Urinary Vanillylmandelic, Homovanillic, and 5-Hydroxyindoleacetic Acids by LC/MS/MS

By dilution using an Agilent 1290 Infinity LC and 6460 Triple Quadrupole LC/MS

Application Note

Clinical Research

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Abstract

A highly sensitive and specific LC/MS/MS method was developed for the quantitation of vanillylmandelic acid (VMA), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) in urine. The analytical method uses a simple dilution procedure for sample preparation. It achieves the required functional sensitivity, and is capable of quantitating analytes over a sufficiently wide dynamic range. Reproducibility was excellent for all compounds (CV < 6 %), and all calibration curves displayed excellent linearity, with an $R^2 > 0.9998$.

Introduction

Liquid chromatography/triple quadrupole mass spectrometry (LC/MS/MS) is ideally suited for the rapid analysis of multiple analytes. A simple dilution sample preparation procedure was developed for the simultaneous determination of VMA, HVA, and 5-HIAA (Figure 1) in urine. Calibrators were created by spiking clean urine with various concentrations of each analyte. The chromatographic system consisted of a pentafluorophenyl (PFP) column and a mobile phase comprised of methanol and water containing 0.2 % formic acid. Quantifier and qualifier MRM transitions were monitored, and deuterated internal standards were included for each analyte to ensure accurate and reproducible quantitation.
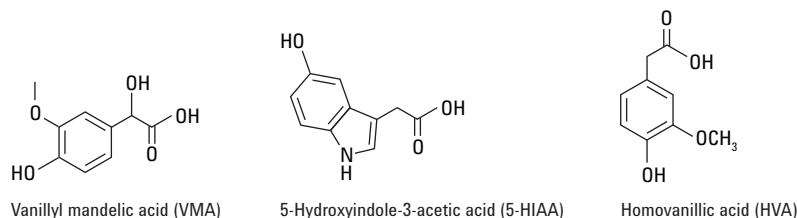

Vanillyl mandelic acid (VMA)   5-Hydroxyindole-3-acetic acid (5-HIAA)   Homovanillic acid (HVA)

Figure 1. Structures of VMA, 5-HIAA, and HVA.

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Experimental

LC method

The LC system consisted of an Agilent 1290 Infinity LC Binary Pump, a well-plate sampler with thermostat, and a temperature-controlled column compartment. If an LC system with different delay volume is used, the gradient may need to be adjusted and verified to reproduce the same chromatography.

MS method

The Agilent MS/MS system consisted of an Agilent 6460 Triple Quadrupole LC/MS with Agilent Jet Stream technology and Agilent MassHunter Software B.06.00.

Chemicals and reagents

Calibrators and internal standards were from Cerilliant, Round Rock, TX. DC Mass Spect Gold urine MSG5000 was from Golden West Biologicals, Temecula, CA. Lyphocheck controls 376 and 377 were from Bio-Rad. Burdick & Jackson. LC/MS-grade methanol was from VWR. Formic acid for mass spectrometry was from Sigma-Aldrich, Corp. St. Louis, MO.

Sample preparation

Standard calibrators were prepared by spiking DC Mass Spect Gold urine with each analyte. Serial dilutions in DC Mass Spect Gold urine were used to achieve the remaining standard calibrator concentrations. Calibrators, controls, and urine samples were diluted 1 in 10 with 0.2 % formic acid in water containing internal standards. Samples should be centrifuged or filtered, or both, before injecting onto the LC/MS/MS system.

Data analysis

Agilent MassHunter Quantitative Analysis (B.06.00) was used for data analysis. A 1/x weighting factor was applied during linear regression of the calibration curves. The quantitation using MassHunter Quantitative Software was performed by comparing chromatographic peak area ratio to a known concentration of the internal standards.

LC method parameters

Parameter	Value
Column	Agilent Pursuit PFP, 2 × 150 mm, 3 µm (p/n A3051150X020)
Guard column	Agilent Pursuit PFP MetaGuard, 200Å, 2 mm, 3 µm (3/pk, p/n A3051MG2)
Mobile phase	A) 0.2 % Formic acid in water B) Methanol
Column temperature	40 °C
Autosampler temperature	4 °C
Injection volume	20 µL
Needle wash	1:1:1:1 MeOH:ACN:IPA:H_2O + 0.1 % formic acid (flush port for 10 seconds)
Flow rate	0.3 mL/min
Gradient	Time (min) %B 0 15 0.5 15 2.5 60 3.0 95 6.0 95 6.1 15 9.0 15

MS method parameters

Parameter	Value
Ion mode	Agilent Jet Stream ESI-
Gas temperature	325 °C
Drying gas (nitrogen)	10 L/min
Nebulizer gas (nitrogen)	50 psi
Sheath gas (nitrogen)	300 °C
Sheath flow	11 L/min
Capillary voltage	3,500 V
Nozzle voltage	1,500 V
Q1/Q3 resolution	0.7 unit
Dwell time	20 ms
Delta EMV	200 V

Table 1. MS transitions.

