

Determination of 5-Fluorouracil in Human Plasma Using LC-MS/MS

Lori D. Payne*; Michael D. Nelson; Michael S. Alexander;
Ryan M. Minikis; John W. Dolan;
Bioanalytical Systems, Inc.

Overview

5-Fluorouracil (5FU), a low molecular weight and highly polar fluorinated pyrimidine, is an antineoplastic antimetabolite given intravenously in chemotherapy. An LC-MS/MS method was developed and validated to quantify 5FU in small volumes (0.1 mL) of human plasma, using 5-bromouracil (5BU) as the internal standard.

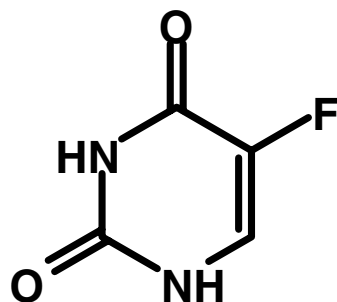
Methods:

5BU internal standard solution is added to 0.1 mL human plasma which is serially extracted with organic solvent. After mixing and centrifugation the solvent is removed and evaporated to dryness, and reconstituted. The sample is injected onto a 2.1 x 50 mm C18 column with a 4.6 x 12 mm C18 pre-column. The pre-column is backflushed with isopropanol after each injection. The mobile phase is 100% aqueous at 0.4 mL/min. Detection is by negative ion electrospray. The range of the method is 10-10,000 ng/mL.

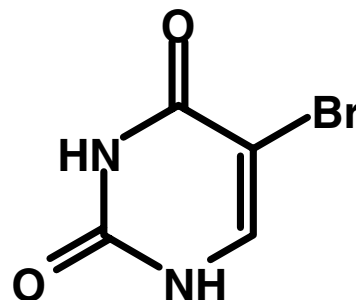
Introduction

5-Fluorouracil (5FU) is used, often in combination with other drugs, in the treatment of certain carcinomas. The compound is a fluorinated pyrimidine, very polar and highly water soluble. The structures of 5FU and the internal standard 5-bromouracil (5BU) are shown in Figure 1.

Figure 1. Structures of 5FU and 5BU



5-fluorouracil



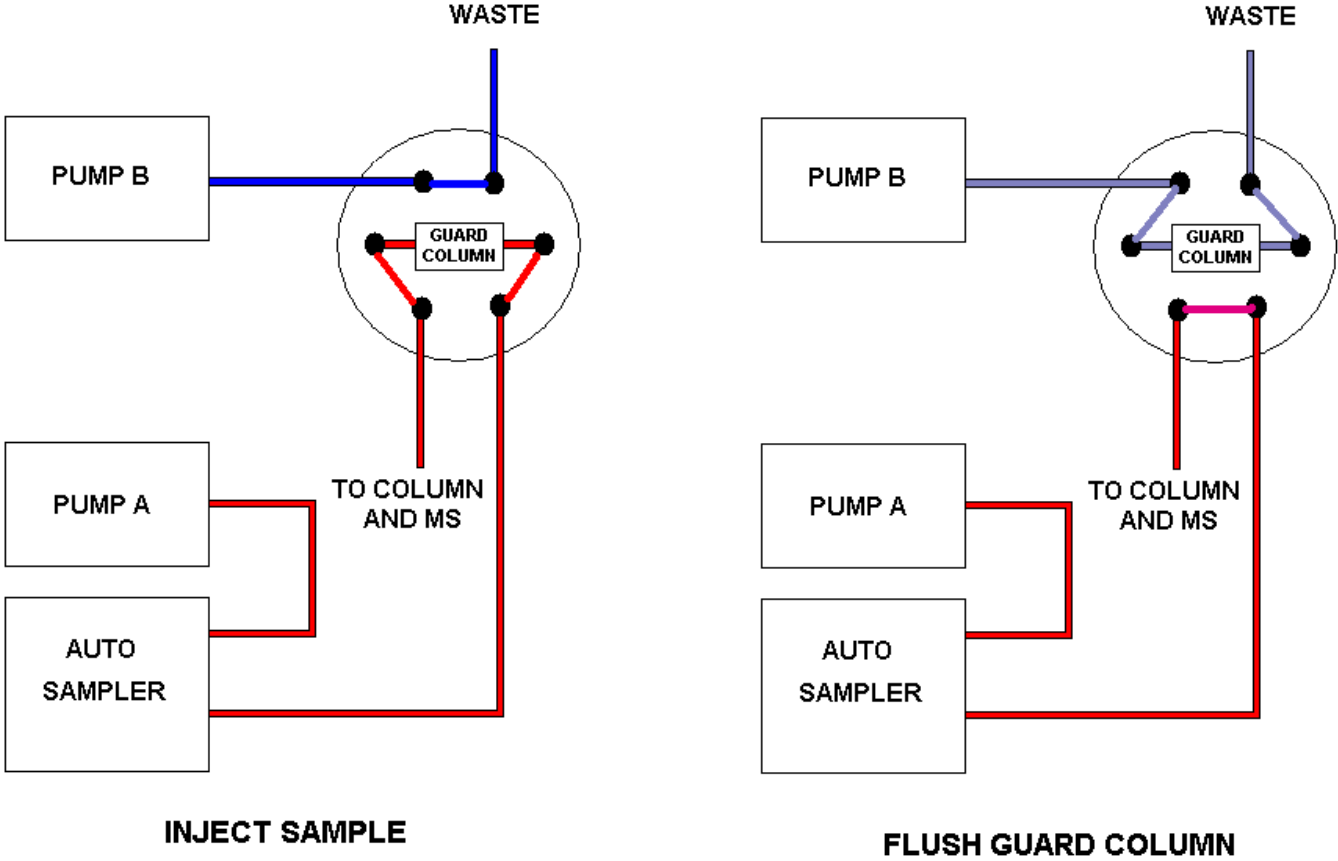
The assay required a relatively wide range with a small sample size; a short run time was also desirable. The LC-MS/MS method described here uses a sample size of 0.1 mL plasma and a calibration range of 10-10,000 ng/mL using two overlapping standard curves (10-600 ng/mL and 600-10,000 ng/mL). Two separate standard curves are required to cover the range due to non-linearity. The analysis time is 7 minutes.

