White cell cystine assay stage 2: Cystine analysis by Tandem MS

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Signature

1. CLINICAL RELEVANCE/PURPOSE OF PROCEDURE

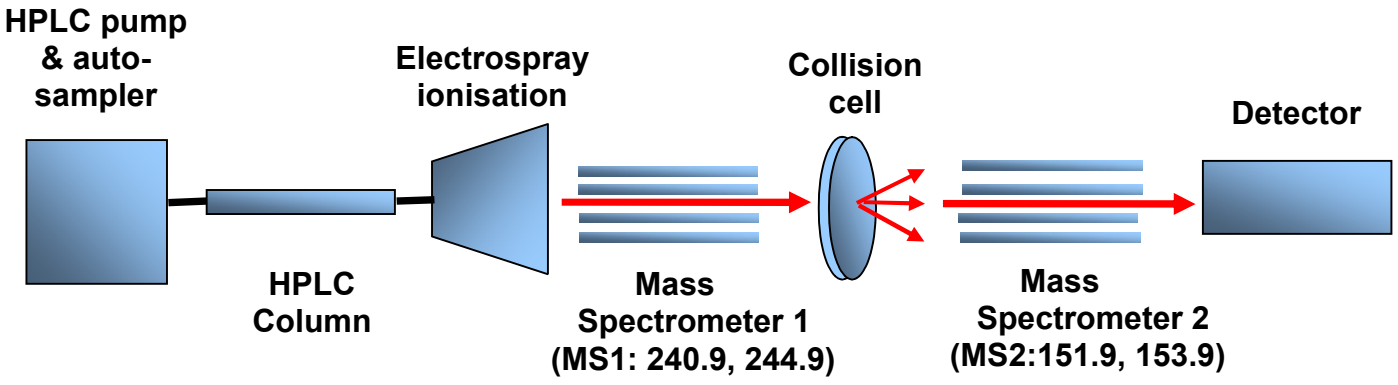
White cell cystine is used to diagnose and monitor the treatment of the inborn error cystinosis. One function of lysosomes is to break down proteins into amino acids for re-use by the cell. These amino acids are then exported from lysosomes via specific transport proteins. Patients with Cystinosis have an inactive lysosomal transport protein for cystine. This causes an accumulation of cystine which disrupts the integrity of the lysosomal membrane leading to cell damage by release of proteolytic enzymes. Tissues and organs of low cell turnover/regenerative ability are the most vulnerable to this damage. Proximal renal tubule cells are particularly vulnerable with significant renal impairment being seen by 1 to 2 years of age. This will cause polyuria, polydipsia, failure to thrive and rickets. A generalised aminoaciduria, glycosuria and phosphaturia is also observed (Fanconi syndrome).

White cells contain lysosomes and are therefore used as a source of cells for diagnosis or monitoring. Cystagon (cysteamine bitartrate) binds with cysteine which is in equilibrium with cystine. The resulting disulphide has a similar size and shape to lysine and is exported from lysosomes via the lysine transport protein. This systemic treatment is for all affected organs, although eye drops are used to avoid cystine crystal formation in the eye. The lower the white cell cystine, the better the treatment, although there have been some reports of side effects in over-treated individuals.

2. PRINCIPLE OF PROCEDURE

Samples are injected into the mobile phase and the HPLC column separates cystine from many other components in the sample. The eluate enters the electrospray ionisation chamber where intact molecules are dried and given a positive charge. These ions pass into MS1 set to allow ions of mass/charge ratio 240.9 (cystine) and 244.9 (deuterated cystine internal standard) to pass to the collision cell. In this cell the molecules are fragmented by argon gas, and the charged fragments (daughter ions) pass to MS2. This was set to detect two daughter ions; 151.9 (cystine) and 153.9 (deuterated internal standard). This measurement mode is called multiple reaction monitoring (MRM).

Ion suppression is a common problem with TMS techniques. It occurs in the electrospray ionisation chamber, reducing the ability of molecules to acquire a charge. Uncharged ions cannot enter the mass spectrometers and result in reduced signal. This competitive process is particularly bad for direct injection techniques, but by using an HPLC column a more refined sample is presented to the ionisation chamber which reduces ion suppression. Use of a stable isotope internal standard, 3,3,3',3'-deuterated cystine, corrects for any remaining ion suppression.



