

ACRIN Study 6689
Separation of FLT and FLT-glucuronide Process
Manual

I. BACKGROUND

Determination of FLT and FLT-gluc in Plasma

The basic hypothesis of FLT PET imaging is that assessment of cellular proliferation will accurately track regional changes in brain tumors before and after therapy (chemo+radiation). Cellular proliferation can be measured directly through a nucleotide base, such as thymidine (top pathway) and into DNA, but ¹¹C-thymidine PET imaging is extremely complicated. Imaging cellular proliferation with FLT tracks just the salvage pathway of nucleotides into DNA. FLT metabolism is less complicated in the cell; however FLT does not proceed beyond the triphosphate pool and is NOT incorporated into DNA (Figure1).

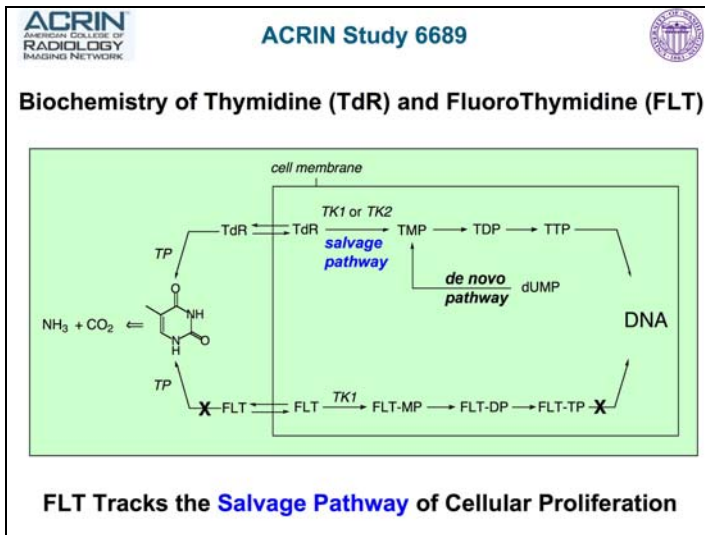


Fig. 1

After injection of ¹⁸F-FLT, the liver metabolizes ¹⁸F-FLT to ¹⁸F-FLT-glucuronide (FLT-gluc). In humans, the fraction of ¹⁸F activity associated with FLT can vary between 40 and 92% at 90 min. If an average profile is used (Black Line on the plot), the variation in the estimation of proliferation (flux) from FLT ranges from 25 to 50% error (average 30% error). Thus FLT and the metabolite of FLT (FLT-gluc) must be measured in order to use compartmental modeling for the separation of transport from proliferation without significant errors in parameter estimation (Figure 2).

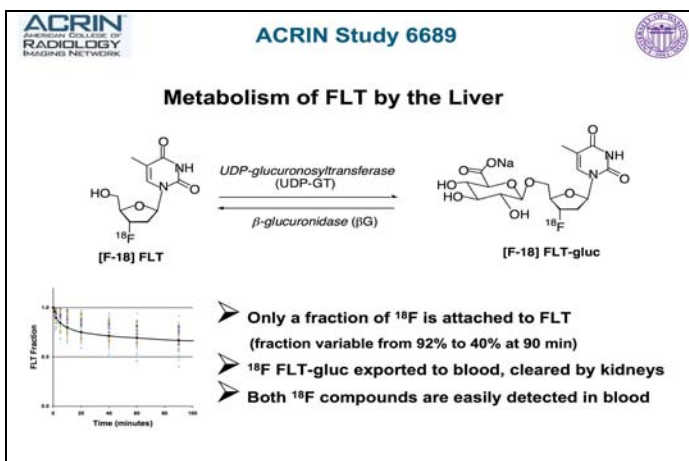


Fig. 2

The kinetic model of FLT metabolism describes the transport of FLT from the blood into the tissue with subsequent phosphorylation and potential de-phosphorylation. For FLT kinetic modeling analysis we use two blood curves; the total blood activity and the fraction of ^{18}F -FLT derived from the FLT metabolite analysis (seen in the lower left plot), and the dynamic imaging data. The FLT model allows us to mathematically separate transport (or K_1) from cellular proliferation (K_i or FLT metabolic flux). In brain, most of the FLT PET signal is related to transport, not cellular proliferation (Figure 3).

