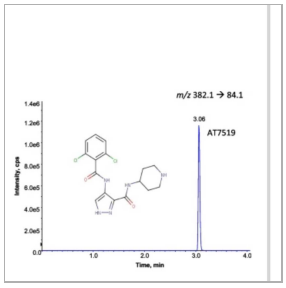

JUL 19, 2023

Analysis of paracetamol and AT7519 in serum using LC-MS/MS

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Joanna Simpson: mass spectrometry, chromatography, drugs

Clinical Mass Spectrometry

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Protocol Citation: Natalie ZM Homer, Joanna Simpson 2023. Analysis of paracetamol and AT7519 in serum using LC-MS/MS. **protocols.io** https://protocols.io/view/analysis-of-paracetamol-and-at7519-in-serum-using-cua8wshw

MANUSCRIPT CITATION: Cartwright JA, Simpson JP, Homer NZM, Rossi AG. Analysis of AT7519 as a pro-resolution compound in an acetaminophen-induced mouse model of acute inflammation by UPLC-MS/MS. Journal of Inflammation (London, England). 2023 Jun;20(1):20. DOI: 10.1186/s12950-023-00345-y. PMID: 37291548; PMCID: PMC10251596.

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

Created: May 16, 2023

Last Modified: Jul 19, 2023

PROTOCOL integer ID: 81984

ABSTRACT

This protocol describes the extraction and targeted mass spectrometry analysis of two drugs - paracetamol and AT7519, a CKDI inhibitor, following enrichment with isotopically labelled internal standards of deuterated versions of the two drugs. Extraction of the drugs was carried out using automated protein precipitation on a liquid handling robot alongside a calibration curve. Analysis of the extract was carried out by liquid chromatography mass spectrometry (LC-MS/MS) in multiple reaction mode. The amount of each drug (APAP and AT7519) in each sample was calculated using linear regression of the peak area ratio of the analytes to the isotopically labelled internal standards.

This protocol was published as part of: J Cartwright, J P Simpson, NZM Homer, Adriano Rossi 'Analysis of AT7519 as a pro-resolution compound in an acetaminophen-induced mouse model of acute inflammation by UPLC-MS/MS', J. Inflammation (2023)

GUIDELINES

Use analytical grade, LC or LC-MS grade solutions and solvents, as detailed in Materials section. Use certified reference materials where detailed. Use the dilution scheme as described to ensure linear calibration curves.

MATERIALS

The following tables detail the materials, equipment and solutions needed to carry out the protocol.

Compound	Abbreviation	Supplier	Catalogue Number	Concentration	Storage Conditions
Paracetamol	APAP	Cerilliant/Merck	A-064-1ML	1 mg/mL in MeOH	-20C
AT7519	AT7519	Astex Pharmaceuticals	///	1 mg/mL in MeOH	-20C
[2H]4-paracetamol	D4-APAP	Cerilliant/Merck	P-909-1ML	1 mg/mL in MeOH	-20C
[2H]8-AT16043M	d8-AT7519	Astex Pharmaceuticals	///	1 mg/mL in MeOH	-20C

Table M1 - Analytical Standards - Name, abbreviation, supplier, catalogue number, concentration and storage conditions

Item	Model	Manufacturer
Plate Shaker	Grant Scientific TS-DW Deepwell	ThermoFisher
Liquid Handling Robot	Extrahera	Biotage
Evaporator	SPE Dry-96 dual	Biotage
Liquid Chromatography Pump	Acquity Binary Solvent Manager	Waters
Column oven	Acquity Column Manager	Waters

Item	Model	Manufacturer
Autosampler	Waters Acquity Sample Manager with Sample Organiser	Waters
Mass spectrometer	QTrap 5500	AB Sciex

Table M2 - Equipment list - All laboratory equipment and instrumentation needed for the extraction and analysis

