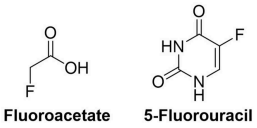
Dec 15, 2023

Targeted analysis of 5-Fluorouracil (5-FU) and Fluoroacetate (FAC) in human plasma by automated PPT+ extraction and LC-HRMS analysis



DOI
dx.doi.org/10.17504/protocols.io.kxygx3p2kg8j/v1

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Protocol Citation: Margaux Billen, Scott G Denham, Joanna Simpson, Natalie ZM Homer 2023. Targeted analysis of 5-Fluorouracil (5-FU) and Fluoroacetate (FAC) in human plasma by automated PPT+ extraction and LC-HRMS analysis. **protocols.io https://dx.doi.org/10.17504/protocols.io.kxygx3p2kg8j/v1**

Protocol status: Working
We use this protocol and it's working

Created: July 14, 2023

Last Modified: December 15, 2023

Protocol Integer ID: 85008

Keywords: 5-fluorouracil, fluoroacetate, capecitabine metabolism, High Resolution Mass Spectrometry, LC-MS method, ThermoFisher Exploris 240 Orbitrap, Human Plasma, catabolite fluoroacetate, fluorouracil, fluoroacetate, labelled lactate, liquid chromatography high resolution mass spectrometry, metabolite, resolution mass spectrometry analysis, liquid chromatography, high resolution mass spectrometry, using automated protein precipitation, human plasma sample

Funders Acknowledgements:
Fonds Wetenschappelijk Onderzoek (FWO) Vlaanderen
Grant ID: V424223N
Biotechnology and Biological Sciences Research Council
Grant ID: BB/V019066/1

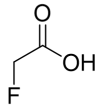
Abstract

This protocol describes the extraction and targeted high-resolution mass spectrometry analysis of two compounds - the anti-cancer drug 5-fluorouracil (5FU) and its catabolite fluoroacetate (FAC), in human plasma samples.

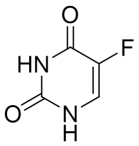
A targeted LC-MS method was developed to measure FAC and 5-FU, by adapting methods from Deenen et al, 2012 and from Leong et al, 2022. Samples were enriched with isotopically labelled lactate and the drug and its metabolite were extracted using automated protein precipitation on an Extrahera liquid handling robot (Biotage) alongside calibration standards.

Analysis of the extract was carried out by liquid chromatography high resolution mass spectrometry (LC-HRMS) in full scan negative mode on an Exploris 240 Orbitrap (ThermoScientific). The amount of each compound (5FU and FAC) in each sample was calculated using linear regression of the peak area ratio of the analytes to the isotopically labelled internal standard.

Analytes

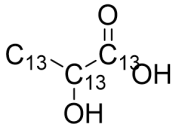


Fluoroacetate



5-Fluorouracil

Internal standard



$^{13}C_3$-lactate

Analyte information

	Name	Abbreviation	Chemical Formula	Monoisotopic mass
	Fluoroacetate	FAC	C2H3FO2	78.0117
	5-Fluorouracil	5-FU	C4H3FN2O2	130.0179
	13C3-lactate	13C3-Lac	[13C]3H6O3	93.042

Guidelines

Ensure all training is up-to-date for operating the laboratory equipment included in this protocol.

Materials

Consumables

	Item	Supplier	Part no.	Quantity
	1.75 mL glass vials with lids	Scientific Laboratory Supplies Ltd	TUB1200	10
	7 mL glass vials with lids	Scientific Laboratory Supplies Ltd	TUB1220	5
	28 mL tall form glass vials with lids	VWR	T008/04	2
	2 mL deep well 96 well collection plate	Biotage	121-5203	1
	Biotage PPT+ plate	Biotage	120-2040-P01	1
	96 Extrahera 1000 mL pipette tips	Biotage	414141	2
	2 mL deep well 96 well collection plate	Waters	186002482	1
	96 well plate sealing film	Merck	Z369659	1
	Adhesive Plate Seal	Waters	186006336	1
	ACQUITY Premier HSS T3 Column 1.8 µm, 2.1 x 150mm	Waters	186009469	1

Chemicals

	Item	Supplier	Article no.
	Water (HPLC grade)	Fisher Scientific UK Ltd (QMRI Stores)	C-10449380-X
	Acetonitrile (HPLC grade)	VWR	C-20060320-X
	Acetonitrile (LC-MS grade)	VWR	83640.320
	Water (LC-MS grade)	VWR	83645.320

