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Identification and Quantification of 22 Benzodiazepines in Postmortem Fluids and Tissues using UPLC/MS/MS

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16. Abstract

Benzodiazepines, a class of drugs known to cause central nervous system depression, are widely prescribed for a variety of different medical conditions such as anxiety, insomnia, and as a preoperative sedative in conjunction with anesthesia. In fact, four are listed in the top 100 most prescribed drugs. Although there are many medicinal benefits with benzodiazepines, there are many impairing side effects that can impact the ability to safely operate an aircraft. Therefore, the Federal Aviation Administration's Forensic Toxicology Research Team has developed and validated a comprehensive method for the analysis of 22 benzodiazepines in postmortem fluids and tissues. The newly developed method uses ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) and a single step "crash-and-shoot" extraction. This new method reduces extraction time, significantly reduces sample volume, and eliminates derivatization steps necessary for commonly employed methods involving solid-phase extraction and GC/MS. For each of the analytes, the linear dynamic range encompasses sub-therapeutic to toxic concentrations. This method proved to be accurate and reproducible with control values not exceeding 20% of the target concentration. Analytes were stable over a 5-day period when stored at 4°C as well as on instrument, post extraction. Additionally, all analytes were stable after three freeze/thaw cycles. Ion suppression was evaluated in a variety of postmortem fluids and tissues. Although there was minimal suppression in fluids, there was suppression observed in tissues. However, this suppression affected the internal standard similarly; therefore, quantitative reliability was maintained. This newly developed UPLC/MS/MS method has been proven to be accurate and precise in the identification and quantification of 22 benzodiazepines in postmortem fluids and tissues.

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INTRODUCTION

Benzodiazepines, a class of drugs known to cause central nervous system depression, are widely prescribed for a variety of different medical conditions including: anxiety, insomnia, convulsion disorders, alcohol withdrawal, and as adjuncts to psychiatric conditions and surgical procedures.(1) While there are many important medicinal uses, benzodiazepines also have the potential for dependence and abuse. There are many impairing side effects such as drowsiness, confusion, dizziness, and lack of coordination.(2,3) Due to the potential impairment associated with these drugs, attempting divided-attention and/or complex activities such as operating a motor vehicle or piloting an aircraft can be especially dangerous.

The Federal Aviation Administration (FAA) is concerned with any drugs/pharmaceuticals used by those certified to operate an aircraft. When fatal civil aviation accidents occur, specimens such as blood, urine, liver, kidney, muscle, heart, brain, and other tissues are submitted to the FAA's Forensic Toxicology Laboratory for analysis. Identification of benzodiazepines and other drugs can provide additional information for accident investigators to aid in their investigations.

Over a 10-year period, 2007-2016, 121 cases out of 2582 fatal aviation accidents were found to be positive for one or more benzodiazepines. Since benzodiazepines are some of the most highly prescribed drugs in America, four of these being in the top 100 most prescribed drugs,(4) it is necessary to have an analytical method capable of identifying a majority of benzodiazepines and corresponding metabolites in postmortem fluids and tissues in an efficient manner.

There are numerous research articles that describe the analysis of multiple benzodiazepines in blood, urine, serum, and even meconium.(5-9) However, none have been applied to postmortem fluids and tissues. In this paper, we describe a simplified extraction and an ultra-performance liquid chromatography-tandem mass spectrometry (UPLC/MS/MS) separation and quantification method for 22 benzodiazepine compounds in postmortem fluids and tissues. The 22 compounds included in this method encompass all of the benzodiazepines we would expect to encounter in our laboratory. Validation of the method was conducted using the Scientific Working Group for Forensic Toxicology (SWGTOX) guidelines and recommendations.(10) We believe this is the first published method that combines a simple "crash and shoot" extraction with UPLC/MS/MS for the analysis of postmortem fluids and tissues.

MATERIALS AND METHODS

Chemicals and Reagents

Benzodiazepine methanolic standards were purchased from Cerilliant (Cerilliant Corp., Round Rock, TX), Lipomed (Arlesheim, Switzerland), and LGC (Lukenwalde, Germany) at 1.00 mg/mL. Methanolic standards were 7-amino-clonazepam, 7-amino-flunitrazepam, alpha hydroxy-alprazolam, alpha hydroxy-midazolam, alprazolam, bromazepam, chlordiazepoxide, clonazepam, desalkylflurazepam, diazepam, estazolam, flunitrazepam, flurazepam, lorazepam, midazolam, nitrazepam, norchlordiazepoxide, nordiazepam, oxazepam, prazepam, temazepam, and triazolam. Deuterated analogues were purchased from Cerilliant, Lipomed, and LGC at 1.0 mg/mL or 100 µg/mL. The deuterated methanolic standards were 7-amino-clonazepam-d_4, 7-amino-flunitrazepam-d_7, alpha hydroxy-alprazolam-d_5, alpha hydroxy-midazolam-d_4, alprazolam-d_5, bromazepam-d_4, chlordiazepoxide-d_5, clonazepam-d_4, diazepam-d_5, estazolam-d_5, flunitrazepam-d_3, lorazepam-d_4, midazolam-d_4, nitrazepam-d_5, norchlordiazepoxide-d_5, nordiazepam-d_5, oxazepam-d_5, prazepam-d_5, temazepam-d_5, desalkylflurazepam-d_4, flurazepam-d_4, and triazolam-d_4. Additionally, lorazepam, oxazepam, and temazepam were purchased as

glucuronides at 100 µg/mL from Cerilliant. *Helix pomatia* (derived β-glucuronidase) was obtained from Sigma Aldrich (Sigma Aldrich, St. Louis, MO). Formic acid, sodium fluoride, LC/MS grade acetonitrile (ACN), isopropanol, and LC/MS grade methanol were purchased from Fisher (Fisher Scientific, Pittsburgh, PA). Double deionized (DI) water was obtained from a Millipore Direct Q-3 UV (Millipore, Continental Water Systems, El Paso, TX). Bovine blood was obtained from Country Home Meat Co. (Country Home Meat Co., Edmond, OK). Immediately upon collection, sodium fluoride and potassium oxalate were added to the blood and mixed to produce a final sodium fluoride/potassium oxalate concentration of 1.0% and 0.2% (w/v), respectively. Human certified negative urine was obtained from UTAK (UTAK Laboratories Inc., Valencia, CA). Serum (bovine) was purchased from Sigma Aldrich. All tissues used for the ion suppression study were obtained from negative cases set for disposal.

Mobile Phase A (MPA) was made with DI water and formic acid (999:1 v/v). Mobile Phase B (MPB) was made with LC/MS grade acetonitrile and formic acid (999:1 v/v). A needle wash solution at a 1:1:1:1 (v/v) ratio was prepared with DI water, LC/MS grade acetonitrile, LC/MS grade methanol, and isopropanol.

Ultra-performance liquid-chromatography-tandem mass spectrometric conditions

All analyses were performed utilizing a Waters Acquity I-class ultra-performance liquid chromatograph (UPLC) connected to a Waters Xevo TQ-S tandem mass spectrometer (MS/MS) (Waters Corporation, Milford, MA). Chromatographic separation was achieved using an Acquity UPLC BEH C_{18} column (2.1 x 100-mm, 1.7-µm; Waters Corporation, Milford, MA). The column manager temperature was set at 60°C. The UPLC was operated at a flow rate of 0.600 mL/min with a gradient program (Table 1). The autosampler temperature was set at 10°C and sample injection volume was 1 µL. The UPLC was equilibrated for approximately 30 minutes prior to use. Typical UPLC pressures observed for these conditions are approximately 9,300 psi.

Table 1. Gradient Program

Time	MPA %	MPB %
0.0	95	5
0.50	95	5
0.51	75	25
2.00	67	33
6.00	65	35
6.10	2	98
7.10	95	5
8.00	95	5

*MPA: DI water with 0.1% FA; MPB: acetonitrile with 0.1% FA

The mass spectrometer portion of the UPLC/MS/MS system was manually optimized for each individual benzodiazepine compound. The source temperature was set at 150°C, capillary voltage at 0.6 kV, desolvation temperature at 550°C, desolvation gas flow at 1000 L/hr, cone flow 150 L/hr, nebulizer 7 bar, and collision flow of 0.15 mL/min. Various ionization modes were evaluated. ESI+ mode was found to provide maximum ionization. Optimized retention times, precursor (parent) and product (daughter) ions, cone voltages, and collision energies for each analyte are listed in Table 2.

There are three criteria set by our laboratory that must be met before an analyte can be reported as positive by UPLC/MS/MS. An analyte's product ions must have a minimum signal-to-noise ratio of 10 (quant ion) and 3 (confirmation ion), a retention time +/- 5% of the average calibrator retention time, and product ion ratio +/- 20% of the average calibrator product ion ratio.

Table 2. Retention times and MS parameters for the benzodiazepines.

