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Ultra-performance liquid chromatography tandem mass spectrometry for determination of Direct Acting Antiviral drugs in human liver fine needle aspirates

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Abstract

An ultra-performance liquid chromatography with triple quadrupole mass spectrometry method was developed and validated for the determination of direct acting antiviral drug concentrations in human liver fine needle aspirates. Liver fine needle aspirate (FNA) biopsy samples were homogenized in acetonitrile to stabilize the analytes and precipitate protein. The acetonitrile supernatants were diluted with internal standards and mobile phase. Separation was achieved with a Waters Acquity BEH C18 column (50 X 2.1 mm, 1.7 μ m) with a gradient elution of 0.1% formic acid in water and acetonitrile. The total run time was 4.25 minutes. Detection of analytes was achieved using electrospray ionization (positive mode) and triple quadrupole selected reaction monitoring. Standard curve concentrations ranged from 12.5 to 5000 ng/mL for dasabuvir and the m1 metabolite of dasabuvir, 1.25 to 2500 ng/mL for ombitasvir and ritonavir, and 5.00 to 5000 ng/mL for paritaprevir. The intra- and inter-day accuracy and precision were less than 13.7% in low, medium, and high quality control samples. The validated method was applied to the analysis of a liver fine needle aspirate of a patient undergoing direct acting antiviral therapy for hepatitis C virus.

Keywords

dasabuvir; ombitasvir; paritaprevir; liver; fine needle aspirate; UPLC-MS/MS

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1. Introduction

The use of direct acting antiviral (DAA) drugs in the treatment of hepatitis C virus (HCV) infection is changing the way patients can live their lives [1–4]. Chronic HCV infection was originally treated with recombinant interferon α in which many patients experience relapse upon cessation of treatment[5]. Interferon based therapies are discarded in favor of DAA therapies. The DAA regimen of ombitasvir (OBV) co-formulated with paritaprevir (PTV) and the pharmacokinetic enhancer ritonavir (RTV) administered with dasabuvir (DSV) (3D regimen, Viekira Pak) has proven to be effective and well tolerated [6]. It is reported that the 3D regimen can produce sustained virologic response rates as high as 100% [4, 7].

Ombitasvir is a non-structural protein 5A inhibitor. Paritaprevir is a potent non-structural protein 3/4A protease inhibitor. Dasabuvir is a non-nucleoside, non-structural protein 5B polymerase inhibitor. Dasabuvir undergoes cytochrome P450 (CYP) mediated hydroxylation at the *tert*-butyl moiety to form the active M1 metabolite of dasabuvir (DSVm1)[8].

Ritonavir (RTV) is a CYP 3A4 inhibitor used to enhance the exposure of PTV which is a CYP 3A4 substrate [9]. RTV increases peak and trough plasma drug concentrations of PTV allowing for once daily administration. OBV, PTV, DSV, and RTV reach maximum serum concentrations at approximately four to five hours, and steady state concentration with approximately twelve days of dosing [10].

Since HCV is characterized by viral infection of the liver, it is important to understand the pharmacokinetic properties of DAAs in liver tissue. The least invasive technique with the lowest associated risk of morbidity for liver biopsy is by fine needle aspiration (FNA) [11, 12]. FNA biopsy can obtain sufficient liver tissue for drug analysis and is best suited for serial sampling versus conventional liver biopsy procedures.

We have further developed our previous publication on the UPLC-MS/MS measurement of PTV and RTV in rat liver [13] to include OBV, DSV, and DSVm1 in human liver FNA. The novelty of our work resides in the measurement of drug concentrations in human liver FNA biopsy. Only one other method has been described to measure PTV, OBV, and DSV in human plasma by UPLC-MS/MS[14]. However, that method lacked the m1 metabolite for DSV and described the application to human plasma where we seek to investigate liver drug concentration. Our group seeks to understand the importance of liver pharmacokinetics and its relation to plasma pharmacokinetics. Our developed and validated UPLC-MS/MS method to measure DSV, DSVm1, OBV, PTV, and RTV is fast, simple, sensitive, and specific for determination in liver tissue. Subsequently, the method was applied to liver tissue acquired by FNA biopsy of a patient undergoing DAA therapy.

