient wash of 25% methanol for 3 min, after 5-FU elution, was introduced to wash away these extracted sample components.

Column life was increased with the 25% methanol gradient, preventing the bonded phase from collapsing. The behavior of C_{18} silica in methanol–water eluants has been reported recently [18]. When the methanol content in the mobile phase is lower than 10% (v/v), the C_{18} silica is not wetted, often causing abnormal chromatographic behavior. This would explain why it was not possible to reproduce literature methods using 0–5% methanol phases. The column performance could be restored by rinsing overnight with a strong methanol wash [19]. However, because of the problem of the late-eluting peaks, it was decided to use a linear 0–25% methanol gradient wash to solve both problems. Dependent on the HPLC condition, 50–65% of methanol was reported to provide a persistent film of the bonded particles [20,21]. However, when column temperature is increased, equilibration and rewetting of the bonded phase is increased [18]. At a column temperature of 40°C, equilibration appeared to be complete, resulting in a reproducible retention of 5-FU and resolution from potential interference peaks. Methanol was chosen over acetonitrile as a washing solvent for a faster equilibration time.

Table 5
Precision and accuracy variations among analysts[a]

Analyst	Parameter	Sample (ng ml^{-1})		
		75	100	225
1	Mean	74.7	99.9	229
	%RSD	8.4	6.2	6.5
	%RE	−0.4	−0.1	+1.9
	N	20	20	20
2	Mean	73.7	98.3	201
	%RSD	1.2	2.1	1.8
	%RE	−1.7	−1.7	−10.8
	N	8	8	8
3	Mean	78.1	104	211
	%RSD	4.9	4.9	4.8
	%RE	+4.2	+3.9	−6.1
	N	18	18	18
4	Mean	74.2	98.8	207
	%RSD	4.9	1.6	2.0
	%RE	−1.1	−1.2	−8.0
	N	2	2	2

[a] The number of validation analytical runs was 5, 2, 3 and 1 for analysts 1, 2, 3, and 4 respectively. They were performed on two HPLC systems. Five analytical columns were tested.

3.3. Linearity and precision

The standard curve for plasma samples was linear over the concentration range 25–300 ng ml^{-1} with a correlation coefficient of $r^2 > 0.999$. The RSD from five validation analytical runs over 5 days was $< 5\%$ for spiked plasma standards and $< 9\%$ for QCs as shown in Tables 2 and 3. The interday variation for plasma QCs was determined by assaying four replicates each of low, high, and medium concentrations. The within-day variation of control samples was 3% or less (Table 4). In addition to the initial method validation, three other analysts conducted pre-study validation analytical runs to validate the method. The RSD and %RE (relative error) for each analyst in Table 5 show that the method is robust and reproducible among four analysts and between two HPLC systems. Five analytical columns with different lot numbers were tested on the HPLC systems. All five columns showed reproducible and similar chromatographic behavior.

3.4. Sensitivity and selectivity

Sensitivity was tested by spiking 25 ng ml^{-1} of 5-FU into six different lots of control plasma and the 5-FU concentrations in these spiked plasma samples were quantified. The RSD from these six determinations was 9.69% and the RE was −0.96%. The signal-to-noise ratio was 23:1 at 25 ng ml^{-1} 5-FU in plasma. 25 ng ml^{-1} was used as the LOQ because this was sufficient for the current study. A lower LOQ could be achieved if there is a need for more sensitivity in the future.

66 out of 66 lots of control plasma and predosed samples were tested and found to be free from interferences for the compound. Known potential metabolites for 5-FU were tested for interference. The 5-fluoro-2-deoxyuridine monophosphate and 5-fluoro-2-deoxyuridine were not back-extracted into the pH 11 phosphate buffer. The 5-fluorouridine was eluted from the column with the 25% methanol wash (Fig. 5). Fig. 6 shows representative chromatograms from volunteers dosed with 5-FU.

3.5. Stability

Stability testing of the analyte was performed on both unextracted and extracted plasma samples and the results are shown in Table 6. At least four QC replicates at each concentration level were determined. Plasma samples containing 5-FU were found to be stable when stored at −20°C for at least 61 days. 5-FU was stable in plasma samples held for at least 4.5 h at room temperature or subjected to three cycles of freezing and thawing. Extracted samples were stable on the HPLC system at room temperature for at least 45.4 h.