Compound	Retention Time (min)	Cone Voltage (V)	Precursor Ion (m/z)	Product Ions (m/z)	Collision Energy (CE)
7-aminoclonazepam	1.19	16	286.0	120.9* 222.1	30 24
7-aminoclonazepam-d_4	1.18	44	290.1	120.9* 254.1	32 18
7-aminoflunitrazepam	1.31	20	284.1	135.0* 226.8	26 24
7-aminoflunitrazepam-d_7	1.30	32	291.1	138.0* 230.1	26 30
Norchlordiazepoxide	1.35	32	286.1	227.0* 241.1	22 14
Norchlordiazepoxide-d_5	1.34	26	291.1	232.1* 246.1	22 14
Chlordiazepoxide	1.44	20	300.1	227.1 283.1*	26 14
Chlordiazepoxide-d_5	1.43	20	305.2	232.1* 288.1	26 14
Alpha OH-Midazolam	1.68	14	341.9	168.0 202.9*	38 24
Alpha OH-Midazolam-d_4	1.67	32	346.0	168.0 202.9*	38 26
Midazolam	1.69	34	326.0	222.9 291.1*	36 24
Midazolam-d_4	1.68	30	330.0	226.9 295.1*	30 26
Flurazepam	1.74	40	388.2	288.1 315.1*	24 22
Flurazepam-d_4	1.72	40	392.2	292.0 319.1*	25 24
Bromazepam	1.86	20	315.9	182.0* 209.0	28 24
Bromazepam-d_4	1.84	10	319.9	186.0* 213.0	32 26
Nitrazepam	2.39	34	282.0	180.0 236.0*	36 22
Nitrazepam-d_5	2.37	26	287.1	185.0 241.1*	34 24
Alpha OH-Alprazolam	2.50	36	324.9	279.1 297.1*	22 24
Alpha OH-Alprazolam-d_5	2.47	38	330.2	284.1 302.1*	22 26
Oxazepam	2.60	20	287.1	104.1 269.1*	32 14
Oxazepam-d_5	2.57	20	292.1	246.1* 274.1	20 14
Clonazepam	2.66	22	315.9	214.0 270.0*	38 24
Clonazepam-d_4	2.64	20	320.0	217.9 274.1*	36 24

*Transition ion used for quantification.

Table 2. Retention times and MS parameters… *Continued*

Compound	Retention Time (min)	Cone Voltage (V)	Precursor Ion (m/z)	Product Ions (m/z)	Collision Energy (CE)
Estazolam	2.67	26	295.1	205.1 267.1*	40 22
Estazolam-d_5	2.64	22	300.1	210.0 272.0*	38 22
Lorazepam	2.77	20	320.9	229.1 274.9*	28 20
Lorazepam-d_4	2.75	18	327.0	235.1 280.9*	30 20
Nordiazepam	2.89	30	271.0	139.9* 165.0	28 28
Nordiazepam-d_5	2.84	18	276.1	140.1* 165.0	26 26
Alprazolam	2.98	30	308.9	204.9 281.1*	40 26
Alprazolam-d_5	2.94	40	314.0	279.2 286.1*	26 26
Flunitrazepam	3.04	34	314.1	239.2 268.1*	32 24
Flunitrazepam–d_3	3.02	22	317.0	242.1 271.1*	34 26
Desalkylflurazepam	3.11	40	289.2	165.1 226.1*	25 25
Desalkylflurazepam-d_4	3.08	40	293.1	149.8 230.0*	30 28
Triazolam	3.17	24	342.9	238.9 308.0*	40 24
Triazolam-d_4	3.14	40	346.9	243.0 312.1*	40 24
Temazepam	3.39	38	301.0	177.1 254.9*	38 20
Temazepam-d_5	3.35	20	306.0	177.1 260.1*	38 18
Diazepam	4.16	32	285.0	153.9* 193.1	26 30
Diazepam-d_5	4.07	32	290.1	154.0* 198.0	26 30
Prazepam	6.56	44	325.1	139.9 271.0*	36 20
Prazepam-d_5	6.55	24	330.0	140.1 276.1*	34 22

*Transition ion used for quantification.

Calibration and control preparation

Calibrators and controls were prepared using separate 1.00 mg/mL methanolic drug standards. All calibrators and controls were prepared using bovine whole blood. Calibration curves were prepared by serial dilution to produce concentrations ranging from 0.78 to 800 ng/mL, except for chlordiazepoxide and norchlordiazepoxide, which ranged from 0.78 to 1600 ng/mL. Controls were prepared at concentrations of 5, 50, 200, and 1000 ng/mL, covering low, medium, and high portions of the calibration curves and were used to determine the accuracy and precision of the method and for various analyte stability studies. A 1000 ng/mL working internal standard solution was prepared in DI water using 100 µg/mL or 1.00 mg/mL methanolic deuterated drug standards. One hundred µL of this working solution was used for each sample (100 ng total).

Select benzodiazepines are present in urine, to some degree, as glucuronide conjugates. Therefore, we hydrolyzed each postmortem urine specimen using the enzyme *β*-glucuronidase in order to identify those analytes as free-drug. A *β*-glucuronidase solution was prepared by the addition of a pH 5, 0.1 M acetate buffer to a lyophilized powder of *β*-glucuronidase, resulting in a 200,000 units/mL mixture. This *β*-glucuronidase solution was stored in a refrigerator at 4°C. Glucuronide controls, prepared as 100 ng/mL drug-glucuronide, were made from 100 µg/mL methanolic standards of lorazepam-, oxazepam-, and temazepam-glucuronide.

Quantification was achieved via an internal standard calibration procedure. Response factors for each compound were determined for every sample analyzed. The response factor was calculated by dividing the area of the analyte quant ion peak by the area of the internal standard quant ion peak. Calibration curves were derived by plotting the analyte/internal standard response factor versus the analyte concentration for each respective calibrator and determining the mathematical model that best fit the calibration data. These calibration curves were then used to determine the concentrations of each benzodiazepine in the prepared controls and biological specimens.

Sample preparation and extraction method

Calibrators, controls, and specimens were prepared using the following procedure. Tissue samples were prepared for homogenization by adding 1.00% NaF solution to the tissue sample in a 2:1 w:w (1% NaF solution:tissue) addition. Tissue samples were homogenized using an OMNI post-mounted mixer homogenizer (Omni International; Kennesaw, GA). To individual 13 x 100-mm polypropylene tubes, 0.5 mL aliquots of each calibrator, control, postmortem fluid, and 1.50 g aliquots of each tissue homogenate (0.5 g wet tissue) were transferred. To each tube, 100 µL of 1000 ng/mL internal standard was added (100 ng total). Urine specimens were hydrolyzed by adding 50 µL of β-glucuronidase (10,000 units) and 1 mL of pH 5 0.1 M acetate buffer to each urine sample and incubated for 2 h at 70°C. Two mL of ice cold ACN was added to each tube, capped, and thoroughly vortexed. The tubes were then centrifuged at 4000 x *g* for 10 minutes in a Thermo RC4 Centrifuge (Thermo Electron Corp.; Chateau-Gontier, France). Centrifugation removed the proteins and cellular debris from the samples. Once centrifuged, supernatants from each tube were transferred to 16 x 100-mm round bottom tubes. Two mL of DI water was added to each tube and vortexed. Three hundred µL of the extract was transferred to a 450 µL fill-volume, 0.2 µm PVDF filter vial (2 mL size, Thomson; Oceanside, CA). The vials were transferred to the autosampler set at 10°C. All specimens were analyzed at one time to avoid inter-assay variations.

RESULTS AND DISCUSSION

The method developed and described herein is a "crash-and-shoot" extraction followed by the separation, identification, and quantitation of 22 benzodiazepines compounds using a UPLC/MS/MS. It was extensively validated following SWGTOX recommended guidelines. One half mL of fluids or 0.50 g tissue was used for analysis. Specimen proteins and particulate matter were precipitated following the addition of 2 mL ACN to each sample. Following centrifugation, samples were further purified using a 0.2 µm PVDF filter vial. This filtration step was effective in avoiding blockage of the UPLC column. UPLC columns are prone to clogging when used for postmortem specimen analysis, due to the small particle size associated with UPLC. UPLC separation was achieved

using a gradient flow (Table 1) with a total run time of 8 min. This gradient flow was effective in "washing" the column of fats and proteins, thus, providing a clean column for each run. This is very important when analyzing postmortem fluid and tissue specimens. A typical calibrator chromatogram is shown in Figure 1. The MS was manually optimized to produce the highest abundance of precursor and product ions. The optimized MS parameters and retention times for the benzodiazepines are listed in Table 2. This simple extraction method and UPLC separation is very fast compared to our previous solid phase extraction/derivatization protocol, which was time consuming and labor intensive. This new procedure cut the analysis time by more than 50% compared to our previous method.

