HHS Public Access

Author manuscript

Rapid Commun Mass Spectrom. Author manuscript; available in PMC 2015 October 07.

Published in final edited form as:

Rapid Commun Mass Spectrom. 2013 August 15; 27(15): 1697–1704. doi:10.1002/rcm.6621.

Comparison of High Resolution and Tandem Mass Spectrometry for the Analysis of Nerve Agent Metabolites in Urine

Elizabeth I. Hamelin[1,*], **William Bragg**[2], **Rebecca L. Shaner**[1], **Leigh L. Swaim**[1], and **Rudolph C. Johnson**[1]

[1] Emergency Response Branch, Division of Laboratory Sciences, National Center for Environmental Health, Centers for Disease Control and Prevention, Atlanta, GA 30341

[2] Oak Ridge Institute for Scientific Education, Oak Ridge, TN 37831

Abstract

Rationale—Although use is prohibited, concerns remain for human exposure to nerve agents during decommissioning, research, and warfare. High-resolution mass spectrometry (HRMS) was compared to tandem mass spectrometry (MS/MS) analysis for the quantitation of five urinary metabolites specific to VX, Russian VX, soman, sarin and cyclosarin nerve agents. The HRMS method was further evaluated for qualitative screening of metabolites not included in the test panel.

Methods—Nerve agent metabolites were extracted from urine using solid phase extraction, separated using hydrophilic interaction chromatography and analyzed using both tandem and high resolution mass spectrometry. MS/MS results were obtained using selected reaction monitoring with unit resolution; HRMS results were obtained using a mass extraction window of 10 ppm at a mass resolution of 50,000. The benchtop Orbitrap HRMS instrument was operated in full scan mode, to measure the presence of unexpected agents.

Results—The assessment of two quality control samples demonstrated high accuracy (99.5-104%) and high precision (2-9%) for both HRMS and MS/MS. Sensitivity, as described by the limit of detection, was overlapping for both detectors (0.2-0.7 ng/mL). Additionally, the HRMS method positively confirmed the presence of a nerve agent metabolite, not included in the test panel, using the accurate mass and relative retention time.

Conclusions—The precision, accuracy, and sensitivity were comparable between the current MS/MS method and this newly developed HRMS analysis for five nerve agent metabolites. HRMS showed additional capabilities beyond the current method by confirming the presence of a metabolite not included in the test panel.

[*]Corresponding Author Elizabeth I. Hamelin, Centers for Disease Control and Prevention, 4770 Buford Hwy, Mailstop F44, Atlanta, GA 30341, United States, Tel.: +1 770 488 7082, ehamelin@cdc.gov.

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Keywords

LC-MS/MS; HRMS; organophosphorus nerve agents; exposure assessment

Introduction

Nerve agents have been created and stockpiled for warfare purposes since their discovery in the 1930s. Although use of these compounds in recent warfare has been limited and is discouraged by the Chemical Weapons Convention [1], concerns remain that nerve agents will be used for non-sanctioned warfare or terrorist activities. Stockpiles throughout the world are gradually being decommissioned [1] and further laboratory research to improve treatments is being pursued [2, 3]. These activities may result in human contact with nerve agents; therefore, the continued ability to assess human exposure to nerve agents is needed.

The most common mass spectrometric approach for determining exposure to these specific compounds measures urinary nerve agent metabolites, which are hydrolysis products of the parent compounds [4-10]. Separation, identification and quantitation of these compounds has been reported using gas chromatography coupled with tandem mass spectrometry (GC-MS/MS), and GC coupled with single quadrupole mass spectrometry (GC/MS) [6,11]. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has also been used with either hydrophilic interaction liquid chromatography (HILIC) [7,8] or anion exchange chromatography [9]. All of these methods measured multiple nerve agent metabolites in urine at part per billion (ppb) to part per million (ppm) levels.

The majority of mass spectrometric methods used to identify exposure are targeted analyses, detecting only specific nerve agent metabolites. While this approach is selective and often sensitive, only compounds included in the method will be identified. Exposure assessment to sarin (GB), soman (GD), cyclosarin (GF), VX, and Russian VX (rVX) is common [7-9], but other nerve agent metabolites may be excluded, such as the metabolite from the nerve agent tabun (GA). As exposure symptoms are not specific to individual nerve agents, screening for metabolites not included in the quantitative panel may be needed to confirm nerve agent exposure. One such qualitative screen for nerve agent metabolites in urine has been reported using high resolution mass spectrometry [12].

