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Ultra Performance Liquid Chromatography with Tandem Mass Spectrometry for the Quantitation of Seventeen Sedative Hypnotics in Six Common Toxicological Matrices

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Abstract

An ultra performance liquid chromatography triple quadrupole mass spectrometry (LC–MS-MS) method for the quantification of 14 benzodiazepines and three sedative hypnotics is presented. The fast and inexpensive assay was developed for California's Orange County Crime Lab for use in antemortem (AM) and postmortem casework. The drugs were rapidly cleaned up from AM blood, postmortem blood, urine, liver, brain and stomach contents using DPX® Weak Anion Exchange (DPX WAX) tips fitted on a pneumatic extractor, which can process up to 48 samples at one time. Assay performance was determined for validation based on recommendations by the Scientific Working Group for Forensic Toxicology for linearity, limit of quantitation, limit of detection, bias, precision (within run and between run), dilution integrity, carry-over, selectivity, recovery, ion suppression and extracted sample stability. Linearity was verified using the therapeutic and toxic ranges of all 17 analytes. Final verification of the method was confirmed by four analysts using 20 blind matrix matched samples. All results were within 20% of each other and the expected value.

Introduction

Benzodiazepines and sedative hypnotics, such as zolpidem, zaleplon and zopiclone are some of the most commonly prescribed and abused drugs in the USA (1–4). Approximately 20 benzodiazepines have been approved for use in the USA and are prescribed as anxiolytics, muscle relaxants, anesthetic adjuncts, anticonvulsants, insomnia treatment and treatment for obsessive-compulsive disorders (1, 2). In forensic toxicology, this means that benzodiazepines and sedative hypnotics can been seen in antemortem (AM) cases, such as driving under the influence of drug(s) and drug-facilitated sexual assaults where both blood and urine could be analyzed (1, 5–9). They are also important in postmortem (PM) toxicology casework, where these same drugs can be used in suicides and analyzed in blood and/or tissue samples. For crime laboratories that analyze both AM and PM casework, it is ideal to have an extraction method that can treat all sample matrices: blood, urine, liver, brain and stomach contents, the same for shorter turnaround times. Due to benzodiazepine and sedative hypnotics' popularity and frequency of abuse, forensic toxicology laboratories need a rapid, sensitive and inexpensive method for determining these drug concentrations in various matrices at the same time (10).

Traditionally, benzodiazepine analysis was performed using gas chromatography–mass spectrometry (GC–MS). Unlike GC–MS, ultra performance liquid chromatography triple quadrupole mass spectrometry (LC–MS-MS) can tolerate injections with aqueous content. Therefore, the high aqueous content found in urine, AM blood, PM blood and tissue homogenate samples is not detrimental to benzodiazepine analysis by LC–MS-MS. In addition, only minimal amounts of specimen were needed to achieve the sensitivity required with elimination of matrix interferences (10). Sample preparation methods, therefore, focused on providing clean, non-aqueous extracts for derivatization. While liquid–liquid extractions (LLE) had been historically

used for the isolation of benzodiazepines and sedative hypnotics from biological samples, solid-phase extraction (SPE) became popular in the 1980s. SPE methods proved to be faster, more efficient and more selective, than LLE methods (11–14). However, both LLE and SPE are time-intensive, hands-on methods that consume a lot of chemical reagents to complete extractions. Since the removal of water is not required when converting methods from GC–MS to LC–MS-MS, a rapid 'clean-up' method using DPX® Weak Anion Exchange (DPX WAX) tips was developed (13, 15, 16).