Item	Supplier	Part number
Methanol (HPLC grade)	VWR	C-20864320-X
Water (HPLC grade)	Fisher Scientific UK Ltd	C-10449380-X
Acetonitrile (HPLC grade)	VWR	C-20060320-X
Water (LC-MS grade)	VWR	83645.320
Methanol (LC-MS grade)	VWR	83638.320
Acetonitrile (LC-MS grade)	VWR	83640.320
1.75mL glass vials with lids	Scientific Laboratory Supplies Ltd	TUB1200
2 mL deep well 96 well collection plate	Biotage	121-5203
Biotage PPT+ plate	Biotage	120-2040-P01
96 Extrahera 1000 □L pipette tips	Biotage	414141
2 mL deep well 96 well collection plate	Waters	186002482
96 well plate sealing film	VWR	391-1250
Zone-free 96 well plate sealing film	Sigma-Aldrich	Z721646-50EA
Waters BEH C18 100 x 2.1 mm, 1.7□m LC column	Waters	

Table M3 - **Consumables and Chemicals** - Item, Supplier, Part number

Solution concentration	Volume of stock to add	Concentration of Stock solution	Volume of Methanol (uL)	Final volume (uL)
50 µg/mL	50 µL	1 mg/mL	900	1000
5 µg/mL	100 µL	50 µg/mL	950	1000
500 ng/mL	100 µL	5 µg/mL	900	1000
50 ng/mL	100 µL	500 ng/mL	900	1000
5 ng/mL	100 µL	50 ng/mL	900	1000

Table M4 - **Preparation of stock solutions** - combined AT7519 and APAP - volumes and concentrations

Standard Name	Amount (ng)	Internal Standard Vol (µL)	Standard Volume mixture (µL)	Standard concentration
0 Std	0	20 µL	0	0
0.05 std	0.05	20 µL	10	5 ng/mL
0.10 std	0.50	20 µL	20	5 ng/mL
0.25 std	4.50	20 µL	5	50 ng/mL
0.50 std	0.10	20 µL	10	50 ng/mL

	Standard Name	Amount (ng)	Internal Standard Vol (µL)	Standard Volume mixture (µL)	Standard concentration
	1.00 std	1.00	20 µL	20	50 ng/mL
	2.50 std	5.00	20 µL	5	500 ng/mL
	4.50 std	0.25	20 µL	10	500 ng/mL
	5.00 std	2.50	20 µL	20	500 ng/mL

Table M5 - **Preparation of Calibration Standards** - aliquot directly into 96-well PPT+ plate. Use solutions prepared as detailed in Table M4.

	QC Level	Standard Volume of APAP and AT7519 (µL)	Volume of 5% BSA
	0.5 ng/mL	100 µL x 5 ng/mL	900
	0.75 ng/mL	150 µL x 50 ng/mL	850
	5 ng/mL	100 µL x 50 ng/mL	900
	40 ng/mL	80 µL x 500 ng/mL	920

Table M6 - Preparation of Quality Control solutions - prepared in bulk in 2% BSA and frozen in 100 µL aliquots. Use solutions prepared as detailed in Table 4

PROTOCOL MATERIALS

d4-acetaminophen (d4-APAP) certified reference material **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P-909-1ML**

Step 1

Acetaminophen (Paracetamol) certified reference material **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A-064-1ML**

Step 1

LC-MS grade water **VWR International Catalog #83645.320** Step 24.1

LC-MS grade acetonitrile **VWR International Catalog #83640.320** Step 24.1

BEFORE START INSTRUCTIONS

Ensure you have all Materials as detailed in Table 1 (Analytical standards), Table 2 (Equipment), Table 3 (Chemicals and Consumables).
Prepare solutions for extraction according to Table 4
Prepare solutions for liquid chromatography according to Table 5

Prepare calibration standard and Quality Control stock solutions

1 The following table details the analytical standards needed for this protocol

	Compound	Abbreviation	Supplier	Catalogue Number	Concentration	Storage Conditions
	Paracetamol	APAP	Cerilliant/Merck	A-064-1ML	1 mg/mL in MeOH	-20C
	AT7519	AT7519	Astex Pharmaceuticals	///	Powder	-20C
	[2H]4-paracetamol	D4-APAP	Cerilliant/Merck	P-909-1ML	1 mg/mL in MeOH	-20C
	[2H]8-AT16043M	d8-AT7519	Astex Pharmaceuticals	///	Powder	-20C

Table 1- Analytical Standards - Name, abbreviation, supplier, catalogue number, concentration and storage conditions

Acetaminophen (Paracetamol) certified reference material **Merck MilliporeSigma (Sigma-Aldrich) Catalog #A-064-1ML**

d4-acetaminophen (d4-APAP) certified reference material **Merck MilliporeSigma (Sigma-Aldrich) Catalog #P-909-1ML**

2 Take the 1 mg /mL **stock solution of APAP** in methanol

2.1 Prepare a 10 µg /mL **stock solution of APAP** in LC grade methanol by taking 10 µL x [M] 1 mg/mL APAP and adding 990 µL x LC grade methanol.