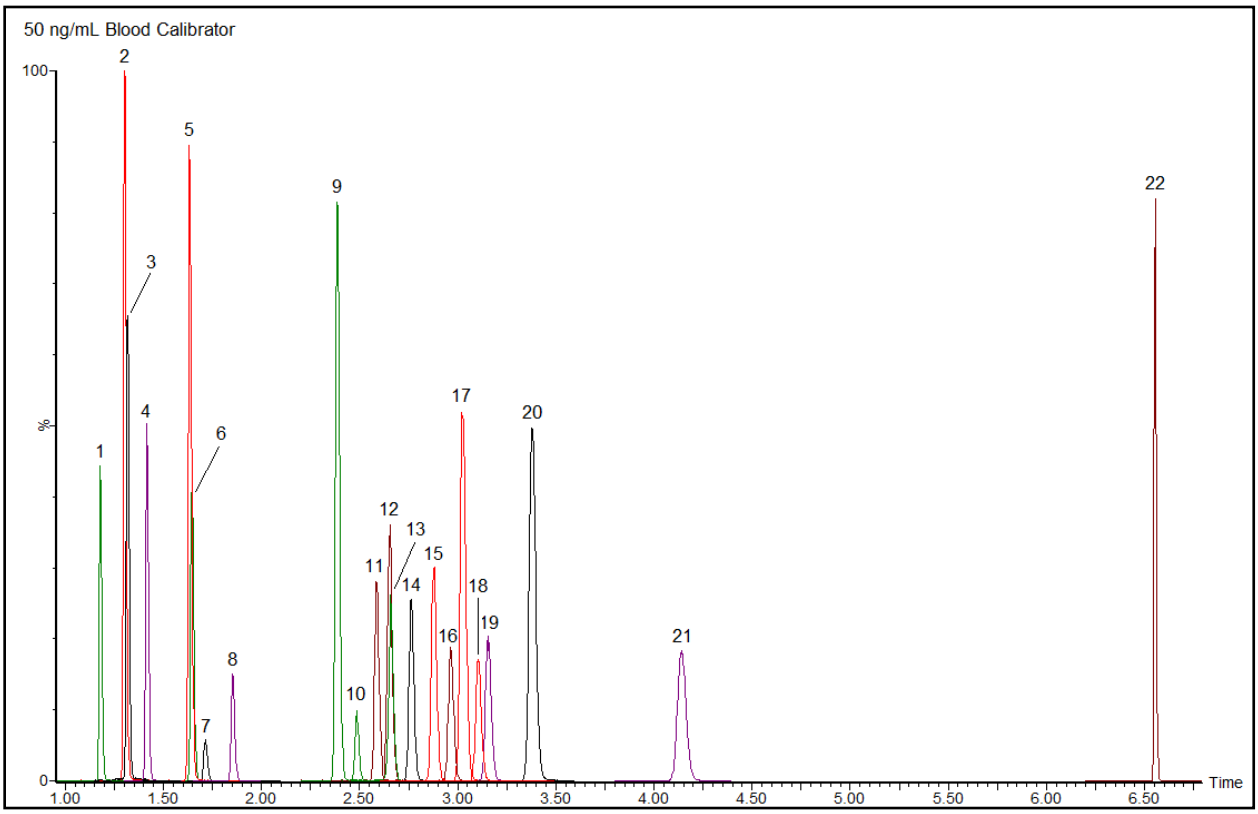

Figure 1. A chromatogram of the 100 ng/mL calibrator. The peak identification are as follows: (1) 7-amino-clonazepam, (2) 7-amino-flunitrazepam, (3) norchlordiazepoxide, (4) chlordiazepoxide, (5) alpha hydroxy-midazolam, (6) midazolam, (7) flurazepam, (8) bromazepam, (9) nitrazepam, (10) alpha hydroxy-alprazolam, (11) oxazepam, (12) clonazepam, (13) estazolam, (14) lorazepam, (15) nordiazepam, (16) alprazolam, (17) flunitrazepam, (18) desalkylflurazepam, (19) triazolam, (20) temazepam, (21) diazepam, and (22) prazepam.

The limit of detection (LOD), limit of quantitation (LOQ), calibration model, and linear dynamic range (LDR) for the method was determined for each analyte. The calibration model for an analyte is the mathematical representation that best fits the correlation between the analyte/internal standard ratio and the analyte concentration. A linear regression, with 1/x weighting, provided the best mathematical fit for all analytes tested. A minimum of 8 calibrators was used to construct each calibration curve. The LDR for each analyte encompasses the benzodiazepine concentrations we expect to encounter in our laboratory. The correlation coefficients (r^2) for all curves exceeded 0.98. The LOD is the lowest concentration of the drug that meets the identification criterion described in the materials and methods section. The LOQ is the lowest concentration that meets the same criteria as the LOD, plus it has an experimentally determined value within ± 20% of its target concentration. A summary of the LOD, LOQ, LDR, and r^2 for all 22 benzodiazepine analytes is displayed in Table 3.

Table 3. LOD, LOQ, and LDR data for 22 benzodiazepines.*

Compound	LOD** (ng/mL)	LOQ (ng/mL)	LDR (ng/mL)	r^2
7-aminoclonazepam	1.56	3.13	3.13-400	0.999
7-aminoflunitrazepam	0.78	1.56	1.56-200	0.999
Norchlordiazepoxide	25	50	50-1600	0.988
Chlordiazepoxide	25	50	50-1600	0.995
Alpha OH-Midazolam	3.13	3.13	3.13-400	0.999
Midazolam	1.56	3.13	3.13-400	0.999
Flurazepam	1.56	3.13	3.13-400	0.999
Bromazepam	3.13	6.25	6.25-400	0.999
Nitrazepam	0.78	1.56	1.56-200	0.999
Alpha OH-Alprazolam	1.56	3.13	3.13-400	0.999
Oxazepam	1.56	3.13	3.13-400	0.999
Clonazepam	1.56	3.13	3.13-400	0.999
Estazolam	1.56	3.13	3.13-400	0.999
Lorazepam	3.13	3.13	3.13-400	0.999
Nordiazepam	1.56	3.13	3.13-400	0.999
Alprazolam	1.56	3.13	3.13-400	0.999
Flunitrazepam	0.78	1.56	1.56-200	0.999
Desalkylflurazepam	1.56	3.13	3.13-400	0.999
Triazolam	3.13	3.13	3.13-400	0.999
Temazepam	1.56	3.13	3.13-400	0.999
Diazepam	3.13	3.13	3.13-400	0.999
Prazepam	1.56	3.13	3.13-400	0.999

* A blood matrix was used for all benzodiazepines in the determination of each analytical parameter above.
** Concentrations below 0.78 ng/mL were not examined.

Carryover was evaluated by analyzing a mobile phase blank injection following the highest calibrator. No carryover was observed for any analyte following the injection of the highest calibrator. Since we analyzed postmortem fluids and tissues with this method, we also injected a mobile phase blank between specimens to ensure no carryover occurred. No carryover was observed at any time throughout the validation process.

Accuracy and precision were evaluated. Accuracy, expressed as relative error (%E), was calculated by determining the difference between the target concentration and the measured concentration. Precision, closeness of individual measurements to one another, was expressed as the coefficient of variation (CV). Control values for Days 1, 2, 3, 4, and 5 were processed from calibration curves prepared fresh on each day. Controls were prepared at 5, 50, 200, and 1000 ng/mL in large lots to ensure a sufficient amount of each control was available for the entire accuracy, precision, and storage stability (refrigerated and freeze/thaw) study. The 5, 50, and 200 ng/mL controls were used for all benzodiazepines except chlordiazepoxide and norchlordiazepoxide. For these two analytes, the 50, 200, and 1000 ng/mL control levels were used, due to the higher therapeutic range of chlordiazepoxide.

The accuracy and precision for all control levels on each day were within 20% of the calculated value from Day 1. Norchlordiazepoxide was outside of 20% of the 1000 ng/mL target value on Day 5. This is likely due to an error in the initial control preparation. However, it is still considered precise due to the reproducibility of the calculated value over the 5 days. Outside of this control, the largest %E for all other analytes tested was 16%. Additionally, all CV were below 12%, which demonstrates the robustness of this new procedure. The accuracy and precision for each analyte over all 5 days is shown in Table 4.

Drug concentrations measured in actual case work must be within the LDR. If concentrations fall above the LDR, such values should not be reported. In such an instance, a specimen may require dilution. Therefore, we evaluated the dilution integrity of the benzodiazepines. Five 800 ng/mL controls were diluted 1:10. All dilutions were within 20% of the expected dilution concentration of 80 ng/mL.

Analyte stability was evaluated at refrigerator temperatures, multiple freeze/thaw cycles, and post-extraction conditions. Refrigerator stability of the benzodiazepines in whole bovine blood stored at 4°C was evaluated by analyzing the 5, 50, 200, and 1000 ng/mL controls, over a 5-day period. All analytes over this 5-day period were within 20% of the Day 1 concentrations, indicating short-term stability at 4°C. Even though the 1000 ng/mL norchlordiazepoxide was outside of the 20% of the target concentration on Day 5, it was only 14% above the Day 1 value. Freeze/thaw stability was evaluated by freezing multiple Day 1 controls, thawing all of the controls for 1 h on Day 2, analyzing 5 of each control level, refreezing all of the remaining tubes, and repeating this freeze/thaw cycle on Days 3 and 4; for a total of 3 freeze/thaw cycles. The freezer temperature was -20°C. All analyte concentrations remained within 20% of their target concentration through all 3 of the freeze/thaw cycles, except for 1000 ng/mL norchlordiazepoxide. However, this concentration was within 20% of the Day 1 control concentration. Post-extraction or on-instrument stability was determined by re-injecting Day 1 controls, left on the instrument at 10°C, on Days 2, 3, 4, and 5. All analytes were within 20% of Day 1 results for the full 5 days. A summary of the stability data is displayed in Tables 4, 5, and 6.

In urine, many benzodiazepines exist as glucuronide conjugates. Therefore, glucuronide controls were prepared and hydrolyzed to evaluate the efficiency of the β-glucuronidase hydrolysis procedure. Lorazepam, oxazepam, and temazepam were the only analytes commercially available as glucuronides. A 100 ng/mL glucuronide control was prepared for each of the analytes in urine, resulting in a free-drug concentration of 67 ng/mL for lorazepam and 62 ng/mL for oxazepam and temazepam. All controls were within 12% of the target concentrations, indicating efficient hydrolysis. Hydrolysis efficiency can be seen in Table 7.

Ion suppression/enhancement was determined by analyzing multiple analyte-spiked solvent samples and spiked post-extraction fluid and tissue specimens and comparing their response. Ion suppression/enhancement was evaluated for each analyte and its internal standard in 5 different sources of blood, urine, serum, liver, lung, muscle, brain, and kidney (each tissue type was a homogenous mixture of 5 separate tissues). No ion enhancement was observed for any specimen type tested. Although no criteria exist for the acceptance of ion suppression, SWGTOX recommendations state that ion suppression greater than 25% be further evaluated to ensure quantitative validation parameters are not negatively impacted. No significant ion suppression was observed for any analytes in blood, urine, or serum. However, ion suppression was observed for analytes in tissues, but similar suppression for the internal standard factored out any quantitative variation. Ion suppression, which can be seen in Tables 8 and 9, was calculated by taking the difference between the suppression of the internal standard and the suppression of the corresponding analyte. We did not observe any total ion suppression greater than 25%. Incorporation of deuterated benzodiazepine analogues as internal standards eliminated concerns of possible matrix effects and allowed for accurate quantitation in specimen types other than blood while using a whole-blood calibration curve. However, caution should routinely be used when interpreting results from postmortem tissues, as putrefactive byproducts may cause ion suppression or enhancement.

