

DEGRADATION RATE OF 5-FLUOROURACIL IN PERIPHERAL BLOOD
MONONUCLEAR CELL (PBMC) BY LC-MS/MS
Codice LC85010

Biochemistry

5-Fluorouracil (5-FU) is currently considered the most effective and widely used antineoplastic agent for the treatment of tumors of the gastrointestinal tract, breasts and ovaries.

5-FU is an analogue of uracil and pyrimidine acts as an antagonist.

The metabolism of this drug interferes with the normal metabolism of pyrimidine and therefore needs to be incorporated at the nucleotide level in order to exert its effect.

5-FU performs its function mainly by acting as an antagonist to the thymidylate synthase (TS).

Its conversion to 5-Fluoro-2'-deoxy-5'-monophosphate (FdUMP) leads to inhibition of TS thus blocking the synthesis of DNA and interrupting the whole transcriptional process and protein synthesis.

The great efficacy of this drug, however, goes hand in hand with its low therapeutic index.

5-FU is catabolized primarily by the enzyme dihydropyrimidine dehydrogenase and patients with complete or partial functional deficiency of this enzyme are likely to develop side effects such as cardiac toxicity, myelosuppression and renal failure.

The present method allows the evaluation in a preventive manner of the rate of degradation of 5-fluorouracil, in order to highlight the toxic risk to an individual before administration of the therapy.



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CE

This product meets all the requirements of the Directive 98/79/EC of 27/10/1998 and 08/09/2000 D.L. 332 of the in vitro diagnostic medical devices (IVD). The EC declaration of conformity is available on request.

N° 005	5-FLUOROURACIL IN PMBC BY LC-MS/MS	06/2025
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TECHNICAL FEATURES

Principle of the Method

This method allows to determine, ex-vivo, the rate of degradation of the drug 5-fluorouracil (5-FU). After purification of peripheral blood lymphocytes, the cells are incubated with a known amount of 5-FU, and the amount of drug metabolised after 2 hours is determined by LC-MS/MS.

<u>Recovery:</u>	100 %
<u>Accuracy:</u>	>85%
<u>Precision:</u>	5.2 %
<u>LOD:</u>	0,0625 µg/ml
<u>LOQ:</u>	0,156 µg/ml
<u>Linearity:</u>	0,0635 – 40 µg/ml
<u>Evaluation of the degradation rate (V_d):</u>	ng/ml/min/million cells

Interpretation of the result:

V_d minor of 0.68 = severe deficit
 V_d between 0.68 and 1.12 = moderate deficit
 V_d between 1.12 and 1.36 = slight deficit
 V_d between 1.36 e 1.9 = normal
 V_d greater than 1.9 = rapid metabolizer

Components of the kit:

Reagent A – Diluent Solution, 2 x 300 ml	
Reagent B – Extracting Solution, 2 x 500 ml	<u>After opening store at 2-8°C</u>
Reagent C – Chemical Standard, 1 x 3 ml	<u>Store at -20°C</u>
<u>(50 µg/ml)</u>	
Serum Calibrator – Level 0, 1 x 1 ml	<u>Store at -20°C</u>
Serum Calibrator – Level 1, 1 x 1 ml	<u>Store at -20°C</u>
Serum Calibrator – Level 2, 1 x 1 ml	<u>Store at -20°C</u>
Serum Calibrator – Level 3, 1 x 1 ml	<u>Store at -20°C</u>
Serum Calibrator – Level 4, 1 x 1 ml	<u>Store at -20°C</u>
Serum Calibrator – Level 5, 1 x 1 ml	<u>Store at -20°C</u>
Serum Calibrator – Level 6, 1 x 1 ml	<u>Store at -20°C</u>
Reagent D – Internal Standard, 1 x 20 ml	<u>Store at room Temperature</u>
Reagent E – Stabilizing solution, 1 x 10 ml	
Reagent M1 – Mobile Phase A, 1 x 500 ml	
Reagent M2 – Mobile Phase B, 1 x 500 ml	
SepMate Tubes 50 pcs	

Minimum Instrumental equipment required:

Centrifuge for 50 ml falcon
counter lymphocytes
Shaker with incubation temperature controlled
LC-MS/MS system
ESI positive

Blood Collection Procedure:

2 test tubes with 7.5 ml EDTA anticoagulant, to be processed the same day.

