Sensitive, high-throughput LC-MS/MS analysis of homovanillic acid in human urine

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This technical note demonstrated a simple 1000-fold dilution sample preparation and LC-MS/MS method for the analysis of homovanillic acid in urine samples. Using the SCIEX QTRAP 6500+ system, the in-sample equivalent limit of quantification (LOQ) was 0.25 µg/mL for the quantifier transition and 0.50 µg/mL for qualifier transition **(Figure 1)**. Further, the method showed good linearity across the calibration range of 0.25-150 µg/mL, with an r^2 value of 0.989. Matrix quality control (QC) standards evaluated at 1.5, 20, and 120 µg/mL (n=5 per level) showed mean accuracy from 77.7% to 100% and mean precision <9.8%CV. The Phenomenex Synergi Polar-RP column showed good peak shape and retention from the void volume using the rapid 5 min gradient.

Key benefits of the analysis of homovanillic acid in urine samples using the QTRAP 6500+ system

- **Good sensitivity in urine matrix-spiked calibration standards:** Using the SCIEX QTRAP 6500+ system, the LOQ was 0.25 µg/mL for the quantifier transition with a mean LOQ accuracy of 106% and precision of 11%CV (n=3)
- **Accurate and precise quantitation in urine matrix-spiked QC standards:** QC standards spiked at 1.5, 20 and 120 µg/mL (n=5 per level) showed mean accuracy from 77.7 to 100% with mean precision <9.8%CV
- **Good analyte peak shape and void volume separation:** The Phenomenex Synergi Polar-RP column and method gradient conditions achieved good peak shape and excellent retention from the void volume within the 5 min runtime, allowing for high sample throughput