2. Experimental

2.1. Chemicals and reagents

Dasabuvir, *C13D3*-dasabuvir, m1 metabolite of dasabuvir, *D6*-m1 metabolite of dasabuvir, ombitasvir, *D13*-ombitasvir, paritaprevir, *D8*-paritaprevir, and ritonavir, were obtained from AbbVie (North Chicago, IL). *D6*-Ritonavir was purchased from Toronto Research Chemicals (Toronto, Ontario, CA). LC-MS OPTIMA grade solvents included water, acetonitrile, and methanol obtained from Fisher Scientific (Pittsburgh, PA). Ultra-pure grade

(>99.9%) nitrogen gas and argon gas were obtained from Jackson Welding and Gas Products (Buffalo, NY). Human liver used for method development, matrix studies, standard, and QC preparation was obtained from BioreclamationIVT (Westbury, NY).

2.2. Preparation of calibration standards and quality control (QC) samples

Each analyte (DSV, DSVm1, OBV, PTV, RTV, and internal standards) was dissolved in suitable solvent to obtain a 1.0 mg/mL stock solution; DSV and DSVm1 were dissolved in 80:20 acetonitrile:water, OBV was dissolved in 0.01% ammonium hydroxide in 80:20 acetonitrile:water, PTV was dissolved in 50:50 acetonitrile:water, and RTV was dissolved in methanol. Stock solutions were then mixed with 50:50 acetonitrile:water to prepare intermediate stock solutions from which acetonitrile based calibration standards were prepared at 10X final concentration. The 10X acetonitrile based standards were diluted 1:10 into blank liver homogenate for each chromatographic run to create standards at concentrations of 12.5, 25, 125, 250, 1250, 2500, 4250, and 5000 ng/mL for DSV and DSVm1, 5.00, 12.5, 50.0, 250, 1250, 2500, 4250, 5000 ng/mL for PTV, and 1.25, 2.50, 25.0, 125, 625, 1250, 2125, 2500 ng/mL for OBV and RTV. The blank liver homogenate was prepared by homogenizing approximately 10 mg human liver tissue in 400 μ L acetonitrile with the Next Advance Bullet Blender® Storm (Averill Park, NY) in the same fashion as study samples described subsequently. Four quality control samples (LLOQ, LQC, MQC, and HQC) were made by spiking with appropriate volumes of separate 1.0 mg/mL stock solutions into blank liver tissue homogenate supernatant to achieve concentrations of 12.5, 37.5, 1000, 4000 ng/mL for DSV and DSVm1, 5.00, 15.0, 1000, 4000 ng/mL for PTV, and 1.25, 15.0, 2000, 7500 ng/mL for OBV and RTV. All stock solutions, calibration standards, and quality control samples were stored at -70°C .

2.3. Sample preparation

Liver FNA samples (2–35 mg wet weight) were homogenized with a Next Advance Storm Bullet Blender® at ambient temperature. Tissue was added to a 1.5 mL Eppendorf® Safe-lock tube, the weight was recorded, and 200 μ L of acetonitrile was added along with 1 scoop (approximately 100 μ L) of 1.0 mm diameter zirconium oxide beads. Tissue was homogenized with the Bullet Blender® Storm at setting 12 for five minutes. The samples were centrifuged post homogenization at 10,000 g for 5 minutes at ambient temperature. Clear supernatant was transferred off the spent liver tissue and bead mixture. Samples were processed for UPLC-MS/MS analysis by adding 25 μ L internal standard (150 ng/mL *C13D8*-DSV, *D6*-DSVm1, *D13*-OBV, *D8*-PTV and *D6*-RTV) to 100 μ L of crystal clear centrifuged homogenate supernatant and lastly diluted with the addition of 125 μ L of mobile phase A.

2.4. Instrumentation

The samples were analyzed with a Waters Acquity UPLC H-Class system (Milford, MA) which included an ultra-performance quaternary pump, autosampler, column manager, and an Acquity TQD triple quadrupole mass spectrometer. Data acquisition and signal output were controlled by Empower 3 software build v3471.

