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Research Article

Despite the improved clinical sensitivity of the Roche benzodiazepines II assay it cannot replace mass spectrometry in all patient populations

Nicole V. Tolan [a,b], Sacha Uljon [a,b], M. Lauren Donnelly-Morell [a,b], Melissa Zhao [a,b], Grace K. Mahowald [a,b], Marion L. Snyder [c], Lindsey Contella [c], Elizabeth D. Urwiller [a], Maria Daluz Fernandes [a], Phillip Kang [a], Stacy E.F. Melanson [a,b,*]

[a] Department of Pathology, Mass General Brigham, Boston, MA, United States
[b] Harvard Medical School, Boston, MA, United States
[c] Luxor Scientific, Greenville, SC, United States

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ABSTRACT

Introduction: Benzodiazepines are frequently prescribed and misused therefore urine drug screening (UDS) is performed in many patient populations. Most current benzodiazepine immunoassays have poor sensitivity, particularly for detecting the metabolites of newer benzodiazepines such as lorazepam in urine.
Objectives: We aimed to verify the clinical performance of the new qualitative Roche Benzodiazepines II (BNZ2) immunoassay, as well as compare its performance to the Roche Benzodiazepines Plus (BENZ) assay in two patient populations: UDS in the emergency department (ED) and compliance monitoring.
Methods: An initial verification study was performed, selecting for samples containing clonazepam and lorazepam metabolites. Performance of the BNZ2 and BENZ assays was compared to liquid chromatography-tandem mass spectrometry (LC-MS/MS) as the reference method. Sensitivity, specificity, false positive rate (FPR) and false negative rate (FNR) were determined.
Results: We verified the performance claims in the initial verification and demonstrated similar precision, with coefficient of variations (CVs) of 12.8% and 7.7% for negative and positive controls, respectively. Furthermore, we observed higher clinical sensitivity and lower FNR with the BNZ2 assay in both the ED and compliance monitoring populations due to improved cross-reactivity for lorazepam and clonazepam metabolites. Despite these improvements, the BNZ2 assay was unable to detect 27% of specimens positive by LC-MS/MS, including specimens from patients using benzodiazepines without prescription.
Discussion: Due to its improved performance and rapid turnaround time, the BNZ2 assay should be implemented for UDS in the ED. However, the assay should not replace LC-MS/MS testing for compliance monitoring, as unsuspected benzodiazepine use may go undetected.

Introduction

Benzodiazepines, a group of psychoactive drugs, are increasingly prescribed in many clinical settings due to their anxiolytic and muscle relaxant properties [1,2]. The rate of benzodiazepine abuse is rising, and the number of emergency department (ED) visits related to benzodiazepines is reaching a level similar to opioids, illustrating a nationwide problem with benzodiazepine misuse [3–6]. This trend suggests that patients should be screened for benzodiazepine use when presenting to the ED with altered mental status, trauma, and other indications for drug testing, as well as for compliance monitoring to assess adherence and detect possible diversion. Furthermore, the increase in

Abbreviations: ALD, automated liquid dispenser; BNZ2, Benzodiazepines II assay; BENZ, Benzodiazepines Plus assay; BWH, Brigham and Women's Hospital; CRR, clinical reportable range; CEDIA, Cloned Enzyme Donor Immunoassay; CV, coefficient of variation; ED, emergency department; FNR, false negative rate; FPR, false positive rate; IS, internal standard; HCL, hydrochloric acid; KIMS, Kinetic Interaction of Microparticles in Solution; LDT, laboratory developed test; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MGB, Mass General Brigham; MGH, Massachusetts General Hospital; SDs, standard deviations; UDS, urine drug screening.

* Corresponding author at: Brigham and Women's Hospital, Department of Pathology, Boston, MA 02115, United States.
E-mail address: semelanson@bwh.harvard.edu (S.E.F. Melanson).

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the use of synthetic benzodiazepines, such as bromazolam, flualprazolam, and flubromazepam, highlights the need to consider the performance of benzodiazepine assays in patients abusing these substances [7,8].

