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High-throughput LC-MS/MS analysis of homovanillic acid in human urine

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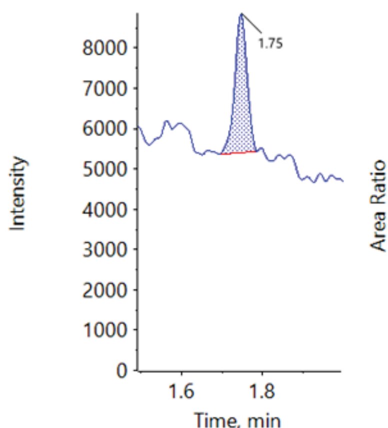
This technical note describes a method for the LC-MS/MS analysis of homovanillic acid (HVA) in urine using a streamlined 1000-fold dilution sample preparation approach. Using the SCIEX QTRAP 4500 system the method achieved an in-sample limit of quantification (LOQ) of 0.5 µg/mL in urine-spiked calibration standards. At the LOQ, the method demonstrated a mean accuracy of 100% and a mean precision of 13%CV. Good quantitative performance was shown across the 0.5-150 µg/mL calibration range, with an r^2 value of 0.992 for the quantifier transition. Urine matrix QC standards (n=5), evaluated at 1.5, 20 and 120 µg/mL, showed mean accuracies ranging from 100% to 106% with a mean precision <5% CV. In addition, the Phenomenex Synergi Polar-RP column provided excellent peak shape and analyte retention using a 5 min gradient.

Key benefits of the analysis of homovanillic acid in urine using the SCIEX QTRAP 4500 system

- **Low µg/mL sensitivity in urine matrix calibration standards:** Using SCIEX QTRAP 4500 system, the in-sample LOQ was 0.5 µg/mL in the urine-spiked calibrators with the mean accuracy of 100% and precision of 13% CV in triplicate samples
- **Good quantitative performance in spiked urine QC standards:** QC standards were evaluated at 1.5, 20, and 120 µg/mL (n=5) and showed mean accuracy ranging from 100% to 106% and mean precision ranging from 3.7%CV to 4.3%CV
- **Chromatographic separation from the void volume:** The combination of Phenomenex Synergi Polar-RP column and gradient conditions achieved good peak shape and excellent retention from the void volume with a retention factor (k') of 2.98 (Retention time ~1.75 min) within the 5 min runtime

Urine sample dilution

LOQ 0.5 µg/mL



Calibration curve 0.5 – 150 µg/mL

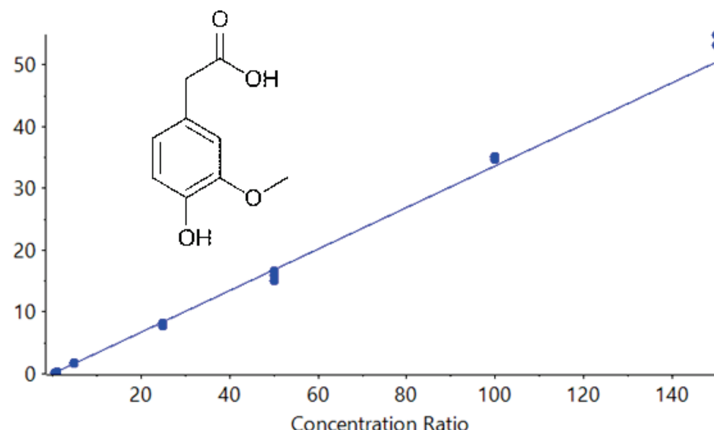


Figure 1. Extracted ion chromatogram (XIC) of the LOQ (0.5 µg/mL) and calibration curve for the analysis of homovanillic acid in urine using the QTRAP 4500 system. Data was from the extracted urine matrix calibration standards and the quantifier transition (m/z:181.0/ 137.0) is shown.

Introduction

Homovanillic acid (HVA) is the principal metabolite of dopamine in humans and serves as an indirect indicator of cerebral dopamine turnover.[1,2] It is produced from dopamine via sequential enzymatic reactions involving monoamine oxidase and catechol-O-methyltransferase.[2] Due to its high water solubility, HVA is efficiently eliminated by the kidneys, making urinary measurement a convenient monitoring approach. Urinary HVA concentrations, often evaluated alongside other metabolites such as vanillylmandelic acid (VMA), have been widely used as non-invasive biomarkers for catecholamine-secreting tumors, including neuroblastoma and pheochromocytoma.[3] Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is well suited for HVA analysis in biological matrices because of its sensitivity, selectivity, and robustness. In this technical note, a straightforward dilution-based sample preparation method was applied to achieve accurate and precise quantification of HVA in urine using the SCIEX QTRAP 4500 system.