2.4.1. UPLC—Analytes were separated on a Waters Acquity BEH C18 (2.1 × 50 mm, 1.7 μm) analytical column, connected to a VanGuard C18 (2.1 X 5 mm, 1.7 μm) precolumn at 45°C. The mobile phases consisted of 95:5:0.1 water:acetonitrile:formic acid v/v/v (mobile phase A) and acetonitrile with 0.1% formic acid (mobile phase B). The flow rate was 300 μL/min with a gradient of 40% solvent A and 60% B for 0–0.5 min, followed by a linear gradient to 85% B from 0.5–1.0 min, then to 95% B from 1.0–1.1 min, held at 95% B for 0.9 min then set to initial conditions from 2.01 to 4.25 min. Processed samples were placed in the autosampler at 10 C and had 10 μL injection volume using a flow through needle.

2.4.2. Mass Spectrometry—Analytes were detected in positive electrospray ionization (ESI+) mode using tandem mass spectrometry with selected reaction monitoring (SRM). The spray voltage was set to 3200V with desolvation gas set at 800 L/hr. The vaporizer temperature and source temperature were set to 400°C and 150°C, respectively. Collision gas (argon) pressure was set at 1.5 mTorr. The ion transitions, collision energies, and cone voltages are shown in Table 1.

2.5. Assay Validation

2.5.1. Calibration and linearity—Calibration curves were constructed using eight samples run in singlicate each increasing in concentration of each analyte (DSV, DSVm1, OBV, PTV, and RTV). For each standard curve, the peak-area ratios of the analyte to their corresponding stable isotope labeled internal standard were calculated and plotted against the nominal analyte concentration. Calibration standards ranged from 12.5 to 5000 ng/mL for DSV, 1.25 to 2500 ng/mL for OBV and RTV, and 5.00 to 5000 ng/mL for PTV. Calibration curves were constructed with linear $1/x^2$ weighted regression using pooled blank human liver homogenate supernatant as matrix.

2.5.2. Accuracy and precision—The inter-day and intra-day accuracy and precision were measured using three quality control concentration sets of six prepared and frozen control samples each analyzed on four different days. The concentrations used were: 37.5, 1000, and 4000 ng/mL for DSV and DSVm1, 3.75, 500, 1875 ng/mL for OBV and RTV, and 15.0, 1000, and 4000 ng/mL for PTV designated as LQC, MQC, and HQC respectively and were prepared in blank human liver homogenate. Accuracy and precision at the lower limit of quantification (LLOQ) was assessed at 12.5 ng/mL for DSV and DSVm1, 1.25 ng/mL for OBV and RTV, and 5.00 ng/mL for PTV and analyzed on the same runs as the three QCs. The calculated mean concentration relative to the nominal concentration was used to express accuracy (% bias). The relative standard deviation (RSD) was calculated from the QC values and used to estimate the precision. Accuracy and precision were determined acceptable at QC concentrations ±15% (±20% at LLOQ).

2.5.3. Matrix Effects—Matrix effect (ME), process efficiency (PE), and recovery efficiency (RE) were tested for each analyte and their internal standards as described previously [15]. Six different lots of blank human liver were tested while spiking analyte at three different concentrations (low range, middle range, and high range) in triplicate. Set one was prepared as a neat sample (analytes spiked into acetonitrile without matrix). Set two was prepared by spiking the analytes into the liver homogenate mix prior to bead

homogenization. Set three was prepared by spiking the analytes into the supernatants of the human liver homogenates after the centrifugation step in the sample processing step. ME was calculated at each concentration level by dividing the mean peak area ratio of set three by set one (set 3/set 1 X 100). Recovery (RE) was calculated at each concentration level by dividing mean peak area ratios of set two by set three (set 2/set 3 X 100). Processing efficiency (PE) was calculated by dividing mean peak area ratios of set two by set one (set 2/set 1 X 100). The peak area ratios in the six lots of human liver were graphed versus the nominal concentration at the three different concentrations. The variation of the slopes using the peak area ratios from the six different lots of liver homogenate were examined to investigate if matrix effects occur over the different concentrations. Negligible variability is determined if the relative standard deviation of the slopes is less than 5.0%.