CONSTRUCTION OF THE CALIBRATION CURVE

STEP 1: Prepare 7 conical tubes (eppendorf type), call them C0, C1, C2, C3, C4, C5, C6 and exempt:

	<u>Serum Calibrator – Level 0</u>	<u>Serum Calibrator – Level 1</u>	<u>Serum Calibrator – Level 2</u>	<u>Serum Calibrator – Level 3</u>	<u>Serum Calibrator – Level 4</u>	<u>Serum Calibrator – Level 5</u>	<u>Serum Calibrator – Level 6</u>
<u>Serum Calibrator – Level 0</u>	80 µl	-	-	-	-	-	-
<u>Serum Calibrator – Level 1</u>	-	80 µl	-	-	-	-	-
<u>Serum Calibrator – Level 2</u>	-	-	80 µl	-	-	-	-
<u>Serum Calibrator – Level 3</u>	-	-	-	80 µl	-	-	-
<u>Serum Calibrator – Level 4</u>	-	-	-	-	80 µl	-	-
<u>Serum Calibrator – Level 5</u>	-	-	-	-	-	80 µl	-
<u>Serum Calibrator – Level 6</u>	-	-	-	-	-	-	80 µl
<u>Reagent D – Internal Standard</u>	150 µl	150 µl	150 µl	150 µl	150 µl	150 µl	150 µl

Close tubes with lid - Vortex for 1 minute

STEP 2: Centrifuge at 15.000 rpm for 5 minutes

STEP 3: Take 100 µl of supernatant and add 100 µl of Reagent E - Stabilizing Solution

Vortex for 20 seconds

INJECTION:

Inject 20ul of solution into the chromatograph LC and wait for the release of the chromatogram.

ANALYTICAL PROCEDURE

STEP 1 : Place 15 mL of whole blood in a 50 mL falcon (in case the blood volume was lower, bring up to volume directly in the tube with the **Reagent A – Diluent Solution**)

STEP 2 : Add 15 mL of **Reagent A – Diluent Solution** to the blood and mix for aspiration / dispense using a pasteur pipette .

STEP 3 : In a test tube SepMate add 15 mL of **Reagent B - Extraction Solution** on the bottom of the tube, through the central hole of the septum (The level of **Reagent B** will exceed the septum).

STEP 4 : Gently add the blood previously diluted (STEPS 1-2), using an automatic pipette and leaning on the edge of the tube SepMate .

STEP 5 : Place the tube SepMate in centrifuge for 10 minutes at 1200 rpm at room temperature .

STEP 6 : Transfer the content over the septum of the tube SepMate in a new 50-mL falcon (not supplied in kit) decanted quickly (to avoid keeping the tube SepMate inverted for more than 2 seconds) .

STEP 7 : Make up to volume (50 mL) with **Reagent A – Diluting Solution** .

STEP 8 : Centrifuge for 10 minutes at 320 g at room temperature

STEP 9 : Remove the falcon from the centrifuge and discard the supernatant by inversion, so that only the pellet of lymphocytes remains. Repeat steps 7-8-9 a second time.

STEP 10 : Resuspend the pellet in the residual liquid (250-290 uL), possibly adding **Reagent A – Diluting Solution** to bring the indicated volume.

STEP 11 : In eppendorf prepare the dilution for the lymphocyte count : 20 of resuspended + 980 ul of **Reagent A**

STEP 12 : Make lymphocyte counts

STEP 13 : Calculate the volume of resuspended cells in STEP 10 necessary to obtain a number of lymphocytes between 2.7×10^6 and 3.8×10^6 . This volume should be between 20 and 64 μ l .

Nr. Cell/ μ l	Cell volume resuspended in Step 10	Reagent A Volume
850-1100	64 μ l	-
1110-1500	50 μ l	14 μ l
1510-1800	40 μ l	24 μ l
1810-2500	30 μ l	34 μ l
2510-3000	24 μ l	40 μ l
3010-3800	20 μ l	44 μ l

Then transfer in each of 3 eppendorf (times 0, 1 and 2), the calculated volume . Note the volume withdrawn and the number of cells obtained in this volume.

STEP 14 : Set the volume of 64 μ l with 3 eppendorf **Reagent A – Diluting Solution**

STEP 15 : Add to each of the 3 eppendorf 16 μ l of **Reagent C - Chemical Standard**

STEP 16 : In the first eppendorf (time 0), immediately add 150 μ l of **Reagent D - Internal Standard**. Centrifuge at 15.000 rpm for 5 minutes.

STEP 17 : Place the other two eppendorf (time 1 and 2) in a shaker for eppendorf thermostated at 37 ° C, to the speed of 1300 rpm .

STEP 18 : After 1 hour, remove the eppendorf " time 1 " and add 150 μ l of **Reagent D - Internal Standard**. Centrifuge at 15.000 rpm for 5 minutes.

STEP 19 : After 2 hours, remove the eppendorf " Time 2 " and add 150 μ l of of **Reagent D - Internal Standard**. Centrifuge at 15,000 rpm for 5 minutes.

STEP 20 : From each of the eppendorf (times 0, 1 and 2) transfer 100 μ l of supernatant into vials for autosampler and add 100 μ l of **Reagent E – Stabilization Solution**.

INJECTION :

Inject 20 μ l of solution into the LC SYSTEM and wait for the release of the chromatogram.