Table 4. Accuracy and precision data and refrigerator stability.*

		Day 1			Day 2			Day 3			Day 4			Day 5		
	Target (ng/mL)	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E
7-aminoclonazepam	5	5.6 ± 0.1	+2	+12	5.7 ± 0.4	+2	+14	5.5 ± 0.3	+6	+11	5.3 ±0.2	+4	+5	5.27 ± 0.08	+2	+5
	50	49.8 ± 0.9	+2	0	49.8 ±0.5	+1	0	50 ± 0.9	+2	0	48. ± 2	+3	0	50.2 ± 0.05	+1	0
	200	198 ± 2	+1	-1	201 ± 2	+1	0	199 ± 3	+2	0	202 ± 6	+3	+2	204 ± 3	+1	+2
7-aminoflunitrazepam	5	5.31 ± 0.08	+2	+6	5.6 ± 0.1	+2	+13	5.5 ± 0.1	+2	+10	5.2 ± 0.5	+9	+4	5.3 ± 0.2	+4	+5
	50	49.3 ± 0.8	-1	-1	50.4 ± 0.5	+1	+1	49.6 ± 0.8	+2	-1	50 ± 1	+3	-1	49.1 ± 0.6	+1	-2
	200	199.4 ± 0.8	+1	0	202.1 ± 0.8	0	+1	198 ± 3	+1	-1	200 ± 6	+3	0	200 ± 4	+2	0
Norchlordiazepoxide	50	47 ± 1	+2	-6	54.5 ± 0.8	+1	+9	55 ± 1	+2	+10	48 ± 3	+7	-4	46.6 ± 0.6	+1	-7
	200	198 ± 6	+3	-1	191 ± 2	+1	-4	188 ± 3	+1	-6	192 ± 10	+5	-4	196 ± 2	+1	-2
	1000**	1112 ± 24	+2	+12	1129 ± 16	+1	+13	1161 ± 16	+1	+16	1162 ± 15	+1	+16	1264 ± 24	+2	+26
Chlordiazepoxide	50	46 ± 1	+2	-7	52.4 ± 0.6	+1	+5	48.1 ± 0.3	+1	-4	51 ± 2	+4	+2	53.3 ± 0.6	+1	+7
	200	200 ± 1	+1	0	195 ± 2	+1	-3	193 ± 2	+1	-4	205 ± 3	+2	+3	188 ± 4	+2	-6
	1000	1028 ± 13	+1	+3	1068 ± 10	+1	+7	1102 ± 19	+2	+10	1150 ± 9	+1	+15	1025 ± 9	+1	+3
Alpha OH-Midazolam	5	5.2 ± 0.2	+3	+3	5.1 ± 0.2	+4	+2	5.1 ± 0.3	+5	+2	5.4 ± 0.2	+4	+8	5.3 ± 0.3	+5	+6
	50	49.3 ± 0.8	+2	-1	50 ± 1	+2	0	50 ± 1	+2	0	50 ± 1	+2	-1	49.7 ± 0.5	+1	-1
	200	201 ± 3	+2	+1	198 ± 3	+1	-1	201 ± 2	+1	+1	197 ± 3	+1	-1	203 ± 2	+1	+2
Midazolam	5	5.20 ± 0.08	+2	+4	5.58 ± 0.3	+5	+12	5.5 ± 0.2	+4	+10	4.9 ± 0.3	+5	-2	5.7 ± 0.1	+2	+15
	50	48.9 ± 0.8	+2	-2	51.1 ± 0.6	+1	+2	50.9 ± 0.3	+1	+2	50 ± 1	+3	-1	50.7 ± 0.8	+2	+1
	200	197 ± 2	+1	-1	205 ± 2	+1	+3	204 ± 2	+1	+2	199 ± 3	+1	-1	206 ± 2	+1	+3
Flurazepam	5	4.5 ± 0.2	+3	-11	4.50 ± 0.06	+1	-10	4.4 ± 0.2	+4	-11	4.2 ± 0.1	+2	-17	5.3 ± 0.5	+9	+5
	50	49.9 ± 0.9	+2	0	50.8 ± 0.4	+1	+2	50.1 ± 0.9	+2	0	50.2 ± 0.6	+1	0	51 ± 1	+2	+3
	200	196 ± 2	+1	-2	195 ± 1	+1	-3	201 ± 2	+1	0	197 ± 3	+2	-2	195 ± 2	+1	-2
Bromazepam	50	47.8 ± 0.4	+1	0	45.9 ± 0.9	+2	-8	46 ± 1	+3	-8	46.8 ± 0.7	+2	-7	46 ± 2	+1	-8
	200	207 ± 1	-4	+3	197 ± 3	+1	-1	198 ± 1	+1	-1	200 ± 3	+1	0	202 ± 1	+1	+1
Nitrazepam	5	5.3 ± 0.1	+3	+6	5.46 ± 0.08	+2	+9	5.3 ± 0.3	+5	+6	5.2 ± 0.2	+4	+4	5.3 ± 0.1	+2	+5
	50	50.5 ± 0.8	+2	+1	50.5 ± 0.8	+2	+1	49.3 ± 0.6	+1	-1	50 ± 2	+3	-1	49.6 ± 0.8	+2	-1
	200	201 ± 1	+1	0	204 ± 2	+1	+2	2000 ± 1	+1	0	199 ± 2	+1	-1	199 ± 1	+1	-1
Alpha OH-Alprazolam	5	4.7 ± 0.2	+3	-5	5.7 ± 0.2	+4	+13	5.4 ± 0.3	+6	+9	4.8 ± 0.5	+10	-5	5.4 ± 0.4	+6	+9
	50	49.9 ± 0.9	+2	0	50 ± 1	+2	-1	50.5 ± 0.9	+2	+1	48 ± 3	+6	-3	51 ± 1	+3	+2
	200	208 ± 2	+1	+4	200 ± 3	+2	0	201 ± 2	+1	+1	196 ± 3	+2	-2	200 ± 2	+1	0
Oxazepam	5	5.5 ± 0.3	+5	+9	5.1 ± 0.4	+8	+3	5.2 ± 0.3	+5	+5	4.9 ± 0.2	+5	0	5.4 ± 0.3	+5	+9
	50	49.7 ± 0.5	+1	-1	50.5 ± 0.8	+2	+1	49.1 ± 0.9	+2	-2	50 ± 1	+2	-1	50.6 ± 0.7	+1	+1
	200	202 ± 2	+1	+1	206 ± 5	+2	+3	198 ± 1	+1	-1	196 ± 4	+2	-2	201 ± 3	+1	0

* $n = 5$ for all measurements. Accuracy was measured as the relative error (%E) from the target concentration. Precision was measured as the CV obtained from 5 replicate measurements. Blood was used as the matrix for all benzodiazepine compounds.

** Day 5 value outside 20% of target value, but within 20% of the value calculated on Day 1. Deviation is likely due to preparation error.