2.5.4. Stability—Analyte stability tests were conducted to evaluate the accuracy of determining DSV, DSVm1, OBV, PTV, and RTV under different conditions at LQC and HQC concentrations. Freeze-thaw stability was assessed for extracted homogenate based QC samples that were subjected to three freeze-thaw cycles from -70°C for 24 hours to room temperature for at least one hour prior to analysis. Bench-top stability was tested by analyzing extracted homogenate based QC samples that were left out on the bench top at room temperature for five hours before processing them for an analytical run. Autosampler stability was determined for processed samples stored at 10°C within the autosampler, which were re-injected and analyzed against the initial standard curve at 24 hours post-processing. Freeze-thaw, bench-top, and autosampler stability samples were compared to the same lot of LQC and HQC that did not undergo the stability test. The percent differences between the initial results and the stability results were calculated.

2.5.5. Dilution Study—Three replicate samples with over the curve concentrations (7500 ng/mL for OBV and RTV and 15000 ng/mL for DSV, DSVm1, and PTV) were diluted 1:5 in blank human liver homogenate supernatant and processed for analysis. The correct dilution factor (5X) was entered into the chromatography software and results were automatically calculated and corrected for the dilution factor.

2.6. Measurement of DAAs in human liver FNA

The method described was applied to an FNA biopsy of human liver from a subject undergoing DAA therapy as part of a clinical study (NCT02493855). The clinical study protocol was approved by the University at Buffalo Institutional Review Board (IRB). After written consent was obtained, the subject received 25/150/100 mg OBV/PTV/RTV QD with 250 mg DSV BID. The subject had the FNA procedure 1.63 hours after the dosing on day 7. FNA was performed by placing a 20G spinal needle into an anaesthetized region of the abdomen. Utilizing ultrasound guidance, multiple oscillations were performed for 5–10 seconds under negative pressure. The needle was removed and the material in the needle bore was expunged. This entire procedure is called a single FNA pass and was repeated once more to obtain enough aspirate for drug analysis. After the sample was collected into a 200 μL Eppendorf tube, it was snap frozen immediately and stored at -70°C until analysis. The liver FNA sample was homogenized in the presence of acetonitrile and concentrations of

DSV, DSVm1, OBV, PTV, and RTV were reported as described in the prior experimental sections.

3. Results and Discussion

This is the first method developed and validated to assess feasibility of measuring the 3D regimen in human liver tissue collected via FNA. The method was validated with consideration of the US FDA guidelines for bioanalytical method validation [16] and was applied to a patient with HCV infection who received the 3D regimen in a clinical trial (NCT02493855).

3.1. Method development

This method was further developed from an initial method to measure PTV and RTV in rat liver tissue [13] to include DSV, DSVm1, and OBV. In the earlier method, 400 μ L of acetonitrile was used to homogenize liver tissue obtained from a rat that received a dose of 30 mg/kg PTV and 15 mg/kg RTV. Doses of the 3D regimen in humans are much smaller. Therefore, we studied the effect of lowering the acetonitrile volume to 200 μ L which would double the amount of drug measured by UPLC-MS/MS in the samples homogenate supernatant. Drug concentrations obtained by UPLC-MS/MS are normalized by tissue weight in g per mL of homogenizing solvent (acetonitrile) and therefore only an adjustment to the calculation was required. The drug amount (ng/g) is obtained by dividing the ng/mL drug concentration by the gram of tissue per mL of homogenate supernatant.

3.2. Chromatographic separation

The five analytes and their stable isotope internal standards were separated on C18 reversed phase UPLC column with a mobile phase gradient. Representative chromatograms of blank liver homogenate and the LLOQ are shown in figure 1. Due to scaling differences, the internal standard peaks were not included in the figure. The analytes were identified based on retention times and mass spectra of individual analytical standards. The retention times were approximately 0.738, 1.18, 1.45, 2.15, and 2.30 min for DSVm1, DSV, RTV, OBV and PTV, respectively. The peaks of interest were well separated and without interference from other compounds. A total run time of 4.25 min allowed for equilibration of the column to the initial gradient conditions.