	Item	Supplier	Article no.
	Formic acid (LC-MS grade)	Fisher	10596814
	2-Propanol (LC-MS grade)	VWR	84881.320
	5-fluorouracil - 500MG	Merck Life Sciences	PHR1227-500MG
	Fluoroacetate - 250MG	Merck Life Sciences	796875-250MG
	L-Lactic acid-13C3 (100 mg)	Merck Life Sciences	746258-100MG

Equipment

	Item	Model	Supplier
	Liquid Handling Robot	Extrahera	Biotage
	Evaporator	TurboVap 96 Dual sample concentrator	Biotage
	Microtube centrifuge	1-15	Sigma
	96 well plate centrifuge	Heraeus Megafuge 16R	Thermo
	Deepwell Plate Thermoshaker	TS-DW	Grant Scientific
	Liquid Chromatography Pump	Vanquish	Thermo
	Autosampler	Vanquish	Thermo
	Column oven	Vanquish	Thermo
	Mass spectrometer	Exploris 240 Orbitrap+	Thermo
	Balance	PS-100	Fisher Scientific

Troubleshooting

Safety warnings

Adhere to local laboratory rules.

Ethics statement

When handling human clinical samples, ensure you are following local guidelines including adherence to Good Clinical Practice. In particular, ensure that data files of samples analysed do not contain identifiable patient information.

Solvent preparation

15m

1 **Mobile Phase A: H_2O + 0.1% Formic Acid**

- Add 1 L of LC-MS grade H_2O to a 1L glass bottle.
- Add 1 mL of LC-MS grade Formic Acid to the H_2O.
- Mix thoroughly.

2 **Mobile Phase B: Acetonitrile**

- Add 1 L of LC-MS grade Acetonitrile to a 1L glass bottle.

3 **Autosampler Seal Wash: 10% Acetonitrile**

- Add 100 mL LC-MS grade Acetonitrile to 900 mL LC-MS grade H_2O in a 1L glass bottle.
- Mix thoroughly.

Internal standard preparation

20m

4 All solutions (internal standards and calibration standards) are kept at -20°C after preparation until use.

5 Prepare Internal standard stock solution.

$^{13}C_3$-Lactate stock solution (1 mg/mL)

Using the PS-100 balance, weigh out approximately 2 mg of $^{13}C_3$-Lactate into a 7 mL glass vial, add appropriate volume of water (HPLC grade) and vortex thoroughly to give a 1 mg/mL $^{13}C_3$-Lactate stock solution e.g. 2 mL for 2 mg.

6 Prepare Internal Standard dilutions according to the table below in:

- 1 × 1.75 mL glass vials labelled '50 µg/mL 13C3-Lactate in water'.
- 1 × 3.5 mL glass vials labelled '5 µg/mL 13C3-Lactate in water'.

	Stock conc	Amount of stock	Vol water (µL)	Final vol (µL)
	50 mg/mL	50 µL x 1 mg/mL 13C3-Lactate	950	1000
	5 µg/mL	200 µL x 50 mg/mL 13C3-Lactate	1800	2000

Calibration standards mix preparation

30m

7 Prepare calibration standard stock solutions.

7.1 **FAC stock solution (1 mg/mL)**
Using a PS-100 balance, weigh out approximately 2 mg of FAC into a 7 mL glass vial, add appropriate volume of water (HPLC grade) and vortex thoroughly to give a 1 mg/mL FAC stock solution e.g. 2 mL for 2 mg.
Store at -20 °C.

7.2 **5-FU stock solution (1 mg/mL)**
Using the PS-100 balance, weigh out approximately 2 mg of 5-FU into a 7 mL glass vial, add appropriate volume of water (HPLC grade) and vortex thoroughly to give a 1 mg/mL 5-FU stock solution e.g. 2 mL for 2 mg.
Store at -20 °C.

8 Prepare Calibration Standard mix dilutions according to the table below, in:

- 4 × 1.75 mL glass vials labelled: 50 µg/mL, 5 µg/mL, 500 ng/mL, 50 ng/mL.

