One SLE to measure them all: Two-part elution for analysis of urinary HIAA, HVA and VMA

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BACKGROUND:

The monoamine acids 5'-hydroxyindoleacetic (HIAA), homovanillic (HVA), and vanillylmandelic (VMA) are metabolites of serotonin and the catecholamine neurotransmitters epinephrine, norepinephrine, and dopamine. Laboratory measurement of these acids in urine is used to assess overproduction of the parent amines due to carcinoid and neuroendocrine tumors (neuroblastoma, pheochromocytoma, paraganglioma).

In our laboratory, urinary HIAA, HVA, and VMA are measured using liquid chromatography with electrochemical detection after sample filtration and dilution. The test is easy to perform; however, the assay utilizes outdated instrumentation and is subject to interference, in part due to lack of selectivity in sample preparation and limited specificity of the electrochemical detection method. Moreover, the long injection to injection time of 21 minutes limits capacity (compromises turn-around time).

OBJECTIVE:

To develop a robust, high-throughput, selective and cost-effective method for the simultaneous analysis of urinary HIAA, HVA and VMA by LC-MS/MS.

RESULTS:

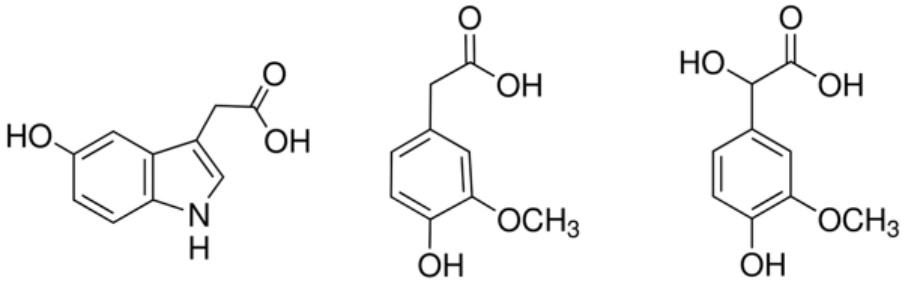
1. Method development

1.1. Sample preparation

All three analytes are small polar acids (see **Figure 1**), which suggests an anion exchange sorbent as the sample cleanup material of choice. As a starting point, we followed the Biotage application note for simultaneous extraction of HIAA, HVA and VMA from synthetic urine on

Evolute® Express AX. We also tested several additional combinations of dilution and wash solvents. All of the tested conditions yielded total process recoveries no higher than 25%. Additionally, signal for the internal standard (IS) HIAA-d5 was disproportionally low and many extra peaks were observed on several analyte and IS transitions.

Figure 1. Compound structures, pK_a and logP values.



HIAA	HVA	VMA
pK_a 4.22	pK_a 3.74	pK_a 3.11
logP 1.41	logP 1.15	logP 0.43

As the goal was to extract all three analytes simultaneously, we decided to explore supported liquid extraction (SLE). The SLE process is analogous to traditional liquid-liquid extraction (LLE) and utilizes the same water immiscible solvent systems for analyte extraction. However, instead of shaking the two immiscible phases together, the aqueous phase is immobilized on an inert diatomaceous earth based support material and the water immiscible organic phase flows through the support, alleviating many of the liquid handling issues associated with traditional LLE such as emulsion formation. Using a fast, simple **load-wait-elute** procedure, supported liquid extraction using ISOLUTE® SLE+ products provides inherently cleaner extracts than other simple sample preparation techniques such as 'dilute and shoot.' Due to the rapid partition and equilibration of analytes into fresh solvent as the elution solvent passes through the SLE column, extraction efficiency compared to LLE is increased. As a result recoveries are often higher and demonstrate better reproducibility from sample to sample. (1)

In order to promote partitioning of analytes into the water-immiscible organic phase in supported liquid extraction, the charge on any acidic or basic groups should be suppressed wherever possible. For analytes with acidic functional groups, such as our analytes of interest, this generally involves sample pretreatment with abundant amount of acid (typically formic acid, FA)

prior to loading. pH control is most important for polar analytes that are only sparingly soluble in solvents when charged. Using a saturated salt solution, e.g. sodium chloride (brine), is an additional strategy for forcing polar analytes into the organic phase and thus increasing extraction efficiency (1).