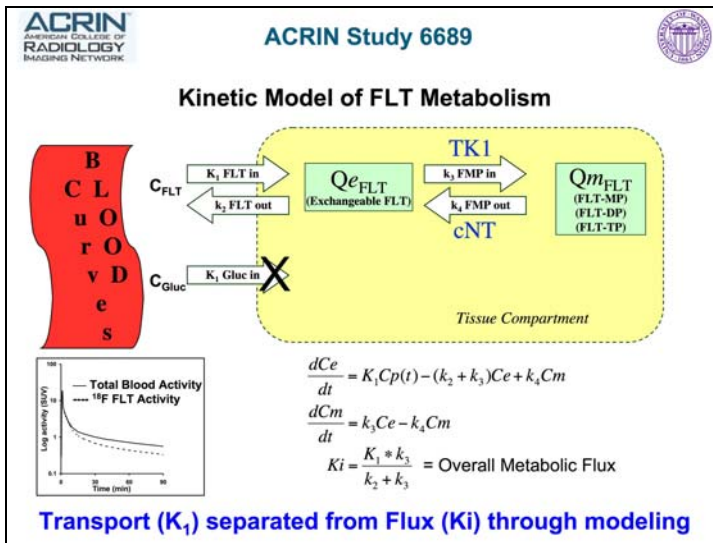


Fig. 3

In these high-grade glioma patient examples, we can see little FDG uptake in the tumor area as indicated by contrast enhanced MRI. For glioma patients with a compromised Blood Brain Barrier (BBB), as in patient A, transport dominates the imaging signal. An FLT SUV image includes both the dominant transport effects and the more subtle cellular proliferation. In patient B, FLT transport is restricted due to an intact BBB and very little ^{18}F -FLT uptake is observed. The time-activity curves to the right reveal the tumor uptake profile relative to contralateral brain regions. Through modeling, estimates of proliferation (flux, K_i) can be separated from transport (Figure 4).

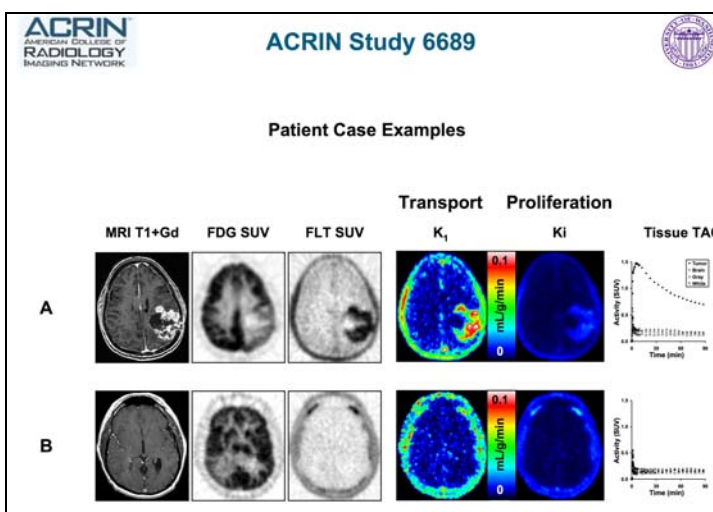


Fig. 4

II METABOLITE PROCESS CRITERIA

A. Blood Collection Time Points

Blood samples will be obtained from each participant at the time of dynamic PET imaging post injection of ^{18}F -FLT at each time point of 15, 30 and 60 minutes for the assessment of cellular proliferation to accurately track regional changes in brain tumors before and after therapy (chemo+radiation).

