Journal of Analytical Toxicology, 2016;40:201–207
doi: 10.1093/jat/bkv140
Advance Access Publication Date: 10 January 2016
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Comparison of LC–MS-MS and GC–MS Analysis of Benzodiazepine Compounds Included in the Drug Demand Reduction Urinalysis Program

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Abstract

Liquid chromatography–tandem mass spectrometry (LC–MS-MS) offers specific advantages over gas chromatography–mass spectrometry (GC–MS) such as the ability to identify and measure a broader range of compounds with minimal sample preparation. Comparative analysis of LC–MS-MS versus GC–MS was performed for urinalysis detection of five benzodiazepine compounds currently part of the Department of Defense (DoD) Drug Demand Reduction Program (DDRP) testing panel; alpha-hydroxyalprazolam, oxazepam, lorazepam, nordiazepam and temazepam. In the analyses of internally prepared control urine samples at concentrations around the DDRP administrative decision point for benzodiazepines (100 ng/mL), both technologies produced comparable results with average accuracies between 99.7 and 107.3% and average coefficients of variation (%CV) <9%. Analysis of service member specimens that screened positive for benzodiazepines using both technologies produced comparable results for all analytes. Different degrees of matrix effect were observed for all analytes in the LC–MS-MS analysis. However, the effects were controlled by using deuterated internal standards (ISTDs). Additionally, there was a 39% increase in nordiazepam mean concentration analyzed by LC–MS-MS due to suppression of the ISTD ion by the flurazepam metabolite 2-hydroxyethylflurazepam. The ease and speed of sample extraction, the broader range of compounds that can be analyzed and shorter run time make the LC–MS-MS technology a suitable and expedient alternative confirmation technology for benzodiazepine testing.

Introduction

While gas chromatography–mass spectrometry (GC–MS) has been widely recognized as the 'gold standard' in forensic testing, use of liquid chromatography–tandem mass spectrometry (LC–MS-MS) is becoming more common. This is because LC–MS-MS offers several advantages over GC–MS such as quicker and less extensive extraction procedures and the ability to identify and measure a broader range of compounds. LC–MS-MS has been authorized for use in federally regulated workplace drug testing (WPDT) since 2008 (1) and its use is also expanding in the Department of Defense (DoD) Drug Demand Reduction Program (DDRP), most recently for the analysis of benzodiazepines.

Benzodiazepines remain one of the most widely prescribed classes of drugs to manage anxiety, insomnia, seizures, muscle relaxation and a myriad of other conditions. Five benzodiazepines in particular are among the 200 most commonly prescribed drugs in the USA, alprazolam (Xanax®), lorazepam (Ativan®), clonazepam (Klonopin®), diazepam (Valium®) and temazepam (Restoril®) (2). Benzodiazepines are commonly abused due in part to their availability and strong sedative properties. Additionally, benzodiazepines are often used in combination with illicit drugs to enhance their effects making them a target for drug abuse monitoring programs (3, 4).

In 2012, five benzodiazepine compounds were added to the DDRP urinalysis drug testing panel; nordiazepam, alpha-hydroxyalprazolam, oxazepam, lorazepam and temazepam. WPDT platforms commonly employ an initial screening test followed by a confirmatory test by either GC–MS or LC–MS-MS. However, in high-throughput testing

operations with strict turnaround requirements, such as DDRP laboratories, benzodiazepine detection and measurement by GC–MS is not temporally optimal as polarity and thermal stability issues can require extensive sample preparation and long run times.

Some of these issues can be avoided using LC–MS-MS. Unlike GC analysis, sample volatilization is not required for LC which avoids problems associated with chemical degradation and the formation of new products common under high heat conditions. Particularly attractive to high volume laboratory environments is the fact that LC–MS-MS specimens typically require no derivatization and minimal sample preparation. In some cases, the specimens can be diluted and directly injected into the LC–MS-MS, which significantly increases throughput.

While previous studies have compared the performance of LC–MS-MS versus GC–MS for detecting and measuring various drugs in human urine (1, 5), there are currently no studies that compare the performance of these two analytical methods in the detection and analysis of benzodiazepines. In this study, we compared the performance of LC–MS-MS versus GC–MS technology for detecting, in urine, five benzodiazepines currently part of the DDRP testing panel. Benzodiazepine confirmation testing was carried out according to DoD requirements (6) using GC–MS and LC–MS-MS methods certified by the Armed Forces Medical Examiner System (AFMES). Both methods were compared with respect to their linearity, precision, accuracy and reproducibility of internally prepared quality control materials in the detection of benzodiazepines.