Solution name / Concentration	Amount of stock	Vol water (µL)	Final vol (µL)
50 µg/mL	50 µL x 1 mg/mL **FAC** + 50 µL x 1 mg/mL **5-FU**	900	1000
5 µg/mL	100 µL x 50 µg/mL of **FAC + 5-FU mix** above	900	1000
500 ng/mL	100 µL x 5 µg/mL of **FAC + 5-FU mix above**	900	1000
50 ng/mL	100 µL x 500 ng/mL of **FAC + 5-FU mix above**	900	1000

Extraction Procedure

2h

9 Acquire or build a sample list for the samples in Microsoft Excel including experimental details and unique sample identification codes.

10 Complete plate map for standards and samples (**make sure to place them column-wise**) using the design as shown. The number of samples that can be analysed per batch is 83, alongside a 10-point calibration curve.

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1 Double Blank	A2 25.0 STD	A3 Sample 5	A4 Sample 13	A5 Sample 21	A6 Sample 29	A7 Sample 37	A8 Sample 45	A9 Sample 53	A10 Sample 61	A11 Sample 69	A12 Sample 77
B	B1 0 STD	B2 50.0 STD	B3 Sample 6	B4 Sample 14	B5 Sample 22	B6 Sample 30	B7 Sample 38	B8 Sample 46	B9 Sample 54	B10 Sample 62	B11 Sample 70	B12 Sample 78
C	C1 0.250 STD	C2 100 STD	C3 Sample 7	C4 Sample 15	C5 Sample 23	C6 Sample 31	C7 Sample 39	C8 Sample 47	C9 Sample 55	C10 Sample 63	C11 Sample 71	C12 Sample 79
D	D1 0.500 STD	D2 Double Blank	D3 Sample 8	D4 Sample 16	D5 Sample 24	D6 Sample 32	D7 Sample 40	D8 Sample 48	D9 Sample 56	D10 Sample 64	D11 Sample 72	D12 Sample 80
E	E1 1.00 STD	E2 Sample 1	E3 Sample 9	E4 Sample 17	E5 Sample 25	E6 Sample 33	E7 Sample 41	E8 Sample 49	E9 Sample 57	E10 Sample 65	E11 Sample 73	E12 Sample 81
F	F1 2.50 STD	F2 Sample 2	F3 Sample 10	F4 Sample 18	F5 Sample 26	F6 Sample 34	F7 Sample 42	F8 Sample 50	F9 Sample 58	F10 Sample 66	F11 Sample 74	F12 Sample 82
G	G1 5.00 STD	G2 Sample 3	G3 Sample 11	G4 Sample 19	G5 Sample 27	G6 Sample 35	G7 Sample 43	G8 Sample 51	G9 Sample 59	G10 Sample 67	G11 Sample 75	G12 Sample 83
H	H1 10.0 STD	H2 Sample 4	H3 Sample 12	H4 Sample 20	H5 Sample 28	H6 Sample 36	H7 Sample 44	H8 Sample 52	H9 Sample 60	H10 Sample 68	H11 Sample 76	H12 Solvent blank

11 Defrost control pooled human male plasma for calibration standard and blanks preparation.
Defrost calibration standard mixes, internal standards and samples.

12 Take a 2mL deep well 96 well collection plate (Biotage) and add 100 µL control pooled to the calibration standard and blank wells (A1-D2).

13 Add required amount of standards to the plasma according to the table shown here. Due to the small volumes of water being pipetted ensure that the standard is pipetted **into** the plasma.

Standard name	Amount (ng)	Concentration (ng/mL) for 100 µL standard volume	STD vol (µL)
0 STD	0	0	0
0.250 STD	0.250	2.50	5 µL x 50 ng/mL
0.500 STD	0.500	5.00	10 µL x 50 ng/mL
1.00 STD	1.00	10.0	20 µL x 50 ng/mL
2.50 STD	2.50	25.0	5 µL x 500 ng/mL
5.00 STD	5.00	50.0	10 µL x 500 ng/mL
10.0 STD	10.0	100	20 µL x 500 ng/mL
25.0 STD	25.0	250	5 µL x 5 µg/mL
50.0 STD	50.0	500	10 µL x 5 µg/mL
100 STD	100	1000	20 µL x 5 µg/mL

14 Add 100 µL of each plasma sample to the appropriate wells.

15 Add 20 µL of the 5 µg/mL internal standard solution to all wells, except for Double Blank and Solvent Blank wells.

16 Seal the plate using a 96 well plate sealing film (Merck) and shake the plate on a plate shaker for 00:05:00 to ensure that the standards and internal standards are sufficiently mixed.