With the advent of bench top HRMS instruments, cost is no longer prohibitive for the acquisition of this technology [13]; hence, many comparisons of MS/MS and HRMS have recently been reported in the literature [14-19]. The results indicated that the tandem MS and HRMS analyses were very comparable [14]. HRMS methods have been able to match the validation acceptance criteria for accuracy, precision, selectivity, sensitivity and matrix effects previously established using LC/MS/MS [17]. Not only can HRMS be used to quantitate using reference materials, it has also been reported to qualitatively screen for compounds without the use of reference materials. The confirmation of the presence of these unknowns was based on exact mass plus characteristic fragments [20] or based on search criteria including a specific elemental composition coupled with a mass confidence level of 95% [12].

This study compared the precision, accuracy, sensitivity and specificity of ion trap HRMS to quadrupole tandem MS for the analysis of five nerve agent metabolites in urine. A nerve agent metabolite, not included in the standard solution, was evaluated to qualitatively confirm the presence of this compound in urine using HRMS.

Experimental

Materials

Solvents used included HPLC-grade methanol and HPLC-grade acetonitrile, both purchased from Fisher Scientific (Pittsburgh, PA). Deionized water used was obtained from an in-house water purifier by Aqua Solutions (Jasper, GA). Ammonium acetate at 5M concentration was purchased from EMD Biosciences (LaJolla, CA). The following solutions were prepared volumetrically with Class I glassware and used for the extraction process: 90% acetonitrile/10% water; 75% acetonitrile/25% water and 95% acetonitrile/5% water. Mobile phase was prepared by mixing 86% acetonitrile with 14% 20 mM ammonium acetate prepared in DI water.

Calibrators and quality control samples were prepared by Cerilliant (Round Rock, TX) in synthetic urine containing the following compounds: VX acid, ethyl methylphosphonic acid; GB acid, isopropyl methylphosphonic acid; GD acid, pinacolyl methylphosphonic acid; GF Acid, cyclohexyl methylphosphonic acid, and rVX acid, 2-(methyl) propyl methylphosphonic acid. Structures are presented in Figure 1. The concentrations of the calibrators were 1, 2, 5, 10, 25, 50, 100 and 200 ng/mL. The quality control sample concentrations were 15 and 75 ng/mL and a blank quality control sample was also included. Proficiency testing materials were provided by O2Si (Charleston, SC) in pooled urine. Individual urine samples and pooled urine were purchased from Tennessee Blood Services (Memphis, TN).

Internal standard was also provided at a concentration of 500 ng/mL prepared in water containing the following compounds: ethyl-D_5 methylphosphonic acid; isopropyl-$^{13}C_3$ methylphosphonic acid; pinacolyl (trimethylpropyl-$^{13}C_6$) methylphosphonic acid; cyclohexyl-$^{13}C_6$ methylphosphonic acid; and 2-(methyl)propyl (methylphosphonyl-^{13}C, D_3) methylphosphonic acid.

N,*N*-dimethylethylphosphoramidic acid (GA acid) was purchased from Los Alamos National Laboratory (Los Alamos, NM). A stock solution was prepared by the dilution of 1.6 mg of GA acid into 1.6 mL of methanol. This solution was diluted into pooled urine, creating spiked matrix samples at the following concentrations: 100, 25 and 5 ng/mL. All calibrators and solutions were stored at −70 °C prior to use.

Sample preparation

Urine samples were diluted in acetonitrile and separated using solid phase extraction. One hundred microliters of urine sample or calibrator was added to 25 μL of isotopically labeled internal standard in a 2-mL 96 well plate. This mixture was placed on a Caliper i1000 Sciclone (Hopkington, MA) for automated extraction. The i1000 incorporated positive pressure and evaluated the SPE plate following solvent addition for residual solvent,