Unlike the traditional bind and elute methods for SPE, the DPX-WAX clean-up method focuses on removing matrix interferences, while keeping the benzodiazepines in the sample solution. The DPX-WAX tips are modified pipette tips containing loose weak-anion exchange sorbent similar to that found in SPE columns. The poly-amino functionalized resin removes phospholipids and small non-amphoteric acids that can cause ion suppression. By simply aspirating and dispensing the sample solutions in the DPX WAX tips, matrix interferences are removed with minimal losses of drug (15, 16). This reduces the time required for extraction by minimizing several conditioning and washing steps, reducing the volume of solvent required for elution and eliminating analyst error with multiple transfer steps (10). The initial addition of acetonitrile to the sample solutions keeps the benzodiazepines and sedative hypnotics from binding to the resin. The end result is a clean-up method that can process up to 48 samples consisting of six common toxicological matrices for LC–MS-MS analysis in just a few minutes.

Methods

Reagents

HPLC grade water, methanol, acetonitrile, isopropanol and formic acid were purchased from Fisher Scientific (Waltham, MA). Ammonium hydroxide was purchased from Sigma-Aldrich Co. (Atlanta, GA). All drug standards were purchased from Cerilliant Corp. (Round Rock, TX). The blood, urine, liver, brain and stomach contents samples were from drug negative cases obtained from the Orange County Coroner's Office. The porcine's blood was obtained from Farmer John's (Vernon, CA). DPX WAX tips were obtained from DPX Labs (Columbia, SC).

Sample preparation and extraction

Prior to analysis, brain samples were prepared by weighing out 20 g of the sample, adding 20 g of deionized water and grinding the mixture in a blender until homogenized. Liver samples were prepared the same way, except 40 g of deionized water was used. Stomach contents were blended in their entirety and then diluted 1:100 with deionized water. Initially, 250 µL of sample (blood or urine) or standard or weigh 250 mg of diluted tissue was pipetted or weighed into a test tube. Urines were hydrolyzed at 55°C for 3 h prior to extraction using β-glucuronidase type HP-2 from *Helix pomatia*. Then 50 µL of deuterated internal standard (ISTD) and 750 µL of acetonitrile were added to each test tube. Samples were briefly vortexed and centrifuged for approximately 4 min. Using the DPX WAX tips, aspirate and dispense the supernatent in the tips three times on the pneumatic extractor, holding the supernatant for 15 s each time. Transfer 50 µL of elutant into LC vials and dilute with 800 µL of mobile phase.

LC conditions

Chromatographic separation was accomplished using the Waters Acquity UPLC. The autosampler was set at 4°C, and optimized gradient chromatographic conditions were identified using the Waters BEH C18 1.7 µL column (2.1 × 100 mm) held at 40°C. The mobile phase A and B were water with 0.1% formic acid and acetonitrile with 0.1% formic acid, respectively. The LC conditions started at 80% A with a linear decrease to 60% A at 1.75 min which was held until 3.5 min. A linear decrease to 15% A to 5 min and then an automatic jump to 0% A for 2 min to wash any organic off the column. From 7.5 to 9.5 min, the system re-equilibrated to the initial conditions of 80% A. Retention times (RTs) and instrument conditions for each analyte can be seen in Table I.

MS–MS conditions

Detection of the target analytes was optimized using a Waters XeVo TQ-S triple quad mass spectrometer with positive electrospray ionization. The mass transitions and MS parameters for each drug are shown in Table I.

Method validation

The evaluation of the assay was conducted over 8 months. Sample batches were analyzed as recommended by SWGTOX for biological assay validation for calibration model, lower limit of quantitation (LOQ), lower limit of detection (LOD), accuracy, precision, dilution integrity, carry-over, selectivity, absolute recovery, ion suppression and stability (17). The final validation test was for multiple trained analysts to conduct analysis on 20 blind samples to determine if their results could be within 20% of each other and the expected value.

Absolute recovery

The absolute percent recovery was determined at middle concentrations for each drug in each matrix. It was determined by spiking blank matrices with this concentration and extracting them without ISTD. The ISTD was added after the final elution off the DPX WAX tips. The ratio of the drug quantifier ion area to the same area of the ISTD was calculated and compared with the same ratio in diluted non-extracted standards. The average of four recovery extractions in all matrices can be seen in Table II.