DATA PROCESSING

Consider the result for each sample relative to the concentration of 5FU at time 0 and at time 2 (120 minutes). The assay result is obtained by applying a formula that takes into account the two above values and the number of lymphocytes considered:

$$\underline{V_d} = [\text{quantity of 5FU at time 0 (ng/ml)} - \text{quantity of 5FU at time 2 (ng)}] / 120 \text{ min} / \text{n. cells (10}^6\text{)} \times 11.5$$

The result will be expressed in ng / min / million cells.

Note: The result of the time 60 minutes is not included in the formula, but it's determined to better assess the reaction of metabolism.

5-FLUOROURACIL IN PBMC BY LC-MS/MS - Warnings

ANALYTICAL COLUMN TREATMENT

Install the new Polaris C18 analytical column 100 x 2 mm - 3 µm.
Disconnect the detector and pass a solution of H₂O: Mobile Phase M2 (20: 80 v / v) to the flow of 300 µl / min for 15 minutes. **Do not recycle the wash.** Condition further with a solution of H₂O: Mobile Phase M1 (5 : 95 v / v) for 10 minutes.

It is NOT possible to make analysis at recycling phase.

If the room temperature is T > 25 ° C is recommended to store mobile phases at 2-8 ° C between two analytical sessions.

COLUMN WASH

Disconnect the detector. Flux a solution of H₂O: Mobile Phase M2 (5: 95 v / v) for 10 minutes at 300 µl / min and discharge. Store the column in a solution of H₂O: Mobile Phase M2 (5: 95 v / v).

WASH NEEDLE INJECTION

Wash the injection needle with Methanol.

GRADIENT			
TIME (min)	% M1 (PUMP A)	% M2 (PUMP B)	FLOW (µl/min)
0	100	0	200
4.0	100	0	200
4.1	25	75	200
7.0	25	75	200
7.1	100	0	200
16.0	100	0	200

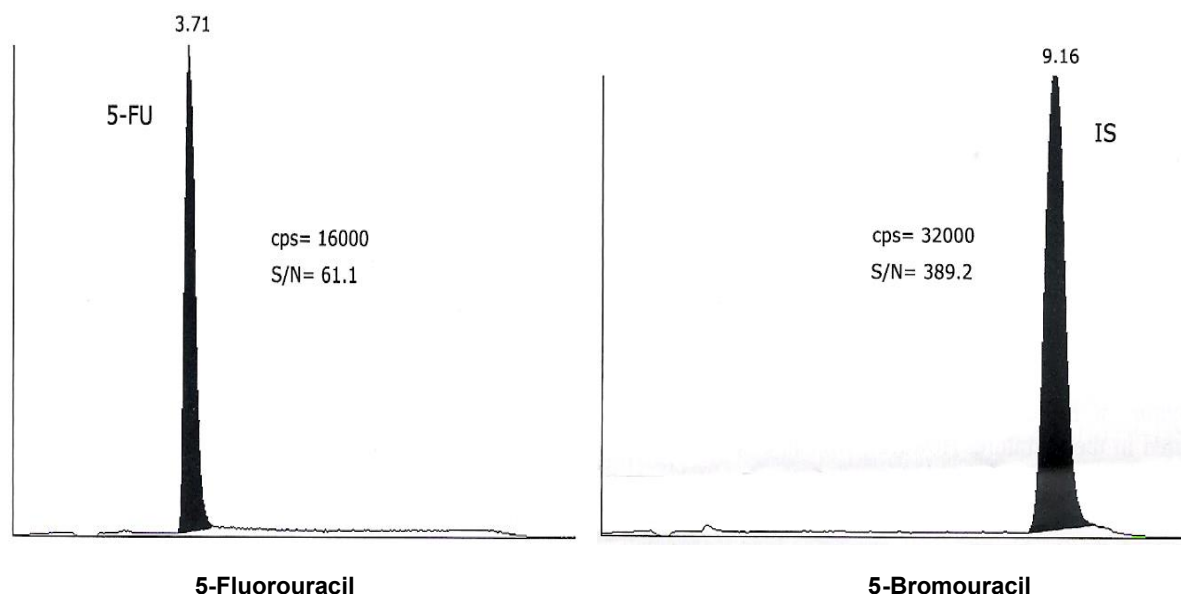
ESI SCIEX 4500 Triple Quadrupole Parameters

Curtain Gas (CUR): 20
Collision Gas (CAD): 9
Ion Spray Voltage: (IS): 4500 V
Temperature (TEM): 600°C
Ion Source Gas 1 (GS1): 80
Ion Source Gas 2 (GS2): 60

ANALYTE	PARENT ION / FRAGMENTS
5 – FLUOROURACIL	131,0 / 88,1-58,1-113,8
5 - BROMOURACIL	192,7 / 175,9

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(Reference chromatograms)



Bibliography

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