3 Prepare a **1 mg/mL stock solution of AT7519** in LC grade methanol using a microbalance (e.g. 2.5 mg AT7519 into 2500 µL methanol).

3.1 Prepare a **10 µg/mL stock solution of AT7519** in LC grade methanol by taking 10 µL x 1 mg/mL AT7519 and adding 990 µL x LC grade methanol.

4 Take the **1 mg/mL stock solution of d4-APAP** in LC grade methanol

4.1 Prepare a **10 µg/mL stock solution of d4-APAP** in LC grade methanol by taking 10 µL x 1 mg/mL and adding 990 µL x LC grade methanol.

5 Prepare a **1 mg/mL stock solution of d8-AT7519** in methanol using a microbalance and using LC grade Methanol (e.g. 2.5 mg AT7519 into 2500 µL methanol).

5.1 Prepare a **10 µg/mL stock solution of d8-AT7519** in LC grade methanol by taking 10 µL x 1 mg/mL and adding 990 µL x LC grade methanol.

6 Prepare a **combined 50 ng/mL Internal standard solution**, d4-APAP and d8-AT7519, by adding 40 µL x 10 µg/mL d4-APAP and 40 µL x 10 µg/mL d8-AT7519 into 8 mL methanol to give a 50 ng/mL stock solution.

7 Prepare a **combined 50 µg/mL APAP and AT7519 standard solution**, by adding 50 µL x **1 mg/mL APAP** and 50 µL x **1 mg/mL AT7519** into 1 mL methanol to give a **50 µg/mL APAP + AT7519 stock solution**.

Use this **50 µg/mL APAP + AT7519** stock solution to prepare four further stock dilution solutions

	Solution concentration	Volume of stock to add	Concentration of Stock solution	Volume of Methanol (uL)	Final volume (uL)
	5 µg/mL	100 µL	50 µg/mL	950	1000
	500 ng/mL	100 µL	5 µg/mL	900	1000
	50 ng/mL	100 µL	500 ng/mL	900	1000
	5 ng/mL	100 µL	50 ng/mL	900	1000

Preparation of stock dilution solutions - combined AT7519 and APAP - volumes and concentrations

8 Prepare **quality control** solutions as below using the combined standard solutions above and 2% BSA solution. Prepare in bulk and sub-aliquot into 110 µL volumes into eppendorfs (0.5 mL) and freeze (-20^{o}C). Defrost one from each level before each extraction.

	QC Level	Standard Volume of APAP and AT7519 (µL)	Volume of 5% BSA
	0.5 ng/mL	100 µL x 5 ng/mL	900
	0.75 ng/mL	150 µL x 50 ng/mL	850
	5 ng/mL	100 µL x 500 ng/mL	900
	40 ng/mL	80 µL x 50 ng/mL	920

Table Preparation of Quality Control solutions - prepared in bulk in 2% BSA and frozen in 110 µL aliquots. Use solutions prepared for calibration solutions.

9 Prepare **calibration standards** using the following table:

	Standard Name	Amount (ng)	Int Std (uL)	Std Vol (uL)
	0 STD	0	20uL (50ng/mL)	0
	0.0500 STD	0.0500	20 uL (50ng/mL)	10 uL (5 ng/mL)
	0.100 STD	0.100	20 uL (50ng/mL)	20 uL (5 ng/mL)
	0.250 STD	0.250	20 uL (50ng/mL)	5 uL (50 ng/mL)
	0.500 STD	0.500	20 uL (50ng/mL)	10 uL (50 ng/mL)
	1.00 STD	1.00	20 uL (50ng/mL)	20 uL (50 ng/mL)
	2.50 STD	2.50	20 uL (50ng/mL)	5 uL (500 ng/mL)
	4.50 STD	4.50	20 uL (50ng/mL)	9 uL (500 ng/mL)
	5.00 STD	5.00	20 uL (50ng/mL)	10 uL (500 ng/mL)

