



Bridging the Gap: D-Penicillamine Measurement in VAMS with Parallel Whole Blood and Plasma Quantification

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INTRODUCTION

- D-penicillamine (DPA) is an established copper-chelating agent used in Wilson's disease. Its quantitative determination remains analytically challenging because DPA occurs in several redox forms and may undergo thiol-disulfide exchange during sample collection, storage and preparation.
- Volumetric absorptive microsampling (VAMS) could facilitate low-volume and outpatient monitoring, particularly in paediatric patients, provided that pre-analytical stability and comparability with conventional matrices are demonstrated.
- The study aims to develop a stabilised LC-MS/MS method for the determination of total reducible DPA in VAMS, whole blood and plasma and to establish quantitative relationships between these matrices using paired clinical samples.

METHODS

- The LC-MS/MS method was validated independently in VAMS, whole blood and plasma.
- VAMS calibration standards and quality-control samples were prepared by applying 10 µL of fortified whole blood onto Mitra™ devices, followed by drying under controlled conditions.
- Whole-blood and plasma standards were prepared by fortifying the corresponding blank matrices.
- D-penicillamine-d₆ was added as the internal standard before analyte extraction (in case of VAMS, after dried blood reconstitution with LC-MS grade water).
- Total reducible D-penicillamine was released by reduction with dithiothreitol, followed by protein precipitation with acidified acetonitrile.
- Quantification was performed using a SCIEX Citrine™ QTRAP® system based on the QTRAP 6500 platform, operating in positive electrospray ionisation and multiple-reaction monitoring mode.
- The optimised transitions were m/z 150.1→104.1 for D-penicillamine quantification, m/z 150.1→56.1 for confirmation and m/z 156.1→110.1 for D-penicillamine-d₆.
- For chromatographic separation, the Amide BEH column (1.7 µm, 2.1 mm x 100 mm) was used in HILIC-mode.
- The gradient mode was applied during entire 4-min run, using mobile phases: A) 10 mM ammonium formate with 0.1% formic acid, and B) acetonitrile with 0.1% formic acid.
- Compound-dependent parameters were optimised by direct infusion and product-ion scanning. Separate matrix-matched calibration curves were used for VAMS, whole blood and plasma.
- Validation included calibration performance, selectivity, carry-over, within-run and between-run accuracy and precision, extraction recovery, matrix effect, dilution integrity and stability. VAMS-specific experiments additionally assessed the effects of haematocrit, drying time, storage conditions and extraction completeness.
- All validation experiments were performed according to ICH M10 bioanalytical methods validation guidelines.

CONCLUSION

A three-matrix strategy combining VAMS, whole blood and plasma may support the clinical implementation of low-volume DPA monitoring. Its success will depend primarily on rigorous control of thiol chemistry and robust demonstration of agreement between sampling matrices.

The success of D-penicillamine microsampling depends less on sample volume than on controlling thiol chemistry and proving matrix comparability.

RESULTS

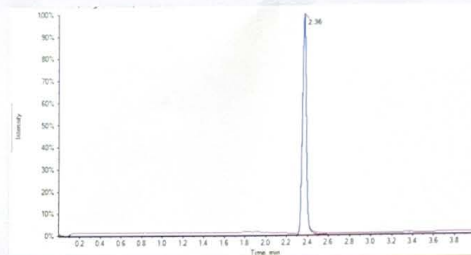


Figure 1. Representative chromatogram of DPA and DPA-d₆ quantification in clinical VAMS sample. Retention time = 2.36 min.



Figure 2. Sample-preparation workflow for the LC-MS/MS determination of total reducible D-penicillamine in VAMS, whole blood and plasma. D-penicillamine-d₆ was added as the internal standard, followed by reduction with dithiothreitol for 30 min at 37°C. Proteins were precipitated with acidified acetonitrile, and the samples were vortex-mixed and centrifuged. The clear supernatant was transferred to an autosampler vial and analysed by HILIC-LC-MS/MS.

Validation parameter	Plasma	Whole blood	VAMS
Calibration range	15–10,000 ng/mL	15–10,000 ng/mL	15–10,000 ng/mL
Calibration model	Linear, 1/x ²	Linear, 1/x ²	Linear, 1/x ²
Coefficient of determination	≥0.9982	≥0.9979	≥0.9976
LLOQ accuracy	102.7%	96.8%	104.2%
LLOQ precision	7.8% CV	8.6% CV	9.4% CV
Within-run precision	≤5.9% CV	≤6.7% CV	≤7.8% CV
Between-run precision	≤6.8% CV	≤7.5% CV	≤8.8% CV
Accuracy across QC levels	94.1–105.8%	92.8–106.4%	91.6–107.3%
Extraction recovery	93.1 ± 4.2%	88.4 ± 5.1%	84.3 ± 5.8%
IS-normalised matrix factor CV	≤5.8%	≤7.2%	≤8.4%
DTT reduction efficiency	98.1–103.4%	96.9–104.2%	96.1–104.8%
Dilution integrity accuracy	97.2–103.8%	95.8–104.5%	94.9–105.7%
Dilution integrity precision	4.3% CV	5.1% CV	6.2% CV
Carry-over after ULOQ	<20% of LLOQ	<20% of LLOQ	<20% of LLOQ
Processed-sample stability	95.1–104.6%	93.8–105.7%	92.9–106.1%
Three freeze-thaw cycles	94.3–105.2%	92.7–106.4%	Not applicable
Haematocrit-related bias	Not applicable	Not applicable	–7.8% to +8.9%
Dried-sample stability	Not applicable	Not applicable	93.1–105.6%

Figure 3. Illustrative summary of LC-MS/MS validation performance for total reducible D-penicillamine in plasma, whole blood and VAMS. Matrix-matched calibration was applied independently to each matrix. VAMS-specific validation additionally included the effects of haematocrit and dried-sample stability.

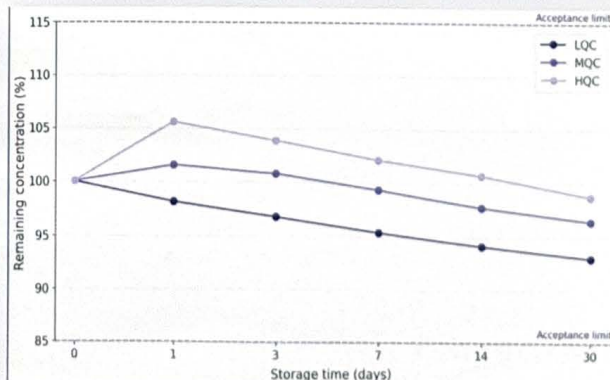


Figure 4. Stability of total reducible D-penicillamine in dried VAMS samples stored at room temperature. Results are expressed as the percentage of the initial concentration at low, medium and high QC levels [n=6].