Calibration model

SWGTOX guidelines require the calibration curve to have at least six standards and one quality control (QC) (17). For this method, seven standards and two QCs were used to cover the concentration ranges needed to cover therapeutic and toxic concentrations for each drug (18–21). The calibration standard range was determined for all drugs being quantitated by reviewing toxicologically important concentrations found in literature and by the ease of making standards. For the majority of the drugs, the entire therapeutic range is covered up to fatal levels (18–21). Linearity was verified from a seven-point calibration curve for each drug. Diazepam, nordiazepam, temazepam, oxazepam and chlordiazepoxide ranged from 100 to 6,400 ng/mL. Midazolam, estazolam, zopiclone, zolpidem, clonazepam, alprazolam and lorazepam concentrations ranged from 10 to 640 ng/mL, while zaleplon was 4–256 ng/mL. Flunitrazepam, flurazepam and phenazepam ranged from 2 to 128 ng/mL, while triazolam ranged from 1 to 64 ng/mL. The final regression model was determined by following the SWGTOX guidelines to be a quadratic $1/x^2$ calibration curve with no forcing through the origin (17).

Table I. Analytes, ISTD, RT, MRM Transitions, Dwell Time, Collision and Cone Energies for the Quant Ion

Analyte	ISTD	RT (min)	Transition	Dwell Time (sec)	Cone (V)	Collision (V)
Alprazolam	Alprazolam-d5	3.05	309>>281	0.009	36	40
			309>>205			
Chlordiazepoxide	Chlordiazepoxide-d5	1.68	300>>227	0.025	32	25
			300>>241			
Clonazepam	Oxazepam-d5	3.01	316>>270	0.009	42	34
			316>>241			
Diazepam	Diazepam-d5	4.33	285>>193	0.044	44	32
			285>>154			
Estazolam	Estazolam-d5	2.85	295>>267	0.009	46	38
			295>>205			
Flunitrazepam	Flunitrazepam-d7	3.39	314>>268	0.025	60	34
			314>>239			
Flurazepam	Midazolam-d4	2.16	388>>315	0.025	44	24
			388>>288			
Lorazepam	Oxazepam-d5	3.01	321>>275	0.009	38	42
			321>>194			
Midazolam	Midazolam-d5	2.07	326>>291	0.025	52	38
			326>>249			
Nordiazepam	Nordiazepam-d5	3.08	271>>140	0.009	46	26
			275>>208			
Oxazepam	Oxazepam-d5	2.87	306>>260	0.009	44	32
			306>>177			
Phenazepam	Diazepam-d5	4.07	349>>206	0.044	62	28
			349>>184			
Temazepam	Temazepam-d5	3.64	301>>255	0.040	28	34
			301>>177			
Triazolam	Nordiazepam-d5	3.19	343>>308	0.009	46	26
			343>>315			
Zaleplon	Midazolam-d4	2.54	306>>236	0.025	44	26
			306>>260			
Zolpidem	Zolpidem-d6	1.54	308>>235	0.032	40	34
			308>>263			
Zopiclone	Zopiclone-d4	1.20	389>>245	0.032	16	30
			389>>217			

Underlined ions were used as quantifier.

Table II. Average Recoveries for the Benzodiazepine and Z-Drug Method in Various Matrices

