CEMAS

SOP No. CEM-3329/001

1 INTRODUCTION

This SOP describes the procedures for determining levels of MTF-753 and five metabolites (753-A-OH; PAM; 753-T-DO; PCA; DM-PCA) in water. For MTF-753, 753-T-DO and 753-A-OH; water samples are diluted with methanol and analysed directly. For PAM, PCA and DM-PCA the water is acidified and extracted with ethyl acetate, the extract is evaporated and the residue is re-dissolved in methanol/Milli-Q water prior to LC-MS/MS analysis. The Limit of Quantitation (LOQ) for each analyte is 0.05µg/L.

2 SAFETY PRECAUTIONS

Please refer to the relevant COSHH assessments and material safety data sheets (MSDS).

3 MATERIALS AND EQUIPMENT

- 15 mL polypropylene centrifuge tubes
- Techne Dry Block Heater
- 250 mL separating funnels
- 50ml polypropylene centrifuge tubes
- HPLC vials
- Measuring cylinders
- Volumetric glassware
- Beakers
- Gilson Microman pipettes
- HPLC column, Synergi 4μ POLAR-RP 80Å 150 x 4.6 mm, Part No. 00F-4336-E0 (Phenomenex)

Note: Equivalent glassware and equipment may be substituted where appropriate.

4 REAGENTS AND SOLUTIONS

- Methanol (Fisher Scientific; HPLC grade Code: M/4056/17)
- Acetic Acid A/0400/PB15
- Ethyl Acetate (Fisher Scientific; HPLC grade Code: E/0906/17)
- MTF-753 reference material, obtained from Mitsui Chemicals Inc., Shiodome City Center 1-5-2, Higashi-Shimbashi, Minato-ku, Tokyo 105-7117 Japan
- DM-PCA reference material, obtained from Mitsui Chemicals Inc., as above
- PCA reference material, obtained from Mitsui Chemicals Inc., as above
- 753-T-DO reference material, obtained from Mitsui Chemicals Inc., as above
- 753-A-OH reference material, obtained from Mitsui Chemicals Inc., as above
- PAM reference material, obtained from Mitsui Chemicals Inc., as above

4.1 Diluting Solvent: 1:1 Methanol/ Milli-Q water

Separately measure 500 mL methanol and 500 mL Milli-Q water and mix in a 1000 mL glass bottle.

4.2 HPLC Mobile Phases
Mobile Phase A1: 10mM ammonium acetate in Water

Mobile Phase B1: Methanol
A1/B1 are used for the determination of MTF-753, 753-A-OH and 753-T-DO

Mobile Phase A2: Water + 0.1% Formic acid

Mobile Phase B2: Methanol + 0.1% Formic acid
A2/B2 are used for the determination of PAM, PCA, DM-PCA

5 REFERENCE ITEMS FOR CALIBRATION AND FORTIFICATION

The preparation of these standard solutions may be achieved by the use of alternative dilutions if necessary. Solutions of reference items should be stored at 4 °C and have a maximum expiry date of 3 months from preparation.

5.1 1000 µg/mL Stock Standard Solutions
Weigh accurately 10 mg (adjusted for purity) of each standard into separate 10 mL volumetric flasks. Dissolve and make up to volume with methanol.

5.2 10 µg/mL Mixed Standard
Pipette 1.0 mL of each 1000 µg/mL stock standard into a 100 mL volumetric flask and make to volume with methanol.

5.3 0.1 µg/mL Mixed Fortification Standard
Pipette 1 mL of the 10 µg/mL mixed standard into a 100 mL volumetric flask and make to volume with 1:1 methanol: Milli-Q water..

5.4 0.01 µg /mL Mixed Fortification Standard
Pipette 10.0 mL of the 0.1 µg/mL mixed standard into a 100 mL volumetric flask and make to volume with 1:1 methanol: Milli-Q water.

The following standard solutions will be used as calibration standards.