Materials and methods

Chemicals and reagents

All solvents used were of analytical grade or better. Sodium acetate buffer, carbonate buffer, acetonitrile, methylene chloride, methanol, ethyl acetate and formic acid were purchased from Fisher Scientific (Fair Lawn, NJ). β-glucuronidase (type HP-2), anhydrous ethanol, ammonium hydroxide and *N-tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) (w/1% *N*-methyl-*N-tert*-butyldimethylsilytrifluoroacetamide, MTBDMCS) were purchased from Sigma-Aldrich (St. Louis, MO). Solid-phase extraction (SPE) columns (CEREX® CLIN II) for the GC–MS method were purchased from SPEware (San Pedro, CA) and SPE columns (Clean Screen® XCEL I) for the LC–MS-MS method were purchased from United Chemical Technologies (Bristol, PA).

Preparation of standards, calibrator, controls and samples

Reference material of alpha-hydroxyalprazolam (AHAL), AHAL-d5, Oxazepam (OXAZ), OXAZ-d5, Lorazepam (LORA), LORA-d4, Nordiazepam (NORD), NORD-d5, Temazepam (TEMA) and TEMA-d5 used in the preparation of control and internal standard (ISTD) solutions and sample material were obtained from Cerilliant (Round Rock, TX). Reference material of AHAL, OXAZ, LORA, NORD and TEMA used in the preparation of the calibrator solution were obtained from Alltech (Deerfield, IL).

The calibrator was prepared by fortifying 1,000 mL of certified drug-free urine with 0.1 mg of AHAL, OXAZ, LORA, NORD and TEMA, for a final concentration of 100 ng/mL. The low quality control (LQC) was prepared by fortifying 1,000 mL of certified drug-free urine with 0.04 mg of AHAL, OXAZ, LORA, NORD and TEMA, for a final concentration of 40 ng/mL. The high quality control (HQC) was prepared by fortifying 500 mL of certified drug-free urine with 0.5 mg of AHAL, OXAZ, LORA, NORD and TEMA, for a final concentration of 1,000 ng/mL. The blind quality control (BQC) was prepared by fortifying 1,000 mL of certified drug-free urine with 0.125 mg of AHAL, OXAZ, LORA, NORD and TEMA, for a final concentration of 125 ng/mL. The ISTD used for the GC–MS method was prepared by fortifying 1,000 mL of anhydrous ethanol with 1 mg of AHAL-d5, OXAZ-d5, NORD-d5 and TEMA-d5, for a final concentration of 1,000 ng/mL. The ISTD used for the LC–MS-MS method was prepared by fortifying 1,000 mL of anhydrous ethanol with 0.5 mg of AHAL-d5, OXAZ-d5, LORA-d4, NORD-d5 and TEMA-d5, for a final concentration of 500 ng/mL. All solutions were aliquoted and stored at −30°C.

Sample material was prepared by fortifying 100 mL of certified drug-free urine with AHAL, OXAZ, LORA, NORD and TEMA at 20, 40, 75, 100, 125, 200, 500 and 1,000 ng/mL. The material was aliquoted into 1 and 0.5 mL aliquots for GC–MS and LC–MS-MS analysis, respectively. The aliquots were stored at −30°C. A total of ten independent batches of four replicates of sample material for each analyte concentration were analyzed by GC–MS and LC–MS-MS. Calibrators and controls were analyzed with each batch.

GC–MS analysis

Sample preparation and extraction

A 1 mL urine aliquot, 0.100 mL of ISTD (final concentration of 100 ng/mL of AHAL-d5, OXAZ-d5 (used for OXAZ and LORA), NORD-d5 and TEMA-d5), 2 mL 0.1 M sodium acetate buffer (pH 4.75) and 0.050 mL of β-glucuronidase (type HP-2) were combined. Tubes were capped, vortexed and incubated for 60 min at 55°C. Tubes were allowed to cool and then centrifuged for 5 min at 3,000 rpm. Samples were transferred to 3 mL CEREX® CLIN II cartridges, and positive pressure was applied at a rate of 1 mL/min using CEREX® System 48-11 from SPEware Corporation (Baldwin Park, CA). The cartridges were then washed with 1 mL of pH 9 carbonate buffer, followed by 1 mL of water–acetonitrile (80:20), followed by 1 mL of water. The cartridges were dried for 15 min at 50 psi. Analytes were eluted with 1 mL methylene chloride–methanol–ammonium hydroxide (85:10:2) and then evaporated to dryness at 55°C. Derivatization was accomplished by adding 0.050 mL of ethyl acetate and 0.050 mL of MTBSTFA (w/1% MTBDMCS) to the dried extracts. Tubes were capped, vortexed and incubated for 20 min at 65°C. Samples were removed from the heat block, allowed to cool and transferred to the GC–MS.

