LC-MS/MS assay method for determining voriconazole levels in serum or plasma

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Introduction

Voriconazole is a broad spectrum antifungal agent used in the treatment of invasive fungal infections such as those caused by Aspergillus and Candida, particularly respiratory infections. It is the drug of choice for invasive aspergillosis. Voriconazole has an extensive list of drug interactions and is metabolized at different rates in patients with cytochrome P450 polymorphisms. In addition, metabolism is fast in children but slow in the elderly. Pharmacokinetics is non-linear in adults and adolescents, and linear in children. It has been shown that there is a risk of therapeutic failure in levels lower than 1.3 mg/L and risk of toxicity in levels greater than 5.7 mg/L. There is strong evidence that therapeutic drug monitoring of patients is beneficial. Titration of doses, based on serum or plasma trough levels, has been suggested.

High performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) is considered to be the gold standard in analysis.

Hazards

Use appropriate personal protective equipment – lab coat and gloves. Safety glasses are recommended.

Check plugs and leads of electrical equipment before turning on for damage or other faults.

All samples must be treated as potentially high risk.

Consult the relevant Material Safety Data Sheets (MSDSs) in Q-Pulse to ascertain the hazards and risks associated with all chemicals and reagents prior to use.

The heater used to seal plates is very hot; take care.

Specimens

At least 2 mL of blood in yellow top (clotted blood) or green top (lithium-heparin) evacuated tubes (Vacutainers BD™) should be withdrawn by venepuncture. Samples should not be obtained through the central line used for giving voriconazole.

Samples should be taken pre-dose, preferably not more than 12 hours since the last dose.

Clotted or whole blood (lithium heparin) should be centrifuged at 1650 g for 10 minutes to obtain serum or plasma respectively.
Minimum quantity required for analysis:

Serum - minimum volume 10µl

Plasma - minimum volume 10µl

**Materials**

- Voriconazole powder (Hope and Pfizer).
- Use a fume cupboard to prepare stock standard. The Powder is toxic; be careful while handling
- Methanol, mass spectrometer grade. (Sigma product No. 34966, 2.5 L).
- Ammonium acetate (Sigma product No. 14267)
- Formic Acid, Analar 500 mL (BDH, Product No. 101154E)
- d3 voriconazole as the internal standard, Toronto Research Chemicals Inc. (Cat. No. V760002)
- Zinc Sulphate heptahydrate Analar, VWR Product No 29253.260. The Powder is toxic; be careful while handling
- Fresh frozen plasma obtained from blood bank (kept frozen).

**Equipment and other materials:**

- Waters Xevo TQD mass spectrometer with Waters Acquity UPLC system and Mass-Lynx software
- Waters Immunosuppressant column (2.1x10 mm) NovaPak C18 6 µm. Product No. 186003523
- Polypropylene 2 mL deep well plate, (Porvair, Product No. 219009)
- Gilson microman M10 positive displacement pipette
- Eppendorf repeater pipette.
- Peelable heat sealed foil sheets from FisherScientific (catalogue No 1197-9355)

**LC conditions**:%

Mobile phase (see Appendix)

Solvent delay: 0.5 minutes

Source: ESI positive mode

Separation: Gradient

0 minute: Mobile phase A: 80%, Mobile phase B: 20%

0.5 minute: Mobile phase A: 0%, Mobile phase B: 100%

1 minute: Mobile phase A: 80%, Mobile phase B: 20%

Flow rate: 0.6mL/min throughout
**MS/MS conditions**:

Parent voriconazole mass: 349.95 Da  
Daughter voriconazole mass: 223.95 Da  
Cone voltage: 22KV  
Collision voltage: 18KV  
Parent internal standard (d3-voriconazole) mass: 353.00 Da  
Daughter internal standard (d3-voriconazole) mass: 223.95 Da  
Cone and collision voltages: As for the parent ion  
ESI in positive scan mode  
Desolvation temperature: 550°C  
Desolvation flow: 700L/Hour  
Cone flow: 50L/Hour  
Low molecular resolution of parent: 14.5  
Low molecular resolution of internal standard: 14.8

† These conditions have been standardized at the Department of Clinical Biochemistry, Wythenshawe hospital, Manchester. Please tune your instrument to conditions specific for your laboratory.

**Quality Control**

- Run internal quality controls with each batch of analysis.
- Peak shapes and retention times should be closely inspected.
- Consistency of internal standard area counts should be monitored.
- Any deterioration in counts identified in the maintenance file should be actioned.
- Any deviation in expected retention time and or peak width (in maintenance file) should be actioned.
- Apply Westgard’s rules to detect IQC failure

An external quality assurance scheme is recommended. Laboratories not having access to such a scheme may opt for inter-laboratory comparison or a split sample testing.