Methods

Reagent and standard preparation: The analyte and internal standard (IS) were purchased from LGC Standards. Intermediate stocks solutions were initially prepared in methanol and stored at -20°C for the analyte and IS. The IS working solution was prepared at 50 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 standards 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 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 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.

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 7	1 mg/mL	75	425	500	150
STD 6	1 mg/mL	50	450	500	100
STD 5	STD 7	300	600	900	50
STD 4	STD 5	300	300	600	25
STD 3	STD 5	50	450	500	5
STD 2	STD 4	20	480	500	1
STD 1	STD 3	50	450	500	0.5
High QC	1 mg/mL	60	440	500	120
Mid QC	High QC	100	500	600	20
Low QC	Mid QC	45	555	600	1.5

Chromatography: Chromatographic separation was performed using an ExionLC AD system and a Phenomenex Synergi Polar-RP column (2.5 µm, 100 x 2.0 mm, P/N: 00D-4371-B0), with a SecurityGuard Guard Cartridge Kit, P/N: KJ0-4282, and a pre-filter Polar-RP cartridge 4 x 2.0 mm, P/N AJ0-6075. Mobile phase A was water with 0.025% (v/v) formic acid, and mobile phase B was acetonitrile 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 15 µL, and the column oven temperature was set to 45°C.

Table 2. LC gradient conditions for the analysis of homovanillic acid in urine

Time	Mobile phase A (%)	Mobile phase B (%)
0.0	95	5
3.0	2	98
3.5	2	98
3.6	95	5
5.0	95	5

Mass spectrometry: Samples were analyzed using the SCIEX QTRAP 4500 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 4.0.0.8559). The raw homovanillic acid area count was normalized to the IS response.

Table 3. Optimized source and gas parameters for the analysis of homovanillic acid in urine using the QTRAP 4500 system

Parameter	Value
Polarity	Negative
Ion source gas 1	60 psi
Ion source gas 2	45 psi
Curtain gas	35 psi
Source temperature	550°C
Ion spray voltage	-4500 V
CAD gas	8

Table 4. Optimized compound-specific MRM parameters for the analysis of homovanillic acid in urine using the QTRAP 4500 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)
Homovanillic acid_1	Negative	181.0	137.0	-30	-10	-10	-11
Homovanillic acid_2	Negative	181.0	122.0	-30	-10	-25	-11
Homovanillic acid_D5	Negative	186.0	142.0	-15	-10	-10	-9

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

Overall, the 1000-fold dilution sample preparation method minimized potential matrix effects while maintaining adequate sensitivity. The combination of the Phenomenex Synergi Polar-RP column and gradient conditions achieved good peak shape and retention from the void volume within the 5 min runtime. Specifically, the retention time was ~1.75 min with a retention factor (k') of 2.98.

Sensitivity, accuracy, precision, and linearity were evaluated using triplicate samples of each urine-spiked calibration standard. Overall, the in-sample LOQ was 0.5 µg/mL, and the linearity ranged from 0.5-150 µg/mL in the urine-spiked calibrators (see **Figure 1** for LOQ XIC and calibration curve). The LOQ was determined using two selective MRM transitions, achieving a signal-to-noise (S/N) ratio≥10 for both MRMs, accuracy of <30%, precision of <15%, and ion ratio tolerance of ±30%. The matrix-spiked calibration standards yielded an r^2 of 0.992 with the $1/x^2$ weighing factor. The accuracy of the full calibration standard set ranged from 89.9% to 115%. The mean accuracy at the 0.5 µg/mL LOQ standard was 100% with a mean precision of 13%CV. 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 (150 µg/mL). Across all analytical batches, no homovanillic 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 method quantitative performance was further evaluated through analyzing urine-spiked QC standards (n=5) at 1.5 µg/mL (low), 20 µg/mL (mid), and 120 µg/mL (high) and quantified against the matrix-spiked calibration standards. The internal standard normalized mean accuracy ranged from 100% to 106% and the mean precision ranged from 3.7% to 4.3%CV. Results for the urine-spiked QC standards for the quantifier MRM transition are shown in T**able 6**. Overall, these results demonstrate the ability of the QTRAP 4500 to quantify homovanillic acid in urine with good accuracy and precision.