3.3. Assay validation

3.3.1. Calibration and linearity—Eight calibration standards were used which had concentrations in the range of 12.5 to 5000 ng/mL for DSV and DSVm1, 1.25 to 2500 ng/mL for OBV and RTV, and 5.00 to 5000 ng/mL for PTV. The calibration curves were generated using the response of peak area ratios (y) versus analyte concentration (x) and regressed with a linear $1/x^2$ weighted relation. Signal-to-noise between the lowest calibrator and blank liver homogenate was greater than 5:1 when compared to blank liver homogenate samples. The intra- and inter-day accuracy and precision were 11.4% for all levels of calibration standards and r^2 values were greater than 0.994.

3.3.2. Accuracy and precision—The intra-day and inter-day accuracy (% bias) and precision (RSD) were determined at QC concentrations of 37.5, 1000, and 4000 ng/mL for DSV and DSVm1, 3.75, 500, and 1875 ng/mL for OBV and RTV, and 15.0, 1000, and 4000 ng/mL for PTV. Assay accuracy (% bias) for all analytes ranged from -7.70 to 13.7%, and precision (RSD) ranged from 0.210 to 10.0% as shown in Table 2. Accuracy and precision at the LLOQ concentration was less than 14.0% for all analytes.

3.3.3. Matrix Effect—Assay selectivity was evaluated by analyzing 6 blank lots of human liver tissue homogenates. Each lot of human liver was processed with and without internal standards and showed no peaks at the retention times for any of the analytes or their internal standards. Matrix effects were assessed quantitatively by comparing average peak area ratios of analytes spiked into liver homogenate to neat. ME ranged from 97.6% to 102% showing no significant matrix effect. In addition to matrix effects, recovery and processing efficiency were assessed by comparing peak area ratios of analytes spiked before homogenization, after homogenization, and neat. RE ranged from 95.4% to 96.7% while PE ranged from 94.3% to 98.2%.

Slopes were generated by plotting the average ratio of analyte to internal standard area versus the theoretical concentration and obtaining a best fit line from the three concentrations in each matrix. RSDs were calculated from the average of the slopes for both pre-preparative and post-preparative and found to be less than 4.44%.

There was no evidence of carryover for DSV, DSVm1, OBV, PTV, RTV or their internal standards as found by injecting mobile phase after the highest calibrator sample.

3.3.4. Sample Stability—The stability of DSV, DSVm1, OBV, PTV, and RTV were evaluated at LQC and HQC concentrations under various conditions. Stability was assessed by comparing samples undergoing a stability test to a QC sample from the run and considered stable if the percent difference was within 15% of the QC response. Freeze-thaw percent difference ranged from -3.29% to 8.71%, bench-top stability percent difference ranged from -1.84% to 2.38%, and autosampler stability percent difference ranged from -7.93% to 4.82% as shown in Table 3.

3.3.5. Dilution Study—In the event that an unknown sample would have a concentration higher than the standard curve, a dilution study was performed to report accuracy and precision of a diluted sample. Percent bias was 12.2%, 0.935, 4.90%, 0.050%, and -5.13% for DSV, DSVm1, OBV, PTV, and RTV respectively and with precision (RSD) less than 6.65% for all analytes. This concludes that concentrations above the curve can accurately and precisely be determined when diluted 1:5 in blank human liver homogenate.

3.4. Application of the method

The described method was applied to the measurement of DSV, DSVm1, OBV, PTV, and RTV in a human liver sample obtained by FNA biopsy. Figure 2 shows the chromatogram for the patient sample. Concentrations observed in the patient's liver FNA homogenate were: 101 ng/mL DSV, 123 ng/mL DSVm1, 5.05 ng/mL OBV, 318 ng/mL PTV, and 84.5 ng/mL RTV. The FNA biopsy had a wet tissue weight of 23.4 mg which was homogenized in 0.2

mL of acetonitrile and therefore the normalized concentrations were: 863 ng/g DSV, 1050 ng/g DSVm1, 43.2 ng/g OBV, 2717 ng/g PTV, and 721 ng/g RTV.