Guard Column and Backflushing:

During method development it was observed that column performance degraded significantly over time (smaller retention volumes, decreased detector response). This was traced to buildup of nonpolar material on the column from the ethyl acetate extracts.

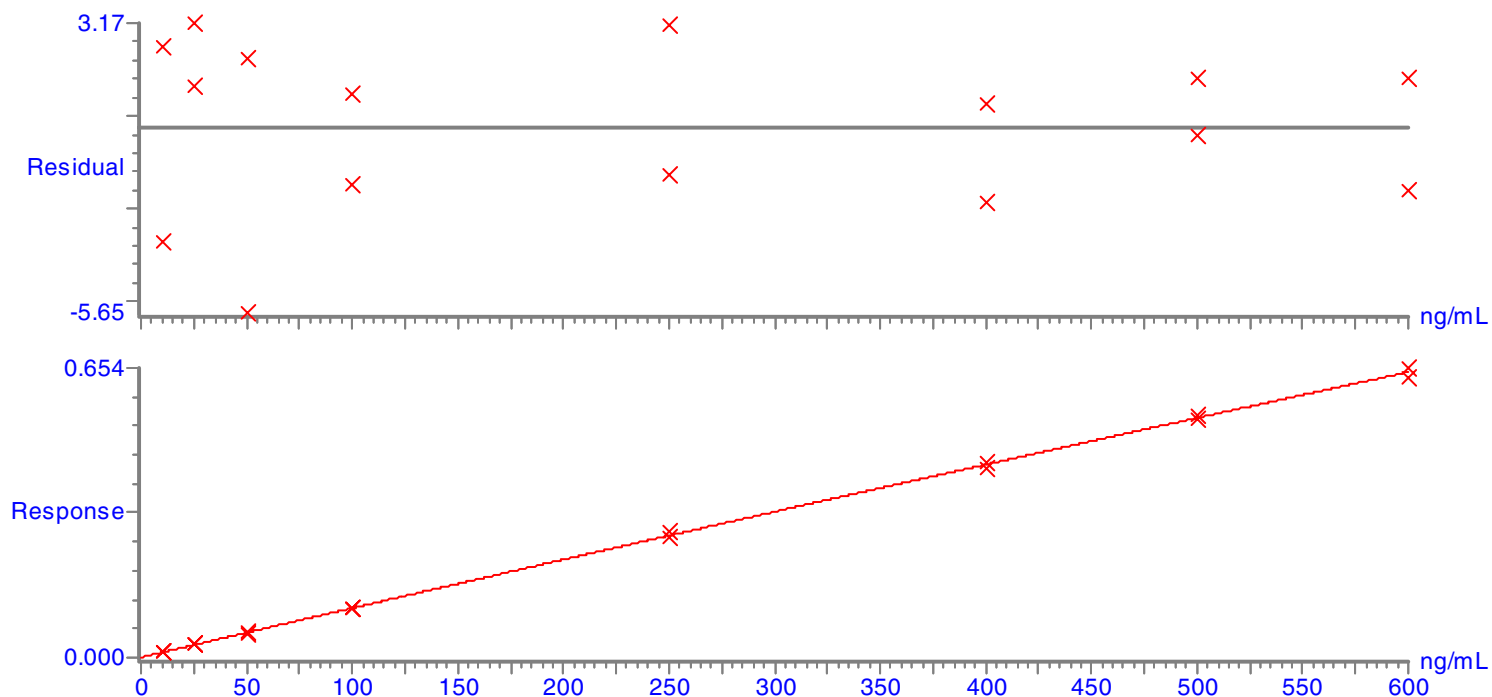
To minimize this problem a guard column was added, along with a switching valve (see Figure 2). At sample injection the guard column traps the hydrophobic material while the 5FU and 5BU pass through essentially unretained to the analytical column. Once the sample reaches the analytical column the guard column is backflushed offline with isopropanol for one minute, then re-equilibrated inline with the analytical column following the elution of 5BU.

