Development of a Method for the Quantification of Fluorouracil and Flucytosine in Human Plasma

Lee Winchester; John Rollag; Kayan Harris; Bridget Prenosil
Celerion, Lincoln, NE

OBJECTIVE:

A method for the quantitation of flucytosine and fluorouracil in human plasma was required to characterize the pharmacokinetics of flucytosine in patients dosed with modified-release test formulations.

INTRODUCTION:

Flucytosine is prescribed to treat fungal infections such as *Candida and Cryptococcus*. Fungal cells take in flucytosine and use cytosine deaminase, an enzyme present in bacteria and fungi but not in mammalian cells to convert it to fluorouracil. Fluorouracil disrupts DNA replication through inhibition of thymidylate synthetase and RNA transcription through incorporporation.

Selective, accurate and precise determination of both flucytosine and fluorouracil was complicated by environmental/reagent interferences in the mass transitions monitored. Chromatographic separation of flucytosine and fluorouracil was also necessary to analyze these compounds of similar mass to avoid isotopic contribution of flucytosine into the fluorouracil signal.

METHODS AND INSTRUMENTATION:

Flucytosine:

- Extraction of flucytosine from human EDTA plasma (50 µL) was by acetonitrile precipitation.
- The precipitated samples were injected onto a HILIC HPLC system consisting of a 50 x 3.0 mm 5 µm Si column with a mobile phase of 90:10 ACN:10 mM Ammonium Acetate pH 3.0 at 1.0 mL/min. Flucytosine effluent was detected using an API 4000 in negative ion mode with an APCI interface and monitoring ions at 128 & 85.2 m/z for flucytosine and 131 & 87.2 m/z for its internal standard ^{13}C-$^{15}N_2$-5-flucytosine.

Fluorouracil:

- Fluorouracil was extracted from plasma (100 µL) by an ethyl acetate liquid/liquid extraction. The extracts were separated on an HPLC system consisting of a 100 x 4.6 mm 3 µm aminopropyl column using a mobile phase of 97:3 ACN:0.1% HCOOH at 1.5 mL/min. Fluorouracil components were detected using an API 4000 in negative ion mode with electrospray ionization. Ions monitored were 129 & 86.1 m/z for fluorouracil and 132 & 88.0 m/z for its internal standard ^{13}C-$^{15}N_2$-5-fluorouracil.

Table 1. Interday Summary for Flucytosine Quality Control Samples in Human (EDTA) Plasma

Batch	LLOQ QC 0.500 µg/mL	QC A 1.50 µg/mL	QC B 15.0 µg/mL	QC C 75.0 µg/mL
Inter-Batch Mean	0.471	1.49	15.7	74.2
Inter-Batch SD	0.0379	0.0629	1.07	3.17
Inter-Batch % CV	8.0	4.2	6.8	4.3
Inter-Batch % Bias	-5.8	-0.7	4.7	-1.1
n	18	18	18	18

Table 2. Interday Summary for Fluorouracil Quality Control Samples in Human (EDTA) Plasma

Batch	LLOQ QC 25.0 ng/mL	QC A 75.0 ng/mL	QC B 750 ng/mL	QC C 3750 ng/mL
Inter-Batch Mean	25.6	75.1	743	3740
Inter-Batch SD	2.05	4.01	18.7	155
Inter-Batch % CV	8.0	5.3	2.5	4.1
Inter-Batch % Bias	2.4	0.1	-0.9	-0.3
n	18	18	18	18

Table 3. Matrix Effect for 5-Fluorocytosine in Human Plasma (EDTA)

		LLOQ		High	
	Lot#	0.500 µg/mL	% Dev.	75.0 µg/mL	% Dev.
	1	0.391	-21.8	66.9	-10.8
	2	0.392	-21.6	62.5	-16.7
	3	0.509	+1.8	74.5	-0.7
	4	0.467	-6.6	65.1	-13.2
	5	0.403	-19.4	66.3	-11.6
	6	0.465	-7.0	70.0	-6.7
Mean		0.438		67.6	
% CV		11.3		6.2	
% Theoretical		87.6		90.1	
n		6		6	

Table 4. Matrix Effect for 5-Fluorouracil in Human Plasma (EDTA)

		LLOQ		High	
	Lot#	25.0 ng/mL	% Dev.	3750 ng/mL	% Dev.
	1	22.0	-12.0	3500	-6.7
	2	22.2	-11.2	3400	-9.3
	3	23.6	-5.6	3500	-6.7
	4	22.4	-10.4	3570	-4.8
	5	24.1	-3.6	3590	-4.3
	6	24.4	-2.4	3420	-8.8
Mean		23.1		3500	
% CV		4.5		2.2	
% Theoretical		92.4		93.3	
n		6		6	

Figure 1. Example Regression for Flucytosine Standards Extracted from Human Plasma.