Table 4. Accuracy and precision data… *Continued*

		Day 1			Day 2			Day 3			Day 4			Day 5		
	Target (ng/mL)	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E
Clonazepam	5	5.4 ± 0.2	+3	+8	5.7 ± 0.3	+5	+14	5.1 ± 0.2	+3	+2	5.6 ± 0.3	+5	+13	5.6 ± 0.2	+3	+12
	50	49.2 ± 0.7	+2	-2	51 ± 1	+2	+3	51 ± 1	+2	+2	48.5 ± 0.8	+3	-3	49.7 ± 0.8	+2	-1
	200	200 ± 3	+1	0	204 ± 2	+1	+2	205 ± 1	+1	+3	199 ± 2	+3	-2	199 ± 2	+1	-1
Estazolam	5	5.5 ± 0.3	+5	+10	5.5 ± 0.4	+6	+11	5.0 ± 0.5	+10	+1	5.0 ± 0.4	+7	0	4.9 ± 0.3	+6	-3
	50	49 ± 1	+2	-3	49 ± 1	+3	-2	50 ± 1	+3	0	49 ± 1	+2	-1	49.4 ± 0.6	+1	-1
	200	200 ± 2	+1	0	201 ± 1	+1	+1	202 ± 3	+2	+1	191 ± 3	+2	-5	198 ± 2	+1	-1
Lorazepam	5	5.4 ± 0.4	+7	+8	5.8 ± 0.2	+4	+15	5.3 ± 0.1	+3	+5	5.4 ± 0.2	+5	+8	5.6 ±0.3	+5	+12
	50	49 ± 2	+3	-2	48.0 ± 0.8	+2	-4	48.6 ± 0.6	+1	-3	48.9 ± 0.7	+1	-2	49.5 ± 0.5	+1	-1
	200	202 ± 2	+1	+1	201 ± 4	+2	0	200 ± 2	+1	0	197 ± 5	+3	-2	201 ± 3	+2	0
Nordiazepam	5	5.0 ± 0.1	+3	0	5.3 ± 0.2	+4	+6	5.4 ± 0.1	+2	+8	5.4 ± 0.3	+6	+7	5.0 ± 0.4	+7	0
	50	50.2 ± 0.9	+2	0	50.5 ± 0.9	+2	+1	50.3 ± 0.8	+2	+1	50 ± 2	+3	0	49.6 ± 0.7	+1	-1
	200	203 ± 2	+1	+1	198.7 ± 0.7	0	-1	200 ± 2	+1	0	202 ± 2	+1	+1	199 ± 3	+2	-1
Alprazolam	5	5.4 ± 0.2	+3	+7	5.3 ± 0.1	+2	+6	5.6 ± 0.2	+4	+11	5.3 ± 0.6	+12	+7	5.0 ± 0.4	+7	+1
	50	48.6 ± 0.5	+1	-3	49.9 ± 0.9	+2	0	50 ± 1	+2	0	50.8 ± 0.4	+1	+2	49 ± 1	+2	-1
	200	197 ± 2	+1	-2	200 ± 2	+1	0	207 ± 3	+2	+3	200 ± 2	+1	0	198 ± 4	+2	-1
Flunitrazepam	5	5.1 ± 0.2	+4	+3	5.3 ± 0.1	+3	+6	5.2 ± 0.2	+3	+4	5.1 ± 0.2	+3	+2	5.3 ± 0.1	+2	+6
	50	50.1 ± 0.5	+1	0	50.1 ± 0.5	+1	0	50.1 ± 0.6	+1	0	48.7 ± 0.7	+1	-3	50.5 ± 0.5	+1	+1
	200	201 ± 2	+1	+1	205 ± 1	+1	+2	203 ± 1	+1	+2	195 ± 2	+1	-2	201.8 ± 0.6	0	+1
Desalkylflurazepam	5	4.6 ± 0.2	+5	-9	4.1 ± 0.1	+3	-18	4.5 ± 0.2	+5	-9	4.3 ± 0.2	+5	-14	4.6 ± 0.5	+10	-9
	50	50.1 ± 0.8	+2	0	49.7 ± 0.9	+2	-1	49.6 ± 0.2	0	-1	50.2 ± 0.9	+2	0	50 ± 1	+2	0
	200	173 ± 3	+2	-14	172 ± 1	+1	-14	171 ± 3	+2	-14	169 ± 2	+1	-15	173 ± 2	+1	-13
Triazolam	5	5.27 ± 0.1	+3	+5	5.6 ± 0.4	+7	+11	5.6 ± 0.2	+4	+7	5.3 ± 0.4	+7	+7	5.2 ± 0.3	+5	+4
	50	49.5 ± 0.6	+1	-1	49.6 ± 0.7	+1	-1	49.2 ± 0.3	+1	-2	50.3 ± 0.9	+2	+1	50.7 ± 0.6	+1	+1
	200	198.3 ± 0.7	0	-1	199 ± 2	+1	0	199 ± 1	+1	0	202 ± 2	+1	+1	200 ± 3	+1	0
Temazepam	5	5.08 ± 0.09	+2	+2	5.3 ± 0.1	+2	+7	5.13 ± 0.05	+1	+3	5.1 ± 0.1	+2	+2	5.11 ± 0.09	+2	+2
	50	49.1 ± 0.3	+1	-2	49.6 ± 0.3	+1	-1	49.7 ± 0.2	0	-1	50.0 ± 0.7	+1	0	49.6 ± 0.2	0	-1
	200	200.2 ± 0.4	0	0	199.9 ± 0.6	0	0	201 ± 1	+1	0	202 ± 3	+1	+1	201 ± 2	+1	0
Diazepam	5	5.3 ± 0.1	+2	+6	5.64 ± 0.07	+1	+13	5.41 ± 0.04	+1	+7	5.4 ± 0.2	+4	+8	5.36 ± 0.09	+2	+7
	50	50.4 ± 0.5	+1	+1	50.3 ± 0.4	+1	+1	50.2 ± 0.4	+1	0	49.8 ± 0.8	+2	0	50.3 ± 0.2	0	+1
	200	200.5 ± 0.7	0	0	201.7 ± 0.7	0	+1	199.9 ± 0.5	0	0	202 ± 2	+1	+1	203 ± 1	+1	+2
Prazepam	5	5.3 ± 0.1	+3	+6	5.65 ± 0.07	+1	+13	5.20 ± 0.04	+1	+4	5.6 ± 0.4	+7	+11	5.30 ± 0.05	+1	+6
	50	50.6 ± 0.7	+1	+1	50.6 ± 0.9	+2	+1	50.4 ± 0.8	+2	+1	50 ± 2	+4	-1	49.9 ± 0.4	+1	0
	200	203 ± 2	+1	+1	205 ± 3	+1	+3	208 ± 4	+2	+4	198 ± 8	+4	-1	201 ± 2	+1	0

* n = 5 for all measurements. Accuracy was measured as the relative error (%E) from the target concentration. Precision was measured as the CV obtained from 5 replicate measurements. Blood was used as the matrix for all benzodiazepine compounds.

Table 5. Freeze/thaw stability.

		Day 1			Day 2			Day 3			Day 4		
	Target (ng/mL)	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E
7-aminoclonazepam	5	5.6 ± 0.1	+2	+12	6.4 ± 0.4	+6	+28	5.6 ± 0.4	+8	+13	5.77 ± 0.09	+2	+15
	50	49.8 ± 0.9	+2	0	50 ± 1	+3	-1	49.4 ± 0.6	+1	+1	49 ± 2	+4	-1
	200	198 ± 2	+1	-1	199 ± 3	+2	0	201 ± 4	+2	0	201 ± 5	+2	+1
7-aminoflunitrazepam	5	5.31 ± 0.08	+2	+6	6.7 ± 0.3	+4	+34	5.8 ± 0.1	+2	+16	5.4 ± 0.3	+5	+9
	50	49.3 ± 0.8	-1	-1	51 ± 1	+2	+2	49 ± 1	+2	-2	49 ± 1	+2	-3
	200	199.4 ± 0.8	+1	0	204 ± 3	+2	+2	203 ± 4	+2	+2	199 ± 7	+4	0
Norchlordiazepoxide	50	47 ± 1	+2	-6	54 ± 1	+3	+8	56 ± 1	+2	+12	51 ± 2	+4	+1
	200	198 ± 6	+3	-1	190 ± 5	+2	-5	189 ± 3	+1	-6	193 ± 9	+4	-3
	1000**	1112 ± 24	+2	+12	1176 ± 15	+1	+18	1169 ± 28	+2	+17	1319 ± 31	+2	+32
Chlordiazepoxide	50	47 ± 1	+2	-7	51 ± 1	+2	+3	48.6 ± 0.2	0	-3	50.8 ± 0.6	+1	+2
	200	200 ± 1	+1	0	190 ± 2	+1	-5	193 ± 3	+2	-4	200 ± 4	+2	0
	1000	1028 ± 13	+1	+3	1094 ± 9	+1	+9	1094 ± 5	0	+9	1147 ± 31	+3	+15
Alpha OH-Midazolam	5	5.2 ± 0.2	+3	+3	5.1 ± 0.3	+6	+3	5.1 ± 0.3	+6	+1	5.1 ± 0.3	+5	+2
	50	49.3 ± 0.8	+2	-1	50 ± 1	+2	-1	49.9 ± 0.6	+1	0	49 ± 2	+4	-3
	200	201 ± 3	+2	+1	200 ± 4	+2	0	200 ± 1	+1	0	200 ± 4	+2	0
Midazolam	5	5.20 ± 0.08	+2	+4	5.7 ± 0.1	+2	+15	5.6 ± 0.1	+2	+11	5.1 ± 0.5	+10	+1
	50	48.9 ± 0.8	+2	-2	51.6 ± 0.6	+1	+3	50.8 ± 0.5	+1	+2	51 ± 1	+2	+1
	200	197 ± 2	+1	-1	209 ± 3	+1	+4	207 ± 3	+1	+3	201 ± 2	+1	+1
Flurazepam	5	4.5 ± 0.2	+3	-11	4.3 ± 0.1	+3	-14	4.2 ± 0.1	+2	-16	4.2 ± 0.1	+3	-16
	50	49.9 ± 0.9	+2	0	50.1 ± 0.6	+1	0	49.1 ± 0.5	+1	-2	49.1 ± 0.3	+1	-2
	200	196 ± 2	+1	-2	196 ± 2	+1	-2	198 ± 4	+2	-1	198 ± 2	+1	-1
Bromazepam	50	47.8 ± 0.4	+1	0	46 ± 1	+2	-8	46.5 ± 0.9	+2	-7	47 ± 2	+3	-6
	200	207 ± 1	-4	+3	199 ± 2	+1	-1	201 ± 2	+1	0	202 ± 5	+2	+1
Nitrazepam	5	5.3 ± 0.1	+3	+6	5.5 ± 0.1	+2	+11	5.3 ± 0.2	+3	+5	5.2 ± 0.1	+3	+3
	50	50.5 ± 0.8	+2	+1	50 ± 1	+2	0	49.6 ± 0.2	0	-1	50 ± 1	+2	-1
	200	201 ± 1	+1	0	206 ± 3	+2	+3	199 ± 2	+1	0	199 ± 2	+1	0
Alpha OH-Alprazolam	5	4.7 ± 0.2	+3	-5	5.9 ± 0.5	+8	+18	5.3 ± 0.3	+5	+6	5.3 ± 0.9	+17	+6
	50	49.9 ± 0.9	+2	0	52.1 ± 0.9	+2	+4	50 ± 1	+2	0	48 ± 2	+5	-3
	200	208 ± 2	+1	+4	201 ± 7	+3	0	205 ± 3	+1	+2	201 ± 2	+1	+1
Oxazepam	5	5.5 ± 0.3	+5	+9	5.4 ± 0.2	+3	+8	5.5 ± 0.2	+4	+10	5.4 ± 0.4	+8	+8
	50	49.7 ± 0.5	+1	-1	51.2 ± 0.5	+1	+2	49.5 ± 0.3	+1	-1	49 ± 2	+4	-2
	200	202 ± 2	+1	+1	199 ± 1	+1	0	199 ± 2	+1	0	199 ± 5	+3	-1

* $n = 5$ for all measurements. Accuracy was measured as the relative error (%E) from the target concentration.
Precision was measured as the CV obtained from 5 replicate measurements. Blood was used as the matrix for all benzodiazepine compounds.
** 3rd freeze/thaw cycle was outside 20% of target value, but within 20% of calculated Day 1 value. Deviation is likely due to preparation error.