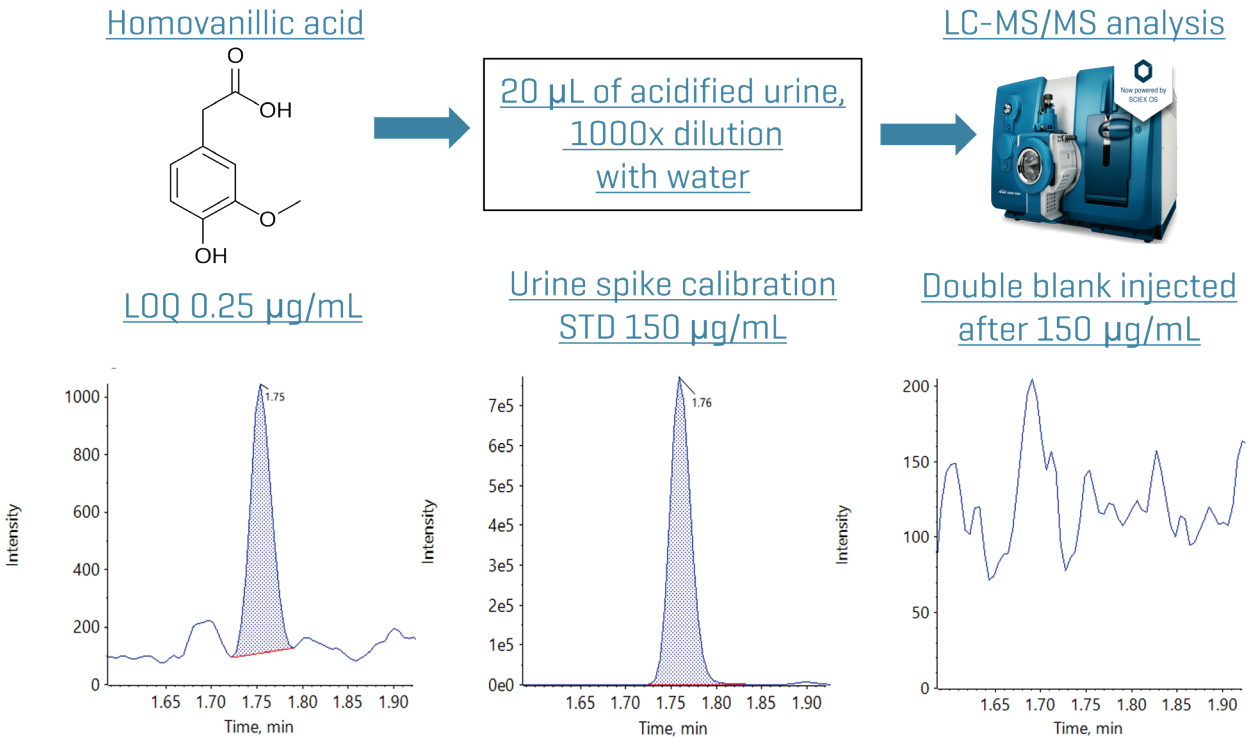

Figure 1. Extracted ion chromatograms (XICs) of the LOQ (0.25 µg/mL), calibration standard 150 µg/mL and double blank for the analysis of homovanillic acid in urine using the QTRAP 6500+ system. Data were from the extracted urine matrix calibration standards, and the quantifier transition (m/z:181.0/122.0) is shown. Carryover was not observed in the double blank after the higher calibration standard.

Introduction

Homovanillic acid (HVA) is the primary metabolite of dopamine in the human body and acts as an indirect marker of dopamine turnover in the brain.[1,2] HVA is formed from dopamine through consecutive reaction with monoamine oxidase and catechol-*O*-methyltransferase.[2] HVA is highly water-soluble and readily excreted by the kidneys, and therefore, conveniently monitored in urine. HVA urinary levels have been used as a non-invasive biomarker, alongside other metabolites such as vanillylmandelic acid (VMA), for certain catecholamine-secreting tumours, such as neuroblastoma and pheochromocytoma.[3] LC-MS/MS is the ideal technique for the HVA analysis in biological samples due to the sensitivity, selectivity and robustness. In this technical note, a simple dilution sample preparation procedure was used to accurately and precisely quantify HVA in urine using the SCIEX QTRAP 6500+ system.

Methods

Reagent and standard preparation: The analyte and internal standard (ISD) were purchased from LGC Standards. Intermediate stock solutions were initially prepared in methanol and stored at -20°C. The ISD working solution was prepared at 50 ng/mL in methanol.

Urine-spiked calibration standards and QC sample preparation: Sigmatrix Urine Diluent (Millipore Sigma) was used as the matrix to prepare the calibration standards (n=3) and QC samples (n=5). Prior to use, a stock of acidified urine was prepared by adding 0.125 mL of acetic acid 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 sample preparation procedure consisted of a 2-stage dilution with HPLC water, ultimately resulting in a 1000-fold dilution of the original urine sample. To prepare the calibration standards and QC samples, 20 µL of the corresponding urine-spiked calibration standard or QC sample was added to 980 µL of HPLC-grade water in 2 mL microcentrifuge tubes. The tubes were vortexed for 10 s and 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. Finally, 50 µL of the ISD solution was added and the tubes vortexed. To prepare the double blank and blank samples, 20 µL of the acidified control urine was added to 980 µL of HPLC-grade water in 2 mL microcentrifuge tubes and the tubes vortexed for 10 s. The blank was further diluted by aliquoting 50 µL of the initial diluent into a clean tube containing 900 µL of HPLC grade water, vortexed for 10 s, 50 µL of the ISD solution added and the tubes vortexed again. The double blank was further diluted by aliquoting 50 µL of the initial diluent into a clean tube containing 950 µL of HPLC grade water and then vortexed.

Table 1. Urine-spiked calibration standard and QC sample preparation scheme for the analysis of homovanillic acid in urine using the QTRAP 6500+ system

Calibration standard/ QC sample	Solution used	Volume unit (µL)			In-sample conc (µg/mL)
		Spike volume	Urine volume	Final volume	
STD 8	1 mg/mL	75	425	500	150
STD 7	1 mg/mL	50	450	500	100
STD 6	STD 7	300	600	900	50
STD 5	STD 5	300	300	600	25
STD 4	STD 5	50	450	500	5
STD 3	STD 4	20	480	500	1
STD 2	STD 3	50	450	500	0.5
STD 1	STD 2	250	250	500	0.25
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.