ensuring that each SPE well had been evacuated before continuing to the next step. A Strata Si 96-well plate SPE by Phenomenex (Torrance, CA) with a 100 mg bed of 55 μm particle size SPE sorbent was conditioned by the i1000 with 1 mL of 75% acetonitrile/25% water followed by a second conditioning step of 1 mL of acetonitrile. The sample mixture was then diluted with 1000 μL of acetonitrile and mixed by drawing up the sample three times into the pipette tips. This mixture was loaded onto the conditioned SPE plate. The impurities were eluted from the SPE with two wash steps: 1) 1 mL acetonitrile and 2) 1 mL of 90% acetonitrile/10% water. Following the wash steps, a fresh 2-mL 96-well plate was placed under the SPE plate to receive the eluted sample. The cleaned sample was eluted with 1 mL of 75% acetonitrile/25% water from the SPE plate. The sample plate was then placed in a Biotage 96-Well Turbovap for concentration at 70 °C; the nitrogen flow was started at a low flow of ~15 standard cubic feet per hour and gradually increased to 70 standard cubic feet per hour to facilitate the dry down process without causing well-to-well contamination. The dried extracts were reconstituted using 100 μL of 95% acetonitrile/5% water and vortexed to mix using a plate vortexer (Wellmix, Thermo Labsystems, Waltham, MA). The reconstituted samples were then transferred to a 300 μL well plate and sealed using heated foil (Thermo Scientific, Waltham, MA) in preparation for chromatographic separation.

Instrumental analysis-Tandem MS

The reconstituted samples were injected onto the LC system consisting of two 1100 LC pumps, two degassers, a 10-port switching valve, column oven and autosampler by Aglient (San Jose, CA). The isocratic mobile phase was 84% acetonitrile mixed with 16% 20 mM ammonium acetate. A column flow rate of 500 μL/min with a ramp in flow rate to 1000 μL/min was used to clear the column following the elution of the compounds. The HILIC column was a 2.1 × 50 mm Waters HILIC column with a 3 μm particle size maintained at a temperature of 40 °C. A 5 μL injection volume was used, which was lower than the previously published method using this sample preparation protocol [7] due to the translation from an AB Sciex 4000 MS/MS to an AB Sciex 5500 MS/MS.

The analytes were eluted into the AB Sciex 5500 tandem mass spectrometer (Framingham, MA) and ionized using negative electrospray ionization. Each compound was identified using multiple reaction monitoring (MRM) mass spectrometry, with the following parameters independently optimized: collision energy, declustering potential, cell exit potential and entrance potential. Two transitions per analyte were monitored to ensure specificity. Stable isotopically labeled internal standards were used to increase precision and accuracy by compensating for preparation losses and ionization suppression experienced by each analyte. The internal standards are identified by one MRM transition. Transitions are identified in Table 1.

Instrumental analysis-HRMS

The reconstituted samples were injected onto the LC system consisting of two LC pumps, degasser, column oven and autosampler by Shimadzu (Columbia, MD). The LC parameters were set the same as for the tandem MS analysis with the exception of a 35 μL injection volume. The analytes were eluted onto a Thermo Exactive (Waltham, MA) including a Heated Electrospray Ionization source (HESI-I) operated in negative ion mode. The heater

temperature for ionization was set at 300 °C, with sheath flow rate at 60 and auxillary flow rate at 20. The following voltages were used: spray voltage (4.5 eV), capillary voltage (−25 eV), tube lens voltage (−70 eV), and skimmer voltage (−22 eV) as determined through infusion of least sensitive analyte with automated optimization. Automated gain control (AGC) set at Balanced (1 E6), with the maximum injection time at 100 ms. Resolution was maintained at high (50,000) throughout the study. The instrument was externally calibrated every three days as recommended by the manufacturer. Full scan data was captured for each run and a mass extraction window of 10 ppm around the calculated exact mass, presented in Table 1, was used for identification and quantitation of all compounds.

Quantitation

Quantitation was based on a standard curve comprised of eight calibrators ranging from 1-200 ng/mL. The standard response was divided by the internal standard response to normalize any sample losses that occurred during preparation, separation and ionization. This value was charted against the known concentration of the calibrators. Each calibration curve must have attained a correlation coefficient of 0.990 or greater to be accepted. Quality was assured through the analysis of two positive quality control samples and one negative quality control sample. The positive quality control samples were characterized with a minimum of 20 independent analyses to determine the acceptable limits for each compound and level.

