

OnDose™ Technical Specifications

Myriad Genetic Laboratories, Inc. 2009

TEST RESULTS SHOULD BE USED ONLY AFTER REVIEW OF THE FOLLOWING SPECIFICATIONS:

Indications and Use

Intended Use

This assay is intended for *in vitro* diagnostic use for the quantitative determination of 5-fluorouracil (5-FU) in human plasma using an immunoassay method on an automated clinical chemistry analyzer in patients receiving 5-FU chemotherapy regimens.

Summary and Explanation

5-FU is a chemotherapy agent that has been in use for many decades. It is used for many types of cancer, principally for colorectal, head and neck, and pancreatic cancers. Side effects of this agent include diarrhea, mucositis, dermatitis, cardiac toxicity, and myelosuppression. These adverse effects can be quite severe even leading to death in cases of DPD deficiencies, an enzyme that catabolizes the drug. In its infusional form, toxicities occur in 10-20% (continuous) to 30-40% (bolus) of cases. Blood levels of 5-FU between individuals can exhibit a 10-50 fold variability despite equal dosing. As a result, approximately 75% of patients receiving 5-FU require dose adjustments with at least 50% requiring an increase in order to improve success of the chemotherapy regimen. Studies have shown that monitoring blood levels of 5-FU during chemotherapy for colorectal cancer reduces both under and overdosing of 5-FU, reduces toxic side effects, and increases success of treatment.

The OnDose Assay provides a rapid and reliable method to monitor blood levels of 5-FU in patients receiving the chemotherapeutic agent.

Description of Method

Sample collection consists of blood drawn at the clinician site 2 hours between after initiation of 5-FU treatment and before the end of drug infusion to ensure steady state levels (the $\tau_{1/2}$ for 5-FU is ~13 minutes). The sample is placed on ice immediately and held for no longer than 1 hour. The sample is then centrifuged (1500g, 4°C for 15min) as soon as possible within that period, to avert 5-FU catabolism by cells within the blood. Plasma is transferred to a plasma tube, labeled with a barcode, then placed in a freezer to freeze solid overnight, and shipped chilled to Myriad Genetic Laboratories, Inc. the next business day for continued analysis.

Upon receipt, the sample bar code is scanned and tracked. The samples are centrifuged through a filter membrane with the resultant filtrate used for analysis. The OnDose Assay is a competitive homogenous two-reagent nanoparticle agglutination immunoassay. The first reagent contains 5-FU conjugate with the second reagent consisting of 5-FU-directed, antibody-conjugated nanoparticles. The amount of free 5-FU in the plasma inhibits the aggregation of the two assay reagents. This absorbance is compared to a standardized calibration curve for quantitation. The amount of light absorbance at certain wavelengths from nanoparticle agglutination depends on the amount of drug in the plasma. The quantitative target range, as expressed by Area Under the Curve (AUC), is calculated from the measured concentration of 5-FU and the infusion duration provided by the clinician on the test request form based on Gamelin E, et al, J Clin Oncol. 2008 May 1;26(13):2099-105.

Quality Control Measures

The OnDose assay is calibrated every 2 weeks using a set of 6 calibrators, which are human plasma spiked at known concentrations of 5-FU (0, 150, 300, 600, 1200, and 1800 ng/mL). QC is run daily with a set of three controls (low, medium, high). Controls are human plasma spiked at known concentrations of 5-FU (225, 450, and 900 ng/mL).

Performance Characteristics/Limitations

Replication

A set of 24 samples were analyzed consisting of pooled patient samples with three tested 5-FU concentrations at levels having clinical significance (e.g. high, med, low). These samples were analyzed in duplicate on two runs each day for a period of 20 working days for each concentration. More than one reagent lot was used with lot-to-lot validation occurring. With this, with-in run, with-in day, and long term imprecision were calculated. Trueness was calculated from the difference of the averaged observed measurements with the true or given value.

Control	Low (225ng/mL)	Medium (450 ng/mL)	High (900 ng/mL)
Trueness	99.98%	100.03%	102.16%
Mean	225 ng/mL	450.2 ng/mL	919.5 ng/mL
SD	12.5 ng/mL	24.9 ng/mL	53.4 ng/mL
CV	5.54%	5.54%	5.80%

Linearity/Reportable Range

Two sets of solutions were examined. The first, a series of samples free of matrix effects of seven known equally spaced concentrations of 5-FU and the second, consisting of calibrating solutions. In both sets, the levels of 5-FU ranging from below to substantially above the expected target range were assayed in quadruplicate. The measured test values were compared to the assigned or dilution values and the reportable range assessed and determined by the linear range of the observed and known concentrations. The upper reportable limit was determined at the point where linearity is no longer maintained, with the CLIA criterion for the allowable total error of 10% at that point. Linearity was exhibited over the entire tested range.