Table 5. Freeze/thaw stability… *Continued*

		Day 1			Day 2			Day 3			Day 4		
	Target (ng/mL)	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E
Clonazepam	5	5.4 ± 0.2	+3	+8	5.7 ± 0.3	+5	+14	5.2 ± 0.1	+2	+4	5.5 ± 0.4	+6	+10
	50	49.2 ± 0.7	+2	-2	51.5 ± 0.8	+2	+3	50.4 ± 0.5	+1	+1	48 ± 1	+2	-4
	200	200 ± 3	+1	0	204 ± 1	+1	+2	205 ± 1	+1	+3	199 ± 7	+3	-1
Estazolam	5	5.5 ± 0.3	+5	+10	5.6 ± 0.3	+6	+12	5.7 ± 0.3	+4	+14	5.4 ± 0.4	+8	+9
	50	49 ± 1	+2	-3	49.7 ± 0.7	+1	-1	50.9 ± 0.9	+2	+2	49 ± 2	+4	-2
	200	200 ± 2	+1	0	200 ± 4	+2	0	200 ± 5	+2	0	201 ± 2	+1	0
Lorazepam	5	5.4 ± 0.4	+7	+8	5.3 ± 0.4	+8	+5	5.2 ± 0.4	+7	+3	5.2 ± 0.5	+9	+5
	50	49 ± 2	+3	-2	48 ± 1	+3	-4	48.8 ± 0.9	+2	-2	49 ± 2	+4	-2
	200	202 ± 2	+1	+1	198.8 ± 0.7	0	+1	199 ± 3	+2	-1	197 ± 2	+1	-1
Nordiazepam	5	5.0 ± 0.1	+3	0	5.4 ± 0.2	+4	+7	5.2 ± 0.1	+2	+4	5.2 ± 0.2	+3	+4
	50	50 ± 1	+2	0	49 ± 1	+2	-3	49.8 ± 0.7	+1	0	50.5 ± 0.9	+2	+1
	200	203 ± 2	+1	+1	200 ± 1	+1	0	200 ± 2	+1	0	2023 ± 2	+1	+1
Alprazolam	5	5.4 ± 0.2	+3	+7	5.1 ± 0.4	+8	+3	5.4 ± 0.3	+6	+9	5.2 ± 0.7	+13	+5
	50	48.6 ± 0.5	+1	-3	50.1 ± 0.5	+1	0	49.9 ± 0.5	+1	0	51 ± 2	+3	+3
	200	196.5 ± 2	+1	-2	200 ± 2	+1	0	204 ± 3	+1	+2	202 ± 6	+3	+1
Flunitrazepam	5	5.1 ± 0.2	+4	+3	5.3 ± 0.1	+2	+7	5.3 ± 0.1	+3	+6	5.0 ± 0.2	+3	0
	50	50.1 ± 0.5	+1	0	50.4 ± 0.4	+1	+1	50.1 ± 0.4	+1	0	49.1 ± 1	+2	-2
	200	201 ± 2	+1	+1	205.4 ± 0.9	0	+3	202.4 ± 1	+1	+1	196 ± 1	+1	-2
Desalkylflurazepam	5	4.6 ± 0.2	+5	-9	4.02 ± 0.04	+1	-20	4.2 ± 0.2	+4	-15	4.2 ± 0.2	+4	-16
	50	50.1 ± 0.8	+2	0	50.2 ± 0.8	+2	0	49.6 ± 0.5	+1	-1	48.5 ± 0.7	+1	-3
	200	173 ± 3	+2	-14	173 ± 2	+1	-14	174 ± 2	+1	-13	169 ± 3	+2	-15
Triazolam	5	5.3 ± 0.2	+3	+5	5.5 ± 0.1	+2	+10	5.4 ± 0.2	+4	+8	5.7 ± 0.3	+5	+15
	50	49.5 ± 0.6	+1	-1	49.5 ± 0.6	+1	-1	49.3 ± 0.8	+2	-1	50.1 ± 0.6	-1	0
	200	198.3 ± 0.7	0	-1	200 ± 2	+1	0	200 ± 3	+3	0	202 ± 2	-1	+1
Temazepam	5	5.08 ± 0.09	+2	+2	5.52 ± 0.07	+1	+10	5.14 ± 0.09	+2	+3	5.14 ± 0.07	+1	+3
	50	49.1 ± 0.3	+1	-2	49.8 ± 0.1	0	0	49.7 ± 0.3	+1	-1	49.7 ± 0.7	+1	-1
	200	200.2 ± 0.4	0	0	202 ± 1	+1	+1	200.9 ± 0.7	0	0	202 ± 2	+1	+1
Diazepam	5	5.3 ± 0.1	+2	+6	5.6 ± 0.1	+2	+12	5.4 ± 0.2	+3	+7	5.3 ± 0.3	+5	+7
	50	50.4 ± 0.5	+1	+1	50.4± 0.4	+1	+1	50.0 ± 0.3	+1	0	49.8 ± 0.3	+1	0
	200	200.5 ± 0.7	0	0	201 ± 1	0	+1	200.8 ± 0.5	0	0	199 ± 1	+1	0
Prazepam	5	5.3 ± 0.1	+3	+6	5.55 ± 0.06	+1	+11	5.3 ± 0.2	+3	+5	5.1 ± 0.3	+5	+5
	50	50.6 ± 0.7	+1	+1	50.9 ± 0.5	+1	+2	51.1 ± 0.4	+1	+2	48 ± 1	+3	-4
	200	203 ± 2	+1	+1	205 ± 2	+1	+3	209 ± 4	+2	+4	192 ± 6	+3	-4

* n = 5 for all measurements. Accuracy was measured as the relative error (%E) from the target concentration.
Precision was measured as the CV obtained from 5 replicate measurements. Blood was used as the matrix for all benzodiazepine compounds.

Table 6. On-instrument stability.

		Day 1			Day 2			Day 3			Day 4			Day 5		
	Target (ng/mL)	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E
7-aminoclonazepam	5	5.6 ± 0.1	+2	+12	5.3 ± 0.3	+5	+5	5.5 ± 0.3	+5	+10	5.5 ± 0.2	+4	+10	5.0 ± 0.2	+5	+1
	50	49.8 ± 0.9	+2	0	49 ± 1	+2	-2	51.0 ± 0.8	+2	+2	49 ± 2	+4	-1	49 ± 2	+2	-2
	200	198 ± 2	+1	-1	198 ± 2	+1	-1	199.7 ± 0.7	0	0	202 ± 3	+1	+1	200 ± 1	+1	0
7-aminoflunitrazepam	5	5.31 ± 0.08	+2	+6	5.1 ± 0.2	+5	+1	5.3 ± 0.2	+4	+5	5.1 ± 0.2	+4	+3	4.9 ± 0.3	+6	-1
	50	49.3 ± 0.8	-1	-1	48 ± 1	+2	-4	49.6 ± 0.7	+1	-1	49 ± 3	+5	-1	50 ± 1	+2	0
	200	199.4 ± 0.8	+1	0	196 ± 1	+1	-2	197 ± 4	+2	-2	201 ± 7	+4	0	201 ± 2	+1	0
Norchlordiazepoxide	50	47 ± 1	+2	-6	48 ± 1	+2	-4	47.3 ± 0.7	+2	-5	47.7 ± 0.7	+6	-5	46 ± 3	+6	-7
	200	198 ± 6	+3	-1	196 ± 2	+1	-2	199 ± 4	+2	0	196 ± 4	+2	-2	195 ± 3	+2	-3
	1000	1112 ± 24	+2	+12	1129 ± 20	+2	+13	1130 ± 24	+2	+13	1087 ± 24	+2	+9	1171 ± 14	+1	+17
Chlordiazepoxide	50	46 ± 1	+2	-7	43.6 ± 0.7	+2	-13	42.7 ± 0.5	+1	-15	45.1 ± 0.9	+2	-10	45.5 ± 0.3	+1	-9
	200	200 ± 1	+1	0	19 ± 2	+1	-5	187 ± 1	+1	-7	196 ± 3	+1	-2	196.6 ± 0.9	0	-2
	1000	1029 ± 13	+1	+3	1007 ± 8	+1	+1	993 ± 18	+2	-1	1002 ± 38	+4	0	1140 ± 9	+1	+14
Alpha OH-Midazolam	5	5.2 ± 0.2	+3	+3	4.9 ± 0.5	+10	-3	5.0 ± 0.2	+3	+1	5.3 ± 0.2	+3	+5	5.0 ± 0.2	+3	-1
	50	49.3 ± 0.8	+2	-1	50.1 ± 0.5	+1	0	52 ± 1	+3	+3	50 ± 1	+2	0	51 ± 1	+2	+2
	200	201 ± 3	+2	+1	200 ± 4	+2	0	205 ± 2	+1	+3	207 ± 5	+2	+4	209 ± 2	+1	+4
Midazolam	5	5.20 ± 0.08	+2	+4	5.09 ± 0.05	+1	+2	5.3 ± 0.2	+3	+6	5.03 ± 0.24	+5	+1	5.42 ± 0.09	+2	+8
	50	48.9 ± 0.8	+2	-2	48.5 ± 0.4	+1	-3	50.3 ± 0.7	+1	+1	49 ± 2	+4	-3	51.9 ± 0.6	+1	+4
	200	197 ± 2	+1	-1	196 ± 2	+1	-2	200 ± 1	+1	0	193 ± 4	+2	-4	207 ± 2	+1	+4
Flurazepam	5	4.5 ± 0.2	+3	-11	4.2 ± 0.1	+3	-16	4.1 ± 0.1	+2	-18	4.2 ± 0.1	+2	-17	5 ± 1	+20	+5
	50	49.9 ± 0.9	+2	0	50.0 ± 0.8	+2	0	49.0 ± 0.8	+2	-2	50.2 ± 0.6	+1	0	51 ± 2	+5	+1
	200	196 ± 2	+1	-2	197 ± 3	+1	-2	197 ± 4	+2	-2	197 ± 3	+2	-2	199 ± 5	+3	-1
Bromazepam	50	47.8 ± 0.4	+1	0	46.3 ± 0.8	+2	-7	45.4 ± 0.5	+1	-9	46 ± 1	-8	-8	46.0 ± 0.8	+2	-8
	200	207 ± 1	-4	+3	207 ± 2	+1	+3	205 ± 1	0	+2	200 ± 4	+2	0	201 ± 2	+1	0
Nitrazepam	5	5.3 ± 0.1	+3	+6	5.0 ± 0.2	+3	0	5.1 ± 0.2	+3	+1	5.1 ± 0.1	+2	+1	4.99 ± 0.08	+2	0
	50	50.5 ± 0.8	+2	+1	49.1 ± 0.5	+1	-2	48.7 ± 0.8	+2	-3	47.9 ± 0.3	+1	-4	48.3 ± 0.7	+1	-3
	200	201 ± 1	+1	0	197 ± 1	+1	-1	197 ± 1	+1	-1	197 ± 2	+1	-1	195 ± 2	+1	-3
Alpha OH-Alprazolam	5	4.7 ± 0.2	+3	-5	5.0 ± 0.3	+6	-1	4.8 ± 0.6	+13	-4	5.1 ± 0.6	+12	+2	4.8 ± 0.6	+11	-5
	50	49.9 ± 0.9	+2	0	48.7 ± 0.9	+2	-3	49 ± 1	+3	-2	50 ± 4	+7	-1	50 ± 0.7	+1	0
	200	208 ± 2	+1	+4	201 ± 5	+2	0	199 ± 4	+2	0	202 ± 5	+2	+1	200 ± 4	+2	0
Oxazepam	5	5.5 ± 0.3	+5	+9	5.1 ± 0.2	+5	+2	4.9 ± 0.4	+8	-2	5.0 ± 0.5	+10	0	5.2 ± 0.1	+3	+3
	50	49.7 ± 0.5	+1	-1	48 ± 2	+4	-3	48.4 ± 0.3	+1	-3	48.5 ± 0.3	+1	-3	48 ± 1	+2	-4
	200	202 ± 1	+1	+1	200 ± 4	+2	0	195 ± 5	+3	-2	199 ± 2	+1	-1	194 ± 3	+1	-3