Sensitivity Comparison

The lowest reportable limit for this analysis was 1 ng/mL as defined by the lowest calibrator; the highest reportable limit was defined by the highest calibrator at a concentration of 200 ng/mL. Limits of detection, used for comparison of sensitivity, were determined for all analytes using results obtained for the three lowest standards and the blank quality control sample. The standard deviation of twenty separate analyses of these standards was charted relative to the concentration and the y-intercept of the best fit line was multiplied by three as defined by Taylor [21]. This value was the estimated limit of detection used for this method comparison.

Results and Discussion

The HRMS ionization parameters were established through flow injection analysis of GB Acid, since this compound was the least sensitive in the initial evaluation. The following parameters, integral to ion formation, were optimized in this experiment: HESI heater temperature, sheath gas flow, aux gas flow, capillary temperature and spray voltage. To achieve the high sensitivity necessary, the settings that produced the largest number of ions in the mass spectrometer which still maintained accurate mass measurements were selected. This included the evaluation of the Automated Gain Control (AGC) at the three available settings: Ultimate (5e5), Balanced (1e6), High (3e6). The data obtained with both the High and Balanced AGC settings had the most peak intensity; however, the data obtained with the Ultimate AGC setting resulted in insufficient sensitivity. The Balanced AGC setting was selected since it was the ideal balance between sensitivity and mass accuracy. Resolution was also evaluated at following three settings: Enhanced (25,000 @ 4 Hz), High (50,000 @

2 Hz) and Ultra High (100,000 @ 1 Hz). Complex matrices can result in unresolved interferences; therefore a minimum resolution of 25,000 is required, with a resolution of 50,000 being ideal according to Kaufmann [15]. The resolution setting affects the speed of data collection and directly impacts the number of points across a peak, which is critical for quantitative analyses. As the typical peak for this analysis was approximately eight seconds wide, the High resolution setting was selected in order to acquire the best resolution data with more than ten points across each chromatographic peak.

Calibrators and quality control samples were prepared for analysis by both LC/MS/MS and LC/HRMS instruments. The chromatograms from both instruments are presented in Figure 2. Twenty sets of calibrators with corresponding quality control samples were analyzed over a period of 43 days using both instruments and three analysts (Table 2). The resulting precision and accuracy of the quality control samples for both detectors demonstrates these methods to be within the specifications for bioanalyical methods as defined by the US Food and Drug Administration [23].

The high resolution mass spectrometric scan data was collected from 100 – 1500 m/z. It was noted that the scan range selection can significantly impact the accuracy of quantitative results obtained with this system. Since the instrument automatically scans from the start mass plus 15 times that mass, as indicated by the manufacturer, the selected scan range was not truncated. The automatic gain control, necessary for minimizing space-charge induced mass error [22], can negatively impact the number of desired ions filling the Orbitrap. If an undesired ion dictated the fill time, less of the desired ions would be included for measurement. An improvement in accuracy of 5-10% was observed for both GD Acid and GF Acid when the selected scan range excluded the abundant acetate ion (59 m/z). Additionally, the linearity of the analysis and the detection of the low calibrator, in particular for VX Acid and GB Acid, were also improved.

To ensure comparability between the instruments across the entire reportable range, nineteen pooled urine samples spiked with five nerve agent metabolites were prepared in triplicate and analyzed on both systems. The variability, as described by the relative standard deviation, did not exceed 13.4% for HRMS and 9.4% for MS/MS, indicating the reproducibility of both analytical methods within FDA specifications [23]. The results were compared to one another by the dividing the HRMS result by the MS/MS result and multiplying the resulting ratio by 100; a ratio of 100% indicates perfect reproducibility (Table 3). For all compounds the average ratio was within 90 – 110%, indicating high comparability between instruments.

The mass extraction window (MEW) was established during method development as recommended in the literature [24], to minimize potential interference while maintaining sufficient signal for this application. Xia, et al, incorporated a calculation to determine the maximum MEW required for a given mass as a function of the resolving power [24]. This approach was applied to all compounds included in this method; the calculated maximum MEW ranged from 15-18 ppm. Additionally, the required mass accuracy to result in a single elemental composition for each nerve agent metabolite was determined to be similar to previous studies [25]. This assessment resulted in 5-7 potential compounds within a 20 ppm

mass accuracy; however, with a mass accuracy of 10 ppm only a single elemental composition option remained for all compounds. Urine samples from persons with no exposure to nerve agents were evaluated for potentially interfering masses to the compounds of interest. No contributing species were detected at a MEW of 10 ppm for all five nerve agent metabolites. Given the lack of calculated and measured interferences for these compounds, the use of 10 ppm MEW was selected for quantitation in extracted urine.