17 Set up Extrahera liquid handling robot for PPT+ extraction.

17.1 Turn on Air Compressor. Make sure a pressure of ~9 bar is achieved and that the compressor goes into Standby (indicated by green flashing light).

17.2 Make sure the fume cupboard is switched on and the duct hose is in place in a fume cupboard to ensure proper ventilation.

17.3 Turn on Extrahera and wait for it to boot up.

17.4 From the Maintenance menu select 'Flush Solvent Inlets' and purge the line which contains LC-MS grade Acetonitrile. Throw away the purged liquid and fill up the container with fresh LC-MS grade Acetonitrile.

17.5 Ensure that sufficient number of standard bore solvent tips (deck position 1 and 2) are on the deck.

17.6 Place a PPT+ plate in deck position 3. Make sure that it is in the correct orientation and is properly clicked in place.

17.7 Take a Waters 2mL 96 well collection plate, and mark it with the project title, investigator name, plate number, extraction date and the initials of the person doing the extraction. Place the plate in carousel position A and **make sure that well A1 is on the outside of the carousel next to the A1 label!**

18 Remove the plate seal and place the sample plate on the deck of the Extrahera in position 4.

19 Select 'Run Single Method' from the Extrahera menu and select an appropriate PPT+ extraction method, then press 'Prepare Run'. Select the columns of the PPT+ plate for processing and update the tip numbers/locations if necessary.

20 Press Run. The Extrahera loads 300 µL of Acetonitrile into each well. It then transfers the sample plate contents onto the acetonitrile in the PPT+ plate. The Extrahera applies positive pressure to pass the samples through the PPT+ plate and collects the eluent in the Waters 2mL deep well 96 well plate.

21 Once complete, check the volumes of elution solvent in the collection plate are approximately equal indicating good performance of the positive pressure head. Check that the samples and standards were correctly aspirated from the sample plate.

22 Place the colection plate on the TurboVap Dual 96 sample concentrator with the gas temperature set to 40 °C and the gas flow to 30 mL/min.

23 Once dry, resuspend the dry samples in 80 µL of H_2O and seal the plate with a Waters Adhesive plate sealing film.

24 Shake the plate for 00:10:00 at 600 rpm to ensure the samples are resolubilised.

Set up of FAC and 5FU LC-HRMS method and analysis 30m

25 Put the freshly prepared mobile phases onto the UPLC system. Purge lines with mobile phase A and mobile phase B.

26 Install an **ACQUITY Premier HSS T3 Column 1.8 µm, 2.1 × 150mm** column into the column oven. Set the column temperature to **40°C** and equilibrate at **100% mobile phase A**, **0.3 mL/min** for at least 15 minutes. Ensure that the pressure is stable and there are no leaks detectable on the system.

27 Create an **acquisition method** in Xcalibur for chromatography and mass spectrometry settings. For chromatography include the following chromatographic gradient conditions in the table below.
Add the detail of the column and mobile phases in the method. Make sure the right column position is selected for the valves and the column oven temperature and column pre-heater are set to 40°C.

	Time (min)	Flow (mL/min)	%A	%B	Curve
	Initial	0.300	100	0	Initial
	1.00	0.300	100	0	5
	2.80	0.300	5	95	5
	3.50	0.300	5	95	5
	3.60	0.300	100	0	5
	7.00	0.300	100	0	5

Chromatographic gradient for separation of FAC and 5-FU in extracted plasma samples on an ACQUITY Premier HSS T3 Column 1.8 µm, 2.1 x 150mm using H_2O + 0.1% Formic Acid (Mobile Phase A) and Acetonitrile (Mobile Phase B).

28 Add the following mass spectrometry method parameters to the acquisition method:

	A	B
	Instrument	Thermo Exploris 240 Orbitrap
	Source, Ionisation Mode	Thermo Scientific™ OptaMax™ NG ion source (H-ESI)
	Scan Mode, Polarity	Full Scan, Negative
	Mass range	50 - 200 m/z

	A	B
	Resolution	120 000
	Acquisition time	7.0 min
	Sheath Gas	50
	Aux Gas	15
	Sweep Gas	1
	IonSpray Voltage (IS) (Negative)	-2500 V
	Ion Transfer Tube Temperature	350°C
	Vaporizer Temperature	450°C
	Probe position (x – axis)	2
	Probe position (y – axis)	2

29 Place the sealed 96-well plate into the autosampler of the chromatography system.

30 Create a batch in Xcalibur - use the correct position for the 96-well plate, the correct position of the column, the correct lines for the mobile phases and the correct LC-MS/MS method. Name and save the **Batch acquisition file.** Use the same naming convention to name the resulting **data file**.