We took the above guidelines as a starting point and chose ethyl acetate, thought to be the best match for the highly polar character of the analytes, as an elution solvent. After a few initial experiments, however, we knew this was going to be a challenging method development. Sample pretreatment with 20% FA in water or brine yielded reasonable recovery for VMA, while HIAA and HVA recoveries were low. Conversely, using brine without FA, HIAA and HVA were recovered at 35-40%, whereas VMA recovery was very low. As in the case of anion exchange based sample cleanup, there were significant and disproportional losses of HIAA-d5 signal when 20% FA was in sample pretreatment.

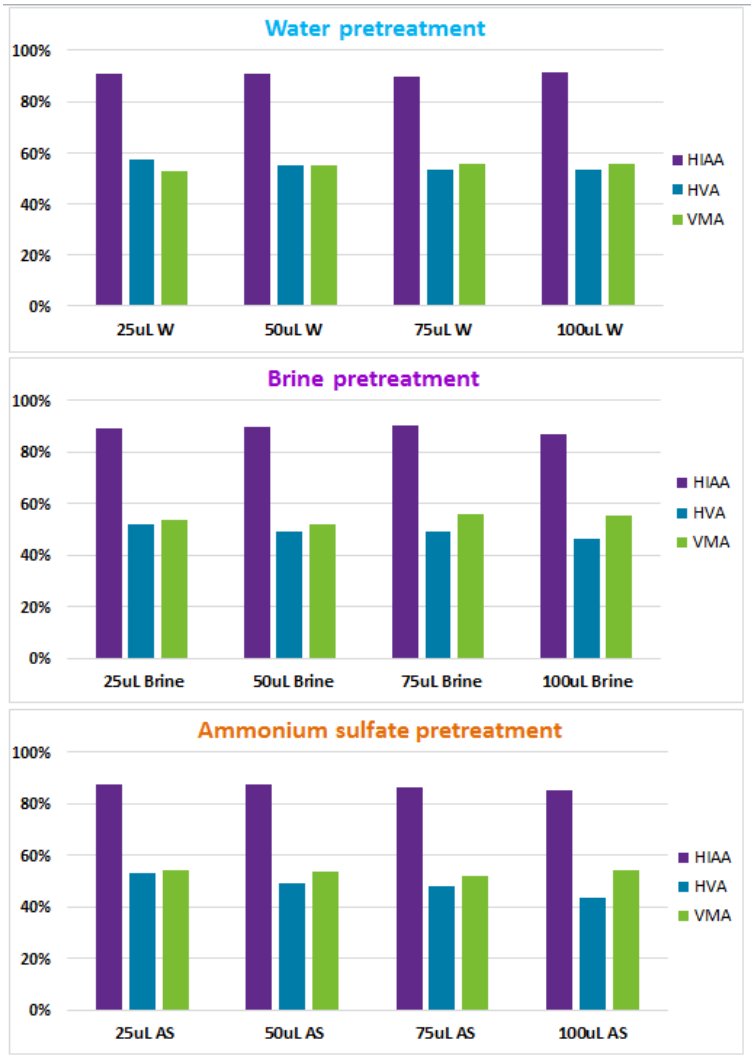
Next, various concentrations of FA (0.1% - 5%) in brine as pretreatment, followed by ethyl acetate elution, as well as pretreatment with brine alone, followed by elution with 5% FA in ethyl acetate, were tested in an attempt to find conditions under which all compounds could be extracted with sufficient recovery. It was found that the recovery of VMA increased with increasing %FA with a plateau at about 2-3%, while HIAA and HVA recoveries continued to decrease in the same direction. Furthermore, HIAA-d5 signal was adversely affected at all FA concentrations used.

Additionally, several other sample pretreatment/wash step/elution combinations tested, including pretreatment with 2% ammonium hydroxide in brine, 2%-20% isopropanol in ethyl acetate elution and wash with 0.1%TEA in hexane, did not yield promising results.