4. Conclusion

We developed and validated a UPLC-MS/MS method for simultaneous determination of DSV, DSVm1, OBV, PTV, and RTV in liver tissue. The method is sensitive enough to measure drug concentrations in the small liver amounts acquired by FNA biopsy which are typically 3–30 mg in weight. We validated the assay with consideration of the FDA guidelines for bioanalytical methods and believe the method to be well suited for pre-clinical and clinical studies.

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Highlights

- UPLC-MS/MS method for measurement of DSV, DSVm1, OBV, PTV, and RTV in human liver FNA
- The method is validated with high accuracy and precision
- The method is simple with excellent recovery and short run-time of 4.25 min
- The method was applied to a patient with HCV undergoing DAA therapy

Fig. 1. Representative chromatograms of blank human liver homogenate and the lower limit of quantification (LLOQ). Figure shows only the data for the analytes dasabuvir m1 metabolite (DSVm1), dasabuvir (DSV), ritonavir (RTV), ombitasvir (OBV), and paritaprevir (PTV). Internal standard chromatograms not shown. LLOQ concentrations were 12.5 ng/mL DSV and DSVm1, 1.25 ng/mL for OBV and RTV, and 5 ng/mL for PTV.

Fig. 2.

Chromatograms of human liver fine needle aspirate (FNA) biopsy obtained 1.6 hours after dose of 250 mg dasabuvir (DSV), 25 mg ombitasvir (OBV), 100 mg ritonavir (RTV), 150 mg paritaprevir (PTV). Homogenate concentrations observed were: 101 ng/mL DSV, 123 ng/mL dasabuvir m1 metabolite (DSVm1), 5.05 ng/mL OBV, 318 ng/mL PTV, and 84.5 ng/mL RTV. Normalized concentrations were as follows: 863 ng/g DSV, 1050 ng/g DSVm1, 43.2 ng/g OBV, 2720 ng/g PTV, and 721 ng/g RTV.

Table 1

SRM transitions

Analyte	Mass transition ^a (<i>m/z</i>)	Cone Voltage	Collision Energy (V)
DSV	494 → 359	42	42
<i>C13D3</i> -DSV	498 → 363	42	34
DSV _{m1}	510 → 412	26	20
<i>D6</i> -DSV _{m1}	516 → 418	28	24
OBV	895 → 70	58	54
<i>D13</i> -OBV	908 → 70	54	56
PTV	766 → 571	42	22
<i>D8</i> -PTV	774 → 571	38	26
RTV	721 → 140	32	70
<i>D6</i> -RTV	727 → 146	34	68

