Quantitative analysis of vanillylmandelic acid in human urine

Sabarinathan[1], Sashank Pillai[1], Craig M. Butt[2]

[1]SCIEX, India; [2]SCIEX, USA

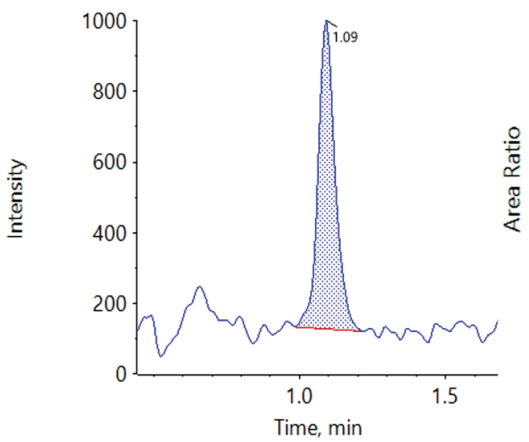
This technical note describes a simple dilution sample preparation method and LC-MS/MS analysis for vanillylmandelic acid in urine samples, achieving a 0.025 µg/mL limit of quantitation (LOQ). Using the QTRAP 6500+ system and 20 µL injection volume, the method demonstrated good quantitative performance in urine-matrix spiked calibration and quality control (QC) standards. Specifically, the 0.025 µg/mL LOQ standard showed a mean accuracy of 111% and precision of 8.7%CV (n=3). Urine-matrix QC standards (n=5) were evaluated at 0.3, 30 and 60 µg/mL and showed mean accuracy between 93.4% and 100.4% with mean precision <3%CV. Good linearity was observed in the urine-spiked calibration standards with an r^2 value of 0.990 for the quantifier transition across the 0.025-70 µg/mL calibration range. Finally, the Phenomenex Synergi Polar-RP column showed good peak shape and analyte retention using the 5 min linear gradient.

Key features of the QTRAP 6500+ system for the quantitation of vanillylmandelic acid in urine samples

- **Sub-ng/mL sensitivity in urine-matrix calibration standards:** Using the QTRAP 6500+ system, the in-sample LOQ was 0.025 µg/mL in the urine-spiked calibrators with mean accuracy of 111% and precision of 8.7%CV (n=3)
- **Good quantitative performance in spiked urine QC standards:** The QC standards were evaluated at 0.3, 30 and 60 µg/mL (n=5) and showed mean accuracies ranging from 93.4% to 100.4% and mean precision ranging from 1.2%CV to 2.4%CV
- **Good analyte peak shape and retention:** The combination of Phenomenex Synergi Polar-RP column and gradient conditions showed good peak shape, retention and void volume separation with a retention factor (k') 1.48 within the 5 min runtime.

SCIEX QTRAP 6500+ system

LOQ = 0.025 µg/mL



Calibration curve (0.025-70 µg/mL

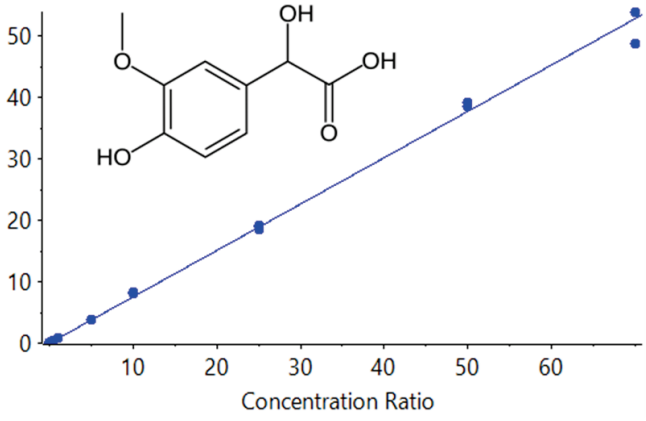


Figure 1. Extracted ion chromatogram (XIC) of the LOQ calibration standard (0.025 µg/mL) and calibration curve for the analysis of vanillylmandelic acid in urine using the QTRAP 6500+ system. Data was obtained from the extracted urine matrix calibration standards, and the quantifier transition (m/z: 197.0/137.0) is shown.