Despite the inclusion of benzodiazepine immunoassays on most urine drug screening (UDS) panels, most immunoassay screens were primarily designed to detect diazepam and its metabolites, resulting in poor sensitivity for detecting the use or misuse of newer benzodiazepines such as lorazepam [1,9–12]. Previous studies have shown high rates of false negative benzodiazepine screening results with both the Kinetic Interaction of Microparticles in Solution (KIMS) and Cloned Enzyme Donor Immunoassay (CEDIA) methods [12]. These false negative results can cause confusion and may lead clinicians to mistakenly believe that patients are diverting their medications when they are actually taking them as prescribed, or can allow patients taking unprescribed benzodiazepines to go undetected.

A beta-glucuronidase enzymatic pre-treatment, aimed at converting glucuronidated benzodiazepine metabolites to their parent form prior to analysis, has been demonstrated to provide improved sensitivity compared to traditional screening assays [12–15]. However, sensitivity and specificity were still higher when using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Additionally, the use of glucuronidase in many immunoassays is considered off-label, requiring validation as a laboratory developed test (LDT).

In this study, our aim is to investigate and compare the performance of the new Roche Benzodiazepines II (BNZ2) assay with our previous Roche Benzodiazepines Plus (BNZ) assay, using LC-MS/MS as the standard reference method. We describe the performance of the BNZ2 assay and determine its sensitivity, specificity, false negative rate (FNR), and false positive rate (FPR) in the ED and for compliance monitoring.

Methods

Study design

This study was conducted with the approval of the Mass General Brigham (MGB) Institutional Review Board. Residual specimens from Brigham and Women's Hospital (BWH) and Massachusetts General Hospital (MGH) were included in the study. An initial verification study was conducted, specifically selecting samples that contained clonazepam and lorazepam metabolites, as these compounds have historically shown low sensitivity in benzodiazepine immunoassays. Additionally, we examined the clinical performance of the BNZ2 and BENZ assays in two distinct patient populations: ED and compliance monitoring. In the ED, rapid UDS is necessary to confirm acute toxicity and/or overdose, where higher concentrations of contributing drugs are expected to be present in the urine. For compliance monitoring, it is important to detect benzodiazepines and/or their metabolites, even at low concentrations, in order to assess compliance and identify undisclosed use. Consecutive urine samples from both the ED (with UDS ordered) and compliance monitoring (with LC-MS/MS ordered) patient populations were collected and stored for subsequent testing.

Immunoassays

The qualitative automated BENZ assay (Roche Diagnostics, Indianapolis, IN, USA) and BNZ2 assay (Roche Diagnostics, Indianapolis, IN, USA) were performed using the Roche c502 analyzer (Roche Diagnostics, Indianapolis, IN, USA) at either BWH Laboratories or MGH Laboratories, following the manufacturer's instructions for use. For both assays, a manufacturer-supplied 100 ng/mL nordiazepam calibrator was utilized to establish the assay cutoff. The absorbance of the calibrator was normalized to a value of 0, and signals equal to or greater than 0 were considered positive. The manufacturers' reported cross-reactivity of the BENZ and BNZ2 assays for select benzodiazepines and their metabolites can be found in Supplemental Table 1.

BWH LC-MS/MS

Benzodiazepines were measured using a previously published dilute and shoot method, originally developed for the determination of 37 prescription and illicit compounds in urine [16], but modified to incorporate a 2D liquid chromatography method. As it pertains to benzodiazepine testing, 100 μL of centrifuged urine is mixed with 700 μL of water and 200 μL of an internal standard (IS) working solution containing 100 ng/mL of aplrazolam-D5, alpha-hydroxyalprazolam-D5, 7-aminoclonazepam-D4, diazepam-D5, nordiazepam-D5, lorazepam-D4, oxazepam-D5, and temazepam-D5 (Cerilliant, Round Rock, TX, USA). The analysis was performed using a Waters ACQUITY UPLC and Xevo TQ-S Micro Triple Quadrupole mass spectrometer (Waters Corporation, Milford, MA, USA), utilizing a 2D trap and back-flush method with positive electrospray ionization and multiple reaction monitoring. A nine-point (5, 25, 50, 75, 100, 250, 500, 1000, and 2000 ng/mL) calibration curve was used for the quantitative determination of alprazolam, 7-aminoclonazepam, lorazepam, nordiazepam, oxazepam, and temazepam where as a seven-point (5, 25, 50, 75, 100, 250, and 500 ng/mL) curve was used for lorazepam glucuronide and oxazepam glucuronide. Qualitative determination of clonazepam and diazepam was performed using a three-point calibration curve (5, 50, and 500 ng/mL). The lower limit of the clinical reportable range (CRR) was 5 ng/mL for all compounds, except for lorazepam glucuronide and oxazepam glucuronide, which had a lower limit of 25 ng/mL, as shown in Supplemental Table 1.

