



***LC/MS/MS Assay Using Traditional  
GC/MS Derivatization to Allow Small  
Polar Compound Analysis***

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Presented at the 2004 ASMS Conference, Nashville, Tennessee

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## Introduction

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The determination of small, polar analytes (MW<200) by LC/MS/MS is problematic because of the high number of background ions present in that region and because the analytes have a tendency for non-specific binding in the ion source. These issues result in lower specificity and higher background noise.

Derivatization of these analytes increases compound mass and makes the compounds more amenable to chromatographic separation. While these advantages have normally been leveraged for GC/MS analysis, these attributes also lend themselves to LC/MS/MS analysis. Using this approach, an LC/MS/MS method was developed for 5-fluorouracil in the presence of one of its prodrugs, Tegafur.

Figure 1. Structures of Analytes

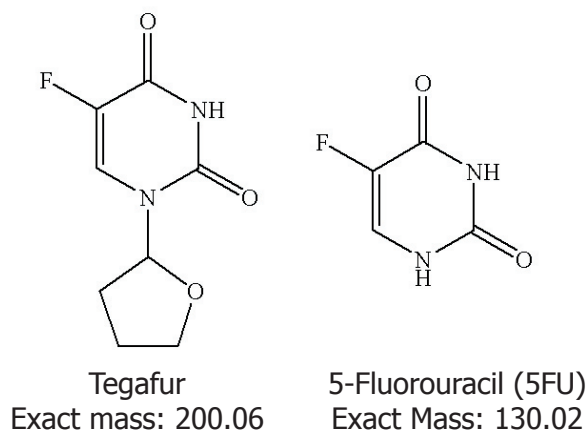
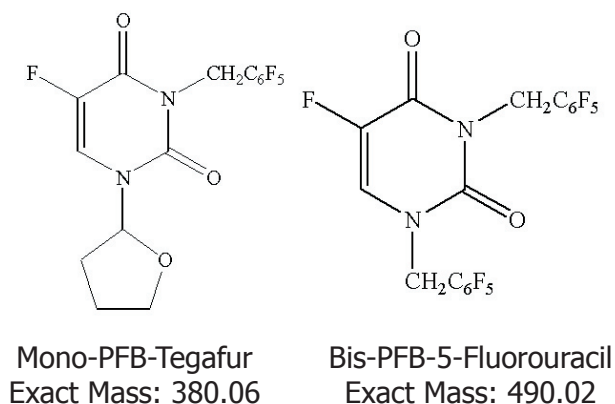


Figure 2. Proposed Structures of PFB Derivatives



## Experimental

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### Extraction Procedure

250  $\mu$ L of plasma were diluted with 500  $\mu$ L of an acidic buffer and extracted with ethyl acetate. The organic layer was transferred to a clean test tube and evaporated to dryness.

### Derivatization Procedure

The dried extracts were dissolved in an aprotic solvent. 2,3,4,5,6-Pentafluorobenzyl bromide (PFBBr) was added to the samples along with an organic base to catalyze the reaction. After derivatization, the analytes were back extracted into hexane/ethyl acetate. The organic layer was transferred to a clean test tube and evaporated to dryness. The samples were reconstituted in methanol.

### LC/MS/MS Conditions

HPLC column:	Luna Phenyl-Hexyl (Phenomenex, Torrence, CA)
Injection volume:	10 - 20 $\mu$ L
LC Program:	6 minute gradient
Ionization Mode:	Negative-Ion APCI
Scan Type:	Multiple Reaction Monitoring (MRM)

### Instrumentation

- LC-10ADvp pumps, Shimadzu (Columbia, MD)
- Rheos 2000 HPLC pump, Flux Instruments AG (Basel, Switzerland)
- LEAP PAL autosampler, CTC Analytics (Zwingen, Switzerland)
- SIL-HTc autosampler, Shimadzu (Columbia, MD)
- API3000 mass spectrometer, PE Sciex (Concord, ON)

## Issues

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- Tegafur and 5-FU are small polar molecules that are weakly retained under both reversed-phase and normal-phase conditions (Figure 1).
- Tegafur loses the tetrahydro-2-furfuryl group to produce 5-FU in the ion source resulting in detected signal for both Tegafur and 5-FU transitions.
- Baseline separation of these compounds minimizes the effects of source fragmentation. However, their tendency to stick in the APCI source increases the baseline noise over time.
- When the underivatized compounds are assayed simultaneously, about 50% of the Tegafur ion signal will be present in the 5-FU chromatogram (Figure 3).
- The most intense peak in the 5-FU chromatogram results from in-source fragmentation of Tegafur because the plasma concentration levels of Tegafur will be ten times greater than that of 5-FU.