**Preparation of reagents:**

Methanolic stock voriconazole standard: Dissolve approximately 10 mg of voriconazole in 1 mL methanol and store it in freezer at -70 to -80°C.
Working standards: Dilute methanolic stock standard in fresh frozen plasma. Levels currently 0, 0.25, 2.50, 5.0, 7.5 and 10.0 mg/L. Make a new batch when down to the last 15 of each. Keep them refrigerated as discussed.

Internal Quality Controls: Dilute methanolic stock QC in fresh frozen plasma. Levels 0.33, 1.33, and 4.0 mg/L would be a suggestion. Make a new batch when down to the last 15 of each.

Stock d3 voriconazole 1 mg/mL in methanol. Keep frozen as discussed.

Working d3 voriconazole internal standard (0.2 mg/L), made by diluting stock 1/5000 in methanol.

0.1 mol/L Zinc sulphate Solution add 2.83 g of zinc sulphate to 100 mL of MS grade deionised water.

Procedure

This procedure is carried out in polypropylene 96 deep well blocks (do not use polystyrene or polycarbonate plates).

1. Ensure the daily MS maintenance has been carried out.
2. If there are enough vials in a previous deep well plate use it. If not, get a new plate.
3. Collect QC and standards from the freezer.
4. Follow the work sheet as described; remember that the standards will go at the start. Write the well number to be used on the worksheet starting with the standards followed by QC and patient samples.
5. Defrost and vortex mix the standards and QC. Invert the patient samples. Pipette 10 µL of each sample into the appropriate well. A positive displacement pipette should be used for maximum accuracy, change the tip daily. Pipette tip must be washed between samples with de-ionised water, then methanol, then fresh de-ionised water.
6. Using the repeat pipette and appropriate tip add 40 µL of 0.1 mol/L ZnSO4 to each well followed by 200 µL of d3 voriconazole internal standard.
7. Seal the plate with heat sealed foil. (TAKE CARE THE HEATER IS VERY HOT). Rub over the surface to ensure that the seal is complete.
8. Vortex mix for 1 minute on the multivortex.
9. Centrifuge the plate at 2500 rpm for 5 minutes in the Eppendorf 5804 centrifuge.
10. Place the samples in the Acquity auto sampler.
11. Put voriconazole column on inside the oven.
12. Operate the instrument from the software instrument.
A description of Mass-Lynx software from Waters is given below. Contact vendor for specifications of your software while working on an assay or project:

In brief, the ratio of voriconazole to d3 voriconazole area count is measured and a calibration curve is constructed by the software. Similar area ratios are constructed for the patient samples and read from the calibration curve. Though this process is automatically processed by the software, the user needs to judge the shape, size and other technical features of each curve before releasing results.

**Opening the machine software:**

In MassLynx click on ‘File’ then ‘Open project’

Click on ‘Yes’ to message ‘When changing to a new project...’

Select _voriconazole.pro.

Open the sample list template by clicking on the open sample list icon

Select the template (_voriconazole template) and click ‘Open’

The sample list templates for the selected project will appear, containing the MS method, inlet method, inject volume, and standards and QCs with their set concentrations.

**Turning instrument on**

Click on tune page icon (At the bottom of the screen). Or click on ‘Instrument’ tab on left of screen, then click on ‘MS tune. This should show the correct tune page for the project (voriconazole.ipr). Ensure the MS is in positive ion mode by selecting 'Ion Mode' > 'Electrospray +' from the window task bar.

Click on Operate icon

‘API gas’ icon

‘Col gas’ icon and minimise tune page. Never close this screen as communication with the MS may be lost.

Right click on inlet file in sample list and click on edit. This brings up the inlet method.
Download inlet method by clicking on

Pump should start, if not click on tap to start pump, and minimise inlet method.

**Creating the sample list from the template**

Click on samples.

Click on add.

Fill in number of patients to add to template.

Click OK.

Change file name; click on box under ‘filename’ and type voriymmdd_001. Then right hand click on filename box and select ‘fill series’. This then fills in all the other filenames and auto increments.

Right click on box under column heading ‘bottle’.

Click on autosampler bed layout.

Select plate position 1 or 2.

Check that the other wells are **UNHIGHLIGHTED (i.e. no green light)**

Click on first position of run e.g. A1 (which then fills in with a green dot),

Click on replace icon (middle one). This fills the first well position.

Click on close.

Right-hand click on the blue ‘bottle’ bar and select fill the series for the plate positions. This should auto-increment the sample wells for all the specimens.

Click on File, and ‘Save as’. Call the sample list voriymmdd.

Double check that the plate is in the autosampler in the correct position and orientation.

**Starting the sample list**

Double check that the plate is in the autosampler in the correct position – A1 to rear left hand side.
Record the back pressure. From inlet method click on ACQUITY Additional Status – the back pressure is displayed in bar. Back pressure and other maintenance data is saved on the network in file.