Instrumental analysis

GC–MS equipment consisted of an Agilent Technologies 7890 GC coupled to a 5975 MS (Palo Alto, CA). A sample volume of 0.5 µL was injected into an Agilent HP-ULTRA 1 (15 M, 0.20 mm, 0.33 µm) column, using a helium carrier gas at a flow rate of 0.9 mL/min and operating in a pulsed splitless mode. Data analysis was done using Agilent Drug Analysis Chemstation, version E.02.02. GC parameters are listed in Supplementary Material, Table SI and MS analysis was conducted in selected ion monitoring (SIM) mode, and ions are listed in Supplementary Material, Table SII. Two benzodiazepine dedicated instruments, ID9252 and ID9700, were used for GC–MS analyses. Three ions for drugs and two ions for ISTD were monitored for compound identification. Furthermore, criteria of peak retention times within ±1% and ion ratios within ±20% of batch calibration standard were used.

LC–MS-MS analysis

Sample preparation and extraction

A 0.5 mL urine aliquot, 0.100 mL ISTD solution (final concentration of 100 ng/mL of AHAL-d5, OXAZ-d5, LORA-d4, NORD-d5 and

TEMA-d5), 1 mL 0.1 M sodium acetate buffer (pH 4.75) and 0.025 mL of β-glucuronidase (type HP-2) were combined. Tubes were capped, vortexed and incubated for 60 min at 55°C. Tubes were allowed to cool and centrifuged for 5 min at 3,000 rpm. Samples were transferred to 3 mL UCT Clean Screen® XCEL I cartridges, and positive pressure was applied at a rate of 1 mL/min. The cartridges were dried for 1 min at 25 psi. The cartridges were then washed with 1 mL of methylene chloride, followed by an additional 5 min dry down at 50 psi. Analytes were eluted with 1 mL ethyl acetate–ammonium hydroxide (100:2) and then evaporated to dryness at 55°C. Samples were reconstituted with 0.200 mL of mobile phase (0.1% formic acid–acetonitrile; 75:25) and transferred to the LC–MS-MS.

Instrumental analysis

LC–MS-MS equipment consisted of a Waters ACQUITY LC (Milford, MA). A sample volume of 10 µL was injected into an ACQUITY UPLC BEH C18 (1.7 µm, 2.1 × 50 mm) column coupled to a Waters Quattro Micro triple quadrupole mass spectrometer with an electrospray source (Milford, MA). Data analysis was performed using Waters MassLynx and TargetLynx, version 4.1 SCN805, with smoothing = mean, iterations = 2 and width = 3. The mobile phase components were (A) 0.1% formic acid and (B) acetonitrile. The LC parameters are listed in Supplementary Material, Table SIII. The analyses were conducted in multiple reaction monitoring (MRM) mode using the MS–MS conditions listed in Supplementary Material, Table SIV with cone voltages and collision energies optimized. The same compound identification criteria were employed for LC–MS-MS with the exception of the retention time, which was ±2%.

Matrix effects

Matrix effects (ME) were assessed using the method described by Matuszewski, *et al.* (7). Two concentrations were used in the evaluation: one at the LQC concentration of 40 ng/mL and one at the administrative decision point of 100 ng/mL. Three sets of samples were prepared. The (A) samples contained the target analytes fortified into the mobile phase. The (B) samples contained the target analytes fortified into negative urine prior to extraction. The (C) samples contained the target analytes fortified into urine after extraction. All the samples also contained 100 ng/mL of ISTD. The mean area responses for the target analytes and the corresponding ISTD in samples A, B and C were evaluated across ten different lots of negative urine. Urine used in the ME studies were randomly selected from service member specimens that screened negative for all drugs tested in the DDRP panel. ME was calculated by taking the ratio of the urine fortified with target analytes after extraction divided by the mobile phase fortified with target analytes $(C/A) \times 100$. Resulting values <100 indicate ionization suppression and values >100 indicate ionization enhancement. Recovery was calculated by taking the ratio of urine fortified with target analytes prior to extraction divided by urine fortified with target analytes after extraction $(B/C) \times 100$. The process efficiency was calculated by taking the ratio of urine fortified with target analytes prior to extraction divided by the mobile phase fortified with target analytes $(B/A) \times 100$.