Schematic representation of the TMS system

3. PERSONNEL / TRAINING REQUIREMENTS

Senior staff of Biochemical Genetics Department and BMS 1/Trainee BMS under supervision of senior staff and or clinical scientists

4. SPECIMEN REQUIREMENTS

Sample type: Sulphosalicylic acid precipitated white cell pellet prepared according to the isolation of a white cell pellet SOP [SLF2BGM016]
Stored at : Frozen at -20^{o}C
Samples are stable for at least 20 days at 2-8^{o}C and at least 5 years at -20^{o}C

5. EQUIPMENT

Quattro Premier XE tandem mass spectrometer.

MDS SCIEX API 4000 LC/MS/MS

6. HEALTH AND SAFETY/RISK ASSESSMENT

Further guidance relating to laboratory accommodation, personal protective equipment and other general safety considerations is available in the Pathology Safety Manual [PHS039].

See COSHH and procedure risk assessments [SLC5CBG014], [SLC5RBG005]

Unless otherwise specified all laboratory work must be performed at containment level 2 [PHS007]
Hand and eye protection must be worn when handling blood and serum/plasma.
Laboratory procedures that may give rise to infectious aerosols must be conducted in a microbiological safety cabinet [PHS006].

High risk samples – Laboratory work must be performed at containment level 2+.
Additional precautions as described in [PHS007]

- Gloves and disposable apron must be worn
- Eye protection must be worn where splashing is assessed as a risk
- Analysis must only be undertaken by experienced BMS staff
- Analysis must be undertaken as a discrete task
- Access by other staff to the area should be restricted

All reagents are disposed of in accordance with the Waste Management procedures outlined in section 15 of the Health & Safety Manual [PHS011]

Any spillages should be dealt with according to the spillage procedures outlined in section 13 of the Pathology health & safety manual [PHS009]

Reagent	Composition	Hazards	Hazard Symbols
3% w/v sulfosalicylic acid Store at 4 °C (fridge 15)	3% w/v sulfosalicylic acid	Corrosive. Harmful by skin, eye contact and ingestion. Wear goggles when using. Wash from body surface with water.	 Corrosive  Harmful
Sigma Amino Acid Std A6407 (acidics and neutrals) Store at at -20 °C (Freezer 21)	Amino acids dissolved in 0.1M hydrochloric acid	Corrosive. Harmful by skin, eye contact and ingestion. Wear goggles when using. Wash from body surface with water.	 Corrosive  Harmful
50% acetonitrile in water with 0.025% formic acid	500mL Acetonitrile 500mL Deionised water 0.25mL formic acid	Highly flammable. Toxic by inhalation, contact with skin and if swallowed. Irritating to eyes.	 Highly Flammable  Toxic  Irritant
Acetonitrile	Component of mobile phase	Highly flammable. Toxic by inhalation, contact with skin and if swallowed. Irritating to eyes.	 Highly Flammable  Toxic  Irritant

Formic acid	Component of mobile phase	Causes burns.	 Corrosive

7. REAGENTS

Sulphosalicylic acid (SSA) solution (3% w/v)

60.00g of sulphosalicylic acid dehydrate (Sigma: S-21300) was dissolved in water in a 2L volumetric flask. Store at 2-8 °C in a plastic bottle. Expiry: 5 years after preparation

Stock internal standard (400 µmol/L) in 0.025% v/v formic acid

0.0980g of 3,3,3,3-d4-cystine (QMX: D5300) was dissolved in deionised water with 250µL of formic acid (Sigma: F-0507) in a 1L volumetric flask. Store in 20mL aliquots at -20 °C. Expiry: 5 years after preparation.

Working internal standard (4 µmol/L)

1.00mL of stock internal standard was added to water with 25µL of formic acid (Sigma: F-0507) in a 100mL volumetric flask. Store in 4mL aliquots at -20 °C. Expiry: 5 years after preparation.

Stock cystine standard (1000µmol/L)

Dissolve 0.2409g of cystine (Sigma: C-8755) in 3% SSA in a 1L volumetric flask.
Store in 20mL aliquots at -20 °C. Expiry: 5 years after preparation

Intermediate stock cystine standard (100µmol/L)

10.00mL of 1000µmol/L cystine stock standard is added to 3% SSA in a 100mL volumetric flask. Store in 20mL aliquots at -20 °C. Expiry: 5 years after preparation

Working cystine standards

Various volumes of 100µmol/L cystine intermediate stock standard were added to 3% SSA in 100mL volumetric flasks as shown:

Cystine standard concentration (µmol/L)	Volume of 100 µmol/L intermediate stock cystine added (mL)
0.4	0.40
4.0	4.00
20.0	20.00

Store in 0.5 mL aliquots at -20 °C. Expiry: 5 years after preparation

Mobile Phase (50% acetonitrile in water with 0.025% formic acid)

The mobile phase used is the same as used for neonatal screening.
Add 500mL of deionised water to 500mL of hypersolve grade acetonitrile (VWR: 20048.320) in the designated 1000mL measuring cylinder. Add 0.25mL of formic acid (Sigma: F-0507).
Cap and mix by inversion several times. The mobile phase is used directly, no separate filtering and de-gassing is required.