Analytical Range: 75-1800 ng/mL

Clinical Reportable Range (w/ sample dilution): \leq 9000 ng/mL

Interference

A pair of aliquots from each validation sample was examined, the first aliquot spiked with a small, known amount of the suspected or reported interfering material and contained 1,000 ng/mL of 5-FU, the second aliquot spiked with pure solvent or a diluting solution that did not contain 5-FU at the same volume as the diluent of the "interferer" of the first aliquot. Both were analyzed in duplicate and the bias, in concentration units, calculated. Several concentrations of the interfering material were tested to establish the level at which the "interferer" invalidates the assay result. No significant interferences were observed from samples with the following conditions:

Total Protein Matrix Effect: 13.1 g/dL

Icteric Interference: 98 mg/dL

Lipemic Interference: 3728 mg/dL

Cross Reactivity

93 compounds that have confirmed or suspected cross reactivity with the assay antibody were tested to ascertain the extent and nature of the reactivity. Compounds were spiked (at a concentration of 10,000 ng/mL for endogenous, 100,000 for exogenous) into 5-FU free samples and tested for reaction with the assay antibody with resultant percent cross reactivity calculated ([measured value/10,000 or 100,000] x100). Cross-reactivity did not exceed 1% with the exception of the three compounds listed below.

Uracil = 11.8%

5,6-dihydro-5-fluorouracil = 2.7%

Theobromine = 1.6%

Theophylline = 2.4%

From these experiments, known interfering substances include Theobromine ingested from chocolates, and Theophylline in therapeutic doses, both of which can falsely elevate an OnDose 5-FU AUC determination.

Recovery

A pair of aliquots types from each sample was examined in duplicate, with the first aliquot spiked with a known small quantity of 5-FU at 250, 500, 1000, or 1500 ng/mL and the second aliquot spiked with pure solvent of the same volume as the first aliquot. The bias was ascertained as the percentage of analyte recovered, ideally 100% which is then converted to proportional error (100-%Recovery).

Recovery: 99%

Detection Limit

The Limit of Blank (LoB), Functional Sensitivity (FS), and Limit of Detection (LoD) were established per CLSI Guidelines. Two different type of samples were analyzed, a "blank" sample with no analyte of interest (e.g. 5-FU), and a spiked sample with a low concentration of 5-FU. Matrix factors were eliminated with pooled plasma used. Progressively higher analyte concentrations were assessed. These samples were performed in repeated fashion similar to a replication type of experiment over approximately a 5 day period to include within-run and day-to-day performance measures, after which the means and standard deviations were calculated and the above parameters calculated.

Limit of Blank

LoB= Blank+ 1.65SD= 14.8ng/mL

Limit of Detection

LoD= LoB + 1.65SD= 37.9ng/mL

Functional Sensitivity

[Analyte] w/ CV of 20%= 63.9ng/mL

Stability

5-FU levels

a. Sample Tube Type and Temperature

Negative plasma samples were drawn in EDTA and Heparin tubes and spiked with 800ng of 5-FU. Each sample tube was split into fresh and frozen sets. The fresh set was split further into 3 sample sets stored at 4C, Room Temperature (RT), and 37C. Similarly, the frozen set was split into 3 sample sets, frozen the day before the study initiation, and then thawed at 4C, RT, and 37C. Daily 5-FU measurements were taken over a 7 day period to assess 5-FU plasma stability under these various tube and temperature conditions. No significant degradation of 5-FU levels was detected.

b. WBC Degradation

The sample sets was created in the same fashion as in part a. These sample were spiked with 800ng of 5-FU and with a standard amount of WBC lysate. These samples were stored at the same temperatures as part a. and measured twice a day for 5-FU over at least a 3 day period. Samples showed significant degradation by 4 hours storage.

c. Shipping Condition Simulation

A sample set identical/similar to that in Part a. was created with samples, frozen and fresh, packed and shipped under a variety of parameters, real time or simulated, to Myriad Genetic Laboratories with subsequent 5-FU measurements performed. No significant 5-FU degradation was found in these specimens for properly drawn/handled specimens.

Comparison of Methods

123 samples were measured in duplicate to assess the specificity of the new versus established method (HPLC or GC-MS). The 5-FU levels covered the entire reportable range. The Deming regression analysis was used to establish correlation. The total error estimate of the assay was calculated to be 1.4%. The regression statistics were used to transfer the target range (X_{lower} and X_{upper}) to the new method ($Y_{lower} = a + bX_{lower}$, $Y_{upper} = a + bX_{upper}$, where a is the y-intercept and b is the slope of the regression line).

Low: 86 ng/mL

High: 1973 ng/mL

Total Error: 1.4%

Calibrators/Controls

The set of calibrators and controls spanning the reportable ranges were tested daily over a 30 day time period and found not to have any significant drift in values indicating no meaningful degradation occurred within the calibrator.

Limitations

As with all analyte determinations, the 5-FU PCM Assay should be used in conjunction with information available from clinical evaluation and other diagnostic procedures. Performance characteristics for the 5-FU PCM Assay have not been established for body fluids other than human plasma containing EDTA or heparin. 5-FU is not stable in whole blood. Hemolysis should be avoided as it interferes with assay results. As with any assay utilizing mouse antibodies, the possibility exists for interference by human anti-mouse antibodies (HAMA) in the sample. Samples containing such antibodies can potentially produce erroneous 5-FU results.

Expected Values/Results

Observed concentrations measured via this immunoassay are collected in context of the duration of 5-FU chemotherapy to derive the Area Under the Curve (AUC) calculation. Using Gamelin E et al, J. Clin Onc. 2008 May 1;26(13): 2099-105 for reference, the target range for 5-FU treatment is 20-24 mg·h/L in order to maximize treatment success while minimizing toxicity. A patient who is optimally treated will have an AUC within this range. Under-treated patients will have results below this range with risk of treatment failure. Over-treated patients will have results over this range with risk of drug toxicity. There are no data presently to support a reportable critical value to which risk of toxicity is definite.