Limit of detection, limit of quantitation and upper limit of linearity

The limit of detection (LOD) and limit of quantitation (LOQ) were established for GC–MS and LC–MS-MS methods. For each analyte, four replicates of the concentration range of 2.0–40.0 ng/mL were quantitated. The LOD was defined as the lowest concentration where the coefficient of variation of the replicates was ≤10%, all ion ratios were ±20% of the calibrator and retention times were within ±1 for GC or ±2% for LC of the averages established by the calibrator. The LOQ was defined as the lowest concentration where the mean concentration of the replicates was within ±20% of the target concentration with coefficient of variation ≤10%, all ion ratios were ±20% of the calibrator and retention times were within ±1 for GC or ±2% for LC of the averages established by the calibrator. Also, all peaks met resolution and symmetry requirements. The upper limit of linearity (ULOL) was assigned based on ten times the benzodiazepine DDRP administrative decision point of 100 ng/mL.

Method comparison

Internally prepared sample solutions containing AHAL, OXAZ, LORA, NORD and TEMA at 20, 40, 75, 100, 125, 200, 500 and 1,000 ng/mL were analyzed by GC–MS and LC–MS-MS. The methods were compared with respect to their linearity, precision, accuracy and reproducibility.

Interferences

Interference samples were prepared by fortifying certified drug-free urine to a concentration of 40 ng/mL of the target compounds and 5,000 ng/mL of the interferant compounds indicated in Supplementary Material, Table SV: (i) structurally similar compounds; (ii) a mix of over the counter (OTC) drugs and (iii) mix of 'club' drugs. The interference samples were then analyzed in five replicates by GC–MS and LC–MS-MS technologies. The interference effect was evaluated based on quantitation values within ±20% of the target concentration and acceptable chromatography.

Service member urine specimens side-by-side comparison

Benzodiazepine concentrations in service member urine specimens were measured using both GC–MS and LC–MS-MS and then compared to assess agreement between the two confirmation technologies. Urine specimens used in this evaluation were anonymously donated from US service members submitted to NDSL Jacksonville for drug screening in accordance with the Military Personnel Drug Abuse Testing Program (MPDATP). As per DDRP procedures, specimens were aliquoted and screened by immunoassay (IA). Screening was conducted on a Roche/Hitachi (Indianapolis, IN) DAT 2400 immunoanalyzer. Urine was screened for benzodiazepines using the Microgenics Corporation CEDIA® (Fremont, CA) DAU immunoassay kit (1775561). Oxazepam in urine at 200 ng/mL was used as reference calibrator using a two-point calibration. Aliquots that screened at or above the cutoff for benzodiazepines were extracted and confirmed for benzodiazepines using both GC–MS and LC–MS-MS as described previously. All identifiers were removed prior to testing.

Statistical analysis

The average, standard deviation (SD), coefficient of variation (%CV), accuracy and mean difference were calculated using Microsoft Excel 2010 (Seattle, WA). Evaluation of the linear regression with analysis of variance (ANOVA) of the regression fit, one-way ANOVA for precision analysis, and Student's *t*-test analyses were conducted using Minitab 17 (State College, PA).

Results and discussion

LOD and LOQ evaluation

Table I summarizes the LOD and LOQ results for the three instruments used in this study. For most analytes, data used to establish

Table I. Evaluation of Instruments LOD, LOQ and ULOL

Instrument	Analyte[a]	LOD (ng/mL)	LOD %CV	LOQ (ng/mL)	LOQ %CV	ULOL (ng/mL)
GC–MS ID 9252	AHAL	5.53	0.9	5.53	0.9	1,000
	OXAZ	19.31	1.7	24.66	0.6	1,000
	LORA	6.13	3.4	6.13	3.4	1,000
	NORD	7.72	0.4	7.72	0.4	1,000
	TEMA	14.99	1.0	14.99	1.0	1,000
GC/MS ID 9700	AHAL	5.70	0.9	5.70	0.9	1,000
	OXAZ	5.62	4.3	15.61	4.9	1,000
	LORA	26.30	4.8	26.30	4.8	1,000
	NORD	14.98	0.9	14.98	0.9	1,000
	TEMA	14.56	1.1	14.56	1.1	1,000
LC–MS-MS ID 1768	AHAL	5.96	3.2	5.96	3.2	1,000
	OXAZ	15.83	1.0	15.83	1.0	1,000
	LORA	2.15	3.4	2.15	3.4	1,000
	NORD	1.96	8.1	1.96	8.1	1,000
	TEMA	2.03	4.1	2.03	4.1	1,000

LOD, limit of detection; LOQ, limit of quantitation; ULOL, upper limit of linearity.