8. CALIBRATION

Working standards are prepared as detailed in section 7.

9. QUALITY CONTROL

Internal Quality Control Material (Patient Pool)

This QC material, even after heat treatment, should be treated as a potential biohazard.
Residual supernatants from patient samples are retained after analysis, and can be used for QC preparation as follows:

Use 10ml glass, screw top tubes
Samples with cystine concentrations in the required QC ranges (low, mid, high) are selected from cystine concentrations by review of results.
Supernatants in the appropriate range are pooled.
The water bath is turned on and allowed to reach 65-70 C.
Place the tubes in a rack in the water bath and leave for 15 minutes to equilibrate.
Keep monitoring the temperature throughout.
Once equilibrated, the sealed tube is heat treated in a water bath at 65-70 C for one hour.
The sample is then cooled and aliquoted.
Store in 200 µl aliquots at -20C. Expire: 5 years after preparation.

Quality Assessment Scheme.

ERNDIM leucocyte cystine QA scheme.

10. COMPUTER / TELEPATH CODES

CYSW1J

11. PROCEDURE OR METHODOLOGY

Sample preparation.

See appendix

Sequence Preparation.

Liase with senior staff in the screening laboratory to see if the instrument is free.

Check that the Quattro premier tandem MS is in standby mode after the previous run.

If so:

In MassLynx select:

PROJECT WIZARD

(Prompts: When changing to a new project some services are automatically closed down)

YES

Type in project name based on date: YYMMDD_WCC

BROWSE

MASSLYNX

WCCYSTINE (sends chromatogram files to this directory)

NEXT

Toggle CREATE USING EXISTING PROJECT AS TEMPLATE

BROWSE

Use previous template

FINISH

A sample list will appear shortly afterwards with the white cell cystine methods defined.

Edit the sample detail fields as per standard, QC and sample details.

Take special care to check the line number, file name, and sample location correspond to the worksheet details and increment correctly.

Inlet method selection

This sets up the HPLC parameters.

Check that there is sufficient mobile phase in the bottle.

Connect the HPLC column to the autosampler injection port and disconnect the post column inlet connection. Place in a glass beaker to pump to waste.

Click on the INLET METHOD tab (extreme left of MassLynx screen)

FILE

OPEN

MASSLYNX

WCCYSTINE

SJUH_Master_WCC.PRO

ACQUDB

InletWCC.w25

OPEN

The inlet method is now set up.

Check that the flow rate is 0.2mL/min constant flow i.e. no gradient.

Toggle the upload tab.

Save the method.

The mobile phase will now be pumping through the column to waste at 0.2mL/min. Switch on the column heater and select MANUAL MODE, 25.0^{o}C on the control pad. Open the heater chamber and check carefully for any leaks. Rectify if there are. Leave for at least 30 minutes and again re-check for leaks.

During this time the cone should be cleaned.

Cone cleaning.

Check that the sytem is in standby (green light shows standby on tandem MS and operate is NOT illuminated.

Lock the laboratory door to avoid accidental damage to the tandem MS.

Unclip and open the cone chamber.

TAKE CARE – THE CONE ASSEMBLY IS HOT!

Put white cotton gloves on (keeps parts clean and protects fingers from heat)

*****TURN THE ISOLATOR LEVER FULLY TO THE RIGHT (isolates the MS and avoids loss of vacuum)****

Carefully unscrew the two cone screws and remove the cone assembly.

Remove the rubber o-ring (do not clean)

Place the inner and outer metal cone sections ONLY into a beaker and add 50% v/v Decon 90 in water.

Sonicate for 15 minutes, drain and rinse with deionised water and then sonicate for a further 15 minutes in deionised water.

Drain, allow to dry for 5 minutes on tissue paper (use white gloves) and then re-assemble the cone and re-fit into the chamber.

*****TURN THE ISOLATOR LEVER BACK TO THE LEFT (i.e. ready for use)****

Close the cone chamber and re-clip.

Connecting the Column. and Running a Sequence.

Connect the post column line to the tandem MS inlet.

Check that there is flow through the inlet (large drops from needle visible through cone chamber window).

Carefully re-check for leaks from any of the connections, particularly those inside the column chamber.

Running the Sequence.

In MS TUNE: Turn on both gases by pressing the tab for each.