As the best recoveries of HIAA and HVA were achieved under neutral conditions and the presence of an acid (in pretreatment or elution) was necessary to extract VMA, we gave up on truly simultaneous extraction of all the analytes and decided to use the next best approach – a two-part elution. The sample was loaded under neutral conditions, HIAA and HVA were eluted with ethyl acetate into one collection plate and VMA was eluted with 2% FA in ethyl acetate,

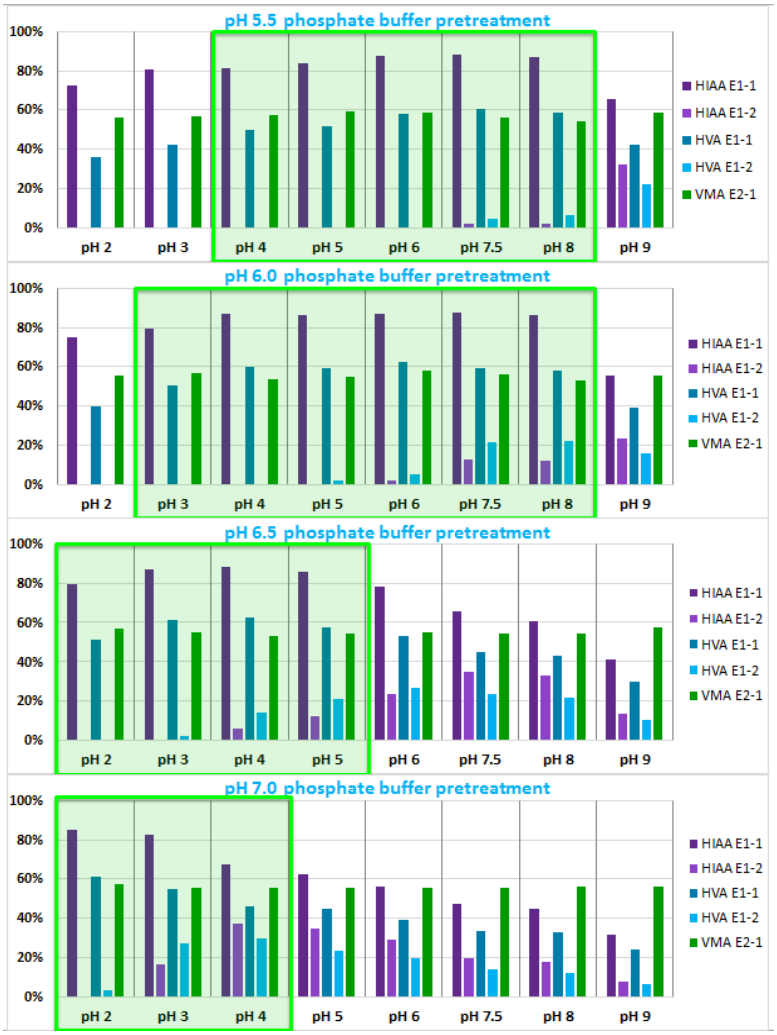
yielding >50% total recoveries (including matrix effects) for all compounds. As HIAA-d5 seemed to be sensitive to the presence of FA, the two sets of reconstituted eluates were analyzed separately using LC-MS/MS. Three solvents (water, brine and saturated ammonium sulfate) were tested for use as aqueous pretreatment at varying volumes (25-100 µL). The results for a 100 µL urine sample on ISOLUTE® SLE+ 200 µL are shown in **Figure 2**. The data clearly shows that there was no appreciable difference in recoveries using the three aqueous pretreatments or the various pretreatment volumes. This indicates that the presumed salting out effect of the saturated salt solution does not play a role in the recovery of HIAA, HVA and VMA in this case.

Figure 2. The effect of pretreatment with water, brine and saturated ammonium sulfate at varying volumes (25-100 µL) on analyte recovery.



Our specimen of choice for this analyte panel is unpreserved urine. The urine specimen pH may vary, however, including pH extremes resulting from clients sending acid- or based-preserved samples, which could affect analyte recoveries. To eliminate the need to test/adjust the pH of each specimen, we explored adjusting the urine pH with a phosphate buffer as part of the pretreatment. A spiked urine pool was divided into several aliquots and each aliquot was pH adjusted to create a pH scale from 2 to 9. Sodium phosphate buffers at several concentrations and pH values were used to pretreat each pH-adjusted urine sample. The optimal buffer concentration was found to be 200 mM. **Figure 3** shows the influence of buffer pH on analyte recoveries at varying urine pH**.** Phosphate buffer at pH 6 facilitated the highest recoveries over the broadest range of urine pH values. Note: Two aliquots were collected and analyzed for both elution 1 (2×1000 µL ethyl acetate) and elution 2 (2×800 µL 2% FA in ethyl acetate).