[a]$n = 4$ for all analytes at all concentrations evaluated.

the LOD values also met the criteria to be established as the LOQ value. For LORA, NORD and TEMA the LC–MS-MS instrument attained lower LOD and LOQ values, ranging from 1.96 to 2.15 ng/mL with CVs between 3.4 and 8.1%, respectively, than the GC–MS instrument, value ranges of 6.13–26.30 ng/mL with CVs between 0.4 and 4.8%, respectively. For AHAL and OXAZ, the LOD and LOQ values were equivalent for both technologies.

Method comparison of GC–MS and LC–MS-MS

Independent sets of samples containing four replicates of the target concentrations: 20, 40, 75, 100, 125, 200, 500 and 1,000 ng/mL were analyzed by GC–MS (10 sets) and LC–MS-MS (9 sets) using a single point calibration at the DDRP administrative decision point of 100 ng/mL. Comparison of GC–MS and LC–MS-MS measured concentrations and descriptive statistics are presented in Table II. Of particular interest is the comparison of both technologies at concentrations around the DDRP administrative decision point of 100 ng/mL. At concentrations of 75, 100 and 125 ng/mL, GC–MS technology had an average accuracy of 102% with CVs < 5% for all analytes across the three concentrations, while the LC–MS-MS technology average accuracy was a comparable 105% with CVs < 7% (Table II). Qualitatively, LC–MS-MS measured concentrations demonstrated a greater spread as compared with GC–MS measurements. However, the % difference between GC–MS and LC–MS-MS at measured concentrations around the DDRP administrative decision point of 100 ng/mL ranged between 0.14 and 5.07% (Table II).

Further, GC–MS analysis across all concentrations for all analytes had a within-run precision average CV < 4%, and a between-run precision average CV < 5% (Supplementary Material, Table SVI). When the measured concentration was compared with the target concentration, the average accuracy for all analytes ranged between 99.3 and 103.8%, with % CVs ranging from 2.93 to 4.93%. Regression analysis was also used to compare measured versus target concentration following the methods used by Stout *et al.* (1, 5). Regression comparison to target concentrations and ANOVA analysis of the line of fit showed that all analytes had a slope close to the ideal value of 1.000 ($r^2 > 0.993$), which was significantly different from 0.000 with all intercepts equal to zero with the exception of NORD and TEMA, suggesting a significant deviation in measured versus target concentration for these two analytes. However, in both cases, the intercepts were below the LOQ established for the two GC–MS instruments used for the analysis (Table I). Further, the within-run precision and between-run precision average CVs were below 2 and 4%, respectively, with average accuracies of 100.8 for NORD and 99.3 for TEMA (Supplementary Material, Table SVI).

LC–MS-MS analysis across all concentrations for all analytes had a within-run precision average CV of <4%, and a between-run precision average CV of <6% (Supplementary Table SVII). The average accuracy for all analytes ranged between 102.5 and 105.9%, with % CVs ranging between 3.77 and 7.14%. Regression comparison to target concentrations and ANOVA analysis of the line of fit showed that all analytes had a slope close to the ideal value of 1.000 ($r^2 > 0.992$), which was significantly different from 0.000 with all intercepts equal to zero with the exception of LORA. Suggesting a significant deviation in measured versus target concentration for LORA. However, LORA within-run precision and between-run precision CV averages were below 3 and 4%, respectively, with an average accuracy of 105.1% (Supplementary Material, Table SVII).

Evaluation of ME

Table III summarizes the LC–MS-MS ME for all analytes at 40 ng/mL and at the DDRP administrative decision point for benzodiazepines of 100 ng/mL in 10 different human urine matrices. For all analytes except NORD, ME led to target ion enhancement ranging from 2 to 52% at both concentrations. Only for NORD at 100 ng/mL did the ME cause target ion suppression by <1%. Target ion recovery (RE) and process efficiency (PE) ranged between 44–83% and 44–95%, respectively. For all analytes at both concentrations, target and ISTD ion responses were similar, suggesting that urine ME effects were appropriately controlled by including compound matched deuterated ISTDs in each analysis. This was further demonstrated by the quantitation analysis of C samples (target analytes fortified into urine after extraction) reported in Table III. For both concentrations, accuracies for AHAL, OXAZ, LORA, NORD and TEMA ranged between 98.3 and 102.5% with CVs <5%.