## Results and Discussion

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- Derivatization of both analytes with PFBBr produces mono-PFB-Tegafur and bis-PFB-[5-FU] (Figure 2).
- Upon ionization, derivatized Tegafur loses the tetrahydro-2-furfuryl group, producing an  $[M-H-C_4H_6O]^-$  ion at **m/z 309** (Figure 4a).
- Derivatized 5-FU produces an  $[M-H]^-$  ion at **m/z 489**, a base ion corresponding to the  $[M-H-HF]^-$  ion at **m/z 469** and an  $[M-H-C_7H_2F_5]^-$  ion at **m/z 309** (Figure 4b).
- Thus, the formation of m/z 309 from derivatized 5-FU should have no effect on Tegafur. However, the formation of m/z 309 from derivatized 5-FU is low (<10% of base ion) and 5-FU plasma concentrations will be lower.
- The derivatization allows for separation while maintaining the needed sensitivity for 5-FU.
- Figure 5 presents a reanalysis of a sample similar to that shown in Figure 3 when derivatization is used. The ability to differentiate the compounds by both mass and chromatography produces chromatograms where the most intense peak corresponds to the analyte of interest. As a result, peak identification and integration become much simpler.
- This approach allowed for the development of an assay for 5-FU in the presence of Tegafur. Some of the statistics from that validation are shown. (Table 1).

Figure 3. Source Fragmentation of Underderivatized Tegafur to 5-FU  
(5-FU at 0.05% of Tegafur)

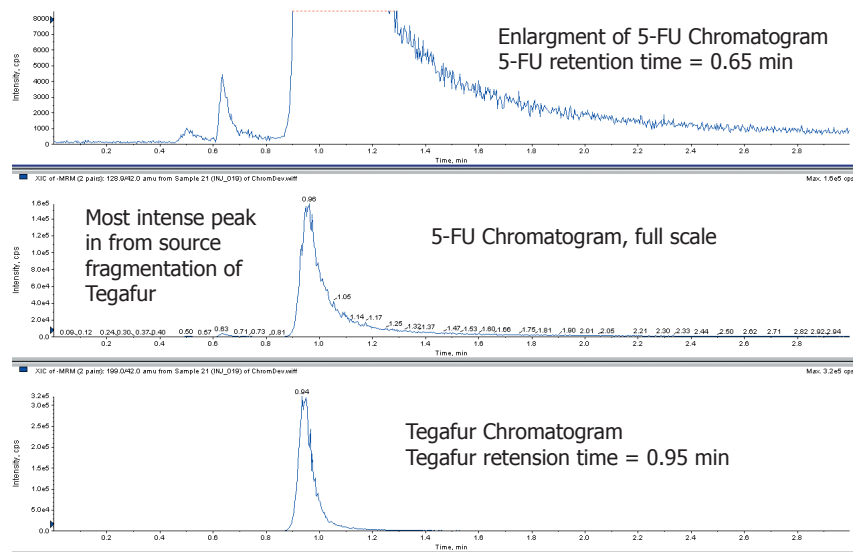


Figure 4a. Q1 Spectrum for Derivatized Tegafur

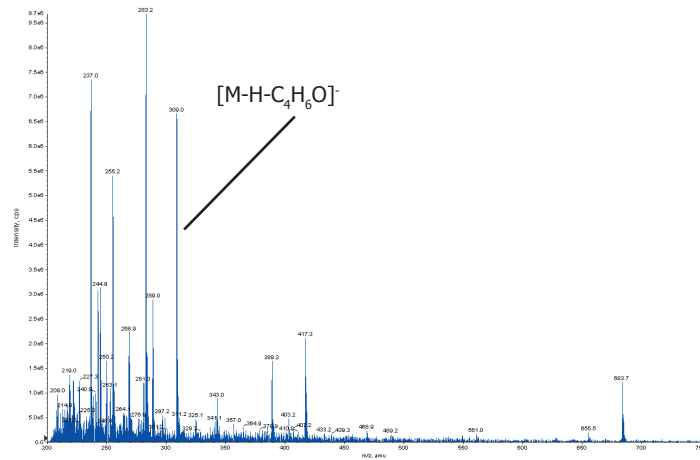


Figure 4b. Q1 Spectrum for Derivatized 5-FU

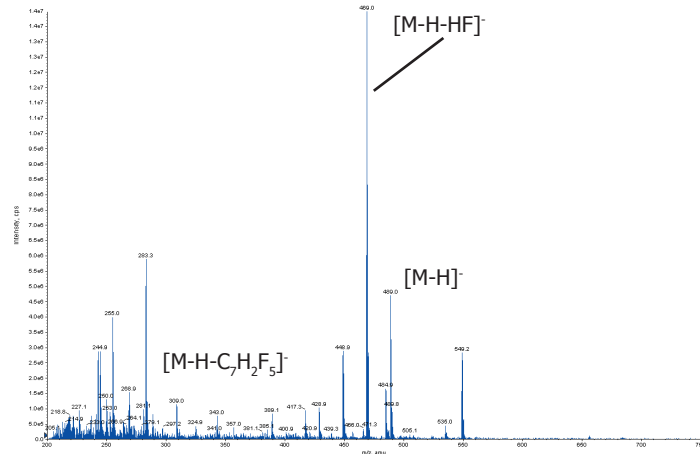


Figure 5. LC/MS/MS Differentiation Between Derivatized Analytes (5-FU at 0.01% of Tegafur)