Background response for the GB-Acid internal standard was observed to increase over time. Since a smaller MEW often minimizes interferences by eliminating adjacent masses, the results were assessed using a 7 ppm MEW (Figure 3), in addition to the established 10 ppm MEW. The internal standard background response was reduced with this selection; however, the evaluation of five calibration curves and quality control samples resulted in no improvement of precision or accuracy. Further investigation determined that the background response could be minimized with regular cleaning of the ion transfer tube.

It is essential in clinical samples to minimize false-positive results which may occur from unknown interferences. Seventy-two individual urine samples with no known exposure to nerve agents were spiked with internal standard, prepared as indicated above, and analyzed using both instruments. The detection of a peak in these urine samples would indicate the presence of an endogenous interference; the quantitation of an interference peak above the lowest standard would result in a measurable false positive. HRMS analysis identified no quantitative responses above the reportable limit for 72 individual urine samples. MS/MS analysis identified one quantitative response at 1 ng/mL for GB Acid, but no other peaks were detected above the reportable limit. Given this information, HRMS resulted in no false positives and MS/MS resulted in one false positive in 72 unexposed urine samples. It should be noted that even though different LC systems were used for this analysis, the retention as measured by the retention factor, k', was the same on both systems, indicating that the differences were a result of the mass spectrometric detection, not the chromatographic separation.

Sensitivity, as defined by the estimated limit of detection (LOD), was calculated for all five analytes for both instruments and is presented in Table 4. All LODs were within the same order of magnitude. With little difference between the estimated LODs for MS/MS and HRMS results, the sensitivity of both detectors can be described as equivalent for this evaluation.

HRMS relied on the exact mass to identify each compound; therefore, mass accuracy was paramount. The mass accuracy was evaluated throughout the concentration range and did not deviate more than 6 ppm from the nominal value. This mass stability indicated that regardless of the concentration, the analyte of interest would be identified correctly and included in the mass extraction window.

Confirmation of the analyte measured is often achieved through the measurement of additional compound specific ions [25, 26]. The tandem MS method measured two separate product ions for each of the five nerve agent metabolites included in this panel; however, the HRMS method only measured the one ion per compound, and lacked the measurement of a

confirmation ion. An additional mass spectrometric period to measure confirmation ions will needed together with the procurement of different isotopically labeled internal standards. The currently available isotopically labeled internal standards form the same product ions as the native compounds, resulting in 95, 77 or 79 m/z, excepting RVX Acid.

The HRMS analysis has the added ability to screen for nerve agent metabolites or similar compounds not included in the current panel. GA acid was spiked into pooled urine at three concentrations of 100, 25, and 5 ng/mL. These samples were spiked with the internal standard mixture, extracted and analyzed in the same manner as indicated previously. The calculated exact mass for the ionized GA Acid, 152.0476 m/z, was extracted for all samples using a MEW of 10 ppm. The extracted chromatograms demonstrated an increase in signal corresponding to spike concentration (Figure 4). A unique product ion of 124.0460 m/z was also identified for this compound. Additional confirmation that this peak resulted from GA Acid was the relative retention time, which fell between GF Acid and GD Acid, correlating with previous studies [13].

Conclusions

The strength of HRMS method developed in this study is the ability to quantitate and screen for additional compounds in a single method. The high resolution mass spectrometer obtained comparable precision, accuracy, sensitivity and specificity to the tandem mass spectrometric method for the quantitation of five nerve agent metabolites resulting from exposure to sarin, soman, cyclosarin, VX and Russian VX. Furthermore, the HRMS was able to identify the presence of another nerve agent metabolite, not included in the standard panel, based upon accurate mass, specific product ion, and relative retention time. Search criteria reported elsewhere [12] may be applied using this instrumentation for the detection of other nerve agent metabolites in the event of a suspected exposure.

The tandem MS method excels at confirmation for this assay, as it meets the criteria for a confirmatory method [26]. Further adjustments to the HRMS method will be required for qualification as a confirmatory method, including the addition of another mass spectrometric period to measure confirmation ions and the synthesis of different isotopically labeled internal standards for four of the five compounds, which is cost prohibitive at this time.