Luxor extended LC-MS/MS panel

Samples that were presumed to be falsely positive by the immunoassay (i.e., positive by one or both immunoassays and negative by the BWH LC-MS/MS assay) were deidentified and sent to Luxor Scientific, LLC for further analysis. Benzodiazepines were measured in an extended panel developed to detect 101 illicit and prescription compounds in urine (Luxor Scientific, LLC, https://luxorscientific.org/test-menu/, Test Code: A001).

For the analysis, 50 μL of spun urine samples were combined with 165 μL of a master mix in a narrow bore extraction column. The master mix consisted of a buffer mixture, LCMS-grade water, working IS, and ICMSzyme RT (IMCS, Irmo, SC, USA). The samples were then incubated for 15 min at room temperature (20–25 °C) to allow the enzyme to release the compounds from their glucuronide state, thus enhancing sensitivity.

Solid phase extraction was performed using an automated liquid dispenser (ALD) (Tecan, Switzerland). The samples were washed with water and 100 mM hydrochloric acid (HCL) in water, and then dried using nitrogen gas. Elution of the samples was carried out into a deep well plate using an elution solvent composed of dichloromethane, isopropanol alcohol, and ammonium hydroxide at 40:10:1, respectively. After elution, the samples were dried using an ultra-vap evaporator and then rehydrated with 200 μL of a reconstitution solution of 10 % methanol and LCMS-grade water.

For separation and detection, samples were run on an Agilent LC 1260 and 6460 triple quad (Agilent, Santa Clara, CA, USA). A five-point calibration curve was used for the quantitative analysis of 7-aminoclonazepam, alpha-hydroxyalprazolam, alpha-hydroxymidazolam, alpha-hydroxytriazolam, lorazepam, nordiazepam, oxazepam, and temazepam at concentrations of 12.5, 25, 50, 200, and 1600 ng/mL. For bromazolam, the calibrator concentrations were 3, 6, 12, 48, and 384 ng/mL. The lower limits of the CRRs for these compounds are shown in Supplemental Table 1.

BNZ2 immunoassay verification

The intra-assay precision of the BNZ2 assay was assessed by repeated same day within-run measurements (n = 21) of negative and positive

quality control materials, which were supplied by the manufacturer and had concentrations within ± 25 % of the 100 ng/mL calibrator. Similarly, inter-assay precision was evaluated over a period of 30 days, with standard deviations (SDs) and coefficients of variation (% CVs) calculated.

To further verify the performance of the BNZ2 assay, 59 residual urine specimens from BWH with clinical UDS or LC-MS/MS orders were selected (Fig. 1). These specimens were chosen to assess the manufacturer's claims of improved cross-reactivity to glucuronidated benzodiazepines and other benzodiazepines, as described in Supplemental Table 1. The selected specimens included known concentrations of lorazepam, clonazepam, and their metabolites, as detected by LC-MS/MS.

Clinical performance of BNZ2 in ED and compliance monitoring

A total of 158 consecutive residual urine specimens from BWH and 137 consecutive residual urine specimens from MGH were included in the study. These urine specimens were collected from patients in the ED who underwent clinical UDS, resulting in a total of 295 ED specimens (Fig. 1). Additionally, 200 consecutive residual urine specimens from BWH submitted for clinical benzodiazepine testing by LC-MS/MS were included. These LC-MS/MS tests were ordered to assess compliance with chronic opioid therapy or monitor patients in addiction management programs. Nine specimens were excluded from the study due to insufficient volume to run one or more methods. Thus, a total of 191 specimens were included for compliance monitoring (Fig. 1).

All three methods (BNZ2, BENZ, and BWH LC-MS/MS) were performed on urine specimens from the ED and compliance monitoring (Fig. 1). The residual urine specimens from BWH and MGH ED were stored at 4℃ and −20℃, respectively. The BNZ2 and BENZ assays were performed on the same day, and the LC-MS/MS testing was conducted within 4 weeks of the immunoassays. The compliance monitoring specimens from BWH, which underwent clinical LC-MS/MS testing, were stored at 4℃ after testing. The BNZ2 and BENZ assays were run on the same day and within 4 weeks of the clinical testing completion.