Mass spectrometry: Samples were analyzed using the SCIEX QTRAP 6500+ system with electrospray ionization operating in negative polarity mode. Data was acquired using multiple reaction monitoring (MRM) with the optimized source and 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 ISD response.

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

Table 3. Source and gas parameters used for the analysis of homovanillic acid in urine using the QTRAP 6500+ system

Parameter	Value
Polarity	Negative
Ion source gas 1	65 psi
Ion source gas 2	60 psi
Curtain gas	40 psi
Source temperature	550°C
Ion spray voltage	-4000 V
CAD gas	8

Table 4. Optimized compound-specific MRM parameters for the analysis of homovanillic 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)
Homovanillic acid_1	Negative	181.0	122.0	-30	-10	-20	-7
Homovanillic acid_2	Negative	181.0	137.0	-30	-10	-10	-7
Homovanillic acid_D5	Negative	186.0	142.0	-30	-10	-10	-7

Method performance in urine-spiked calibration standards: Sensitivity, accuracy and precision

Matrix interferences in biological samples, such as urine and blood, can result in poor quantitative performance, including false positives or negatives. The sensitivity of the QTRAP 6500+ system allowed for a simple 1000-fold dilution sample preparation procedure which minimized potential matrix interferences while maintaining sensitivity to achieve the sub-µg/mL LOQ. Further, the Phenomenex Synergi Polar-RP column provided good retention from the void volume and unretained polar interferences. Specifically, the retention time was ~1.75 min with a retention factor (k') of 2.98.

The quantitative performance of the method, using the QTRAP 6500+ system, was evaluated in a series of urine-matrix spiked calibration standards, prepared in triplicate. Good sensitivity was achieved, as demonstrated by the in-sample LOQ of 0.25 µg/mL for the quantifier transition (m/z 181.0/ 122.0) and 0.50 µg/mL for the qualifier transition (m/z 181.0/137.0). The representative LOQ chromatogram for the quantifier transition is shown in **Figure 1**. Considering the quantifier transition, the mean LOQ accuracy was 106% and the mean LOQ precision was 11%CV. The calibration curves showed good linearity across the range of 0.25-150 µg/mL for the quantifier and qualifier transitions **(Figure 2)**. Specifically, r^2 values were 0.989 and 0.992 for the quantifier and qualifier MRMs, respectively, using the weighting factor of $1/x^2$. The urine-matrix calibration standard LOQ accuracy and precision, and r^2 values are shown in **Table 5**. The method carry-over was evaluated by running a double blank sample immediately after the highest calibration standard. As shown in **Figure 1**, no homovanillic acid peak was detected in the double-blank, demonstrating negligible carryover in the LC-MS/MS system.

Accuracy and precision in urine-spiked QC standards

The method reproducibility was evaluated using urine-spiked QC samples at 1.5, 20, and 120 µg/mL (n=5 per level). The QC area counts were normalized to the ISD response and were quantified against the urine matrix-spiked calibration curve. Overall, the QCs showed good accuracy and precision across 3 spiking levels, demonstrating the ability of the QTRAP 6500+ system to produce good quantitative data for the analysis of homovanillic acid in urine (data presented in **Table 6**). The mean QC accuracy range was 77.7–100% and the mean precision was <9.8%CV.