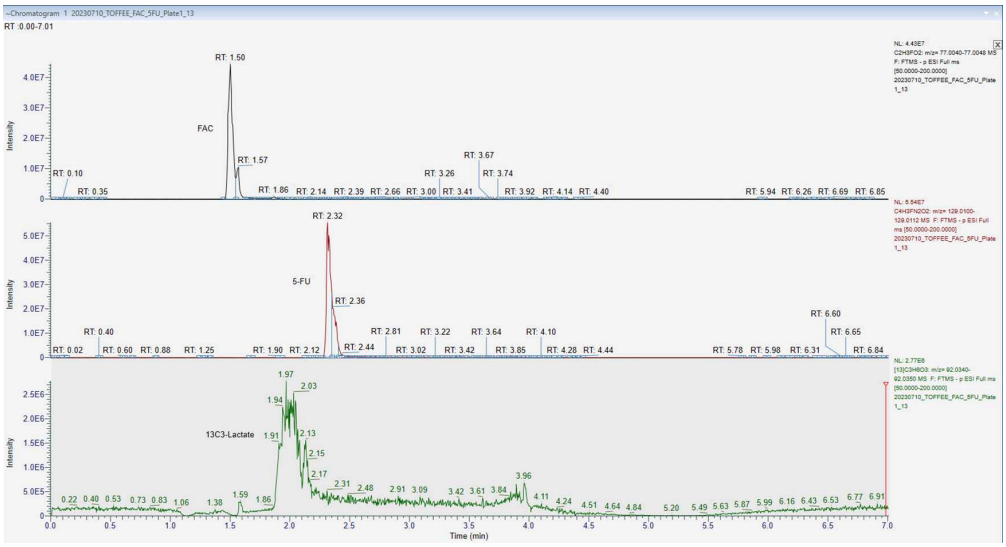
31 Set volume of injection to **20 µL** and submit batch to analyse.
Test the system with a mid-standard curve point injection and then complete the batch in order from A1 to H12.

32 Use the *m/z* $[M-H]^-$ for each compound in the table below to interrogate the data:

		FAC	5FU	13C3-Lactate
	m/z [M-H]-	77.0044	129.0106	92.0345
	Retention time (min)	1.50	2.32	1.97

33 Example chromatography of FAC, 5-FU and $^{13}C_3$-Lactate separation is shown below.
Separation performed on an ACQUITY Premier HSS T3 Column 1.8 µm, 2.1 × 150mm

using a system of H_2O + 0.1% Formic Acid (Mobile Phase A) and Acetonitrile (Mobile Phase B).



Chromatographic Separation of FAC (1.50 mins), 5FU (2.32 mins) and the internal standard $^{13}C_3$-Lactate (1.97 mins) on an ACQUITY Premier HSS T3 (Waters) Column 1.8 µm, 2.1 x 150mm using a system of H_2O + 0.1% Formic Acid (Mobile Phase A) and Acetonitrile (Mobile Phase B). Flow rate 0.3 mL/min, 40°C and a gradient elution over 7 minutes.

Data Analysis using TraceFinder

2h

34 Use the protocol below and the compound specific table details (mass and retention time) in Step 32 to evaluate the data and obtain the FAC and 5FU concentrations in the samples analysed:
Margaux Billen, Scott G Denham, Joanna P Simpson, Natalie ZM Homer 2023. Using TraceFinder and Excel software to evaluate and report multi-analyte targeted LC-MS data acquired on an ThermoScientific Exploris 240 Orbitrap. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n92ldm8z7l5b/v1

Protocol references

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Margaux Billen, Scott G Denham, Joanna P Simpson, Natalie ZM Homer 2023. Using TraceFinder and Excel software to evaluate and report multi-analyte targeted LC-MS data acquired on an ThermoScientific Exploris 240 Orbitrap. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n92ldm8z7l5b/v1