Figure 3. Analyte recovery as a function of pretreatment buffer pH.



Although ethyl acetate as the water-immiscible extraction solvent was quite effective at recovering the analytes, it unfortunately also recovered a fair amount of the signal suppressing sample matrix. We therefore tested methyl-*tert*-butyl ether (MTBE) alone and in mixtures with ethyl acetate for improved extract cleanliness. Underloading of the aqueous content (sample + pretreatment) as another strategy for reducing matrix effects was tested concurrently. **Figure 4** shows that using MTBE total recoveries (including matrix effects) improved for HVA and VMA. The results were further confirmed by a post-column infusion study on the ethyl acetate and MTBE extracts. As can be seen from **Figure 5,** both elution 1 and elution 2 extracts show less signal suppression using MTBE.

Figure 4. Analyte recovery as a function of elution solvent composition.

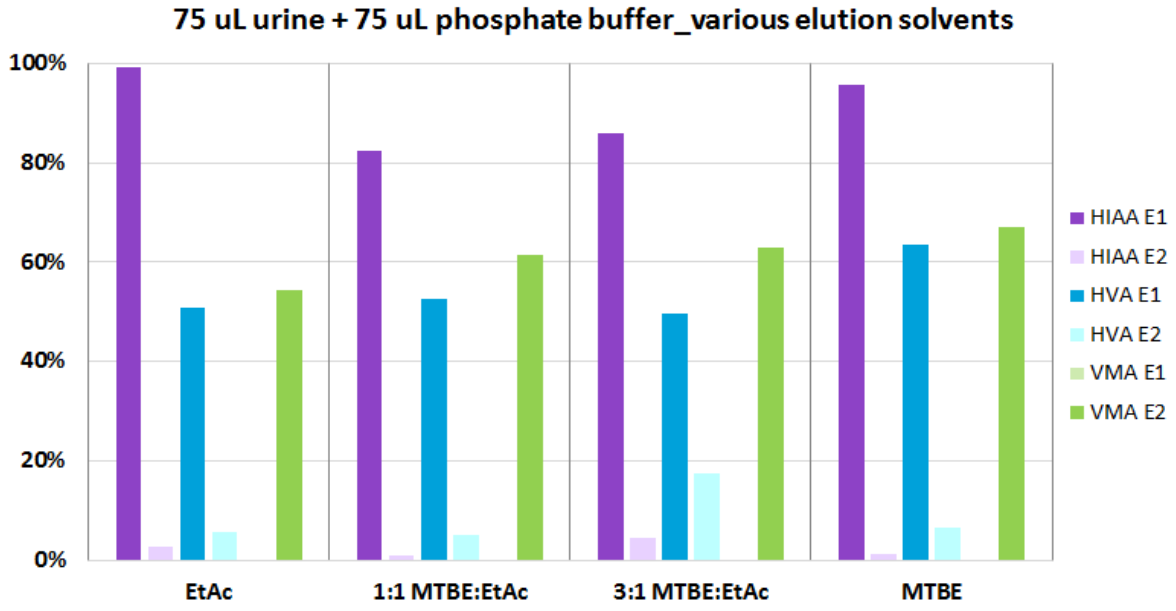
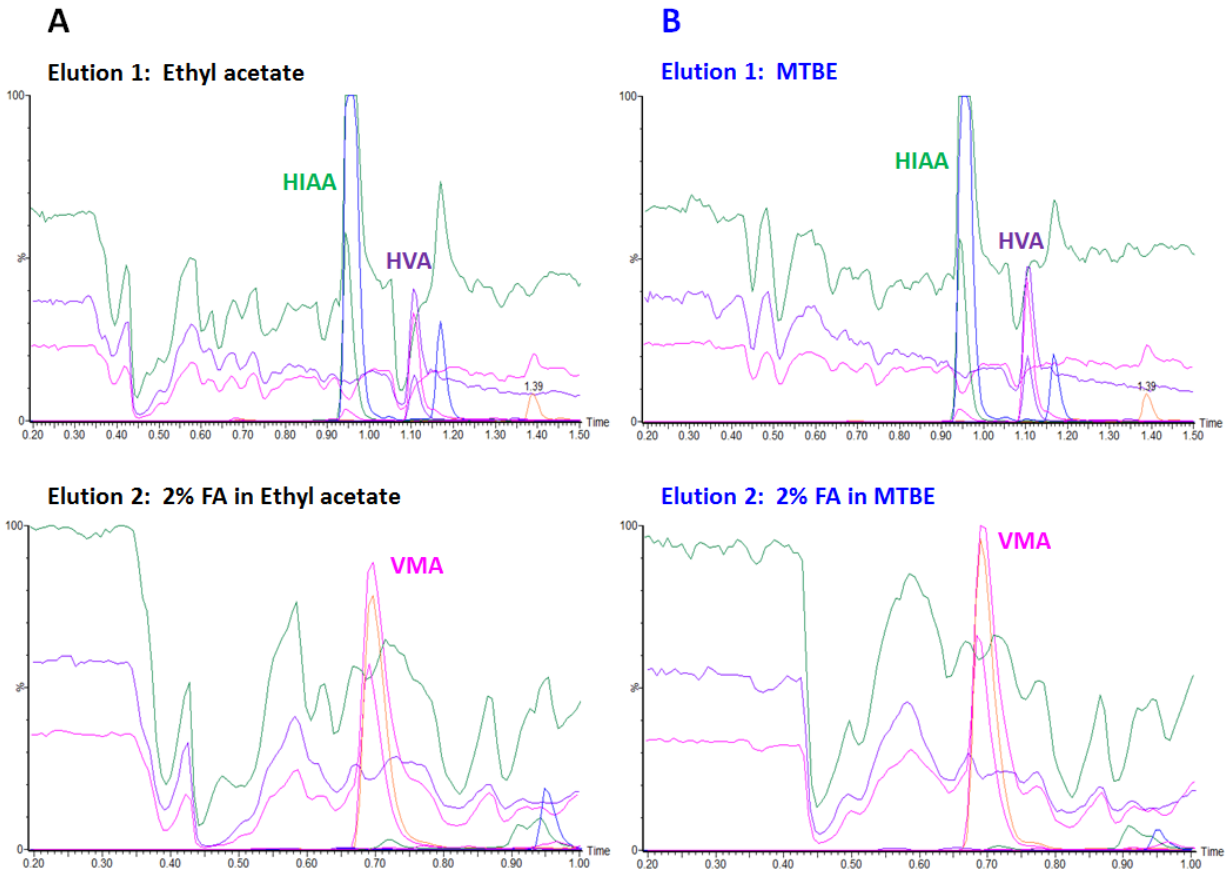