After 5 minutes, Press OPERATE

The tandem MS is ready to start a sequence.

Place the micro-titre plate on the autosampler (CHECK IT IS IN TRAY 1 – the front upper drawer)

Press RUN and check that the sample range is correct.

Viewing and Integrating results during a run.

In the MassLynx sample list, click on the completed sample of interest to highlight in black.

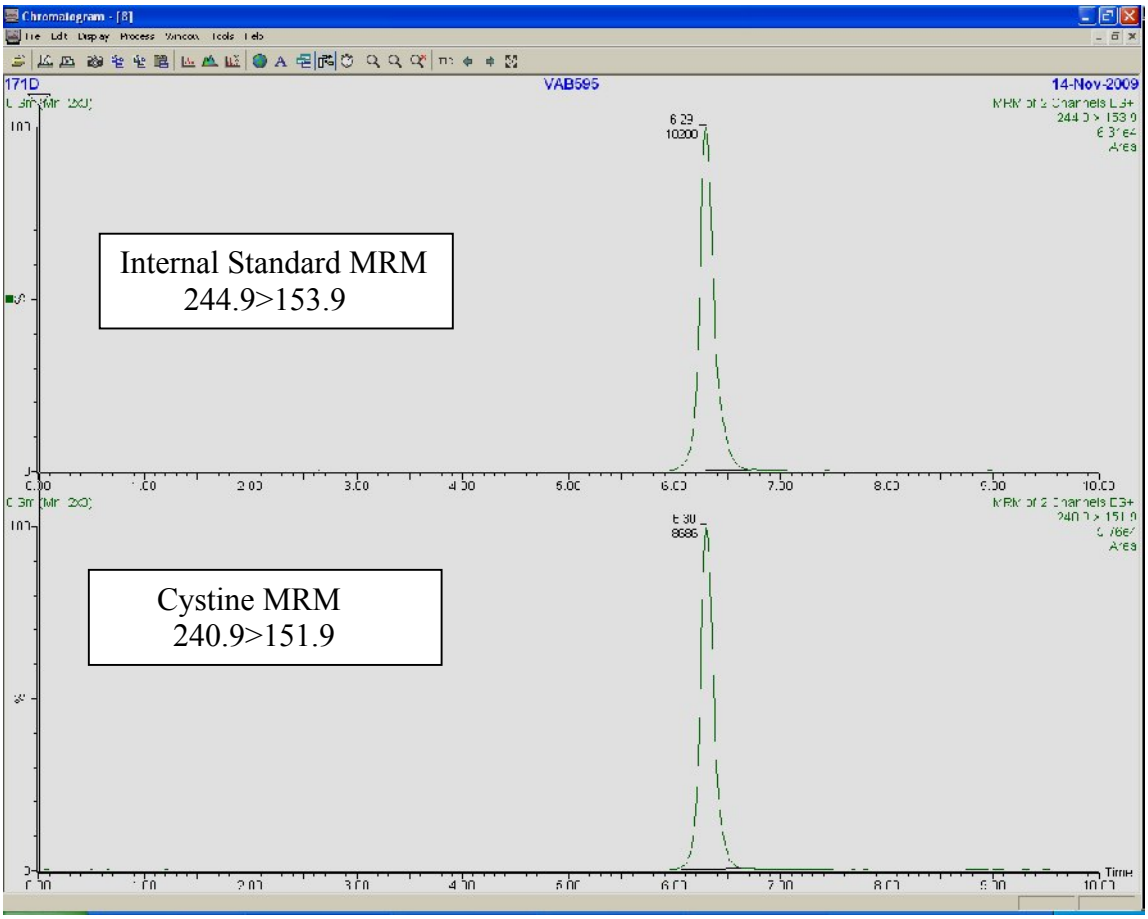
CHROMATOGRAM

DISPLAY

MASS

Double click on each of the 2two MRM's, OK.

This shows a split screen with one MRM in each field as below.



Click on one peak.

PROCESS

INTEGRATE (uses peak smoothing and integration parameter of 600)

The peak is integrated and the area is flagged next to the peak

Repeat for the other peak

PRINT

The areas are then used directly for calculation of the final result.

12. UNCERTAINTY OF MEASUREMENT

Interferences: There is no evidence of cysteamine interference (tested up to 20.0mg/L)

Linearity: The method is linear up to at least 100µmol/L

The lower limit studies revealed a limit of blank of 0.081 µmol/L, a limit of detection of 0.139 µmol/L. A limit of quantification was selected as 0.2 µmol/L, the concentration of the lowest standard, at which there was an acceptable CV of 16.8% and an accuracy of 107%.