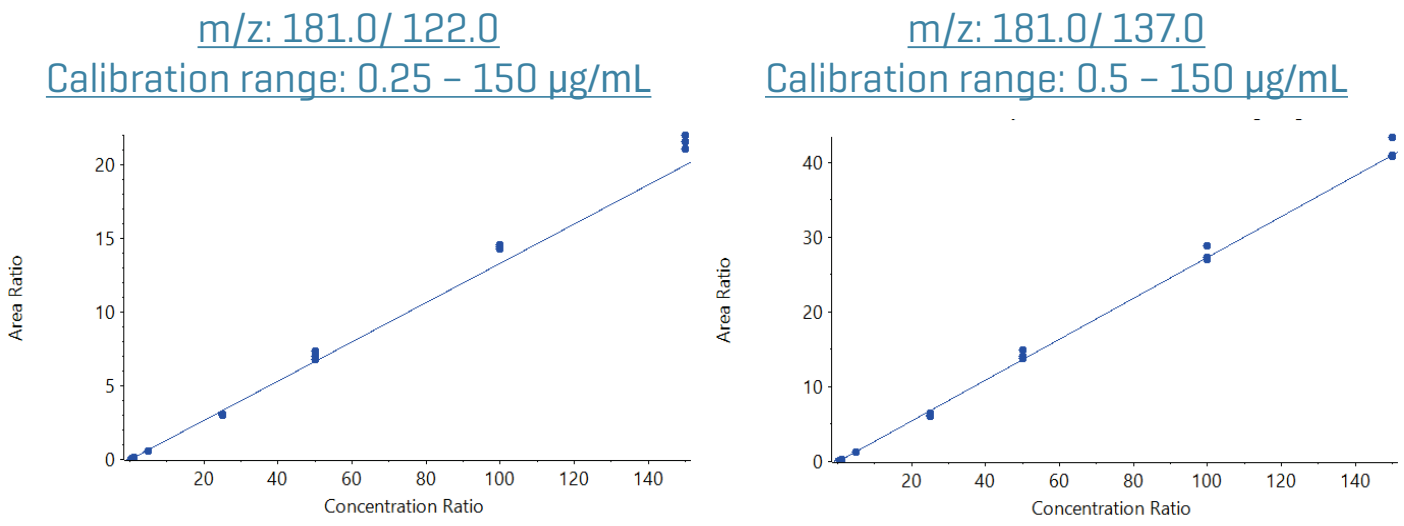

Figure 2. Calibration curves for the analysis of homovanillic acid in the spiked urine-matrix standards using the SCIEX QTRAP 6500+ system. The quantifier transition (m/z 181.0/122.0) showed an r^2 value of 0.989 and the qualifier transition (m/z 181.0/137.0) showed an r^2 value of 0.992.

Table 5. Quantitative performance of the urine-spiked calibration standards for the analysis of homovanillic acid using the QTRAP 6500+ system. The mean LOQ accuracy and precision, mean accuracy across the calibration range and correlation coefficient are shown for both the quantifier ("1") and qualifier ("2") transitions. Triplicate samples were prepared and analyzed for each calibration level. The LOQ was 0.25 µg/mL for the quantifier transition and 0.50 µg/mL for the qualifier transition.

Compound name	Transition (m/z)	LOQ accuracy (%)	LOQ precision (%CV)	Mean accuracy (%) range of calibration standards	Calibration range (µg/mL)	Correlation coefficient (r^2)
Homovanillic acid_1	181.0/ 122.0	106	11	87.7 – 108	0.25-150	0.989
Homovanillic acid_2	181.0/ 137.0	94.5	5.9	91.9 – 113	0.50-150	0.992

Table 6. Quantitative performance of the urine-spiked QC samples for the analysis of homovanillic acid using the QTRAP 6500+ system. Five replicate (n=5) samples were prepared and analyzed at 1.5, 20, and 150 µg/mL and values are shown for both the quantifier and qualifier ion transitions.

Compound name	Transition (m/z)	1.5 µg/mL		20 µg/mL		120 µg/mL	
		Accuracy	Precision (%CV)	Accuracy	Precision (%CV)	Accuracy	Precision (%CV)
Homovanillic acid_1	181.0/ 122.0	80.9	3.5	77.9	4.0	100	9.4
Homovanillic acid_2	181.0/ 137.0	79.4	6.3	77.7	3.6	94.1	9.8

Conclusions

This technical note demonstrated:

- An LC-MS/MS method for the analysis of homovanillic acid in urine using the QTRAP 6500+ system with 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 0.25 µg/mL in the urine-spike calibrators (n=3) with a mean accuracy of 106% and mean precision of 11%CV for the quantifier transition
- Linearity across the 0.25-150 µg/mL calibration range with an r^2 value for the quantifier transition
- Good quantitative performance in the urine matrix QC standards (n=5); mean accuracy was 77.7–100% and precision <9.8%CV

References

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