Figure 5. Post-column infusion profiles for Elution 1 and 2 extract with (A) ethyl acetate and (B) MTBE as the extraction solvents.



1.2. LC parameter optimization

The injection-to-injection time was shortened from 21 min to 4 min for both the Elution 1 and Elution 2 LC methods. After optimizing the sample preparation method, the initial LC gradients were adjusted to minimize the impact of sample matrix on analyte quantitation. Results not shown.

1.3. MS parameter optimization

As all three analytes are very small molecules, the selection of useful multiple reaction monitoring (MRM) transitions was not large. All viable MRM transitions were carefully monitored for interferences and those where interferences were found were eliminated from the MS method. Due to the large variations in signal intensities for the different MRM transitions of the three analytes of interest and their internal standards, the electrospray voltages and the

collision energies were not used at their optimal values for each individual compound, but were adjusted to make the signal intensities more uniform and to provide the highest sensitivity for the compounds/MRM transitions with the lowest signal intensities. This allowed us to use calibration standards with uniform values for all three analytes and thus simplify result reporting.

2. Final method

2.1. Sample preparation

The analytes were extracted from urine matrix using ISOLUTE® SLE+ 200 µL 96-well plate after pretreatment with IS mixture in 0.2 M sodium phosphate buffer, pH 6, and MTBE. HIAA and HVA were eluted in the first elution using MTBE, VMA was eluted in the second elution using 2% FA in MTBE. Eluates 1 and 2 were dried separately using forced nitrogen evaporation at 37 ºC and reconstitute with deionized water.