Recoveries were found to be 96.7% and 96.8% for two different patient sample pools spiked into the lower and upper halves of the standard range.

In the event of a machine breakdown, samples kept sealed in the micro-titre plates could be stored for 1 day at room temperature and a further 5 days at 2-8^{o}C without loss of cystine.

A pool of patient samples showed that samples were stable at 2-8°C for at least 20 days, and that three freeze thaw cycles had no significant effect on the results.

13. REFERENCE RANGE / ACTION LIMITS

N/A

14. REFERENCES

This method is based on a well established but unpublished procedure. Guidance on the initial set-up of this assay was provided by the original workers (Prof Neil Dalton and Dr Charles Turner, Renal Research Laboratory., Evelina Children's Hospital, London).

15. APPENDICES

Short instructions for white cell cystine preparation (tandem mass analysis)

1. Thaw out QC material (low, mid, high QC and ERNDIM QC cystine SPN if needed), standards (0.4, 4.0 and 20.0), working internal standard and patient's samples prepared in the isolation of white cell pellet SOP [SLF2BGM016].

2. Vortex the QC material, standards and patient's samples. Centrifuge them at 10,000 rpm for 5 minutes.

3. Transfer the supernatant of combined QC and patient's samples to 1.5 ml plastic tubes **without removing any of the protein**. **Record the volume of supernatant** in the white cell cystine worksheet [SLFOBGM025]. Keep the pellets of the combined QC and patient's samples for protein analysis [SLF2BGM025].

4. Label 1.5 mL capped plastic tubes for each QC material, standard and patient's samples.

5. Add 50 µL of cystine working internal standard to each tube.

6. Add 50 µL of QC material, ERNDIM SPN if needed, standard or patient's samples to the appropriate tubes.

7. Cap the tubes, vortex and pulse centrifuged for 10 seconds (up to 6000 rpm).

8. Pipette them into micro-titre plate wells and seal the plate. Name the plate with the batch identification.

9. Samples are now ready for tandem mass spectrometry analysis

Short instructions for WCC tandem analysis API 4000

1. Open analyst software
2. Select the WCC\2011_11_10 project
3. Connect HPLC column
4. Equilibrate for at least 20 minutes: Select acquire →View queue → equilibrate →WCC.dam →Time 20 min →OK
5. Check for leaks in the line from the HPLC system to the mass spectrometer
6. Place the WCC plate in position 1
7. Program the samples:
    - Select acquire→ open file→ Select acquisition batch files →Template WCC.dab→ OK.
    - Change the set WCC to WCC batch number (for example, WCC212)
    - Add sample name identification from sample 7
    - Delete extra rows
    - Change data file to the appropriate batch number (for example DataWCC212). Change first column →select data file column →right click to mouse and fill down to all columns
    - Save the acquisition batch as batch number.dab (for example, WCC212.dab)
    - Submit the sequence
8. Run the sequence: Press ready → Press start sample (please make sure the autosampler samples as expected)

9. Quantitation:

- Double click in quantitation wizard → Select data file (for example, DataWCC212) → Select the samples that need quantitation → Press => → Next → Next → Finish
- Change sample type for the standards from unknown to standard. Type their respective analyte concentrations

10. Review the data generated: Use the peak review-pane and calibration review-pane. Go through all the samples to check that the automatic integration of the cystine peaks is correct.
11. Print the result table: Delete the review-peak and chromatogram panes →Move the calculated concentration by the sample name column →Print → Select the printer → OK
12. Save the result table: File → Save as WCCbatch.rdb (for example, WCC212.rdb)
13. Generate PDF report: Double click in reporter 3.0 → Select Analyte report 1.xml → Select output format PDF → create report → type file name as WCCbacth (for example WCC212)→ Save → Close PDF → Close analyst reporter

[SLFOBGM025]- White cell cystine worksheet

Optimized Tandem MS Parameters

TMS Parameter	Setting
Parent and daughter ions used	Cystine: Parent = 240.9, Daughter = 151.9 Internal standard: Parent244.9, Daughter = 153.9
Voltage settings	Capillary: 3.5kV Cone: 29V Extractor: 3V RF lens: 0.1V
Temperatures	Source: 120°C Desolvation: 250°C
Nitrogen flow	Desolvation: 700 L/hr Cone: 20 L/hr
Dwell Time	0.2 seconds
Collision Energy Collision gas flow	Cystine: 13.0 eV Internal standard: 14.0 eV 0.15 mL/min

16. TRAINING

Training in this procedure is recorded in the staff members training competency assessment [SLB9BG001] within the Trust Training Record File which is held centrally at each site and remains the property of the Trust.