Figure 2. LC pump and column configurations for sample injection (left) and sample analysis (right). During analysis the guard column is backflushed with isopropanol.



Low Curve (10-600 ng/mL)

Compound name: 5FU
Coefficient of Determination: 0.999438
Calibration curve: $-7.36947e-008 * x^2 + 0.00111766 * x + 0.000869132$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: $1/x^2$, Axis trans: None

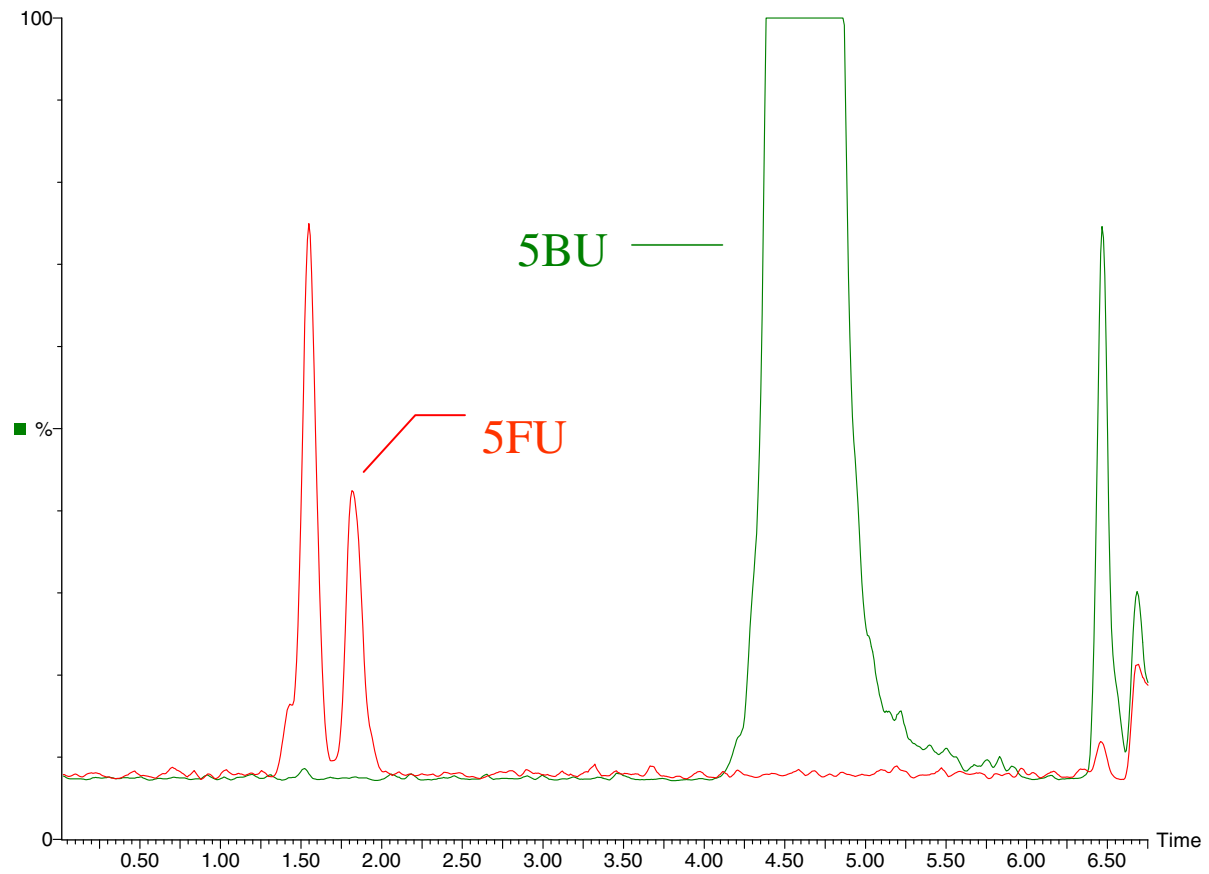


SC Results - Low Curve

| Nominal Concentration (ng/mL) | 0 | 10.0 | 25.0 | 50.0 | 100 | 250 | 400 | 500 | 600 |
|---|----|-------|------|-------|------|------|-------|------|-------|
| Calculated Concentration (ng/mL) | nd | 10.2 | 25.8 | 51.0 | 101 | 258 | 391 | 507 | 588 |
| | nd | 9.65 | 25.3 | 47.2 | 98.3 | 246 | 403 | 499 | 609 |