Drug	Aqueous	Blood	Liver	Brain	Stomach Contents	Urine
Zopiclone	88.07	73.63	74.88	74.40	76.78	87.66
Zolpidem	85.64	75.47	75.08	76.06	77.34	88.19
Chlordiazepoxide	83.01	63.35	63.27	63.15	66.45	74.45
Midazolam	80.08	69.43	70.02	70.99	71.54	81.59
Flurazepam	87.61	69.60	76.31	75.80	80.69	83.59
Zaleplon	91.07	78.78	77.45	78.92	83.67	90.37
Estazolam	86.34	70.41	75.60	75.52	75.44	87.38
Oxazepam	73.87	60.84	63.98	64.40	63.04	72.95
Lorazepam	77.80	64.28	62.85	66.39	66.85	76.25
Clonazepam	91.50	76.98	76.02	81.86	79.57	93.11
Alprazolam	91.02	79.41	79.78	80.32	77.87	93.12
Nordiazepam	65.23	56.00	55.34	55.15	54.30	58.98
Triazolam	76.95	71.78	68.99	72.94	68.06	83.19
Flunitrazepam	72.17	61.80	59.87	60.58	59.83	67.19
Temazepam	79.59	67.59	68.66	69.18	68.76	79.37
Phenazepam	76.55	66.59	64.92	68.41	65.46	71.11
Diazepam	73.40	62.80	63.18	63.61	61.77	66.75

Limit of quantitation and limit of detection

The aqueous standards for the quantitation method were extracted three times along with the QCs and six further dilutions of 1:2 of the lowest standard. The runs were then processed normally to ensure that the standards and QCs made acceptable curves. Then the extra diluted standards were included in the calibration curve one at a time, from the highest concentration to the lowest, until the LOQ was determined. Forensically significant LOQ concentrations were established using literature references and previous cases (18–21). The LOD was determined based on chromatography, including acceptable ion ratio and RT. The concentrations seen in Table III were then fortified into blank AM blood, PM blood, liver (1:3), brain (1:2), stomach contents (1:100) and urine. Each matrix was extracted in triplicate in three runs. All drugs were then analyzed to determine if they met the acceptance criteria of a normal run, which included good chromatography, correct RT and identification ratios within 20% of the standards. On average, all drugs met these criteria in all matrices.

Bias and precision

Bias and precision were determined in five extractions. Each extraction consisted of triplicate sampling of PM blood, AM blood, brain (1:2),

Table III. Concentrations Extracted to Determine LOQ and LOD of the Instrument

Analyte	Reporting LOQ (ng/mL)	LOQ (ng/mL)	LOD (ng/mL)
Chlordiazepoxide	100	25	6.25
Oxazepam	100	50	12.5
Nordiazepam	100	50	6.25
Diazepam	100	50	6.25
Temazepam	100	50	6.25
Midazolam	10	5	1.25
Estazolam	10	2.5	2.5
Zopiclone	10	1.25	1.25
Zolpidem	10	5	1.25
Clonazepam	10	2.5	2.5
Lorazepam	10	5	2.5
Alprazolam	10	5	1.25
Zaleplon	4	2	0.5
Flunitrazepam	2	1	0.25
Phenazepam	2	1	1
Flurazepam	2	1	1
Triazolam	1	0.5	0.5

liver (1:3), stomach contents (1:100) and urine fortified at high, medium and low concentrations for each drug. SWGTOX Guidelines requires that all drugs in all matrices must have a bias and precision less than ±20% at each concentration level (17). All biases were less than +20% on all matrices except zaleplon at the high concentration level in all matrices. Zaleplon also had many individual concentrations greater than +20% of the expected value in the medium and low concentrations in various matrices. All drugs, at all concentrations, in all matrices have a between run precision and within run precision less than +20% except zaleplon in some matrices. Thus, zaleplon cannot currently be accurately quantitated in this method.

Carry-over

The standard set of calibrators was extracted with samples that contained 3, 4, 8 and 10 times the highest standard concentration were extracted. A separate blank was extracted and injected on the instrument to follow each standard to determine if carry-over above the LOD was seen at any concentration. No significant carry-over above the LOD was seen at 10 times any concentrations for all drugs. Possible carry-over was seen for zolpidem, oxazepam, nordiazepam, temazepam and diazepam at concentrations that were 8 and 10 times higher than the highest standard. However, the possible carry-over was less than 10% of the LOQ concentration for these drugs.