Acknowledgements

The authors appreciate the valuable advice of Dr. Samaret Macek and the information provided by Gwen Tran regarding the spiked urine materials.

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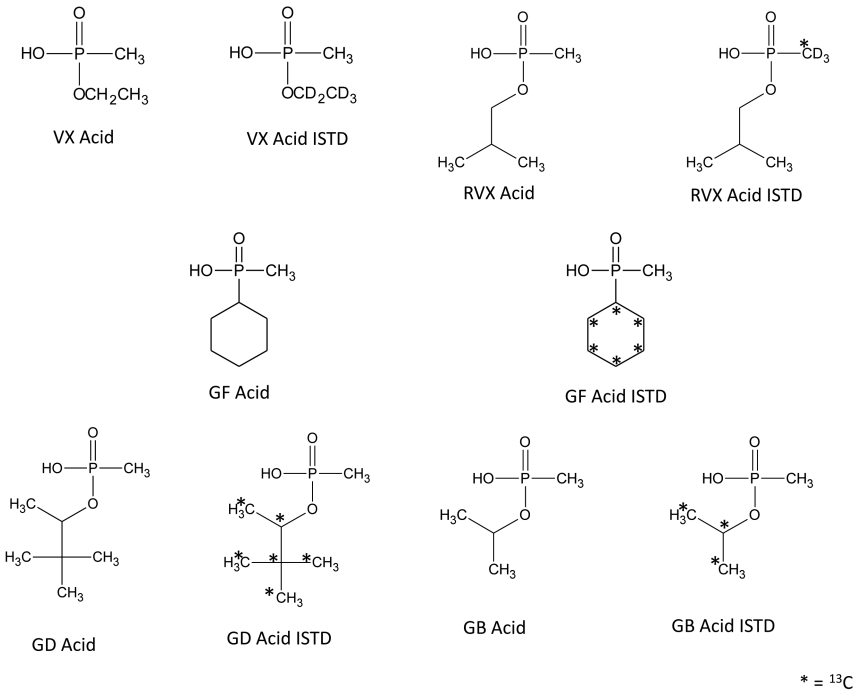

Figure 1.
Structures of nerve agent metabolites and corresponding internal standards.

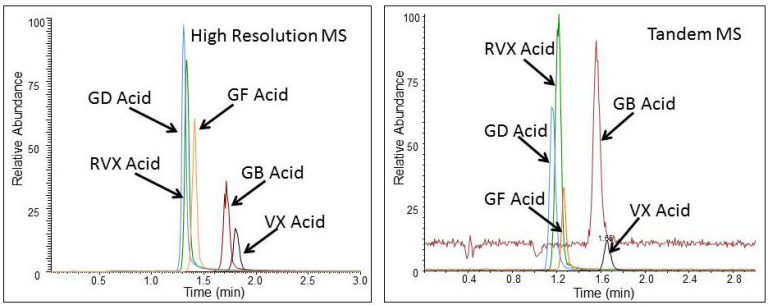

Figure 2.

Chromatograms for quality control sample (15 ng/mL) for both high resolution and tandem mass spectrometry

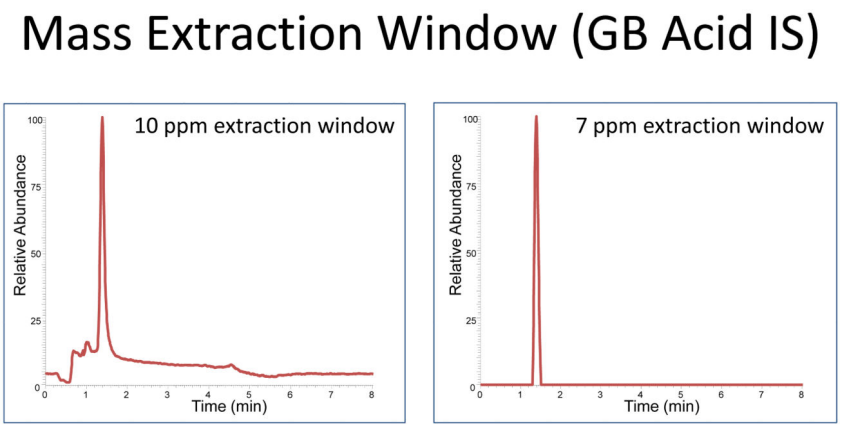

Figure 3.
Mass extraction window of 10 and 7 ppm for GB Acid internal standard (140.0469 m/z) in matrix