| | nd | 10.4 | 26.7 | 50.3 | 95.8 | 256 | 413 | 504 | 608 |
| | nd | 9.43 | 24.6 | 48.4 | 106 | 227 | 388 | 540 | 562 |
| | nd | 10.3 | 25.9 | 51.4 | 101 | 253 | 399 | 503 | 598 |
| | nd | 9.78 | 23.7 | 47.3 | 104 | 239 | 409 | 509 | 585 |
| | nd | 8.97 | 23.2 | 50.4 | 102 | 251 | 387 | 512 | 595 |
| | nd | 11.0 | 26.5 | 51.2 | 99.1 | 238 | 404 | 499 | 611 |
| N | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Average (ng/mL) | na | 9.98 | 25.2 | 49.6 | 101 | 246 | 399 | 509 | 595 |
| SD (ng/mL) | na | 0.645 | 1.26 | 1.75 | 3.14 | 10.5 | 9.71 | 13.3 | 16.3 |
| Deviation (%) | na | -0.24 | 0.85 | -0.71 | 0.88 | -1.6 | -0.17 | 1.9 | -0.90 |
| CV (%) | na | 6.5 | 5.0 | 3.5 | 3.1 | 4.2 | 2.4 | 2.6 | 2.7 |

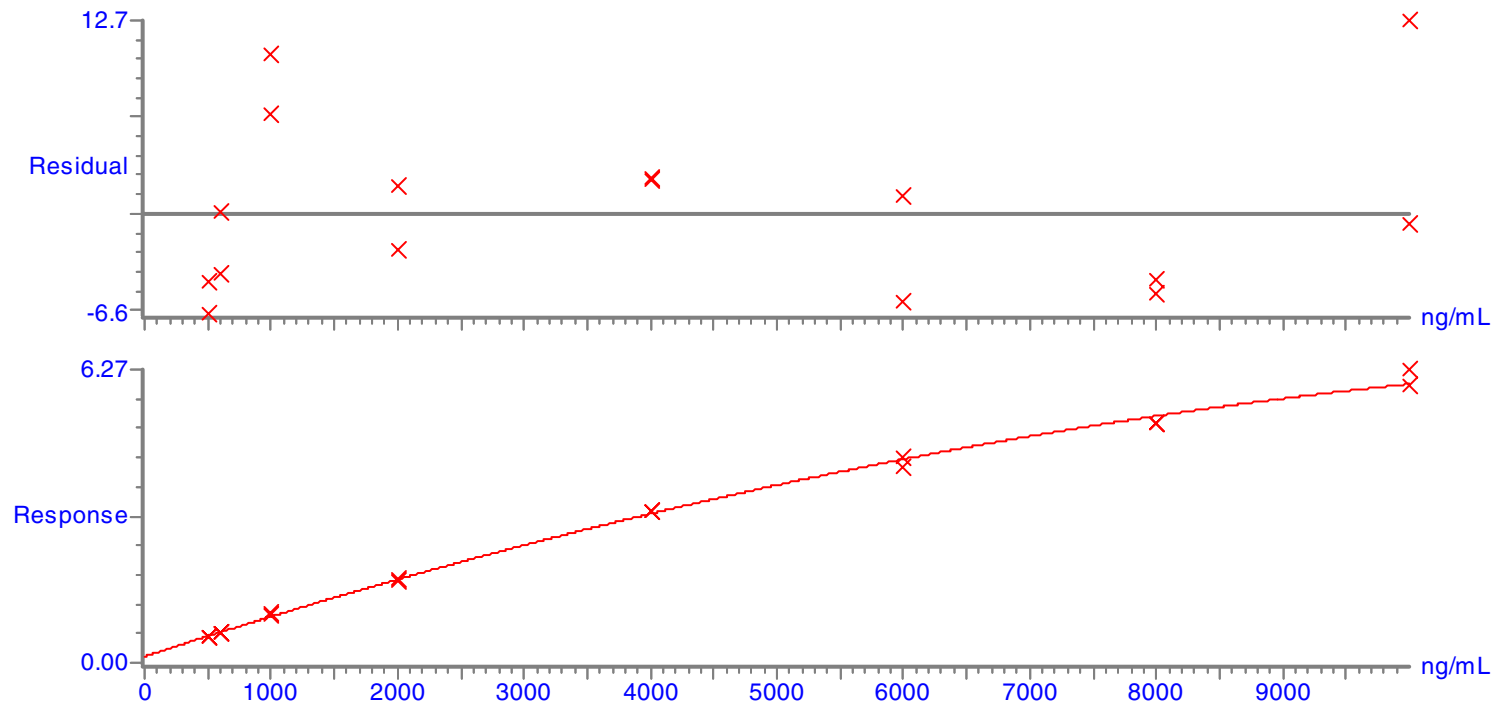
nd = no peak detected or $\leq 20\%$ LLOQ; na = not applicable