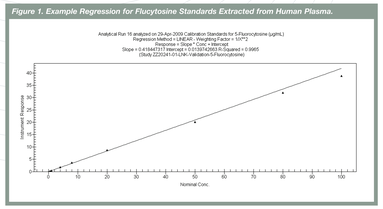


Figure 2. Example Regression for Fluorouracil Standards Extracted from Human Plasma.

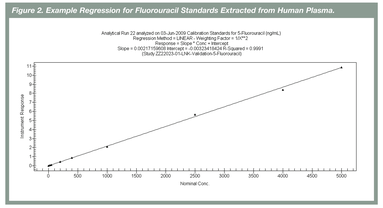


Figure 3. Example Chromatogram at LLOQ Extracted Plasma for Flucytosine.

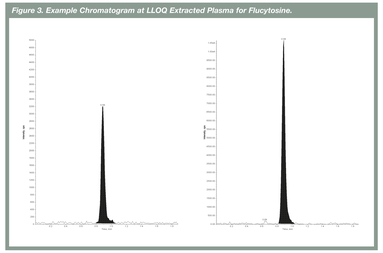


Figure 4. Example Chromatogram of Extracted Blank Plasma for Flucytosine.

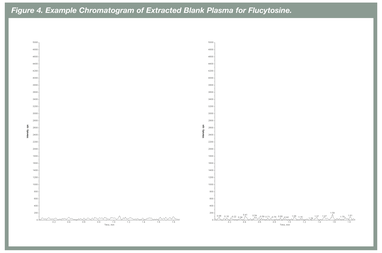


Figure 5. Example Chromatogram at LLOQ Extracted Plasma for Fluorouracil.

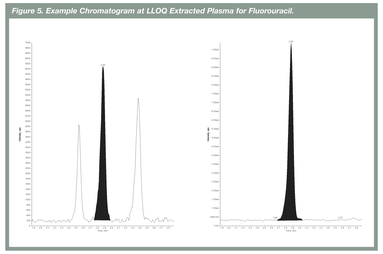
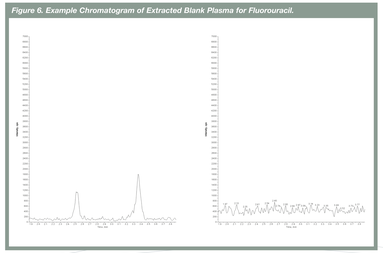


Figure 6. Example Chromatogram of Extracted Blank Plasma for Fluorouracil.



RESULTS:

- A weighted $1/x^2$ linear regression was used for flucytosine & fluorouracil over concentration ranges of 0.500-100 µg/mL (Tables 1 and 2) and 25 to 500 ng/mL (Tables 4 & 5), respectively.
- Inter-batch precision (% CV) and accuracy (% Bias) of flucytosine (Table 1) & fluorouracil (Table 2) quality control samples met predefined validation acceptance criteria.
- Six lots of plasma from different donors were fortified at the LLOQ and at the high quality control concentrations. No significant matrix effect was observed. (Tables 3 & 4).
- Selectivity in hemolyzed plasma was demonstrated.
- Multiple analyte selectivity was demonstrated.
- The average extraction recoveries of flucytosine and ^{13}C-$^{15}N_2$-5-flucytosine were 82% and 87%, respectively.
- The average extraction recoveries of fluorouracil and ^{13}C-$^{15}N_2$-5-fluorouracil were 33% and 82%, respectively.
- Short-term stability in plasma was established for at least 20 hours at ambient temperature under white light for both flucytosine and fluorouracil.
- Freeze and thaw stability in plasma was established for six freeze (-20°C) and thaw (ambient temperature) cycles.
- Post preparative stability in injection solvent (quantitation against freshly extracted standards) was established for 124 hours for flucytosine and 108 hours for fluorouracil at 5°C.
- Processed sample integrity in injection solvent (re-injection stability) was established for 176 hours for flucytosine and 107 hours for fluorouracil at 5°C.
- Method robustness was demonstrated with multiple column lots, mass spectrometers, and extraction scientists.

DISCUSSION & CONCLUSION:

The most challenging aspect of this method development other than separating flucytosine and flurouracil had to do with interfering peaks arising in reagents and materials. All plates and glassware used were rinsed with acetonitrile. Methanol was not used in any extraction step due to interferences showing up in the flucytosine and fluorouracil channels. Acetonitrile was used to wash all materials and was the primary solvent used for most solution preparations.

Sensitive selective methods for flucytosine and fluorouracil were developed. These methods have been demonstrated precise, accurate and method for measurement of flucytosine and its metabolite fluorouracil in human EDTA plasma clinical samples.