* $n = 5$ for all measurements. Accuracy was measured as the relative error (%E) from the target concentration. Precision was measured as the CV obtained from 5 replicate measurements. Blood was used as the matrix for all benzodiazepine compounds.

Table 6. On-instrument stability… *Continued*

		Day 1			Day 2			Day 3			Day 4			Day 5		
	Target (ng/mL)	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E	Mean (ng/mL)	CV	%E
Clonazepam	5	5.4 ± 0.2	+3	+8	5.2 ± 0.3	+5	+5	5.1 ± 0.3	+5	+2	5.1 ± 0.4	+8	+2	4.9 ± 0.3	+6	-3
	50	49.15 ± 0.7	+2	-2	51 ± 2	+3	+1	51 ± 1	+3	+1	49.1 ± 0.9	+2	-2	50 ± 1	+2	+1
	200	200 ± 3	+1	0	203 ± 3	+1	+1	203 ± 1	+1	+1	203 ± 7	+3	+1	201 ± 2	+1	0
Estazolam	5	5.5 ± 0.3	+5	+10	5.51 ± 0.09	+2	+10	5.8 ± 0.2	+3	+15	5.0 ± 0.3	+6	-1	5.3 ± 0.3	+6	+6
	50	49 ± 1	+2	-3	50.2 ± 0.8	+2	0	50.3 ± 0.7	+1	+1	50.6 ± 0.7	+1	+1	50 ± 1	+2	0
	200	200 ± 2	+1	0	201 ± 3	+1	+1	199 ± 2	+1	0	204 ± 2	+1	+2	201 ± 4	+2	0
Lorazepam	5	5.4 ± 0.4	+7	+8	5.6 ± 0.2	+4	+11	5.2 ± 0.2	+4	+3	5.0 ± 0.3	+5	0	5.4 ± 0.3	+5	+8
	50	49 ± 2	+3	-2	50.1 ± 0.9	+2	0	49.6 ± 0.9	+2	-1	49 ± 3	+5	-2	49 ± 1	+3	-2
	200	202 ± 2	+1	+1	201 ± 3	+1	0	200 ± 1	+1	0	198 ± 3	+1	-1	199 ± 4	+2	0
Nordiazepam	5	5.0 ± 0.1	+3	0	5.0 ± 0.3	+5	-1	4.8 ± 0.2	+4	-5	4.8 ± 0.2	+5	-4	4.6 ± 0.3	+6	-8
	50	50 ± 1	+2	0	48.3 ± 0.4	+1	-3	47.8 ± 0.9	+2	-4	48.6 ± 0.7	+1	-3	47 ± 0.8	+2	-6
	200	203 ± 2	+1	+1	198 ± 3	+1	-1	194 ± 3	+2	-3	196 ± 2	+1	-2	190 ± 3	+2	-5
Alprazolam	5	5.4 ± 0.2	+3	+7	5.1 ± 0.2	+3	+2	5.2 ± 0.2	+2	+4	5.0 ± 0.1	+2	0	5.2 ± 0.2	+4	+5
	50	48.6 ± 0.5	+1	-3	49 ± 1	+3	-2	50.0 ± 0.4	+1	0	49 ± 3	+6	-2	48.5 ± 0.5	+1	-3
	200	197 ± 2	+1	-2	198 ± 3	+1	-1	195 ± 4	+2	-2	193 ± 1	+1	-3	194 ± 4	+2	-3
Flunitrazepam	5	5.1 ± 0.2	+4	+3	5.1 ± 0.1	+3	+2	4.96 ± 0.07	+2	-1	4.9 ± 0.2	+4	-2	5.0 ± 0.1	+2	+1
	50	50.1 ± 0.5	+1	0	49.8 ± 0.7	+1	-1	49.5 ± 0.5	+1	-1	48 ± 1	+3	-3	49.0 ± 0.5	+1	-2
	200	201 ± 2	+1	+1	199 ± 2	+1	0	199 ± 0.9	0	0	196 ± 2	+1	-2	197 ± 1	+1	-1
Desalkylflurazepam	5	4.6 ± 0.2	+5	-9	4.1 ± 0.1	+3	-17	4.1 ± 0.1	+3	-17	4.3 ± 0.2	+4	-13	4.3 ± 0.2	+5	-14
	50	50.1 ± 0.8	+2	0	51.0 ± 0.8	+2	+2	50 ± 1	+3	0	50.7 ± 0.5	+1	+1	50 ± 2	+3	0
	200	173 ± 3	+2	-14	176 ± 2	+1	-12	175 ± 3	+2	-13	174 ± 3	+2	-14	171 ± 3	+2	-14
Triazolam	5	5.3 ± 0.1	+3	+5	5.4 ± 0.2	+4	+8	5.31 ± 0.09	+2	+6	5.1 ± 0.3	+5	+3	5.4 ± 0.2	+4	+8
	50	49.5 ± 0.6	+1	-1	49.4 ± 0.8	+2	-1	49.8 ± 0.6	+1	0	49 ± 1	+3	-2	50.6 ± 0.9	+2	+1
	200	198.3 ± 0.7	0	-1	199 ± 1	+1	0	199 ± 2	+1	-1	203 ± 3	+2	+1	200 ± 2	+1	0
Temazepam	5	5.08 ± 0.09	+2	+2	5.12 ± 0.04	+1	+2	5.02 ± 0.07	+1	0	5.0 ± 0.1	+2	0	5.03 ± 0.08	+2	+1
	50	49.1 ± 0.3	+1	-2	49.2 ± 0.4	+1	-2	48.8 ± 0.3	+1	-2	49.0 ± 0.6	+1	-2	48.5 ± 0.5	+1	-3
	200	200.2 ± 0.4	0	0	199 ± 1	+1	0	198 ± 1	+1	-1	202 ± 3	+1	+1	198.0 ± 0.7	0	-1
Diazepam	5	5.3 ± 0.1	+2	+6	5.2 ± 0.1	+2	+5	2.1 ± 0.1	+2	+2	5.0 ± 0.1	+2	+1	5.0 ± 0.2	+4	0
	50	50.4 ± 0.5	+1	+1	50.2 ± 0.3	+1	0	49.4 ± 0.4	+1	-1	50.5 ± 0.6	+1	+1	49.6 ± 0.5	+1	-1
	200	200.5 ± 0.7	0	0	201 ± 2	+1	0	198 ± 2	+1	-1	202 ± 3	+1	+1	200.1 ± 0.5	0	0
Prazepam	5	5.3 ± 0.1	+3	+6	4.91 ± 0.03	+1	-2	5.1 ± 0.1	+2	+2	5.0 ± 0.2	+3	-1	5.2 ± 0.1	+2	+5
	50	50.6 ± 0.7	+1	+1	47.3 ± 0.6	+1	-5	49 ± 1	+3	-1	49 ± 2	+5	-1	49.2 ± 0.7	+1	-2
	200	203 ± 2	+1	+1	191 ± 2	+1	-5	200 ± 3	+1	0	194 ± 4	+2	-3	200 ± 3	+2	0

* n = 5 for all measurements. Accuracy was measured as the relative error (%E) from the target concentration. Precision was measured as the CV obtained from 5 replicate measurements. Blood was used as the matrix for all benzodiazepine compounds.

Table 7. Hydrolysis efficiency.*

Compound	Free-drug Target (ng/mL)	Mean (ng/mL)
Lorazepam	67	57.4
Oxazepam	62	60.1
Temazepam	62	55.4

* 100 ng/mL glucuronide-conjugate control was prepared for each of the analytes in urine.