LLOQ - 10 ng/mL



High Curve (500-10,000 ng/mL)

Compound name: 5FU
Coefficient of Determination: 0.996843
Calibration curve: $-3.00995e-008 * x^2 + 0.000883826 * x + 0.133935$
Response type: Internal Std (Ref 2), Area * (IS Conc. / IS Area)
Curve type: 2nd Order, Origin: Exclude, Weighting: 1/x, Axis trans: None



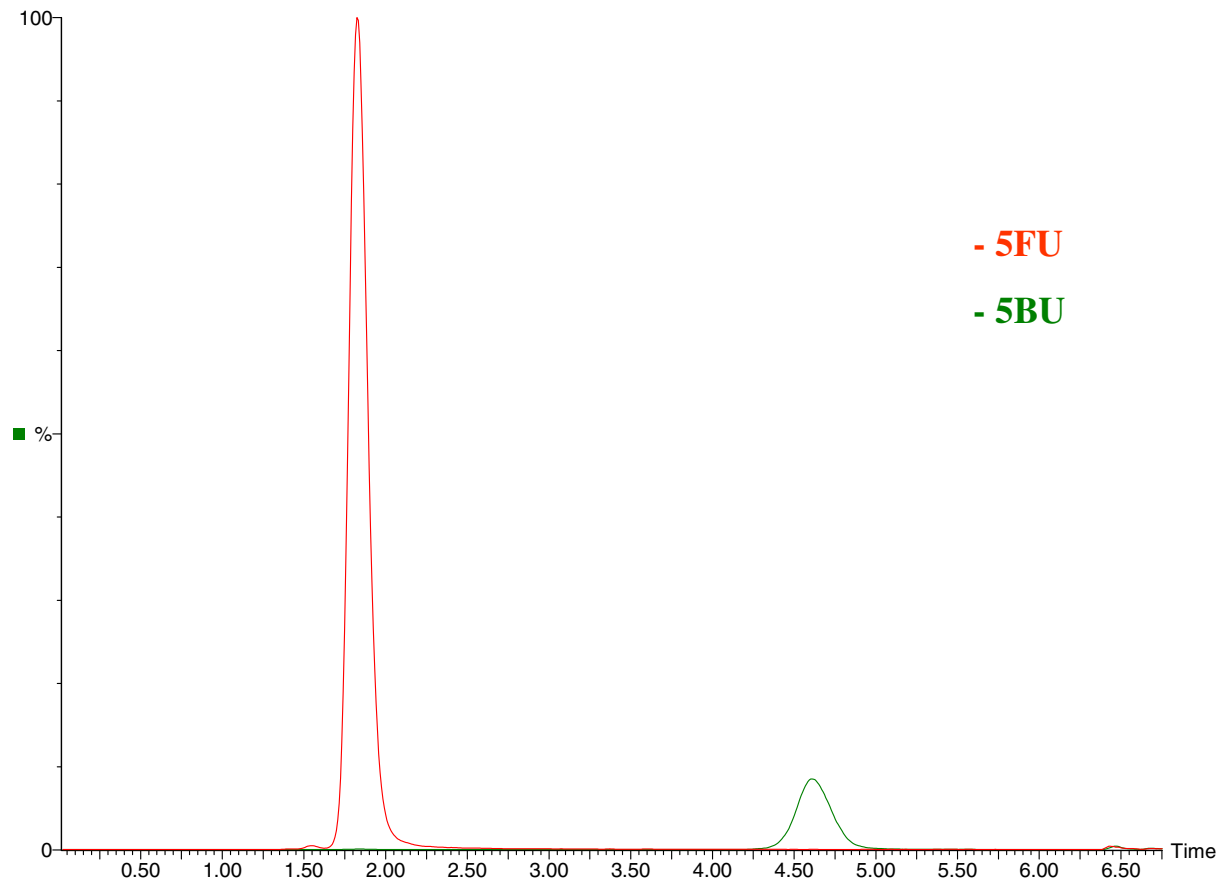
SC Results - High Curve

| Nominal Concentration (ng/mL) | 500 | 600 | 1000 | 2000 | 4000 | 6000 | 8000 | 10000 |
|---|------|------|------|---------|------|-------|------|--------|
| Calculated Concentration (ng/mL) | 477 | 576 | 1065 | 2037 | 4094 | 6068 | 7574 | 11274 |
| | 467 | 601 | 1105 | 1951 | 4089 | 5650 | 7652 | 9935 |
| | 473 | 600 | 958 | 2118 | 3957 | 6516 | 7462 | 11027 |