Data Analysis, visualization and medication review

SQL Server Management Studio (Microsoft, Redmond, WA, USA, version 15.0.18424.0) was utilized to run SQL queries from our laboratory information system (Sunquest Information Systems, Tucson, AZ, USA), in order to retrieve toxicology results. Quantitative data for clonazepam and diazepam (which were reported qualitatively) were obtained from MassTracks (Waters Corporation, Milford, MA, USA). Data analysis and visualizations were performed using Python 3.0 (Python Software Foundation, Wilmington, DE, USA) in MGB JupyterHub, an extension of Jupyter Notebook. Various libraries such as Pandas, NumPy, Matplotlib, and Seaborn were utilized for these purposes. Lucidchart (Lucid Visual Collaboration, South Jordan, UT, USA) was used to create an outline of the study participants.

The BWH LC-MS/MS method was utilized as the standard reference method to determine the sensitivity, specificity, FPR and FNR for both the BENZ and BNZ2 assays in all specimens (including those from the ED and compliance monitoring populations), as well as separately for the ED and compliance monitoring groups. The quantitative results obtained from LC-MS/MS were compared to the qualitative immunoassay results.

In cases where there were discrepant results among the three methods (e.g., LC-MS/MS positive and BNZ2 and BENZ negative, BNZ2 positive and LC-MS/MS and BENZ negative), for the verification and compliance monitoring specimens, the medical records were reviewed by author SEM. The purpose of this review was to determine if the patient had an active benzodiazepine prescription on the day of specimen collection (Fig. 1). If the patient was not prescribed benzodiazepines (including on an as-needed or PRN basis) on the day of specimen collection, positive results were categorized as unsuspected use (i.e., not prescribed). If the date of the prescription started on the day of specimen collection, the positive results were categorized as unknown (i.e., neither prescribed nor not prescribed). For potential false positive immunoassay results, all active medications on the date of collection were noted to identify any drugs that could potentially cross-react. In such cases, the Luxor extended LC-MS/MS panel was performed (Fig. 1) to further investigate and confirm the presence or absence of benzodiazepines or other compounds.

Results

BNZ2 method verification

For the BNZ2 assay, the % CV for the intra-assay precision (n = 21) was determined to be 12.8 % for the low manufacturer-supplied control and 7.7 % for the high manufacturer-supplied control. The low control consistently produced normalized signals below (−49.2 ± 6.3), while the high control consistently produced normalized signals above (41.3 ± 3.2) the threshold signal (0) corresponding to the 100 ng/mL calibrator.

In terms of inter-assay precision (n = 30), the % CV for the low manufacturer-supplied control was found to be 14.8 %, and 12.7 % for