Compound	Precusor	Product	Frag (V)	CE (V)	CAV (V)
HVA*	181.1	137	70	4	2
HVA	181.1	122	70	16	2
HVA-D5	186.1	142.1	70	4	2
VMA*	197	137	100	24	2
VMA	197	138	100	10	2
VMA-D3	200.1	140	100	24	2
5-HIAA*	190.1	146.1	70	6	2
5-HIAA	190.1	144	70	24	2
5-HIAA-D5	195.1	151.1	70	8	2

* = Quantification transition

Results and Discussion

Chromatographic separation of all analytes (Figure 2) was achieved through the use of an Agilent pentafluorophenyl (PFP) column. The separation of interferences present in urine from HVA and 5-HIAA are especially critical. Without proper separation by retention time, these compounds can cause interferences, leading to inaccurate quantitation.

To study matrix effects, a mix of all the analytes at 10 different concentrations were spiked into 0.2 % formic acid in water. Another set was prepared the same way, but spiked into blank urine. All the mixes were subjected to the dilution procedure with the internal standard solution and analyzed. Peak area ratios between the two solutions for each analyte were calculated and were found to vary from 91 to 93 % (Table 2). However, when calculating the concentrations with the internal standards using the peak area ratio corrections, very good corrections were obtained, varying between 93 and 115 % (Table 2). This confirmed that the internal standards corrected for variations.

Calibration standards and Bio-Rad Lyphocheck controls were prepared over a series of three days and three times in one day to establish both inter- and intra-day precision and accuracy. All three analytes had inter- and intra-day accuracies within 10 % and coefficient of variation values were less than 6 % for all concentrations within the linear range (Table 3 and Table 4). This analytical method had excellent linearity within the measured range of 0.078 to 100 mg/L with an R^2 value greater than 0.9998 (Figure 3).
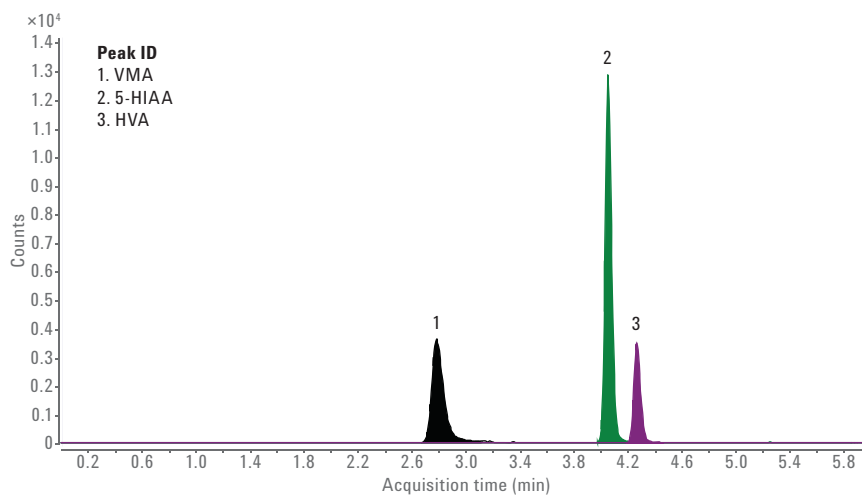

Figure 2. Chromatography for VMA, 5-HIAA, and HVA.

Table 2. Matrix effect measurements done at 10 different concentrations from 0.078 to 100 mg/L.

Compound	Matrix effects %*(n = 10)		Accuracies % with ISTDs corrections**(n = 10)		
	Average	SD	Range	Average	SD
VMA	91.5	6.4	95.6–108.3	100.0	3.9
5-HIAA	93.2	3.3	94.6–115.2	100.0	5.8
HVA	91.3	1.5	92.9–103.9	100.0	3.1

* Peak areas from urine spiked compared with 0.2 % formic acid-H_2O solutions spiked.
** Calculated concentrations of urine spiked with ISTD corrections versus theoretical concentrations.