| | 517 | 544 | 1134 | 1983 | 4057 | 5541 | 7535 | 10074 |
| | 487 | 593 | 1024 | 2084 | 4031 | 6009 | 7543 | 11223 |
| | 493 | 579 | 1079 | 1892 | 4054 | 6029 | 7611 | 11721* |
| | 486 | 589 | 1044 | 2002 | 4008 | 6121 | 7673 | 11432 |
| | 470 | 610 | 1076 | 1931 | 4126 | 5812 | 7713 | 9548 |
| N | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 7 |
| Average (ng/mL) | 484 | 586 | 1061 | 2000 | 4052 | 5968 | 7595 | 10645 |
| SD (ng/mL) | 16.3 | 20.6 | 53.6 | 77.1 | 53.4 | 304 | 82.8 | 767 |
| Deviation (%) | -3.2 | -2.3 | 6.1 | -0.0048 | 1.3 | -0.53 | -5.1 | 6.4 |
| CV (%) | 3.4 | 3.5 | 5.1 | 3.9 | 1.3 | 5.1 | 1.1 | 7.2 |

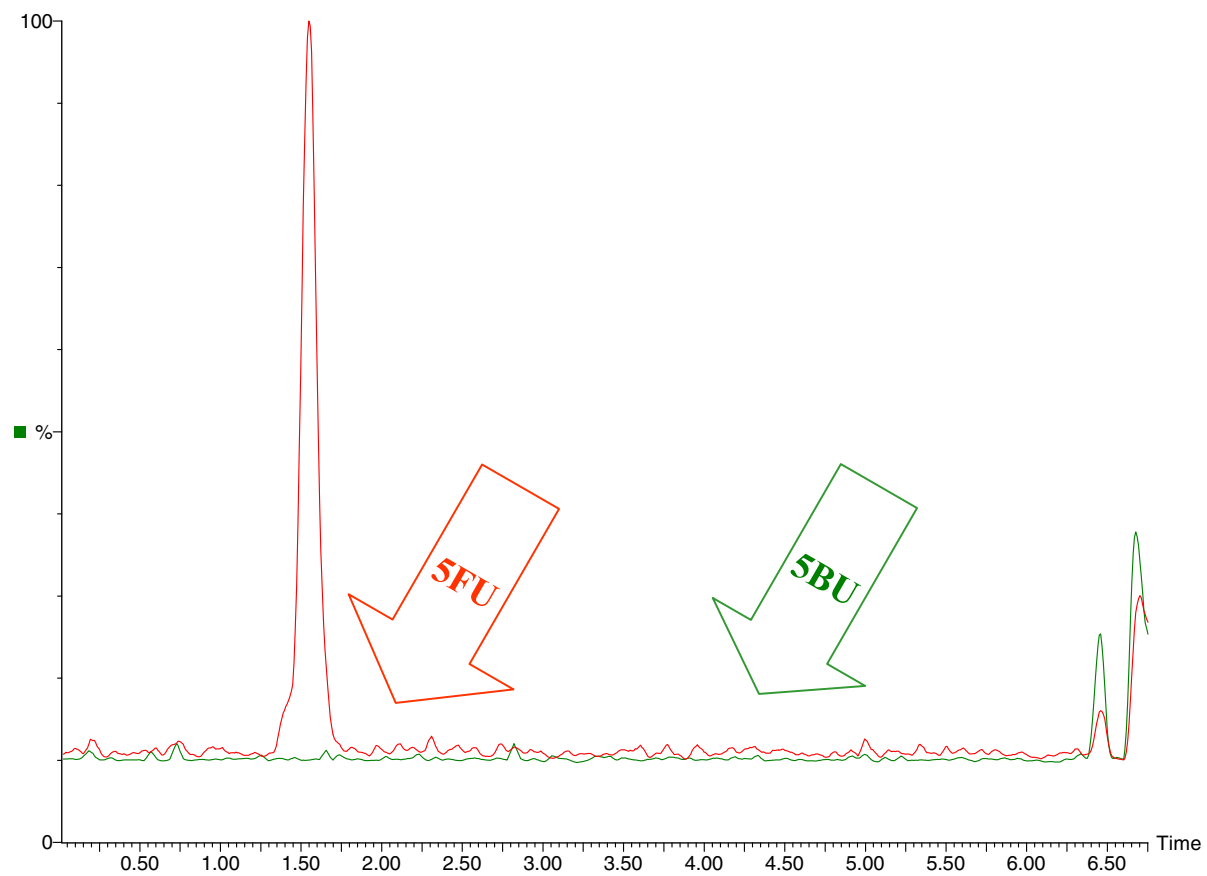
*excluded from standard curve; not included in statistics (did not meet +/-15% criteria)