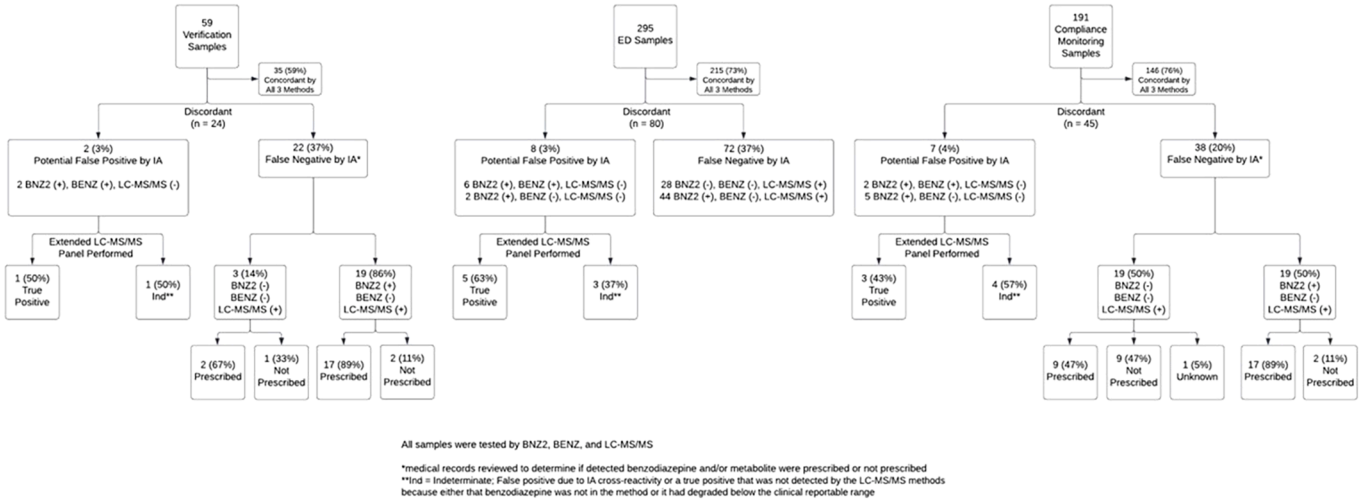

Fig. 1. The groups and sample size included in the study, the testing performed and the cases for which additional testing or medication review was performed are shown.

the high manufacturer-supplied control. Similar to the intra-assay precision results, the low control consistently produced normalized signals below (−50.3 ± 6.4), while the high control produced signals above (36.8 ± 5.4) the threshold signal (0) corresponding to the 100 ng/mL calibrator.

In a sample population that specifically included specimens positive for lorazepam, clonazepam, and their metabolites as confirmed by LC-MS/MS (n = 59), the BNZ2 assay demonstrated a sensitivity of 93.5 % and a specificity of 84.6 %. In comparison, the BENZ assay had a sensitivity of 52.2 % and a specificity of 84.6 %. The FPR for both assays was 15.4 %, and the FNR for the BNZ2 assay was 6.5 % while the FNR for the BENZ assay was 47.8 %. These findings indicate that the BNZ2 assay had improved clinical sensitivity and a decreased FNR in this sample population when compared to the BENZ assay.

There were a total of 24 discrepant specimens, which fell into three distinct groups (Fig. 1):

Group 1 consisted of three specimens that were negative for both the BNZ2 and BENZ assays, but positive by LC-MS/MS. Upon further analysis, one patient was found to be prescribed clonazepam with 151 ng/mL 7-aminoclonazepam. Another patient was using clonazepam without a prescription with 43 ng/mL 7-aminoclonazepam . The third patient had undergone an emergent procedure in the ED, and it was likely that they were administered lorazepam, as evidenced by the presence of lorazepam-glucuronide at 138 ng/mL.

Group 2 consisted of 19 specimens that were positive for BNZ2 and LC-MS/MS but negative for BENZ. Among these specimens, 17 patients were prescribed either lorazepam or clonazepam, while one patient was using lorazepam without a prescription and one patient was both prescribed lorazepam and using clonazepam without a prescription.

Group 3 included two specimens that were positive for both BNZ2 and BENZ, but negative by LC-MS/MS. In one of these specimens, a high level of alpha-hydroxymidazolam (360 ng/mL) was detected by the Luxor extended LC-MS/MS panel, indicitive of a true positive not detected by BWH LC-MS/MS. For the other specimen, medication review did not reveal a known cross-reactant, and the Luxor extended LC-MS/MS panel did not detect any additional benzodiazepines. This suggests that the result may have been falsely positive or a true positive due to the presence of another synthetic benzodiazepine that was not detected by either LC-MS/MS panel.

Overall performance

In the consecutive ED and compliance monitoring populations (n = 486), the BNZ2 assay demonstrated a sensitivity of 73.4 % and the BENZ assay had a sensitivity of 37.8 % (Table 1). The specificity for BNZ2 was 95.1 % and for BENZ it was 97.4 %. The FPR for BNZ2 was 4.9 % and for BENZ it was 2.6 %. The FNR for BNZ2 was 26.6 % and for BENZ it was 62.2 %. These results indicate that the BNZ2 assay had improved clinical sensitivity and a decreased FNR compared to the BENZ assay.

The drug concentrations in specimens containing only the parent drug and/or its metabolite (e.g., lorazepam and metabolite, clonazepam and metabolite, alprazolam, and oxazepam glucuronide) were determined.