Interference study

Results for the interference study are summarized in Table IV. Deviations between target and mean measured concentrations were within ±20% for GC–MS analysis for all analytes. For LC–MS-MS analysis, deviations between target and mean measured concentrations were within ±20% for all analytes, with the exception of NORD in the presence of 2-hydroxy-ethylflurazepam (2HEF). In comparing GC–MS to LC–MS-MS results, only discrepancies greater than 10% were considered of practical interest. GC–MS and LC–MS-MS measured mean concentrations for LORA and NORD differed more than 10% when 2HEF was fortified into 40 ng/mL of these analytes. For LORA, 2HEF caused a 15% difference in measured concentrations between technologies. For NORD, 2HEF increased the LC–MS-MS mean quantitation value by 39% as compared with the results obtained using GC–MS. Supplementary Material, Figure S1 shows an LC–MS-MS response of a representative sample of NORD at 40 ng/mL containing the interferant 2HEF. In the interference sample, NORD ISTD ion response was suppressed by 36% in comparison to the ISTD ion response in the LQC at 40 ng/mL. In contrast, similar responses were observed for the interference sample

Table II. Descriptive Statistics of GC–MS and LC–MS-MS Analysis of Sample Material at Variable Target Concentrations

Analyte	Target concentration	GC–MS average concentration[a]	GC–MS average concentration CV%	GC–MS concentration average % accuracy	LC–MS-MS average concentration[b]	LC–MS-MS average concentration CV%	LC–MS-MS concentration average % accuracy	GC–MS LC–MS-MS % difference
AHAL	20	19.9	2.61	99.5	20.6	6.24	103.0	3.5
	40	39.6	3.08	99.0	40.0	4.86	100.0	1.0
	75	**74.8**	**2.26**	**99.7**	**77.1**	**6.01**	**102.8**	**3.1**
	100	**100.2**	**1.93**	**100.2**	**102.7**	**6.12**	**102.7**	**2.5**
	125	**125.1**	**2.24**	**100.1**	**128.1**	**5.39**	**102.5**	**2.4**
	200	200.3	2.53	100.2	203.2	5.35	101.6	1.4
	500	501.0	3.83	100.2	517.1	5.45	103.4	3.2
	1,000	981.9	3.94	98.2	1,042.3	4.91	104.2	6.2
OXAZ	20	20.5	4.15	102.5	20.8	5.45	104.0	1.5
	40	40.4	3.94	101.0	41.8	5.46	104.5	3.5
	75	**76.9**	**3.50**	**102.5**	**78.7**	**5.72**	**104.9**	**2.3**
	100	**103.1**	**3.75**	**103.1**	**105.7**	**4.21**	**105.7**	**2.5**
	125	**127.0**	**4.91**	**101.6**	**130.6**	**5.26**	**104.5**	**2.8**
	200	202.2	5.38	101.1	207.7	5.42	103.9	2.7
	500	505.0	6.74	101.0	527.6	6.18	105.5	4.5
	1,000	1,005.0	6.06	100.5	1,060.9	7.30	106.1	5.6
LORA	20	20.5	4.45	102.5	21.1	4.37	105.5	2.9
	40	41.1	3.18	102.8	41.6	3.65	104.0	1.2
	75	**78.1**	**3.90**	**104.1**	**80.0**	**3.83**	**106.7**	**2.4**
	100	**105.7**	**4.91**	**105.7**	**105.8**	**4.06**	**105.8**	**0.1**
	125	**130.1**	**4.86**	**104.1**	**132.0**	**3.15**	**105.6**	**1.5**
	200	206.3	5.31	103.2	213.1	3.60	106.6	3.3
	500	523.7	5.76	104.7	520.2	2.89	104.0	−0.7
	1,000	1,034.6	5.82	103.5	1,028.6	3.05	102.9	−0.6
NORD	20	20.4	4.19	102.0	21.2	5.15	106.0	3.9
	40	40.1	3.82	100.3	42.2	4.12	105.5	5.2
	75	**76.6**	**2.59**	**102.1**	**80.5**	**3.68**	**107.3**	**5.1**
	100	**102.4**	**3.04**	**102.4**	**106.1**	**4.03**	**106.1**	**3.6**
	125	**127.3**	**2.75**	**101.8**	**133.2**	**3.33**	**106.6**	**4.6**
	200	202.2	3.36	101.1	212.5	3.60	106.3	5.1
	500	496.8	5.07	99.4	527.7	3.88	105.5	6.2
	1,000	974.2	3.99	97.4	1,041.8	4.05	104.2	6.9
TEMA	20	20.0	3.00	100.0	20.3	4.32	101.5	1.5
	40	39.6	2.48	99.0	41.1	2.68	102.8	3.8
	75	**75.4**	**2.60**	**100.5**	**78.0**	**3.39**	**104.0**	**3.4**
	100	**100.1**	**2.81**	**100.1**	**103.5**	**3.34**	**103.5**	**3.4**
	125	**124.9**	**2.88**	**99.9**	**129.6**	**3.76**	**103.7**	**3.8**
	200	199.1	2.97	99.6	207.6	4.07	103.8	4.3
	500	491.7	4.57	98.3	526.2	3.60	105.2	7.0
	1,000	966.8	3.90	96.7	1,057.9	4.71	105.8	9.4