Selectivity

All drugs and ISTDs in the method were extracted separately at concentrations near the upper limit of linearity to determine if any of the compounds interfered with the transitions of the other compounds in the methods. It was determined that clonazepam-d4 interfered with lorazepam and lorazepam-d4 interfered with alprazolam. Since these two drugs are common benzodiazepines seen in casework, the internal standards were removed from the method and both drugs will use oxazepam-d5 as an ISTD. Chlordiazepoxide-d5 has similar transitions to zolpidem; however, the difference between their RTs is more than 5%. Therefore, both zolpidem and chlordiazepoxide-d5 remained in the method.

Selectivity of the assay was assessed in all six matrices to ensure that endogenous biological components did not interfere with the assay. Five different samples of all possible matrices were extracted with no internal standard to determine if there were any matrix interferences for any of the drugs. The matrices extracted were: AM blood, PM blood, pig's blood (which would be used to dilute a casework blood sample), brain, liver, urine and stomach contents. The PM samples included at least one sample from a decomposition case. No peaks were detected that co-eluted with the drugs or internal standards in the method.

Selectivity from others drugs not detected in this method was evaluated. Since not every drug could be evaluated, 79 of the most commonly confirmed drugs within the lab were evaluated by extracting 10 µg/mL of each drug without internal standard. The drugs tested were acetaminophen, amitriptyline, amphetamine, benzoylecgonine, benztropine, brompheniramine, buprenorphine, bupropion, butalbital, caffeine, carbamazepine, carisoprodol, chlorpheniramine, chlorpromazine, citalopram, cocaine, codeine, cyclobenzaprine, desipramine, dihydrocodeine, diphenhydramine, doxepin, doxylamine, ephedrine, fentanyl, fluoxetine, gabapentin, guaifenesin, hydrocodone, hydromorphone, hydroxychloroquine, ibuprofen, imipramine, ketamine, lamotrigine, lidocaine, methylenedioxyamphetamine, methylenedioxymethamphetamine, memantine, meprobamate, methadone, methadone metabolite (EDDP), methamphetamine, methorphan, methoxetamine, metoprolol, mirtazapine, morphine, naproxen, nicotine, norfluoxetine, norsertraline, nortriptyline, orphenadrine, oxycodone, oxymorphone, paroxetine, PCP, pentobarbital, phenobarbital, phentermine, phenytoin, promazine, promethazine, propofol, propoxyphene, pseudoephedrine, quetiapine, quinidine, quinine, salicylic acid, sertraline, THC, THCA, topiramate, tramadol, trazodone, valproic acid, verapamil and warfarin.

Promazine could interfere with demoxepam and chlorpromazine could interfere with lorazepam; however, in both instances, the ion ratio and RT are incorrect. Imipramine can interfere with nitrazepam; however, the ion ratio is outside of the acceptable ±20%. Citalopram can interfere with midazolam; however, at 10 µg/mL, it is not detected above the cut-off and would not have the same RT. Orphenadrine could interfere with zolpidem as a K^+ adduct; however, at 10 µg/mL, it is not detected above the LOQ. All combinations of possible interference and method analytes were extracted together to ensure that the presence of both drugs in a sample would not affect the results.

Ion suppression and enhancement

Analyte and ISTD concentrations that fell in the middle of the calibration curve were infused, post-column, on the MS while extracted blank matrices were injected on the LC methods. Four blank samplings of AM blood, PM blood, liver (1:3), stomach contents (1:100), brain (1:2) and urine were extracted. For all PM samples, a sample from a decomposed body was included. Suppression was only seen in eight drugs, see Table IV. By the SWGTOX guidelines, there cannot be ion suppression/enhancement >25% averaged across the blank matrices. Thus, there were no ion suppressions or enhancements that required further investigation of the method.