B. Confidentiality

The confidentiality of the participant's identity will be maintained. All collected information will be protected per ACRIN policies and procedures and federal regulatory guidelines. Access to study data will be limited to the RTOG 0837/ACRIN 6689 staff working on the study. All computer data will be maintained in a manner consistent with Title 21 Code of Federal Regulations Part 11 (21 CFR Part 11). In addition, access to the data management system will be limited to designated staff through use of individualized, confidential login ID and password. Designated staff will not share login IDs or passwords.

The data from this study will be maintained until 10 years following completion of the study or until the data are no longer required for research. Data will be destroyed as required by the ACRIN Record Retention Policy and federal regulatory guidelines. Human research subjects are protected in accordance with Title 45 CFR Part 46 and Title 21 CFR Part 50. ACRIN is not a covered entity according to the Privacy Rules of the 1996 Health Insurance Portability and Accountability Act (HIPAA); therefore, HIPAA regulations should be followed according to institutional standards.

C. Informed Consent

Human research subjects are protected by informed consent procedures in accordance with Title 45 CFR Part 46 and Title 21 CFR Part 50. The Biomarker consent component of the institution-specific, IRB-approved informed consent form grants permission for study investigators to request and obtain blood and blood fractionates, and to use those samples for research involving molecular studies on the development of cancers and/or for other diseases.

All participants will already have provided a written consent for the collection of blood for this biomarker assessment using the institution-specific, IRB-approved informed consent form at the time of enrollment (a sample informed consent form template is provided as Appendix I in protocol RTOG 0837/ACRIN 6689). If blood collection was not included in the original institution-specific, IRB-approved informed consent form, please inform the ACRIN Protocol Development and Regulatory Compliance department and refer to your local IRB and institutional policy for further guidance.

II PROCESS FOR SEPARATION OF FLT AND FLT-GLUCURONIDE IN BLOOD

A. List of Laboratory Supplies

1. Equipment
 - a. Gamma Counter (Packard, Beckman, Perkin-Elmer)
 - b. Microcentrifuge (or a bench top centrifuge)
 - c. Pipette, 1 mL adjustable (Gilson, Rainin or similar vendor)

2. Chemicals
 - a. Reagent grade Ammonium Formate (FW 63.06g)
 - b. Absolute ethanol
 - c. Distilled water

3. Solid phase extraction cartridges (Waters Corp., Milford, MA)
 - a. QMA anion exchange (Accell™ Plus Short QMA SepPak, WAT020545)
 - b. C18 octadecylsilane (Reversed Phase Plus Short C18 SepPak, WAT020515)

4. Disposable Supplies
 - a. Blue Pads (adult diapers)
 - b. 1 mL Pipette Tips (filtered or non)
 - c. 2.0 mL Microcentrifuge tubes (VWR, MSI, Cole-Parmer or similar vendor)
 - e. Microcentrifuge tube rack
 - f. Luer-Lok syringes (3 cc heparinized, 5 cc and 10 cc) (B-D, VWR or similar vendor)
 - g. 3-way stopcocks with Luer-lok fittings (B-D, VWR or similar vendor)
 - h. 13 x 75mm Tubes with caps, polypropylene (VWR, MSI, Cole-Parmer or similar vendor)
 - 3 Plasma activity-counting tubes with caps
 - 3 Plasma sample dilution tubes
 - 3 Sets of 4 tubes. where each set is labeled **L** (Load Sample), **W** (Wash sample), **Q** (QMA column eluate) and **C** (C18 column eluate)
 - 3 Empty tubes for determining background activity
 - i. Test tube rack to fit 13x75mm tubes

B. Introduction to the Separation Method

1. Dr. John Grierson, who invented FLT subsequently developed the following procedure to separate FLT from the primary metabolite (FLT-gluc) in plasma samples.