Bolded values denote data for concentrations around the DDRP decision point for benzodiazepines (100 ng/mL).

[a] $n = 40$ for each analyte at each target concentration.

[b] $n = 36$ for each analyte at each target concentration.

and the low quality control NORD target quant ion. These findings are consistent with the observed difference in NORD measured concentration between GC–MS and LC–MS-MS. Although scientifically interesting, the impact of 2HEF on NORD quantitation is of minimal concern for DDRP laboratory operations since the concentration used for the interference study was comparable to urine concentrations found in fatal cases (8), additionally flurazepam is not a commonly prescribed benzodiazepine (9). However, the effect of 2HEF on NORD quantitation should be taken into consideration during method development if the same parental and transition ions are chosen.

Successful analysis of TEMA (40 ng/mL) by GC–MS was not accomplished in three of five replicates containing club drug mixture. In each case, the analysis did not meet GC–MS chromatographic criteria for the qualifier ion 359 *m/z*. No problems were encountered with the analysis of TEMA by LC–MS-MS.

Urinalysis specimens analyzed by both GC–MS and LC–MS-MS

Table V summarizes the results for service member specimens analyzed for benzodiazepines using both GC–MS and LC–MS-MS. The regression analysis suggests a linear relationship between both technologies for all analytes. A *t*-test analysis found no significant difference ($P < 0.05$) between GC–MS and LC–MS-MS quantitation values for NORD, OXAZ, LORA and AHAL. In contrast, TEMA results differed significantly between technologies ($P = 0.025$), however the mean percent difference between both technologies was found to be <3% (data

Table III. LC–MS-MS Matrix Effect Evaluation at 40 and 100 ng/mL

Analyte	Target ion			ISTD ion			Quantitation (type C sample)		
	% ME	% RE	% PE	% ME	% RE	% PE	Average[a]	% Accuracy	% CV
AHAL(40)	151.9	59.7	90.8	154.8	59.7	92.4	39.3	98.3	2.79
AHAL(100)	129.4	63.6	82.2	128.7	65.1	83.8	100.7	100.7	3.39
OXAZ(40)	114.5	82.7	94.7	111.8	83.0	92.8	41.0	102.5	1.39
OXAZ(100)	107.6	81.5	87.7	107.1	81.2	87.0	100.6	100.6	4.21
LORA(40)	108.6	77.9	84.5	109.7	78.4	86.1	39.6	99.0	3.27
LORA(100)	103.0	75.8	78.1	103.1	77.3	79.7	100.0	100.0	3.40
NORD(40)	105.2	80.1	84.2	105.3	80.9	85.2	39.9	99.8	2.94
NORD(100)	99.4	82.7	82.2	100.4	81.9	82.2	99.0	99.0	3.65
TEMA(40)	106.4	53.8	57.3	106.8	54.6	58.3	39.9	99.8	2.88
TEMA(100)	101.9	43.7	44.5	101.0	45.6	46.1	101.0	101.0	3.91

Type C samples contained the target analytes fortified into urine after extraction.
ME, matrix effect; RE, recovery; PE, process efficiency.
[a]$n = 10$ for each analyte at each target concentration.