nd = no peak detected or $\leq 20\%$ LLOQ; na = not applicable

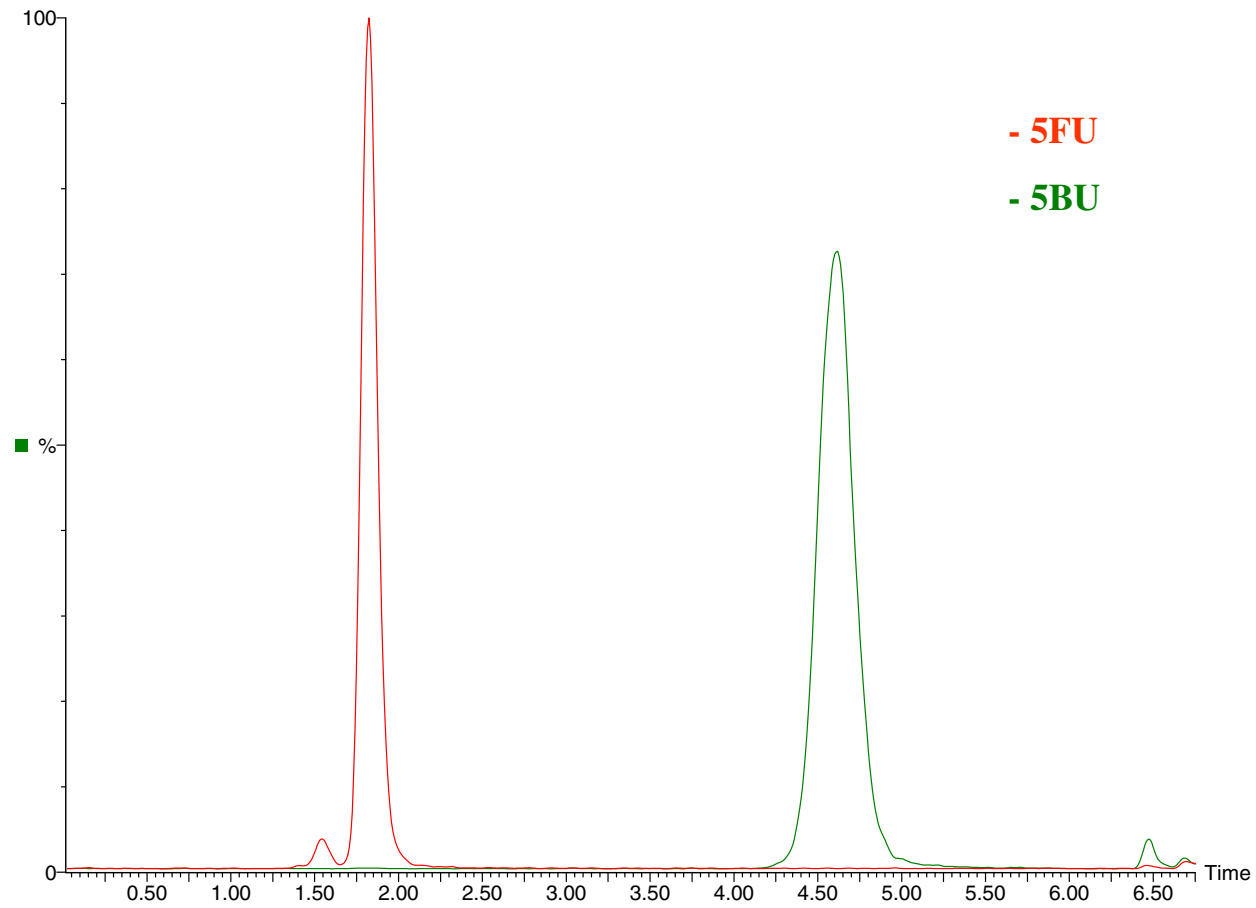
ULOQ - 10,000 ng/mL



Extracted Blank Plasma



Mid Range - 600 ng/mL



Results

The method was validated over three days for accuracy, precision, ruggedness and robustness. Assay range was 10-10,000 ng/mL (up to 1,000,000 ng/mL with dilutions) using two overlapping standard curves (10-600 ng/mL and 500-10,000 ng/mL). Curve fit was nonlinear (weighted quadratic). %RSD ranged from 1.6 to 11% within run (n=6) and 3.1 to 12% between runs (n=18). Accuracy ranged from -9.9 to 11% within run (n=6) and -2.1 to 5% between runs (n=18). Samples were stable through 3 freeze/thaw cycles and heat treatment of 30 minutes at 56°C for deactivation of the HIV virus. Multiple plasma sources showed no interferences >20% of the lower limit of quantification. A panel of 10 co-administered and OTC drugs showed no interferences. The assay has been successfully used to quantify 5FU in clinical samples.