Introduction

Vanillylmandelic acid (VMA) is the terminal metabolite of the catecholamine hormones, epinephrine and norepinephrine, and is primarily excreted in the urine.[1] Elevated urinary VMA levels are associated with catecholamine-secreting tumors, including neuroblastoma, pheochromocytoma, and paraganglioma in children.[2] Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is widely used for the quantitation of urinary VMA levels due to its high specificity, sensitivity, capacity for multiplexed small-molecule biomarkers, and suitability for high-throughput workflows. In this technical note, a simple dilution-based sample preparation procedure and an LC-MS/MS method using the QTRAP 6500+ system were developed for the rapid quantitation of VMA in human urine.

Methods

Reagent and standard preparation: The analyte and internal standard (IS) were purchased from LGC Standards. Intermediate stock solutions were prepared in methanol and stored at -20°C. The IS working solution was prepared at 20 ng/mL in methanol.

Urine-spiked calibration standards and QC sample preparation: The blanks, calibration standards (n=3), and QC samples (n=5) were prepared in acidified control urine using the Sigmatrix Urine Diluent (Millipore Sigma) as the matrix. To prepare the acidified urine, 0.125 mL of acetic acid was added to 50 mL of the Sigmatrix Urine Diluent. Urine-spiked calibration standard and QC samples were prepared using the scheme shown in **Table 1**.

Sample preparation: The double blanks, blanks, calibration standards, and QC samples were prepared by 1000-fold dilution in two stages. First, 980 µL of HPLC-grade water was aliquoted into 2 mL microcentrifuge tubes. For the calibration standards and QC samples, 20 µL of the corresponding urine-spiked calibration standard or QC sample was added, and the tubes were vortexed for 10 s. For the double blank and blank samples, 20 µL of the acidified control urine was added, and the tubes were vortexed for 10 s. Second, the blank, calibration standard and quality control samples were further diluted by aliquoting 50 µL of the initial dilution sample into a clean tube containing 900 µL of HPLC-grade water and vortexed for 10 s. Then, 50 µL of the IS solution was added, and the tubes were vortexed again. The double blank samples were diluted by aliquoting 50 µL of the initial dilution into a clean tube containing 950 µL of HPLC grade water and vortexed. Finally, all samples were transferred to autosampler vials for LC-MS/MS analysis.

Table 1. Urine-spiked calibration standard and QC sample preparation scheme

Calibration standard/ QC sample	Solution used	Volume unit (µL)			In-sample conc (µg/mL)
		Spike volume	Urine volume	Final volume	
STD 10	1 mg/mL	35	465	500	70
STD 9	1 mg/mL	25	475	500	50
STD 8	STD 9	250	250	500	25
STD 7	STD 8	200	300	500	10
STD 6	STD 9	50	450	500	5
STD 5	STD 7	50	450	500	1
STD 4	STD 6	50	450	500	0.5
STD 3	STD 5	50	450	500	0.1
STD 2	STD 3	250	250	500	0.05
STD 1	STD 2	250	250	500	0.025
High QC	1 mg/mL	30	470	500	60
Mid QC	High QC	250	250	500	30
Low QC	Mid QC	10	990	1000	0.3

Chromatography: Chromatographic separation was performed using an ExionAD LC system and a Phenomenex Synergi Polar-RP column (2.5 µm, 100 x 2.0 mm, P/N: 00D-4371-B0). Mobile phase A was water with 0.025% (v/v) formic acid, and mobile phase B was methanol with 0.025% (v/v) formic acid. The runtime was 5 min using the gradient conditions presented in **Table 2**. The flow rate was 500 µL/min, the injection volume was 20 µL, and the column oven temperature was set to 40°C.

Table 2. LC gradient conditions for the analysis of vanillylmandelic acid in urine using the QTRAP 6500+ system

Time	Mobile phase A (%)	Mobile phase B (%)
0.0	80	20
0.5	80	20
3.0	2	98
4.0	2	98
4.1	80	20
5.0	80	20

Mass spectrometry: Samples were analyzed using the QTRAP 6500+ system with electrospray ionization operating in negative polarity mode. Data was acquired using multiple reaction monitoring (MRM) with the optimized source gas parameters shown in **Table 3** and the compound-specific parameters in **Table 4**. Two MRMs per compound were monitored.

Data processing: Data acquisition and processing were performed using the SCIEX OS software (version 3.4.5). The raw peak area count was normalized to the IS response.