Table M5 - **Calibration standard** table - Volumes of Int Std and Std stocks

Extract calibration standard & Quality Control and samples by PPT

10 Complete a plate map for standards, quality controls and samples (MAKE SURE TO PLACE THEM COLUMN-WISE!) using the design as shown below. The attached excel can be downloaded and filled in with sample specific detail for a real plate map and real samples - change the detail in the second tab (Sample List) and it will populate the plate map view, which you can print out and use at the bench. 20230515_APAP_AT_MockPlateMap_Protocol_NZMH.xlsx 15m

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1 Double Blank	A2 BSA STD 5.0	A3 Double Blank	A4 Sample 08	A5 Sample 16	A6 Sample 24	A7 Sample 32	A8 Sample 40	A9 Sample 48	A10 Sample 56	A11 Sample 64	A12 Sample 72
B	B1 BSA STD 0	B2 Double Blank	B3 Sample 01	B4 Sample 09	B5 Sample 17	B6 Sample 25	B7 Sample 33	B8 Sample 41	B9 Sample 49	B10 Sample 57	B11 Sample 65	B12 Sample 73
C	C1 BSA STD 0.05	C2 Double Blank	C3 Sample 02	C4 Sample 10	C5 Sample 18	C6 Sample 26	C7 Sample 34	C8 Sample 42	C9 Sample 50	C10 Sample 58	C11 Sample 66	C12 Sample 74
D	D1 BSA STD 0.1	D2 QC LLOQ	D3 Sample 03	D4 Sample 11	D5 Sample 19	D6 Sample 27	D7 Sample 35	D8 Sample 43	D9 Sample 51	D10 Sample 59	D11 Sample 67	D12 Sample 75
E	E1 BSA STD 0.25	E2 QC LOW	E3 Sample 04	E4 Sample 12	E5 Sample 20	E6 Sample 28	E7 Sample 36	E8 Sample 44	E9 Sample 52	E10 Sample 60	E11 Sample 68	E12 Sample 76
F	F1 BSA STD 0.5	F2 QC MED	F3 Sample 05	F4 Sample 13	F5 Sample 21	F6 Sample 29	F7 Sample 37	F8 Sample 45	F9 Sample 53	F10 Sample 61	F11 Sample 69	F12 Sample 77
G	G1 BSA STD 1.0	G2 QC HIGH	G3 Sample 06	G4 Sample 14	G5 Sample 22	G6 Sample 30	G7 Sample 38	G8 Sample 46	G9 Sample 54	G10 Sample 62	G11 Sample 70	G12 Double Blank

Plate Map Design for 96-well extraction including calibration standards, quality controls, double blanks and up to 77 biological samples per batch

11 Defrost biological Sample and quality control samples (lower limite of quantitation (LLOQ_, low, med and high) 10m

12 Set up Extrahera liquid handling robot for PPT+ extraction: 10m

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

12.2 Turn on fume cupboard and make sure duct hose is in place in fume cupboard to ensure proper ventilation.

12.3 Turn on Extrahera at plug and use twisting switch on right hand side (turn it over 90 degrees to start it), and wait for it to boot up.

12.4 Once the touch screen is active, from the Maintenance menu select **Flush Solvent Inlets** and Purge line S3 with Acetonitrile

12.5 Ensure that a sufficient number of solvent tips (deck position 1) and sample tips (deck position 2) are on the deck. *For a full plate you will need 96 tips.*

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

12.7 Place a Waters 2mL 96 well collection plate in carousel position A (MAKE SURE WELL A1 IS ON THE OUTSIDE OF THE CAROUSEL NEXT TO THE A1 LABEL!).

13 Take a Biotage 2mL deep well 96 well collection plate and add 100 uL 5% BSA solution to each of the standard wells.

14 Add required amount of standards to the BSA according to the PREPARATION OF CALIBRATION STANDARDS table section. Due to the small volumes of standards being pipetted, ensure that the standard is pipetted INTO the BSA. 10m

15 For sample, according to plate map, add 50 uL serum plus 50 uL of water to the appropriate wells. 10m

16 Set up a Repeater pipette for adding internal standard solution to the plate. Choose a 2.5 mL tip and set to 20 uL. Make sure you have enough internal standard solution for the full number of samples and standards (Calculate this by counting the number of standards and samples adding 5 and then multiplying by 20 uL) 3m