4. Conclusion

A sensitive, accurate, specific, rugged, and precise HPLC method was developed and validated for the determination of 5-FU in human heparinized plasma. 5-FU was resolved from the endogenous uracil when the column temperature was increased from 25°C to 40°C. The resolution

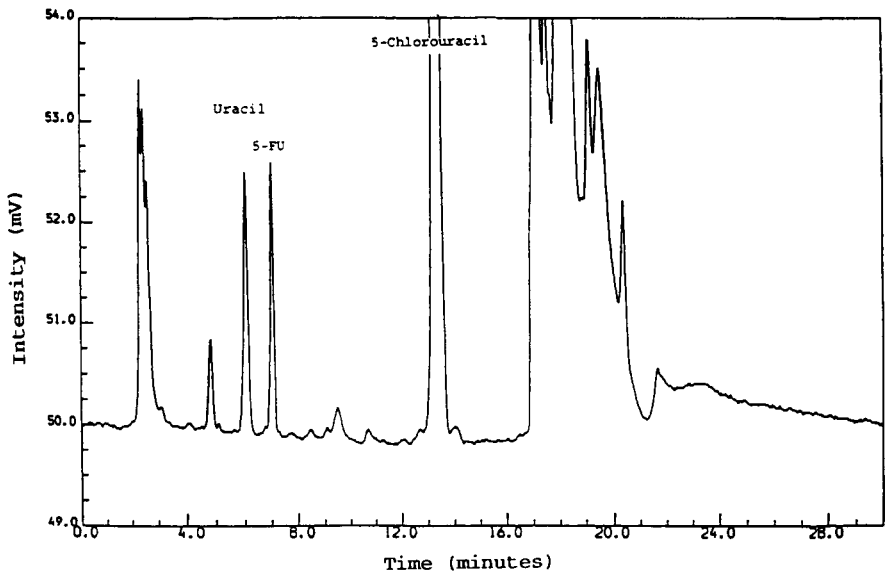


Fig. 5. Selectivity test of plasma extract: uracil, 500 ng ml^{-1}; 5-fluorouracil, 300 ng ml^{-1}; and chlorouracil, 2000 ng ml^{-1}.

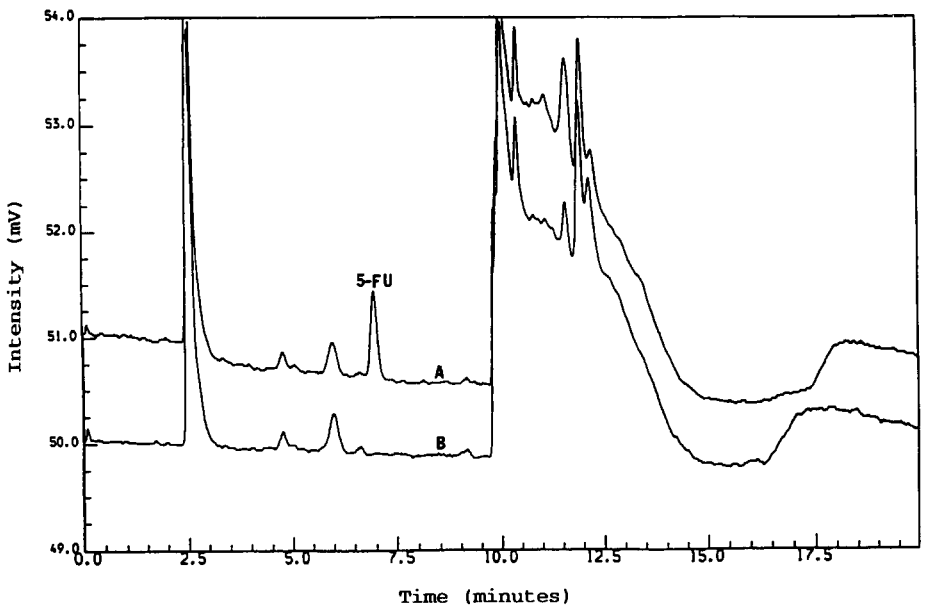


Fig. 6. Chromatograms of clinical sample extracts: (A) 0.75 h post-dose clinical sample; (B) pre-dose sample.

produced chromatographs of very low baseline noise, enabling an improved sensitivity over previously published methods. Washing the analytical column with 25% methanol allowed a run time of 20 min, which is a much shorter time than 30–40 min interjection cycle of previously published methods. The validation of the method and subsequent assay of clinical samples were conducted on two different HPLC systems by at least four scientists, demonstrating the ruggedness of the method.

Table 6
Stability of QC samples

Stability condition	Time	% of control
5-FU in plasma		
Storage, −20°C	61 days	94.2–95.1
Bench top	4.5 h	97.3–102
Freeze/thaw (×2, ×3)	N/A	97.9–106
5-FU in extracted samples		
On-system	45.5 h	90.2–92.8
Refrigeration	39.5 h	92.0–92.3

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