Table 3. Optimized source and gas parameters for the analysis of vanillylmandelic acid in urine using the QTRAP 6500+ system

Parameter	Value
Polarity	Negative
Ion source gas 1	75
Ion source gas 2	65
Curtain gas	45
Source temperature	550°C
Ion spray voltage	-4500
CAD gas	8

Table 4. Optimized compound-specific MRM parameters for the analysis of vanillylmandelic acid in urine using the QTRAP 6500+ system. The quantifier transition is designated as "_1" and the qualifier transition is designated as "_2".

Compound	Polarity	Precursor ion (m/z)	Fragment ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Vanillylmandelic acid_01	Negative	197.0	137.0	-25	-10	-30	-11
Vanillylmandelic acid_02	Negative	197.0	138.0	-25	-10	-15	-9
Vanillylmandelic acid-d3	Negative	200.0	136.9	-25	-10	-30	-11

Sensitivity, accuracy, and precision in urine-spiked calibration standards

The chromatographic conditions were extensively optimized to achieve good peak shape, retention, and separation of the analyte from the void volume. Further, using the Phenomenex Synergi Polar-RP column, the analyte retention time was ~1.1 min, with a retention factor (k') of 1.48, during the 5 min runtime. The enhanced sensitivity of the QTRAP 6500+ system enabled a 1000-fold dilution, reducing matrix effects and improving data quality while maintaining low detection limits.

Triplicate samples of each urine-spiked calibration standard were used to evaluate the method sensitivity, accuracy, precision and linearity. The in-sample LOQ was 0.025 µg/mL, and linearity ranged from 0.025 to 70 µg/mL in the urine-spiked calibrators (see **Figure 1** for the LOQ XIC and calibration curve). The LOQ selection was based on the following criteria for both the quantifier and qualifier transitions: signal-to-noise (S/N) ratio ≥ 10 for both MRMs, accuracy of (±30%), precision %CV (<15%) and ion ratio tolerance of ± 30%. The LOQ mean accuracy was 111%, and the mean precision was 8.7%CV. The matrix-spiked calibration standards yielded a linear dynamic range (LDR) with 3 orders of magnitude and an r^2-value of 0.990 with the $1/x^2$ weighting factor. The internal standard normalized mean accuracy of the full calibration standard set ranged from 77.3% to 119%. The quantitative data for the quantifier transition of the calibration standards are shown in **Table 5**.

Carry-over was evaluated by analyzing a double blank immediately after the highest calibration standard (70 µg/mL). Across the batch, no vanillylmandelic acid peak was detected in the double blank (**Figure 2**), demonstrating negligible carryover in the LC-MS/MS system.

Quantitative performance in urine-spiked QC standards

The QC standards (n=5) were prepared at 0.3 µg/mL (low). 30 µg/mL (mid), and 60 µg/mL (high) to further evaluate the method performance. The QC standards were quantified against the matrix-spiked calibration standards. The IS normalized mean accuracy ranged from 93.4% to 100.4% and the mean precision ranged from 1.2% to 2.4% CV. The urine-spiked QC standard recovery from the quantifier MRM transition are shown in **Table 6**. Overall, these results illustrate the sensitivity and capability of the QTRAP 6500+ system for the quantitative analysis of vanillylmandelic acid in urine with good accuracy and precision.