Validation Samples

Low Curve

| ALL RUNS | Nominal Concentration (ng/mL) | 0 | 10.0 | 25.0 | 100 | 600 |
|-------------------------------|----------------------------------|----|------|------|------|-------|
| | N | 18 | 18 | 18 | 18 | 18 |
| | Average (ng/mL) | na | 9.89 | 26.3 | 103 | 596 |
| Between-run Statistics | SD (ng/mL) | na | 1.19 | 1.44 | 3.62 | 32.0 |
| | Deviation (%) | na | -1.1 | 5.3 | 2.5 | -0.66 |
| | CV (%) | na | 12 | 5.5 | 3.5 | 5.4 |

na = not applicable

High Curve

| ALL RUNS | Nominal Concentration (ng/mL) | 600 | 2000 | 8000 | 9000 |
|-------------------------------|----------------------------------|------|------|-------|------|
| | N | 18 | 18 | 18 | 18 |
| | Average (ng/mL) | 587 | 2051 | 7934 | 9271 |
| Between-run Statistics | SD (ng/mL) | 36.3 | 63.4 | 583 | 767 |
| | Deviation (%) | -2.1 | 2.5 | -0.82 | 3.0 |
| | CV (%) | 6.2 | 3.1 | 7.3 | 8.3 |

Heat Treatment Stability

| Run | Dataset ID | Nominal Concentration (ng/mL) | 0 | 25.0 | 600 ^a | 600 ^b | 8000 |
|-----|---------------------------|-------------------------------|----|------|------------------|------------------|------|
| | | N | 6 | 6 | 5 | 5 | 6 |
| | | Average (ng/mL) | na | 26.1 | 629 | 625 | 8041 |
| | Summary Statistics | SD (ng/mL) | na | 1.49 | 35.2 | 42.1 | 389 |
| | | Deviation (%) | na | 4.3 | 4.9 | 4.2 | 0.51 |
| | | CV (%) | na | 5.7 | 5.6 | 6.7 | 4.8 |

nd = no peak detected or $\leq 20\%$ LLOQ; na = not applicable

^acalculated from low SC curve, ^bcalculated from high SC curve

Freeze-Thaw Stability

| Run | Dataset ID | Nominal Concentration (ng/mL) | 0 | 25.0 | 600 ^a | 600 ^b | 8000 |
|-----|---------------------------|-------------------------------|----|------|------------------|------------------|------|
| | | N | 6 | 6 | 6 | 6 | 6 |
| | | Average (ng/mL) | na | 26.1 | 622 | 619 | 7531 |
| | Summary Statistics | SD (ng/mL) | na | 1.38 | 21.7 | 23.9 | 371 |
| | | Deviation (%) | na | 4.3 | 3.6 | 3.1 | -5.9 |
| | | CV (%) | na | 5.3 | 3.5 | 3.9 | 4.9 |

nd = no peak detected or $\leq 20\%$ LLOQ; na = not applicable

^acalculated from low SC curve, ^bcalculated from high SC curve

Short-term Stability (24 hr Room Temperature)

| Run | Dataset ID | Nominal Concentration (ng/mL) | 0 | 25.0 | 600 ^a | 600 ^b | 8000 |
|-----|------------|-------------------------------|----|------|------------------|------------------|------|
| | | N | 6 | 6 | 6 | 6 | 6 |
| | | Average (ng/mL) | na | 26.3 | 592 | 580 | 7379 |
| | | SD (ng/mL) | na | 2.23 | 28.0 | 33.9 | 376 |
| | | Deviation (%) | na | 5.1 | -1.3 | -3.3 | -7.8 |
| | | CV (%) | na | 8.5 | 4.7 | 5.8 | 5.1 |

nd = no peak detected or $\leq 20\%$ LLOQ; na = not applicable

^acalculated from low SC curve, ^bcalculated from high SC curve

Processed Sample Stability (48 hr Room Temperature)

| Run | Dataset ID | Nominal Concentration (ng/mL) | 0 | 25.0 | 600 ^a | 600 ^b | 8000 |
|-----|------------|-------------------------------|----|------|------------------|------------------|------|
| | | N | 6 | 6 | 6 | 6 | 6 |
| | | Average (ng/mL) | na | 24.6 | 592 | 586 | 7480 |
| | | SD (ng/mL) | na | 1.52 | 14.9 | 16.5 | 440 |
| | | Deviation (%) | na | -1.5 | -1.3 | -2.3 | -6.5 |
| | | CV (%) | na | 6.2 | 2.5 | 2.8 | 5.9 |

nd = no peak detected or $\leq 20\%$ LLOQ; na = not applicable

^acalculated from low SC curve, ^bcalculated from high SC curve

Conclusions

An HPLC column that resists dewetting allows for the use of 100% aqueous mobile phase for 5FU, giving adequate retention and good peak shape.

Liquid/liquid extraction of 5FU gives good extraction efficiency with minimal background and ion suppression.

Use of a C18 guard column equipped with a switching valve and isopropanol backflush protects the analytical column from buildup of hydrophobic residues with no effect on peak shape.

A validated method that is accurate, precise, and robust was developed. The method has demonstrated excellent specificity with no observed stability problems. The method has been successfully used to quantify 5FU in clinical samples.

Ask about other oncology drug assays provided by BASi.