5.5 20 ng/mL Mixed Calibration Standard
Pipette 20 mL of the 0.1 µg/mL mixed standard into a 100 mL volumetric flask; make to volume with 1:1 methanol: Milli-Q water.

5.6 2.0 ng/mL Mixed Calibration Standard
Pipette 2.0 mL of the 0.1 µg/mL mixed standard into a 100 mL volumetric flask; make to volume with 1:1 methanol: Milli-Q water.

5.7 1.0 ng/mL Mixed Calibration Standard
Pipette 1.0 mL of the 0.1 µg/mL mixed standard into a 100 mL volumetric flask; make to volume with 1:1 methanol: Milli-Q water.

5.8 **0.5 ng/mL Mixed Calibration Standard**
Pipette 5 mL of the 10 ng/mL mixed standard into a 100 mL volumetric flask; make to volume with 1:1 methanol: Milli-Q water.

5.9 **0.1 ng/mL Mixed Calibration Standard**
Pipette 1 mL of the 10 ng/mL mixed standard into a 100 mL volumetric flask; make to volume with 1:1 methanol: Milli-Q water.

5.10 **0.05 ng/mL Mixed Calibration Standard**
Pipette 0.5 mL of the 10 ng/mL mixed standard into a 100 mL volumetric flask; make to volume with 1:1 methanol: Milli-Q water.

5.11 **0.01 ng/mL Mixed Calibration Standard**
Pipette 1 mL of the 1.0 ng/mL mixed standard into a 100 mL volumetric flask; make to volume with 1:1 methanol: Milli-Q water.

5.12 **0.005 ng/mL Mixed Calibration Standard**
Pipette 0.5 mL of the 1.0 ng/mL mixed standard into a 100 mL volumetric flask; make to volume with 1:1 methanol: Milli-Q water.

6 SPECIMEN EXTRACTION AND CLEANUP

6.1 Controls and Reagent Blank

At least one untreated control sample must be analysed with each set of samples to ensure that no contamination of the samples has occurred prior to, or during, the analysis from matrix, solvents or materials. A reagent blank may also be included in a batch if deemed necessary.

6.2 Fortification samples

Two appropriately fortified control samples will be analysed with each set of samples to assess the analytical efficiency of the method. One of these will be fortified at the LOQ and the other at 10 times the LOQ or at a higher level anticipated in the samples.

6.3 Extraction for PCA, PAM and DMPCA

6.3.1 Measure 50 mL of water sample into 250 mL separating funnel.
6.3.2 Add 1mL of acetic acid to each sample and mix well.
6.3.3 Add 50 mL ethyl acetate and shake for 5 min, allow to separate.
6.3.4 Draw off the lower aqueous layer into a beaker and collect the ethyl acetate portion into a 250 mL round bottomed flask.
6.3.5 Repeat with a further 50 mL portion of ethyl acetate
6.3.6 Evaporate the combined ethyl acetate using a rotary evaporator to approximately 2 mL.
6.3.7 Quantitativley transfer the extracts to a graduated tube (eg 15 mL graduated centrifuge tube) using methanol
6.3.8 Blow off the methanol under nitrogen at 40 °C or lower. DO NOT blow down to dryness.
6.3.9 Add 2 mL of methanol and blow down as before (to remove all trace of ethyl acetate).
6.3.10 Blow down to a small volume and make to 1.0 mL with methanol and then make up to 2 mL with Milli-Q water and transfer an aliquot into a HPLC vial for LC-MS analysis.

6.4 Direct injection for MTF-753, 753-A-OH and 753-T-DO

6.4.1 Measure 50 mL of water sample into a plastic centrifuge tube.
6.4.2 Transfer 0.5 mL aliquot of the sample into an HPLC vial.
6.4.3 Add 1.0 mL of methanol, mix well.

6.5 LC-MS/MS Analysis

Transfer an aliquot of the prepared samples from Section 6.3 & 6.4 and an aliquot of the calibration standards from Section 5 to HPLC vials and inject using the conditions described in Section 7.