2. Reminder: When working with radioactive blood samples, please follow the standard procedures and safety guidelines defined in your clinic or imaging center.

3. Summary of Process and Supplies
 - a. A 2.0 mL microcentrifuge tube, a microcentrifuge and Pipettes (& tips) are needed to separate RBCs (Red Blood Cells) and plasma.
 - b. For each blood sample there is a set of QMA and C18 SepPak cartridges coupled to each other that is required for the separation process. In the separation process, the QMA cartridge binds the metabolite FLT-gluc, while the C18 column binds FLT. All Sep Pak Cartridges are pre-washed prior to use.

- c. To load and elute the plasma sample on the Sep Pak Cartridges, a minimum of three 5 cc syringes is required filled with the following three solutions: 0.25 M Ammonium Formate, Ethanol and Water.
- d. To collect the SepPak cartridge eluates for each blood sample, 4 gamma-counting tubes (13x75 mm) able to hold a volume of 4 mL or more are needed. For each blood sample, label the tubes with L, W, Q, and C, for Load, Wash, QMA and C18 respectively
- e. To count the samples, a Gamma counter cross-calibrated with a dose calibrator is required.

C. Pre-Study Preparation of QMA and C18 SepPaks

1. Prior to use, QMA SepPak columns are washed with 0.25 M ammonium formate solution and then distilled water.
 - a. To make a 100 mL solution of ammonium formate (FW 63.06) weigh 1.576g and add to an Erlenmeyer flask, graduated cylinder, volumetric flask, or similar vessel capable of accurately measuring a 100 mL volume. Add distilled water to the 100 mL level and stir until dissolved. Label your container appropriately.
 - b. Couple the 3 QMA columns together, attach a 10 cc syringe filled with 0.25 M ammonium formate solution and slowly flush the QMA cartridges at a rate of 3 mL/min.
 - c. Repeat the flush method using a syringe filled with 10 cc of distilled water at a rate of 3 mL/min.
 - d. Uncouple QMA SepPak and set aside
2. Prior to use, the three C18 SepPaks are coupled together and washed with ethanol and then water
 - a. Couple the C18 SepPaks together, attach a 10 cc syringe filled with 10 cc ethanol and slowly flush the C18 SepPaks at a rate of 3 mL/min.
 - b. Repeat the flush method using a syringe filled with 10 cc of distilled water at a similar rate of 3 mL/min.
 - c. Uncouple C18 SepPak and set aside.
3. For separation of FLT from FLT-gluc, one of each cartridge type are coupled together with the QMA first and the C18 SepPak second.
 - a. All three sets of QMA+C18 SepPaks are flushed with 10 cc water at a rate of 3 mL/min prior to use.
4. For all 3 blood samples prepare:
 - a. 3 - 5 cc syringes filled with 4 cc 0.25 M ammonium formate
 - b. 3 - 5 cc syringes filled with 4 cc distilled water
 - c. 3 - 5 cc syringes filled with 4 cc ethanol
5. For each blood sample, label:
 - a. 1 - 2.0 mL microcentrifuge tube
 - b. 7 - 13x75 mm tubes
 - i. 1 - plasma activity tube; to this tube add 0.8 mL of distilled water

- ii. 1 - plasma dilution tube for metabolite analysis; to this tube add 3.6 mL of distilled water
- iii. 1 - empty background activity tube (Background)
- iv. 4 - tubes for the metabolite separation (labeled L, W, Q, and C).