17 To samples and standards add 20 uL of internal standard solution (Prepared according to Table - Internal Standard Preparation) using the repeater pipette (Choose 2.5 mL tip, set to 20 uL as above) to all relevant wells 10m

18 Seal the plate using a VWR 96 well plate sealing film and shake the plate on a plate shaker for 2 mins to ensure that the standards and internal standards are sufficiently mixed (shake speed - 200 rpm). 2m

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

20 Select Run Single Method from the Extrahera menu and select the DCA, PPT+ method then press Prepare Run. Select the columns of the PPT+ plate for processing, update the tip numbers/locations if necessary and top up the solvent reservoirs if necessary (when orange). Here you can also adjust the sample volume to fit with the amount you have used. For 100 uL of sample set to 150 uL

21 Press Run. The Extrahera loads 400 uL of Acetonitrile into each well. It then transfers the sample plate contents onto the acetonitrile in the PPT+ plate. Following a 10 min wait the Extrahera applies positive pressure to pass the samples through the PPT+ plate and collects the eluent in the Waters 2 mL deep well 96 well plate. 20m

22 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. IF the volumes are not equal you may need to push through on the standalone positive pressure unit.

23 Place the extracted samples (in the collection plate) on the SPE Dry 96 Dual Sample Concentrator with the gas temperature set to 40°C and the highest flow it will achieve. This step may take up to 30 mins. Keep an eye on the drying phase and make sure needles 30m

do not go into the solvent if you alter the height of the plate.

24 Once dry, resuspend all the dry standards and samples in a solvent suitable for LC-MS/MS.

24.1 Prepare mobile phase solution for resuspension of samples (90:10 Water/Acetonitrile, v/v). Take 90 mL LC-MS grade water **VWR International Catalog #83645.320** and add to 100 mL glass bottle, then add 10 mL LC-MS grade acetonitrile **VWR International Catalog #83640.320** Mix well. Label bottle. 5m

24.2 Prepare a repeater pipette with 2.5 mL tip and set volume to 100 µL. Pull up full volume into repeater pipette. 3m

24.3 Add 100 µL of 90:10 Water:Acetonitrile to each well on the plate that is part of the experiment and seal the plate with a Zone-free 96 well plate sealing film. 5m

24.4 Shake the plate for 10 minutes on the ThermoShaker (600 rpm) to ensure the samples are resolubilised. 10m

Set up LC-MS/MS for analysis of extracts 35m

25 **Prepare Mobile Phase A - H_2O + 0.1% Formic Acid** 10m

- 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.

26 **Prepare Mobile Phase B - CH_3CN + 0.1% Formic Acid** 10m

- Add 1 L of LC-MS grade CH_3CN to a 1L glass bottle.
- Add 1 mL of LC-MS grade Formic Acid to the H_2O.
- Mix thoroughly.

27 Put freshly prepared mobile phases onto the Acquity UPLC system. Purge lines with mobile phase A and mobile phase B on Acquity UPLC system 5m

28 Install a **Waters Acquity BEH C18 (100 x 2.1 mm; 1.7 µm) column** into correct position for the method, set column temperature to 45 °C and equilibrate at **95% A, 0.5 mL/min** for at least **15 minutes.** Ensure pressure is stable and no leaks on the column. 15m

29 Create an LC-MS/MS method by building the chromatographic method to be as follows:

	A	B	C	D	E	F

	A	B	C	D	E	F
	Step	**Time (min)**	**Flow (mL/min)**	**A (%)**	**B (%)**	**Curve**
	1	Initial	0.5	95	5	Initial
	2	1.50	0.5	95	5	6
	3	5.00	0.5	5	95	6
	4	7.00	0.5	5	95	6
	5	7.10	0.5	95	5	6
	6	9.00	0.5	95	5	6

Table 2 - Chromatographic gradient details

30 Start with **divert valve** going to waste, divert to mass spectrometer at 1 minute, then back to waste at 8 minutes.

31 In Method creation use existing method (or create a method) with the following multiple reaction monitoring (MRM) parameters as mass spectrometry settings.