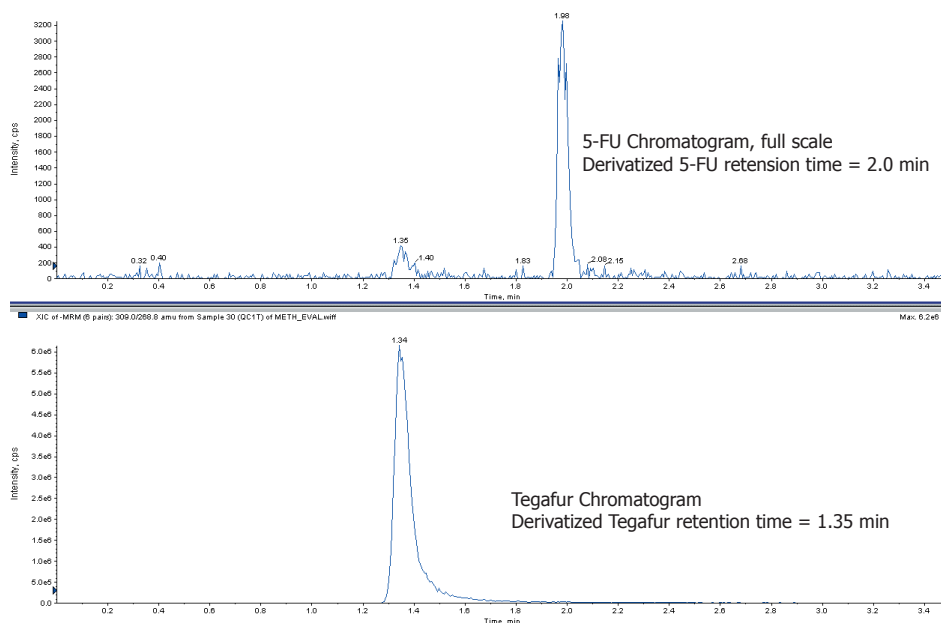


Table 1. Standard and QC Performance for 5-FU Validation

Analytical Run Number	STD A 1.00 ng/mL	STD B 2.00 ng/mL	STD C 5.00 ng/mL	STD D 10.0 ng/mL	STD E 50.0 ng/mL	STD F 100 ng/mL	STD G 250 ng/mL	STD H 500 ng/mL
1	0.958	2.02	4.83	11.0	53.9	92.6	233	502
	1.02	2.08	4.83	9.75	51.9	95.6	240	535
2	1.05	1.92	5.28	10.8	47.4	96.9	251	518
	0.960	1.95	5.01	10.1	49.3	101	238	503
3	0.966	1.87	5.13	10.3	48.5	98.3	240	508
	1.03	2.11	5.23	9.49	51.3	100	250	506
4	0.942	2.33	4.77	10.2	50.8	96.9	240	573
	0.962	2.02	5.46	9.62	48.2	102	209	504
5	0.996	2.00	5.07	10.6	49.2	101	242	493
	1.02	1.91	5.05	9.97	50	100	236	533
Mean	0.990	2.02	5.07	10.2	50.1	98.4	238	518
S.D.	0.0374	0.132	0.220	0.503	1.96	2.94	11.6	23.8
%CV	3.8	6.5	4.3	4.9	3.9	3.0	4.9	4.6
%Bias	-1.0	1.0	1.4	2.0	0.2	-1.6	-4.8	3.6
n	10	10	10	10	10	10	10	10

Nominal Conc.	LLOQ 1.00 ng/mL	Low 3.00 ng/mL	Med 200 ng/mL	High 400 ng/mL	ULOQ 500 ng/mL	Dilution 4000 ng/mL
Mean Observed Conc.	1.05	3.05	206	410	492	4260
%Bias	5.0	1.7	3.0	2.5	-1.6	6.5
Between Run Precision (%CV)	N/A	3.1	4.2	0.0	N/A	N/A
Within Run Precision (%CV)	N/A	8.4	5.9	5.6	N/A	N/A
Total Variation (%CV)	N/A	8.9	7.2	5.2	N/A	N/A
n	6	26	26	26	6	6
Number of Runs	1	5	5	5	1	1
N/A - Not applicable because samples are only included in one run or each run has only one sample.						

## Conclusions

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Analysis of underivatized 5-FU in the presence of Tegafur was complicated by

- (a) source fragmentation of Tegafur to 5-FU.
- (b) unfavorable conditions for separation of compounds and detection by LC/MS/MS.

Derivatization with PFBBr

- (a) reduced source fragmentation of Tegafur to 5-FU.
- (b) produced hydrophobic products that were easily amenable to LC/MS/MS.

This approach has been used successfully to validate assays for these small, polar compounds.