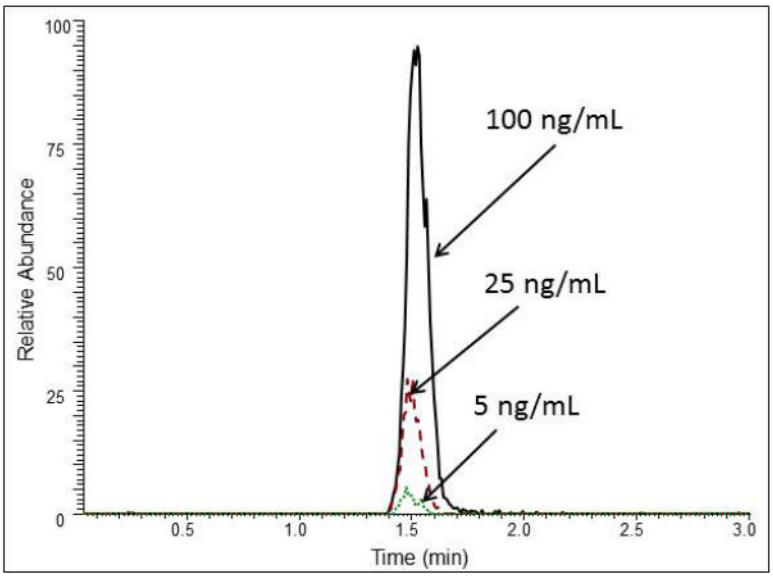

Figure 4.
Extracted urine spiked with GA Acid, mass extraction window of 10 ppm

Table 1

Transitions and exact masses monitored for each analyte

Analyte	Mass Transition		Exact Mass Fragment (m/z)
	Precursor Ion (m/z)	Product Ion (m/z)	
VX Acid	123.0	95.0	123.02111
VX Acid, label	128.0	96.0	128.02857
GB Acid	137.0	95.0	137.03676
GB Acid, label	140.0	95.0	140.04696
RVX Acid	151.0	95.0	151.05241
RVX Acid, label	155 .0	99.0	155.07462
GD Acid	179.1	95.0	179.08371
GD Acid, label	185.1	95.0	185.10411
GF Acid	177.1	79.0	177.06806
GF Acid, label	183.1	95.0	183.08846

Table 2

Reproducibility and bias of quality control samples (n=20) evaluated using high resolution mass spectrometry (HRMS) and tandem low resolution mass spectrometry (MS/MS)

		QL (15 ng/mL)			QH (75ng/mL)		
		Mean	RSD	Accuracy	Mean	RSD	Accuracy
VX Acid	HRMS	15.6	3.9%	104%	74.7	2.5%	99.6%
	MS/MS	15.1	4.9%	100%	76.8	5.6%	102%
GB Acid	HRMS	15.4	7.9%	103%	76.5	9.3%	102%
	MS/MS	15.2	4.5%	101%	75.6	4.8%	101%
RVX Acid	HRMS	15.3	2.8%	102%	74.6	3.6%	99.5%
	MS/MS	15.2	4.2%	101%	76.1	5.5%	102%
GD Acid	HRMS	15.4	2.0%	103%	74.9	3.3%	99.8%
	MS/MS	15.1	4.1%	101%	77.2	5.4%	103%
GF Acid	HRMS	15.5	2.8%	103%	75.0	3.3%	100%
	MS/MS	15.0	3.7%	100%	75.9	5.1%	101%

Table 3

Comparison of the high resolution mass spectrometry result relative to the tandem mass spectrometry result for 19 spiked urine samples; a ratio of 100% indicates perfect reproducibility

	HRMS result/Tandem MS Result Average	RSD
GBAcid	90.5%	9.1%
GD Acid	106%	2.1%
GFAcid	96.5%	1.3%
RVX Acid	101%	5.2%
VX Acid	100%	6.6%

Table 4

Limits of detection (ng/mL) calculated using Taylor method (n=20)

	GB-Acid	GD-Acid	GF-Acid	RVX-Acid	VX-Acid
MS/MS	0.44	0.18	0.18	0.17	0.70
HRMS	0.62	0.14	0.19	0.21	0.40