Table 3. Summary of analyte performance for VMA, 5-HIAA, and HVA.

Compound	R^2	Concentration (mg/L)	Concentration (µM/L)	Accuracy (%) n = 3	Intraday CV (%) n = 3	Interday CV (%) n = 5
VMA	0.9999	0.078	0.39	106.8	3.9	3.9
		12.5	63.1	100.1	1.3	1.7
		100	504.6	100.5	0.6	0.5
5-HIAA	0.9998	0.078	0.41	109.7	3.4	3.4
		12.5	65.4	99.1	2.2	1.7
		100	523.1	100.8	0.1	0.1
HVA	0.9999	0.078	0.43	102.2	4.2	3.2
		12.5	68.6	99.9	1.7	1.3
		100	548.9	100.5	0.4	0.3

Table 4. Results of Bio-Rad QC run by LC/MS/MS (range determined by Bio-Rad using HPLC). All measurements are in mg/L (n = 5 over 3 days).

Compound	Level 1			Level 2		
	Range (HPLC)	Measured	CV (%)	Range (HPLC)	Measured	CV (%)
VMA	2.1–3.1	2.5	2.3	11.2–16.8	14.6	2.4
5-HIAA	2.2–3.4	2.8	2.0	20.8–31.2	27.6	2.8
HVA	1.0–1.4	1.3	5.8	13.0–19.6	15.8	3.9

Conclusions

An analytical method was developed for quantifying vanillylmandelic acid (VMA), homovanillic acid (HVA), and 5-hydroxyindoleacetic acid (5-HIAA) in urine. A simple dilution procedure is used for simultaneous determination of all three analytes with excellent recoveries, reproducibility, and accuracy.
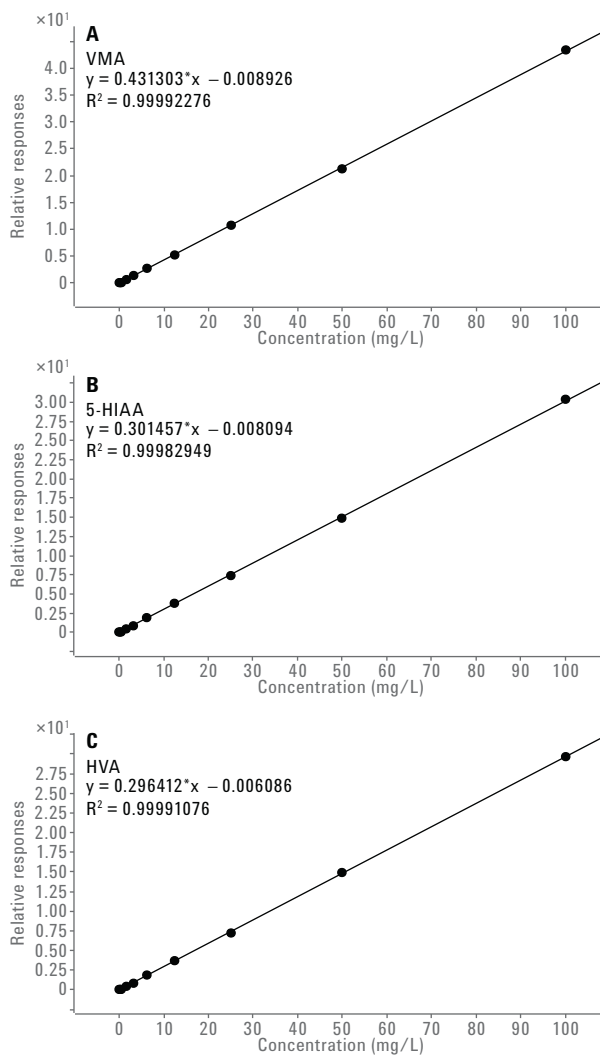
A
VMA
$y = 0.431303*x - 0.008926$
$R^2 = 0.99992276$

B
5-HIAA
$y = 0.301457*x - 0.008094$
$R^2 = 0.99982949$

C
HVA
$y = 0.296412*x - 0.006086$
$R^2 = 0.99991076$

Figure 3. Calibration curves for (A) VMA, (B) 5-HIAA, and (C) HVA.

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