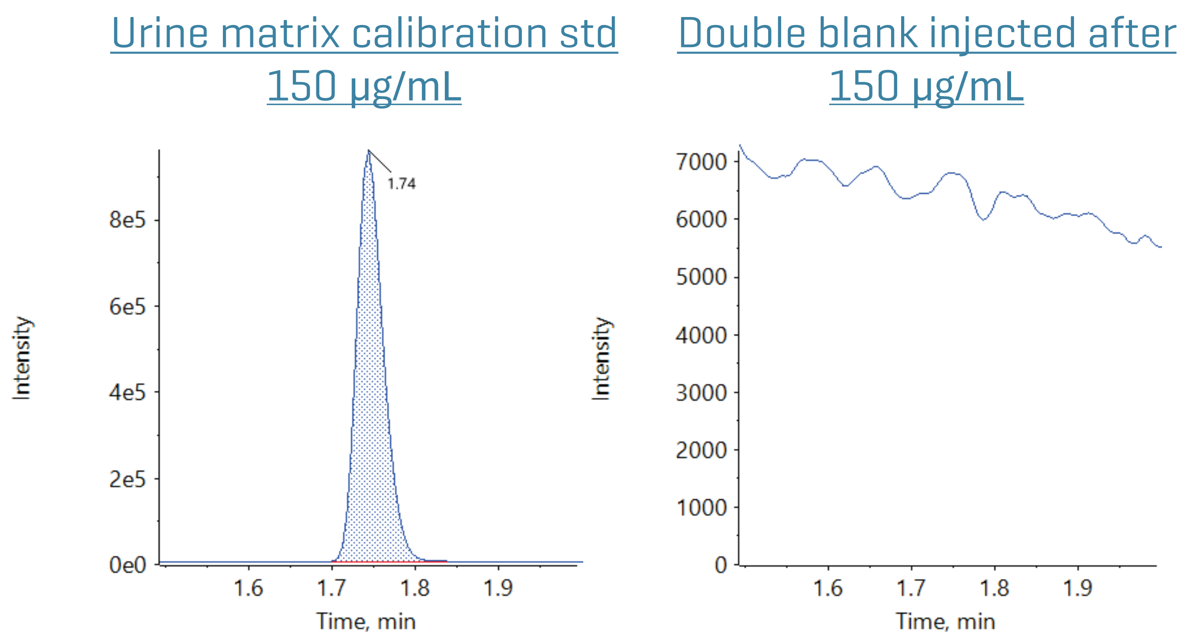


Figure 2. XIC chromatograms for HVA in the 150 µg/ml urine matrix calibration standard and the double blank ran immediately after the 150 µg/ml standard. The traces were shown for the quantifier transition (m/z: 181.0/ 137.0).

Table 5. LOQ, mean LOQ accuracy and precision, correlation coefficient, and accuracy across the calibration range for the analysis of homovanillic acid in urine samples using the QTRAP 4500 system (n=3). Values are shown for the quantifier ion transition

Parameter	Value
LOQ	0.5 µg/mL
LOQ accuracy	100%
LOQ precision	13%CV
Calibration range	0.5-150 µg/mL
Correlation coefficient (r^2)	0.992
Accuracy across calibrators	89.9%-115%

Table 6. Mean accuracy and precision for the urine-spiked QC standards (n=5) at 1.5, 20, and 150 µg/mL. Values are shown for the quantifier ion transition

QC level	Accuracy (%)	Precision (%CV)
1.5 µg/mL	106	4.1
20 µg/mL	103	3.7
150 µg/mL	100	4.3

Conclusions

This technical note demonstrated:

- An LC-MS/MS method for the analysis of homovanillic acid in urine using the QTRAP 4500 system and a simple sample preparation procedure using a 1000-fold dilution with water
- Good peak shape and retention from the void volume using the Phenomenex Synergi Polar-RP column with a 5 min linear gradient.
- An LOQ of 0.5 µg/mL in the urine-spike calibrators (n=3) with a mean accuracy of 100% and mean precision of 13%CV
- Linearity across the 0.5-150 µg/mL calibration curve with an r^2 value of 0.992
- Good quantitative performance in the urine matrix QC standards (3 levels, n=5 per level); mean accuracy was 100-106%, precision <5%CV

References

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