Table 8. Ion suppression/enhancement at 50 ng/mL.*

Compound	Blood	Urine	Serum	Liver	Lung	Brain	Muscle	Kidney
7-aminoclonazepam	-4.6	-3.4	-5.1	-24.2	-19.4	-13.0	-3.9	-21.7
7-aminoflunitrazepam	-8.2	-4.1	-1.8	-23.6	-20.1	-15.3	2.1	-23.5
Norchlordiazepoxide	-4.2	-10.8	-2.8	-9.9	-18.9	-13.4	0.7	-21.8
Chlordiazepoxide	-5.9	-4.7	-3.4	-15.3	-19.6	-17.7	1.8	-23.5
Alpha OH-Midazolam	-7.3	-5.7	-2.0	-13.4	-7.6	-21.7	2.9	-22.4
Midazolam	-5.4	-6.2	-3.3	-9.1	-20.6	-17.9	4.1	-22.3
Flurazepam	-1.0	-1.6	-0.3	-7.8	-6.5	-12.0	2.2	-7.3
Bromazepam	-6.2	-8.5	-6.1	-22.4	-23.2	-16.9	-6.1	-21.4
Nitrazepam	-3.8	-3.6	-2.3	-13.9	-21.2	-18.6	1.7	-23.4
Alpha OH-Alprazolam	-2.2	0.0	1.1	-14.0	-10.7	-11.7	7.9	-18.3
Oxazepam	-5.3	-3.1	-2.4	-19.9	-13.4	-21.4	-0.5	-16.9
Clonazepam	-5.4	-4.7	-1.1	-17.9	-22.5	-17.8	0.4	-20.0
Estazolam	-1.2	-3.9	1.8	-9.7	-7.7	-14.8	2.7	-17.6
Lorazepam	-4.6	-3.8	-1.4	-10.5	-22.5	-17.0	0.3	-23.8
Nordiazepam	-1.1	0.7	3.8	-8.7	-15.7	-11.5	6.9	-14.5
Alprazolam	-7.0	-3.0	-3.2	-19.2	-24.7	-20.5	-2.7	-20.6
Flunitrazepam	-6.0	-6.1	-2.2	-16.6	-15.9	-21.1	-0.9	-20.5
Desalkylflurazepam	-4.7	-7.1	-4.4	-22.8	-12.6	-20.9	0.4	-21.4
Triazolam	-3.7	-3.1	-1.7	-19.7	-20.7	-19.3	-0.2	-21.9
Temazepam	-6.0	-3.8	-2.2	-14.2	-19.9	-17.6	3.5	-24.2
Diazepam	-7.2	-7.8	-8.0	-23.2	-17.4	-22.6	-1.8	-20.6
Prazepam	-2.2	-3.9	-1.8	-10.2	-15.0	-15.9	-1.3	-16.6

* $n = 5$ for all measurements. The matrix effect listed here is the ion suppression /enhancement that is not compensated by the deuterated internal standards.

Table 9. Ion suppression/enhancement at 1000 ng/mL.*

Compound	Blood	Urine	Serum	Liver	Lung	Brain	Muscle	Kidney
7-aminoclonazepam	2.3	-1.7	0.9	0.0	0.0	-0.8	-0.7	0.1
7-aminoflunitrazepam	1.2	0.3	6.7	4.0	3.4	-2.9	4.1	0.6
Norchlordiazepoxide	3.7	-6.6	-1.3	2.2	1.7	-3.8	0.5	-1.1
Chlordiazepoxide	-0.5	-7.6	-0.9	-1.9	1.5	-2.7	0.5	-2.6
Alpha OH-Midazolam	4.6	-7.2	-0.6	1.5	2.7	-4.9	-1.5	2.2
Midazolam	2.0	-7.6	0.9	1.4	1.5	-3.5	0.3	0.8
Flurazepam	-7.7	-20.0	-15.3	1.3	-8.5	-23.8	-16.5	-15.0
Bromazepam	1.4	-5.0	2.9	3.3	-1.1	-0.3	3.1	-1.8
Nitrazepam	0.6	-5.1	2.0	0.9	2.0	-1.4	1.1	0.6
Alpha OH-Alprazolam	2.7	-5.3	-0.5	1.5	1.1	-0.7	2.5	-0.2
Oxazepam	-0.8	-10.9	-4.1	1.2	1.2	-4.2	0.1	-1.9
Clonazepam	-1.6	-7.7	-1.5	0.0	0.6	-2.8	-1.7	-2.4
Estazolam	-0.1	-5.1	0.2	1.5	1.0	-2.9	1.2	1.2
Lorazepam	0.9	-5.4	0.8	4.2	3.3	-2.0	2.9	0.1
Nordiazepam	1.4	-4.8	-1.1	2.4	1.8	-0.1	2.8	0.9
Alprazolam	3.5	-4.5	2.5	1.8	2.8	-3.7	1.9	1.4
Flunitrazepam	0.1	-4.5	0.9	0.8	1.3	-0.5	-0.7	1.1
Desalkylflurazepam	-2.4	-6.0	-4.2	-3.2	-1.4	-2.3	-4.4	-0.7
Triazolam	0.6	-6.2	1.6	0.6	-0.2	-3.7	2.6	-2.0
Temazepam	2.8	-2.9	2.6	4.9	4.1	-0.3	4.7	2.2
Diazepam	0.3	-6.5	-1.5	-0.1	1.0	-3.1	1.1	-2.3
Prazepam	-4.3	-12.5	-4.0	-8.5	-6.0	-16.9	-13.6	-15.3

* $n = 5$ for all measurements. The matrix effect listed here is the ion suppression /enhancement that is not compensated by the deuterated internal standards.

It is common to encounter multiple drugs in a case. Therefore, drug interference needed to be evaluated to determine if common drugs can alter the detection and/or quantification of the 22 benzodiazepine compounds. Drugs commonly encountered in our laboratory were prepared at final concentrations of 5 µg/mL and the benzodiazepine compounds at 80 ng/mL. The drugs included for interference were acetaminophen, atenolol, atorvastatin, citalopram, dextromethorphan, diphenhydramine, hydrocodone, methamphetamine, naproxen, and sertraline. Five of these controls were analyzed and none suffered qualitative or quantitative interference.

This newly developed method analyzes for 22 benzodiazepine compounds—14 more than our previous GC/MS procedure. With the ability to analyze for 14 additional drugs, our laboratory should not miss any potentially impairing benzodiazepine compounds. This new procedure uses significantly less biological specimen than our previous method (83% less) and, with the single-step extraction and UPLC separation/analysis, the analysis time has been cut by more than 50%. The new method provided a wide LDR and very low LOD for all analytes.

Furthermore, the accuracy, precision, and stabilities for the analytes were exceptional. Due to the violent nature of aviation accidents, our laboratory receives blood in only approximately 60-70% of the cases examined; therefore, our laboratory must routinely rely on other biological specimens for toxicological analysis. During the validation of this method, the UPLC provided superior chromatography in all fluid and tissue specimens tested compared to the GC/MS method. The new method experienced minimal compensated ion-suppression in all postmortem fluids and tissues tested. The Forensic Toxicology Laboratory is currently utilizing this new method for the determination of postmortem distribution of benzodiazepines in fluids and tissues from a large number of aviation cases.

CONCLUSION

The use of the simple "crash-and-shoot" extraction and the UPLC/MS/MS provided a rapid, robust, and sensitive method for the analysis of 22 benzodiazepine compounds and metabolites from 0.5 mL/0.5 g samples. Analysis performed with this new procedure offers several advantages over our previous GC/MS SPE procedure. This new procedure utilizes significantly less specimen, and has a much wider LDR and lower LOQ. The single-step extraction method is very fast compared to the previous solid phase extraction/derivatization protocol which was time consuming and labor intensive. The new chromatographic analysis total run time is 8 minutes. While this could be shortened, we found that the high organic wash at the end of each run was important for chromatographic reproducibility. This new procedure allows our lab to cut the analysis time by more than 50%. This method also offers the ability to identify and quantitate 22 benzodiazepine compounds, which will give the toxicologist a more complete picture of the drugs present and aid in the toxicological interpretation and determination of drug use as well as possible impairment/overdose.

REFERENCES

1. Baselt, R.C. (2011) Disposition of toxic drugs and chemicals in man, 9th ed. (Biomedical Publications, Foster City, CA).
2. Levine, B. (2013) Principles of forensic toxicology, Fourth ed. (AACC Press, Washington DC).
3. Prescribers' digital reference, Available at http://www.pdr.net/.
4. Drugs.com, Available at https://www.drugs.com/top200/.
5. Smink, B.E., Brandsma, J.E., Dijkhuizen, A., Lusthof, K.J., de Gier, J.J., Egberts, A.C., and Uges, D.R. (2004) Quantitative analysis of 33 benzodiazepines, metabolites and benzodiazepine-like substances in whole blood by liquid chromatography-(tandem) mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci,* **811**, 13-20.
6. Marin, S.J., Roberts, M., Wood, M., and McMillin, G.A. (2012) Sensitive UPLC-MS-MS assay for 21 benzodiazepine drugs and metabolites, zolpidem and zopiclone in serum or plasma. *J Anal Toxicol,* **36**, 472-476.
7. Simonsen, K.W., Hermansson, S., Steentoft, A., and Linnet, K. (2010) A validated method for simultaneous screening and quantification of twenty-three benzodiazepines and metabolites plus zopiclone and zaleplone in whole blood by liquid-liquid extraction and ultra-performance liquid chromatography-tandem mass spectrometry. *J Anal Toxicol,* **34**, 332-341.
8. Marin, S.J., Coles, R., Merrell, M., and McMillin, G.A. (2008) Quantitation of benzodiazepines in urine, serum, plasma, and meconium by LC-MS-MS. *J Anal Toxicol,* **32**, 491-498.
9. Verplaetse, R., Cuypers, E., and Tytgat, J. (2012) The evaluation of the applicability of a high ph mobile phase in ultrahigh performance liquid chromatography tandem mass spectrometry analysis of benzodiazepines and benzodiazepine-like hypnotics in urine and blood. *J Chromatogr A,* **1249**, 147-154.
10. Scientific Working Group for Forensic, T. (2013) Scientific working group for forensic toxicology (SWGTOX) standard practices for method validation in forensic toxicology. *J Anal Toxicol,* **37**, 452-474.