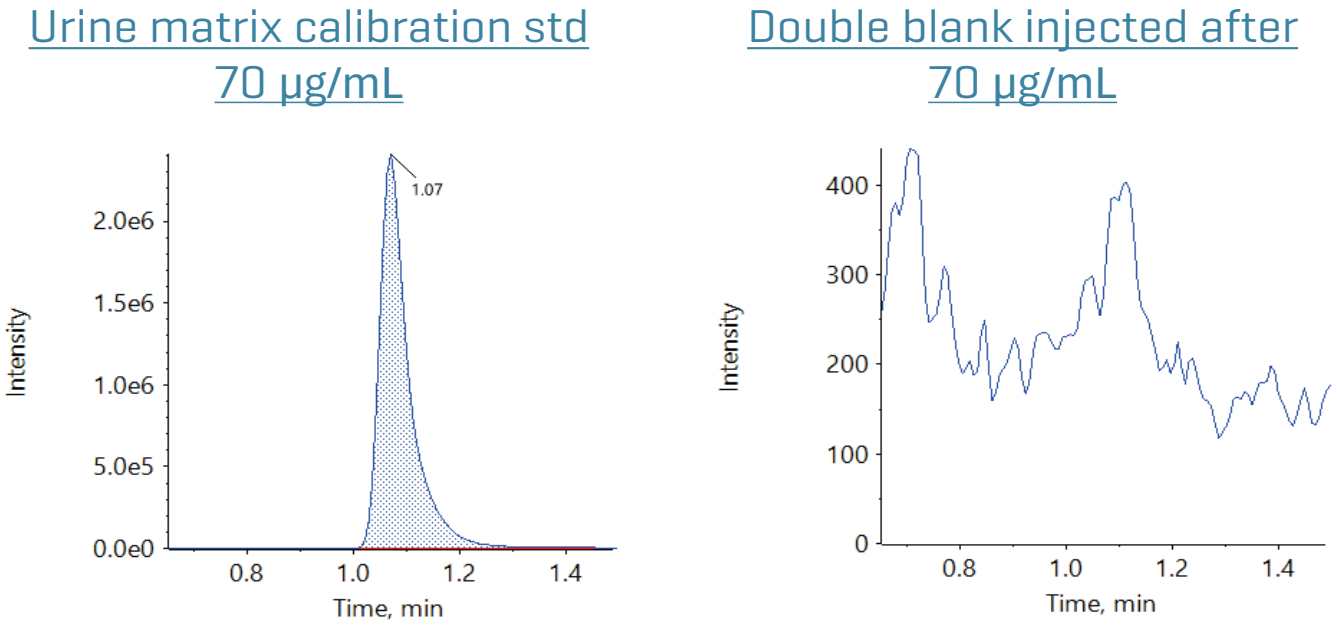


Figure 2. XIC chromatograms of the vanillylmandelic acid quantifier MRM (m/z: 197.0/137.0) in the urine matrix high calibration standard (70 µg/ml) and the double blank injected immediately after.

Table 5. Mean accuracy and precision of LOQ, correlation coefficient, and accuracy and precision across the calibration range for the analysis of vanillylmandelic acid in urine using QTRAP 6500+ system. Triplicate (n=3) samples were analyzed, and values are shown for the quantifier ion transition

Compound name	Calibration range (µg/mL)	LOQ (0.025 µg/mL)		Correlation coefficient (r^2)	Accuracy range (%) across calibrators
		Accuracy (%)	Precision (%CV)		
Vanillylmandelic acid	0.025 – 70	111	8.7	0.990	77.3 – 119

Table 6. Mean accuracy and precision for the vanillylmandelic acid urine-spiked QC standards (n=5) at 0.3, 30, and 60 µg/mL. Values are shown for the quantifier ion transition

Compound name	0.3 µg/mL		30 µg /mL		60 µg /mL	
	Accuracy (%)	Precision (%CV)	Accuracy (%)	Precision (%CV)	Accuracy (%)	Precision (%CV)
Vanillylmandelic acid	93.4	1.6	100.3	2.4	100.4	1.2

Conclusions

This technical note demonstrated:

- An LC-MS/MS method, with a dilution-based sample preparation procedure, for the quantitation of vanillylmandelic acid in urine using the QTRAP 6500+ system
- Excellent peak shape and retention from the void volume using the Phenomenex Synergi Polar-RP column with a short 5 min linear gradient
- An in-sample LOQ of 0.025 µg/mL in the urine spike calibration standards (n=3) with a mean accuracy of 111% and mean precision of 8.7% CV
- The urine-spiked calibration curve showed an r^2 value of 0.990 across the 0.025-70 µg/mL range
- Good quantitative performance in the urine matrix QC standards (n=5); mean accuracy was 93.4-100.4%, precision <3% CV

References

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2. Eisenhofer, G.; Peitzsch, M.; Bechmann, N.; Huebner, A. Biochemical diagnosis of catecholamine-producing tumors of childhood: neuroblastoma, pheochromocytoma and paraganglioma. Front. Endocrinol. 2022, 13, 901760. DOI: 10.3389/fendo.2022.901760

Headquarters
250 Forest Street, Marlborough,
MA 01752 USA
Phone 508-383-7700
sciex.com

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