7 INSTRUMENTATION AND OPERATING CONDITIONS

7.1 Instrumentation

Agilent 1100 series Liquid Chromatography System
API 4000 LC-MS/MS System, Applied Biosystems, MDS SCIEX
Valco switching valve for above.
Applied Biosystems / MDS SCIEX Analyst Software version 1.4.1

7.2 Operating Conditions

Mobile phases for HPLC are in section 4.2

HPLC Column = Synergi 4μ POLAR-RP 80Å 150 x 4.6 mm, Part No. 00F-4336-EO (Phenomenex)
Flow Rate = 1000 µL/min
Column oven temperature = 40°C
Injection Volume = 50 μL
Diverter Valve = Yes
Diverter Valve Positioning:

Time (min)	Position
0.0	B
1.0	A
7.5	B

Gradient:
The same gradient will be used for both groups of analytes but with different mobile phases as described in section 4.2

Total Time (min)	A %	B %
0.0	35	65
7.5	15	85
8.0	35	65
10.0	35	65

Scan Type = MRM
Ion Source = Turbospray
Polarity = Both Negative & Positive (see details below)
Resolution Q1 = Unit
Resolution Q3 = Unit
CAD = 6.0
Curtain = 35.0

Gas 1 = 30.0
Gas 2 = 30.0
IS = -4500.0
Temperature = 400 °C

Compound Specific Parameters:
Note: All compounds are run in negative mode except for PAM, which is run in positive mode.

MTF-753
Q1 Mass = 358.10
Q3 Mass = 149.00
Dwell Time = 50 msec
DP = -95.0
EP = -10.0
CE = -32.0
CXP = -10.0

DM-PCA
Q1 Mass = 179.00
Q3 Mass = 159.20
Dwell Time = 200 msec
DP = -55.0
EP = -10.0
CE = -20.0
CXP = -7.0

753-T-DO
Q1 Mass = 390.19
Q3 Mass = 356.06
Dwell Time = 50 msec
DP = -85.0
EP = -10.0
CE = -20.0
CXP = -11.0

753-A-OH
Q1 Mass = 374.10
Q3 Mass = 149.04
Dwell Time = 50 msec
DP = -85.0
EP = -10.0
CE = -36.0
CXP = -25.0

PCA
Q1 Mass = 193.10
Q3 Mass = 109.00
Dwell Time = 200 msec
DP = -45.0
EP = -10.0
CE = -30.0
CXP = -7.0

PAM (positive mode)
Q1 Mass = 194.10
Q3 Mass = 174.10
Dwell Time = 100 msec
DP = 56.0
EP = 10.0
CE = 15.0
CXP = 12.0

Note: These settings may be instrument specific and can be adjusted for achieving acceptable chromatography and required sensitivity.

8 ANALYSIS

- Using quantitation standards prepared as described in section 5, inject replicate aliquots of an appropriate concentration to obtain a reproducible response before proceeding.
- Inject aliquots of standards 20 ng/mL – 0.005 ng/mL to ensure that the system is linear.
- Inject no more than four samples between an appropriate bracketing standard.

9 CALCULATION

Calculation of residue, expressed as µg/L.

$$\text{Residue (µg/L.)} = \frac{A}{B} \times \frac{C}{D}$$

A = Peak area response for specimen
B = Mean standard peak area response bracketing the specimen
C = Concentration of analyte standard (µg/mL)
D = Specimen concentration (L/mL)

The limit of quantification has been established as 0.05 µg/L. for all compounds.

$$\% \text{ Recovery} = \frac{(\text{Residue, µg/L.}) - (\text{Apparent Residue in Control, µg/L.})}{\text{Fortification Level, µg/L.}} \times 100$$

10 RESPONSIBILITIES

Management are responsible for the implementation of this procedure and for ensuring that operators receive training in its use.

Individual operators are responsible for ensuring that the procedure is followed during use.

Appendix 1 - Typical retention times

Compound	Retention time
DM-PCA	2.1
PAM	2.2
PCA	2.5
753-A-OH	4.7
753-T-DO	5.5
MTF-753	5.9