Table IV. GC–MS and LC–MS-MS Interference Analysis at Target Concentration of 40 ng/mL

Analyte	OTC Mix		Club Mix		AHT		7AC		2HEF		AHM	
	GC	LC	GC	LC	GC	LC	GC	LC	GC	LC	GC	LC
AHAL												
Average	37.8	34.8	37.5	34.2	46.1	47.7	37.7	34.5	37.3	33.7	37.9	40.3
% CV	1.27	3.50	0.21	3.10	4.41	6.23	2.07	3.85	1.16	1.82	0.80	7.30
OXAZ												
Average	40.3	37.8	40.4	37.8	40.5	37.1	40.2	37.4	40.1	37.5	40.6	38.5
% CV	1.25	3.14	0.46	1.50	0.70	2.57	1.06	1.76	1.43	1.89	0.88	1.00
LORA												
Average	38.3	39.7	38.4	39.7	38.5	38.8	38.7	38.7	37.7	43.4[a]	38.2	39.9
% CV	3.63	2.63	0.82	0.70	2.51	2.55	3.34	1.88	1.71	2.63	2.10	2.54
NORD												
Average	40.2	40.1	40.1	39.3	40.3	39.2	40.2	38.8	39.9	55.4[a,b]	40.6	39.7
% CV	1.38	3.73	0.51	2.70	0.62	2.53	1.43	2.17	0.94	1.97	0.79	1.69
TEMA												
Average	40.1	39.6	39.7	39.0	39.4	37.8	39.2	38.4	39.2	38.9	39.7	39.1
% CV	0.60	3.45	1.94	1.43	0.99	2.43	1.36	1.03	1.15	1.81	1.70	2.91

$n = 5$ for each analyte and each interferant.
[a]≥10% deviation between GC–MS and LC–MS-MS measured concentrations.
[b]≥20% deviation between target and measured concentration.

Table V. Side By Side Comparison of GC–MS and LC–MS-MS Results for DoD Service Member Specimens Submitted to NDSL Jacksonville, FL (Nov 2012–Jul 2013)

	NORD	OXAZ	LORA	TEMA	AHAL
N	50	81	7	71	8
r^2	0.991	0.998	0.995	0.998	0.998
Regression slope	1.09	1.02	0.98	1.05	1.03
t-test results (*P*-value)	0.057	0.109	0.052	0.025[a]	0.090
Average GC–MS ISTD response	60,771	11,745	11,661	34,031	31,364
% CV	23.87	30.18	21.21	26.09	30.78
Average LC–MS-MS ISTD response	37,447	101,989	24,302	133,746	18,854
% CV	19.75	11.77	8.30	17.24	20.71
Average GC–MS retention time	5.93	6.68	7.21	6.80	9.10
% CV	0.44	0.53	0.65	0.58	0.82
Average LC–MS-MS Retention time	1.91	1.80	1.87	2.11	1.66
% CV	0.22	0.25	0.26	0.18	0.00

[a]GC–MS and LC–MS-MS results significantly different ($P < 0.05$) for TEMA.

analysis not shown). The ISTD responses for all analytes measured via LC–MS-MS had a tighter distribution as compared with GC–MS. Both technologies had similar and tightly distributed retention time windows.

Conclusion

This study demonstrated that LC–MS-MS technology is comparable with GC–MS and produced confirmatory testing results that meet DDRP guidelines and criteria for urine drug testing without the extensive sample preparation required for GC–MS analysis. Both technologies produced comparable results at concentrations around the DDRP administrative decision point for benzodiazepines (100 ng/mL), with excellent accuracy and precision across the range of concentrations evaluated. Analysis of service member specimens that screened positive for benzodiazepines using both technologies produced comparable results for all analytes. Different degrees of ME were observed for all analytes in the LC–MS-MS analysis. However, these effects were controlled for by using deuterated ISTD resulting in minimal impact on measured versus target quantitation values. Interference in NORD LC–MS-MS quantitation occurred in the presence of flurazepam metabolite 2HEF resulting in a 39% increase in NORD mean measured concentration from suppression of the sample ISTD ion by 2HEF. However, this effect occurred at 2HEF concentrations that were 125 times greater than the sample and in quantities higher than the literature suggests would be physiologically realistic concentrations. This study confirmed that LC–MS-MS technology is a suitable and expedient alternative for DDRP benzodiazepine confirmation testing. LC–MS-MS offers the additional advantages of ease and speed of sample extraction, shorter run times and potentially a broader range of compounds that can be analyzed as compared with GC–MS.

Disclaimer

The views expressed in this article are those of the authors and do not necessarily reflect the official policy or position of the Department of the Navy, Department of Defense, nor the US Government.

Supplementary material

Supplementary Material is available at *Journal of Analytical Toxicology* online.

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