D. Blood Sampling and Processing

1. Each blood sample is used both for the determination of radioactivity and metabolite analysis. Blood samples are drawn from patients using a second venous catheter placed in the opposite arm from the injection site. Blood samples of 2-3 mL each are collected into heparinized syringes (or a heparinized blood vacutainer) at 15, 30 and 60 minutes after ¹⁸F-FLT injection, which is infused over one minute
 - a. Just prior to the actual sample, draw 3 cc of blood waste to clear the line and discard.
 - b. Using a 3 cc heparinized syringe (or a heparinized blood vacutainer) the actual blood sample is drawn. Record the time of the blood draw on the ACRIN 6689 Blood Sampling Form (**BS** form) in hh:mm:ss format.
2. For each collection syringe, 2 mLs of whole blood are dispensed into a 2 mL microcentrifuge tube, and then centrifuged immediately for 3 min at 8000 x g to separate plasma from red blood cells. If a blood vacutainer is used, centrifuge the blood tube at 700g for 20 minutes at 4°C with no break at the end of centrifugation for plasma extraction.
3. The top portion of the centrifuged blood tube is called plasma. Directly from the microcentrifuge tube (or vacutainer), pipette 0.2 mL of plasma directly into the counting tube labeled plasma activity tube containing 0.8 mL water and cap for later counting.
4. Pipette 0.4 mL of plasma into the tube labeled plasma dilution tube containing 3.6 mL of distilled water. The dilute plasma sample is assayed for its relative FLT and FLT-glucuronide content using the SepPak separation methods described in section E.

E. The Separation Procedure

1. Solid phase extraction units are constructed by connecting, in order, an empty 5 mL syringe with plunger removed, a 3-way plastic Luer-Lok stopcock, a QMA SepPak and a C18 SepPak, both SepPaks previously washed as in C.1-3 above. A 5 cc syringe loaded with 4 mL of distilled water is connected to the side-port of the 3-way stopcock, which should be in the closed position for that side port.
2. For each sample, pour the diluted plasma into the empty syringe barrel and insert the plunger collecting the eluate in the tube labeled L (L=load). Slowly force the sample through the attached SepPaks by depressing the syringe plunger at 3 mL/min and collecting the eluate in the tube labeled L. After this procedure, cap the L tube for later counting.
3. When the sample solution is completely expelled from the loading syringe, switch the stopcock to open the syringe with the water wash to the SepPaks. Position the SepPak

over the second tube labeled W (W= wash). Depress the water syringe washing the sample onto both SepPaks at 3 mL/min. Cap the W tube for later counting.

4. The contents of the QMA (¹⁸F-FLT-gluc) and C18 (¹⁸F-FLT) SepPaks are then eluted separately into the next two tubes (Q for QMA and C for C18) with 4 mL each of 0.25 M Ammonium Formate and Ethanol, respectively. First, uncouple the cartridges from the stopcock and from each other for separate elutions described below in E.5 and E.6.

5. Position the QMA SepPak cartridge outflow port over the tube labeled Q (Q= QMA SepPak). Fit the 5 cc syringe containing 0.25 M Ammonium Formate onto the inflow port and apply 4 cc of the formate solution to the column at 3 mL/min collecting the outflow in the Q tube. Remove the formate syringe, and cap the Q tube for later counting.

6. Next, place the C18 Sep Pak cartridge outflow port over the tube labeled C (C= C18 SepPak). Fit the syringe containing Ethanol onto the inflow port and apply 4 cc of Ethanol at 3 mL/min collecting the outflow in the C tube. Remove the Ethanol syringe, and cap the C tube for later counting.

7. An empty blank tube is prepared for each sample set (3 empty tubes for each blood sample) for determining background (Background) activity.

8. The volume of the L, W, Q and C tubes should be approximately the same, since 4 mL of each flush solution was collected. Count all 6 tubes (Plasma activity, L, W, Q, L and Bkg) in the gamma counter recording the start time of counting (hh:mm:ss), the duration of counting (mm:ss) and the counts in the appropriate place on the **BS** form. There should be a total of 18 tubes for all of the three blood samples in the study.

9. The L+W tubes represent sample column breakthrough, indicating the sample and wash were applied too rapidly. The glucuronide activity is collected in the Q tube and the FLT activity in the C tube.

10. The completed ACRIN 6689 BS form is submitted by site research personnel via the ACRIN website.