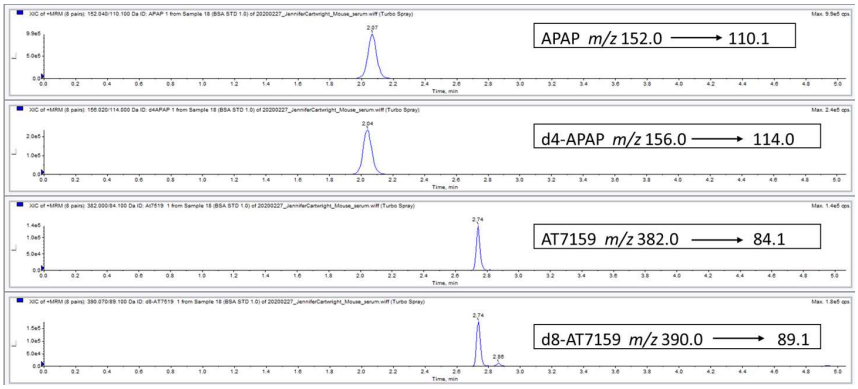
	Analyte ID	Q1 (Da)	Q3 (Da)	DP (V)	CE (V)	CXP (V)
	APAP 1	152.0	110.1	81	23	12
	APAP 2	152.0	92.9	81	31	14
	d4APAP 1	156.0	114.0	81	23	12
	d4APAP 2	156.0	97.0	81	23	12
	AT7519 1	382.1	84.1	66	29	10
	AT7519 2	382.1	136.0	66	51	14
	d8-AT7519 1	390.0	89.1	66	29	10
	d8-AT7519 2	390.0	136.0	66	51	12

Mass Spectrometry parameters for multiple reaction monitoring on an AB Sciex 6500+ Qtrap; Turbo V Source, ESI. Curtain gas 35 units, Collision gas Medium, Ion Source Gas 1 50 and gas 2 60 units with a temperature of 550^{O}C and an ionspray voltage of 5500 V.

Set up LC-MS/MS and analyse extracts

35m

32 Set a 'system suitability test' solution of APAP, AT7519, d4APAP and d8AT719 to run and check chromatography and peak area response. Retention time should be as shown:



Extracted ion chromatograms of APAP, d4-APAP, AT7519 and d8-AT7519 on C18 BEH column

33 Put plate of extracted samples into the correct position and once SST has shown good results, set the batch to run. Inject 10 µL for each sample. Observe pressure on the column and check that it progresses to inject all samples and collect data.

34 Assess chromatography and peak response while batch is underway.

Evaluation of LC-MS/MS data

35 Use Analyst Explore to view the total ion chromatogram. Extract APAP and d4-APAP and check retention times. Extract AT7519 and d8-AT5519 and check retention times.

36 Open **MultiQuant 3.0.3** and create a new result file. Select the data file collected. Save the result file with the file naming convention (yyymmdd_Exxxx_Analyte_initials). Use a method to evaluate the data that integrates APAP at 2.07 mins; AT7519 at 2.74 mins; d4-APAP at 2.04 mins and d8-AT7519 at 2.7 mins.

36.1 **Define sample type** - Blanks, Double Blanks, Standards, Unknowns (samples) and Solvents.

36.2 Insert **standard curve values** and QC valules in 'actual amount' according to the calibration table and QC table.

36.3 **Check all peaks have integrated** for each analyte - use **Metric plots** to assess peak area and retention time to ensure correct peaks have been integrated

36.4 **Check calibration curves** for accuracy (<20%) and exclude those points that are outwith. Ensure there are over 6 points in each calibration curve and a regression coefficient R>0.99

36.5 Copy the data of all peaks and calculated amounts into Microsoft Excel and name the excel file using the file naming convention (yyymmdd_Exxxx_Analyte_initials) and name the first tab 'RawData'

37 Create a new tab. Go back to 'Raw Data' select 'filter' and select the first analyte in the list (alphabetical). Copy this analyte excel data into the new tab and rename the tab with the analyte name. Repeat with all analytes until you have an excel spreadsheet with the following tabs - Raw Data; APAP 1; AT7519 1

38 In the two tabs for the analytes - rename 'calculated amount' on the APAP tab to '[APAP] ng' and rename in the AT7519 tab to '[AT7519] ng'

39 Create a new tab and name it 'Summary'. Copy the first three columns from APAP tab, which contain sample details and calibration level details. Paste into the new tab. Then return to APAP tab and copy the '[APAP] ng' column.