2.2. Instrumental parameters

Instrumental analysis was performed on a Waters Xevo TQMS Acquity® TQD mass spectrometer interfaced with Acquity® LC system consisting of a binary pump, degasser, column manager, sample manager, and autosampler. The instrument utilized an electrospray interface in positive electrospray ionization mode. Data was collected in MRM mode. Reconstituted eluates 1 and 2 were analyzed on a Phenomenex Kinetex XB-C18 3×50 mm column, 2.6 µm particles, using two separate 4-minute LC gradient method, both utilizing 0.05% FA in water as mobile phase A, and 0.05% FA in methanol as mobile phase B.

2.3.Method validation

2.3.1. Linearity, sensitivity, imprecision

Method linearity was evaluated by analyzing HIAA, HVA, and VMA linearity samples prepared by dilution of deidentified specimens with synthetic urine. Observed results were plotted against expected results to give the following regression equations:

HIAA: $y = 1.008x + 0.011$; observed error 3.7%. Linear from 0.4 to 100.0 mg/L.

HVA: $y = 1.028x – 0.021$; observed error 5.9%. Linear from 0.4 to 100.0 mg/L.

VMA: $y = 1.025x – 0.032$; observed error 3.8%. Linear from 0.4 to 100.0 mg/L.

Method sensitivity was evaluated by analyzing HIAA, HVA, and VMA linearity samples prepared by dilution of deidentified specimens with synthetic urine. A curve was fitted to obtain an estimate of the coefficient of variation (CV) as a function of the mean. The target CV was 20%. The limit of quantitation (LOQ) was defined as the lowest concentration for which the CV was within 20% and the concentration was within 20% of the expected value. The LOQ was estimated to be 0.4 mg/L for all three compounds.

Method imprecision was evaluated by analyzing two replicates per run (front and back of the sequence), one run per day, of Level I and Level II controls over 8 days**.** Results are given in **Table 1.**

Table 1. Within-run, between-run/day and total imprecision.

Quality Control	**Mean (mg/L)**	**Within-run CV**	**Between-run/day CV**	**Total CV**
HIAA				
Level I	3.77	2.5%	3.0%	4.0%
Level II	28.92	5.9%	2.9%	6.6%
HVA				
Level I	1.72	3.4%	2.7%	4.4%
Level II	15.44	6.6%	2.8%	7.2%
VMA				
Level I	3.13	3.0%	not calculated	3.0%
Level II	15.04	6.4%	2.3%	6.8%

2.3.2. Method comparison

Accuracy results, determined by a method comparison to the existing LC-ECD assay, are reported in

Table 2.

Table 2. Method comparison results for HIAA, HVA, and VMA.

Analyte	Deming regression	$S_{y/x}$	R	n
HIAA	y = 0.977x – 0.43	121.32	0.9822	74
HVA	y = 0.906x + 0.31	48.20	0.9949	73
VMA	y = 1.020x – 0.19	11.89	0.9966	114

SUMMARY AND CONCLUSIONS:

We have developed and validated a high throughput method for HIAA, HVA and VMA using SLE-based sample preparation and LC-MS/MS analysis. The sample preparation method development required significant diversion from basic manufacturer guidelines, but resulted in the use of a single sample preparation product to extract all three analytes. Sequential two-part elution of the analytes yielded not only cleaner extracts and higher recoveries for each analyte, but also prevented the signal loss of HIAA-d5, which is most likely due to the degradation of the deuterated IS in the presence of FA.

Additionally, all three analytes are not typically ordered together; HIAA-only requests represent approximately three quarters of the assay volume. Triage of specimens to "HIAA-only" (processed only to Elution 1 and analyzed by Elution 1 LC method) and "other analyte combinations" (processed to and analyzed for both elutions), can further decrease the sample extraction and LC analysis time for a majority of specimens submitted for this test.

REFERENCES:

1. Biotage SLE User Guide [http://www.biotage.com/literature/download/sle_user_guide_web.pdf?ref=http%3A%2F%2Fwww.biotage.com%2Fproduct-page%2Fisolute-sle-supported-liquid-extraction-products]