Immediately before running the first sample go back to the inlet method, click on LC and select ‘Run gradient (no injection)’ to equilibrate the column.

In MassLynx click the Start Run icon (play button) to open window headed “Start sample list run”.

Ensure all samples are selected.

Click OK.

Next window opens ‘create TargetLynx data set’.

Click ‘OK’

The sample currently in progress has a green light on the work list. Watch the first sample to ensure that the chromatography is working appropriately. Ensure the internal standard has an adequate area. As soon as the standards have been analysed, process them and observe the standard curve. Check the linearity and $r^2$ value.

To view chromatograms in real time:

Click on chromatogram to show peaks.

Click on stopwatch icon to get real time display.

**Stopping the sequence**

If there is a problem and you need to stop the run click on the stop icon in MassLynx, then go into inlet method and click on the hand icon then click on yes. This will stop the auto sampler and the sequence can be re-started if needed.

**To process and view results:**

When you are about to process results ensure the chromatogram window is closed, as this can interfere with processing.

Click the TargetLynx tab on left-hand side of MassLynx window.

Click on ‘Process Samples’. Then select OK

(You can change each sample type to analyte/standard or QC and reprocess results.

Results can be processed while the samples are still running, only if sample list is in the same project that is running) you need to click on browse to select correct calculation method of vori.mdb.
Select Next compound or

Previous compound until the standard curve appears. Then assess each sample for chromatography and the internal standard,

Next sample.

Previous sample.

Check the correct peaks are identified for each sample. Check retention times

After all changes have been made, print a copy of the results. Exit the results and save as voriYYMMDD.qld.

**Editing the method**

‘Method Edit’ In TargetLynx can change parameters for peak identification, retention times (mins) and peak selection.

This can be changed for the current run by display chromatogram then click right hand mouse drag over the peak starting before the peak starts to rise and finishing after it has returned to baseline.

Then click on modify.

You can also change the integration parameters, by altering the number of smoothes, and changing the threshold for small peaks and noisy baselines.

**To turn the system off**

Always turn the HPLC pump off first; (inlet method) click on tap. Then turn off the API and COLLISION gases (MS tune page). Then click press for standby.

You can have the analyser in automatic shutdown mode. This is only needed if the run is left running and the results are not required until the next day,

Click on bottom right of screen where it says ‘Only Error Shutdown Enabled’ to open shutdown.

Tick box enable shut down after batch.
Then click on save icon. ‘Only Error Shutdown Enabled’ should change to ‘Shutdown Enabled’.

This will turn off the LC then the mass spec 5 mins after the run is finished.

To change this back, reverse the process.

**Calculations:**

The voriconazole drug concentration should be calculated using TargetLynx.

This will automatically generate a calibration line and calculate the drug concentrations using the integrated peak heights of the analyte relative to the internal standard. A linear fit with 1/x weighting is used.

If QCs are within the correct ranges, then the patient results can be entered into Telepath.

**Reporting**

Report as the value of voriconazole obtained in mg/L (or µg/mL) for serum or plasma samples

Minimum reportable range is 0.01mg/L.

Trough level (pre-dose) reference interval is 1.3-5.7 mg/L

Consider does escalation for any level (pre, post or random) < 1.3 mg/L

>5.7 mg/L consider dose reduction, toxic above this level. Phone result

>10 mg/L adverse effects possible, phone physician immediately. Drug should be stopped immediately, and a time interval of 24 hours allowed before restarting at a lower dose, usually 50%.

Where samples are not taken pre-dose (as happens in outpatients and clinics), post-dose levels of <1.3mg/L are too low. A random level >6mg/L should be phoned and voriconazole possibly withheld, depending on the level, the patient status and timing.

**Timetable**

Preparation of QC solutions and standards:

1 hour

Preparation of protein precipitation of the samples:

30 minutes (for a series of 40 samples, standards and the QC)

Interpretation:

30 minutes
Reportable interval of examination results:

0.1-10 mg/L

Samples with voriconazole >10 mg/L may be diluted in zero standard (Plasma) and re-analysed.

Appendix

Mobile phases are made directly into the Winchester bottles.

2 mmol/L Ammonium acetate, 0.1% formic acid in 2.5 L of methanol: To 2.5L Winchester HPLC grade methanol add 2.5 mL of Analar formic acid followed by 0.3854 g Ammonium acetate.

2 mmol/L Ammonium acetate, 0.1% formic acid in 1 L of MS grade deionised water: To 1 L of deionised water add 1 mL of formic acid followed by 0.1542 g Ammonium acetate.

Always check purity of compounds.

This method was develop in-house at the Wythenshawe Hospital, University Hospital of South Manchester by Prog Brian G Keevil and his team at the Department of Clinical Biochemistry.

REFERENCES