^a[M+H]⁺

Table 2

Intra-Day and Inter-Day Accuracy and Precision

Analyte	Level (ng/mL)	Intra-day (n=6)			Inter-day (n=24)		
		Mean $\pm$ SD ng/mL	Accuracy % Bias	Precision RSD	Mean $\pm$ SD ng/mL	Accuracy % Bias	Precision RSD
DSV							
	LLQ (12.5)	13.2 $\pm$ 1.49	5.78	11.3	12.5 $\pm$ 1.63	-0.240	13.0
	LQ (37.5)	40.8 $\pm$ 2.01	8.87	4.92	38.3 $\pm$ 3.68	1.91	9.59
	MQ (1000)	1137 $\pm$ 61.8	13.7	5.44	1124 $\pm$ 64.8	12.4	5.76
	HQ (4000)	4227 $\pm$ 33.0	5.69	0.779	4063 $\pm$ 274	1.56	6.75
DSVml							
	LLQ (12.5)	11.1 $\pm$ 0.870	-11.0	7.82	12.2 $\pm$ 1.81	-2.50	14.8
	LQ (37.5)	38.0 $\pm$ 3.80	1.25	10.0	39.8 $\pm$ 3.28	5.69	8.28
	MQ (1000)	1019 $\pm$ 64.8	1.94	6.35	1020 $\pm$ 53.8	2.00	5.26
	HQ (4000)	3977 $\pm$ 210	-0.590	5.28	3945 $\pm$ 174	-1.37	4.41
OBV							
	LLQ (1.25)	1.07 $\pm$ 0.098	-14.8	9.35	1.09 $\pm$ 0.144	-13.2	13.3
	LQ (3.75)	3.83 $\pm$ 0.194	1.73	5.07	3.75 $\pm$ 0.160	0.210	4.25
	MQ (500)	558 $\pm$ 5.85	11.6	1.07	556 $\pm$ 9.85	11.1	1.77
	HQ (1875)	2050 $\pm$ 27.0	9.34	1.31	2056 $\pm$ 57.2	9.67	2.78
PTV							
	LLQ (5.00)	5.50 $\pm$ 0.229	9.87	4.16	5.00 $\pm$ 0.700	-0.040	14.0
	LQ (15.0)	15.8 $\pm$ 0.620	5.55	3.92	15.5 $\pm$ 1.12	3.34	7.21
	MQ (1000)	1108 $\pm$ 8.00	10.8	0.728	1099 $\pm$ 38.0	9.93	3.46
	HQ (4000)	3692 $\pm$ 164	-7.70	4.43	3723 $\pm$ 193	-6.93	5.17
RTV							
	LLQ (1.25)	1.23 $\pm$ 0.122	-2.01	9.93	1.23 $\pm$ 0.151	-1.68	12.3
	LQ (3.75)	3.68 $\pm$ 0.153	-1.83	4.15	3.78 $\pm$ 0.323	0.505	8.57
	MQ (1000)	513 $\pm$ 9.93	2.58	1.93	516 $\pm$ 12.1	3.13	2.34
	HQ (4000)	1780 $\pm$ 30.0	-5.10	1.68	1744 $\pm$ 45.3	-7.01	2.60

Table 3

Stability of LQC and HQC samples

Test	Analyte	Target ng/mL Mean	Measured ng/mL Mean $\pm$ SD	% Difference
Freeze-Thaw	DSV	38.1	37.6 $\pm$ 4.36	-1.16
-70°C to 20°C		4213	4169 $\pm$ 123	-1.04
3 cycles	DSVml	39.2	41.8 $\pm$ 3.87	6.80
		3903	3774 $\pm$ 211	-3.29
	OBV	3.85	3.88 $\pm$ 0.036	0.841
		2013	2051 $\pm$ 25.5	1.88
	PTV	16.4	16.1 $\pm$ 0.895	-1.98
		3808	3731 $\pm$ 81.6	-2.02
	RTV	3.88	4.22 $\pm$ 0.251	8.71
		1711	1777 $\pm$ 37.0	3.86
Bench-Top	DSV	38.1	38.8 $\pm$ 4.91	1.98
20°C		4213	4224 $\pm$ 139	0.241
5 hours	DSVml	39.2	39.0 $\pm$ 2.22	-0.28
		3903	3996 $\pm$ 289	2.38
	OBV	3.85	3.85 $\pm$ 0.093	0.007
		2013	2059 $\pm$ 50.5	2.32
	PTV	16.4	16.2 $\pm$ 1.39	-1.64
		3808	3738 $\pm$ 222	-1.84
	RTV	3.88	3.82 $\pm$ 0.159	-1.71
		1711	1740 $\pm$ 31.8	1.70
Autosampler	DSV	38.1	35.1 $\pm$ 3.84	-7.93
10°C		4213	4120 $\pm$ 168	-2.20
24 hours	DSVml	39.2	40.0 $\pm$ 1.4	2.09
		3903	3717 $\pm$ 115	-2.19
	OBV	3.85	4.04 $\pm$ 0.076	4.82
		2013	2034 $\pm$ 118	1.05
	PTV	16.4	16.3 $\pm$ 1.60	-0.787
		3808	3607 $\pm$ 182	-5.27
	RTV	3.88	4.06 $\pm$ 0.137	4.66
		1711	1683 $\pm$ 82.3	-1.63