HPLC 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. 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.

Compared to bioassays, high performance liquid chromatography (HPLC) is a reliable and robust platform. Tandem mass spectrometry, which is considered as the gold standard, is costly, requires specialized training and not widely available in routine Biochemistry laboratories.

Hazards

Normal microbiological technique is adequate for safety whilst preparing HPLC extraction of plasma/serum. Gloves should be worn when handling all patient samples to minimise the risk of infection.

Any worker who is, or has reason to suspect that they may be pregnant should not handle acetonitrile.

Specimens

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2 mL of blood in yellow top (clotted blood) or purple top (EDTA blood) evacuated tubes (Vacutainers BD™) should be withdrawn by venepuncture.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

Clotted or whole blood should be centrifuged at 1650 g for 10 minutes to obtain serum or plasma respectively.

Minimum quantity required for analysis:-

Serum - minimum volume 700µl

Plasma - minimum volume 700µl
Materials

Voriconazole (99.8% pure) from Sigma Aldrich, US
Quinoxalone as internal standard (IS) from Sigma-Aldrich (Steinheim, Germany).
Bovine Serum Albumin (BSA), Sigma-Aldrich (Steinheim, Germany).
HPLC grade acetonitrile
HPLC grade methanol
Sodium hydroxide (Merck, Darmstadt, Germany)
Diethyl ether (Merck, Darmstadt, Germany)
Reverse osmosis HPLC grade water using a Millipore system (16-18 MΩ-cm)
Ammonium phosphate monobasic (Riedel-de-Haën, Seelze, Germany)
Acetonitrile (Merck, Darmstadt, Germany)

Equipment

The HPLC system (Beckman-Coulter, Krefeld, Germany) consist of a 126 solvent pump, a 168 UV-VIS photo-diode array detector, an auto-sampler, a chromatography data system (32 Karat software) and a printer.
ReproSil-Pur Basic C18 column, 5 µm (150 mm x 2 mm) (Dr. Maisch GmbH, Ammerbuch, Germany) protected by a C18 guard column (4 mm x 2 mm; Phenomenex, Aschaffenburg, Germany)
10 mL glass tubes
5 mL glass tubes
Clean pipette tips.
Volumetric flasks
Millipore filtration system with PTFE disposable filters (from Sartorius)
Calibrated analytical balance (0.001-1000mg)
Eppendorf centrifuge 5804
HPLC Conditions

Mobile phase (see Appendix)

Flow rate – 0.2 mL/min

Injection volume - 50 µl

Run time - 10 min

Wavelength – 250 nm

Quality Control

Internal Control procedure

Internal quality control samples spiked with fixed quantities of voriconazole are included in each HPLC extraction and run.

It is advisable to run the standards and quality control material in duplicates during initial phases of setting up of the assay method.

Total precision of internal controls should not exceed 10% at all concentration levels.

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 drug solutions

To prepare voriconazole stock solution with a concentration of 50 mg/L, add 5 mg of pure voriconazole in a 100 mL volumetric flask and make the volume up to 100 mL by addition of HPLC grade methanol using a clean pipette.

Quinoxaline working standard is prepared by adding HPLC grade methanol to 2 mg of accurately weighed powder in a 100 mL volumetric flask and making the volume up to 100 mL.

All stock standards can be aliquoted and stored at -20° C for at least 1 month.

Remove voriconazole and quinoxaline stock solutions from the freezer, and allow them to reach room temperature (18-25°C).

When room temperature has been reached, the following voriconazole standards should be prepared fresh in 5% BSA on each occasion: 0.25, 5, 1, 2, 5, and 10 mg/L.
The dilutions can be found by using the law of concentrations; \( C_1V_1 = C_2V_2 \)

Where \( C_1 \) is the stock concentration and \( V_1 \) is the volume of the stock required and \( C_2 \) and \( V_2 \) are the desired working concentration and volume respectively.

For e.g., to obtain a 2mL standard of 5 mg/L add 200µL of the stock to 1800µL of 5% BSA solution.

Similarly prepare 6mL each of 0.50, 2.0, and 5.0 µg/mL of the internal quality control material by diluting the voriconazole stock standard in voriconazole free pooled human plasma.

**Extraction procedure**

Standards, Internal quality controls and, plasma/serum specimens

Mix 500µL aliquots of BSA standards or plasma/serum or control samples with 200 µl of 0.1 M sodium hydroxide in 10-ml glass tubes.

Add 20 µL of IS and then vortex for 30 seconds.

Cap the tubes tightly and extract twice with 3 ml of diethyl ether for 5min, followed by centrifugation at 5000 x g for 5 min.

Transfer the separated organic layers into 5 mL glass tubes and evaporated to dryness at 37°C under a gentle stream of nitrogen.

Dissolve the residue obtained with 250 µl of the mobile phase.

Inject a 50µl aliquot of extracted sample onto the HPLC column for analysis.

Each sample takes 10 minutes to be analysed.

**Interpretations/Calculations**

The ratio of peak height for each calibration standard versus the IS is recorded and a calibration curve for the set of standards is automatically constructed by the software.

The level of voriconazole in each of the patient’s samples (spiked with the IS) can then be established from this calibration curve.

**Limitations**

No significant interference has been documented after comparing for endogenous metabolites or commonly used drugs in the clinics from various studies.

**Trouble-shooting**
A. Observation: A good standard curve is not obtained.

   Cause: Controls are made up incorrectly / HPLC mechanical problems

   Action required: Disregard the results and repeat the extraction with freshly made up calibration standards or if there are mechanical problems, repair these first and then re-run the samples with no re-extraction.

B. Observation: Internal control is not within 10% of the expected value.

   Cause: As above, or internal control has deteriorated.

   Action required: Check previous internal control results for signs of deterioration. If none apparent, take action as above.

Reporting

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

Minimum reportable range is 0.05mg/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 drug solutions:

1 hour

Extraction procedure for plasma/serum:

3 hrs for one patient's specimen (including 6 controls, 3 internal controls, and one patient specimen)

Interpretation / Calculation:
Appendix

Preparation of 5% BSA

5% BSA solution is to be prepared fresh by dissolving 5 g of BSA powder with RO water in a volumetric flask and making the volume to 100 mL.

Mobile Phase –

Weigh out 10.36 g* of ammonium phosphate in an 1L volumetric flask, add 300mL of RO water and dissolve. Then carefully make the volume up to a litre to obtain a 0.09M aqueous ammonium phosphate monobasic solution. Adjust the pH to 5.3 using 1N HCl.

Add 500 mL to 500mL of acetonitrile to make 50%:50% (vol/vol).

*Always check the purity of the compound. Here it is assumed to be 98% pure.

REFERENCES