Table IV. Ion Suppression

Drug	Matrix	Transition	Percentage
Estazolam-d5	Brain	300>>272	<10% in one sample
Oxazepam-d5	Brain	Both	~20% in one sample
Oxazepam	Brain	Both	~10% in one sample
Diazepam	PM blood	Both	<10% in one sample
Diazepam-d5	PM blood	Both	<10% in one sample
Zopiclone-d4	PM blood	Both	~20% in one sample
Midazolam-d4	PM blood	Both	~15% in one sample

Extracted drug stability

A set of calibration standards along with QCs were extracted and injected on an LC–MS-MS on the day of extraction, the following day, 2 days after extraction, 5 days after extraction and 1 week after extraction. Between injections, samples were kept in the 4°C sample manager on the LC–MS-MS. Once all the injections were completed, the runs were processed normally. For no drug, in any injection, was a calibrator removed in the calibration curve. The R^2 values were examined over time to determine if the fit got worse. All $R^2 > 0.99$ and residuals were similar over all injections. Only two QCs were outside of the acceptance criteria on the injections performed a week after extraction. The two QCs that fell outside of acceptable criteria were one for each clonazepam and triazolam, 3% high and 4% low, respectively. However, since the second QC for both drugs was within acceptable range, the calibration curve was still valid. The area counts of each compound in each standard were also tracked after each injection to determine if drug was breaking down while sitting on the instrument. For all drugs in all standards, the area counts increase from the initial injection to the final injection. This is most likely due to solvent evaporating over time, which would cause the samples to become more concentrated creating an increase in area counts.

Dilution integrity

For all drugs quantitated, dilution integrity was determined from samples with a known amount of each drug present. Dilution integrity was investigated only in urine and both types of blood samples. The sample amount for all tissue samples will always be 0.25 mg as any dilution will take place prior to sampling. The samples were diluted 1:2 and 1:5 with either blank porcine blood or deionized water in two separate extractions. All dilutions were within 20% of their non-diluted value and the between run precision did not exceed 10%.

Results and discussion

The presented method demonstrated acceptable reliability, reproducibility and specificity for the quantitation of 17 sedative hypnotics and the detection of nine metabolites in six common toxicological matrices. The assay was free of any significant interference from commonly ingested drugs or matrices and had minimal ion suppression. Minimal carry-over can be seen on five of the drugs, at concentrations 10 times higher than the highest standard and not realistic concentrations seen in casework. Of the 17 drugs that were validated, all of them can be quantitated except for zaleplon. Zaleplon's bias and precision was not less than ±20% as required. This is suspected to be due to a lack of deuterated internal standard at time of validation. Thus, zaleplon is currently reported qualitatively.

As a final test, 30 samples, casework or fortified matrix, were extracted by four different analysts using the final extraction and instrument method. The 30 samples contained 89 drugs in total. The results from each analyst were compared with each other and the true value. There were no unexplainable deviations in all four extractions of each sample. The method has been in practice since October of 2013 and quantitations of 16 drugs, with uncertainty have been reported on all cases since then.

Conclusion

An LC–MS-MS method for the quantitation of 17 sedative hypnotics and identifying nine more in blood, urine and tissues was developed and validated following SWGTOX guidelines. The method uses a fast and simple clean-up procedure prior to chromatographic analysis and is ideal for AM and PM toxicology work. The DPX WAX tips used for extraction show benefits over traditional LLE or SPE by a reduction in extraction time and solvent use. The resulting clean-up method gave rapid extractions (<2 min), high recoveries, 48 sample throughout and negligible solvent waste. The use of LC–MS-MS over GC–MS for analysis allows for smaller sample size and eliminating the need to ensure the final sample is water free. Validation of 14 benzodiazepines and three sedative hypnotics using the DPX WAX tip clean-up method was demonstrated following SWGTOX guidelines for method validation for both AM and PM casework.

In the future, stability of these drugs in the same toxicological matrices will be investigated over a 1 year time frame. Benzodiazepines and sedative hypnotics can be abused in all aspects that concern forensic toxicology. Whether looking for the drugs in blood in DUID cases, blood or tissues in postmortem work, or urine in pain management, DPX® WAX tips offer a fast and simple clean-up method that can be used for blood, urine, brain, liver and stomach contents.

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Conflict of interest

None declared.

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