For specimens with lorazepam and its metabolite, 26 specimens were negative for both BNZ2 and BENZ assays. The majority of these specimens (22 out of 26; 85 %) had concentrations of lorazepam plus lorazepam glucuronide below 200 ng/mL (Fig. 2a). We observed BNZ2 positive and BENZ negative specimens across a range of concentrations (136–5,292 ng/mL; median 1,045 ng/mL). Specimens positive for both BNZ2 and BENZ had concentrations > 200 ng/mL (267–5,973 ng/mL; median 876 ng/mL).

For specimens with clonazepam and/ or 7-aminoclonazepam, 16 specimens were negative for both BNZ2 and BENZ assays. The majority of these specimens (14 out of 16; 88 %) had concentrations of clonazepam plus 7-aminoclonazepam < 200 ng/mL (Fig. 2b). We observed BNZ2 positive and BENZ negative specimens as well as BNZ2 positive and BENZ positive specimens across a range of concentrations for clonazepam plus 7-aminoclonazepam (16–644 ng/mL; median 171 ng/mL and 90–2,779 ng/mL; median 667 ng/mL, respectively).

Most specimens (15 out of 17; 88 %) that only had alprazolam detected by LC-MS/MS (at concentrations ranging from 5 to 90 ng/mL; median 12 ng/mL) were positive by both the BNZ2 and BENZ immunoassays. There were only two specimens that were negative for one or both immunoassays. One sample was negative for both BNZ2 and BENZ (11 ng/mL), and the other was positive for BNZ2 and negative for BENZ (6 ng/mL).

There were nine specimens that only had oxazepam glucuronide detected by LC-MS/MS (35-312 ng/mL; median 113 ng/mL). Among these, three were negative for both BNZ2 and BENZ (35-36 ng/mL; median 36 mg/mL), five were positive for BNZ2 and negative for BENZ (44-139 ng/mL; median 113 ng/mL), and one was positive for both BNZ2 and BENZ (312 ng/mL).

UDS ED population

In the UDS ED population (n = 295), the sensitivity was 73.8 % and 32.7 %, specificity was 95.7 % and 96.8 % with a 4.3 % and 3.2 % FPR and 26.2 % and 67.3 % FNR for BNZ2 and BENZ, respectively (Table 1).

In total, there were 80 discrepant specimens, which could be categorized into four distinct groups (Fig. 1):

Group 1 included 28 specimens that tested negative for both the BNZ2 and BENZ assays but were positive on LC-MS/MS.

Group 2 consisted of 44 specimens that tested positive on BNZ2 and LC-MS/MS, but negative on BENZ.

Group 3 had 6 specimens that tested positive on both BNZ2 and BENZ, but were negative on LC-MS/MS.

Group 4 had 2 specimens that tested positive on BNZ2, negative on BENZ, and negative on LC-MS/MS.

All specimens that contained detectable levels of diazepam and/or its metabolites (nordiazepam, temazepam, oxazepam) by LC-MS/MS were positive on both the BNZ2 and BENZ assays. Fig. 3a illustrates that the BNZ2 assay exhibited higher positivity rates for specimens containing detectable levels of oxazepam glucuronide, alprazolam, clonazepam, 7-aminoclonazepam, lorazepam, and lorazepam glucuronide by LC-MS/MS. However, none of the BNZ2 positivity rates achieved 100 % with the exception of alprazolam.

When considering specimens with only one benzodiazepine or metabolite detected by LC-MS/MS, the BENZ assay was negative for all

Table 1
Sensitivity, Specificity, False Positive and False Negative Rates for Roche Urine Benzodiazepines Assays.

Patient Population	Sensitivity (%)		Specificity (%)		FPR (%)		FNR (%)	
	BNZ2	BENZ	BNZ2	BENZ	BNZ2	BENZ	BNZ2	BENZ
Overall (n = 486)	73.4	37.8	95.1	97.4	4.9	2.6	26.6	62.2
UDS ED (n = 295)	73.8	32.7	95.7	96.8	4.3	3.2	26.2	67.3
LC-MS/MS Compliance Monitoring (n = 191)	72.9	45.7	94.2	98.3	5.8	1.7	27.1	54.3

FPR = false positive rate; FNR = false negative rate; BENZ = Roche Benzodiazepines Plus; BNZ2 = Roche Benzodiazepines II; UDS = urine drug screening; ED = emergency department; LC-MS/MS = Liquid chromatography-tandem mass spectrometry.

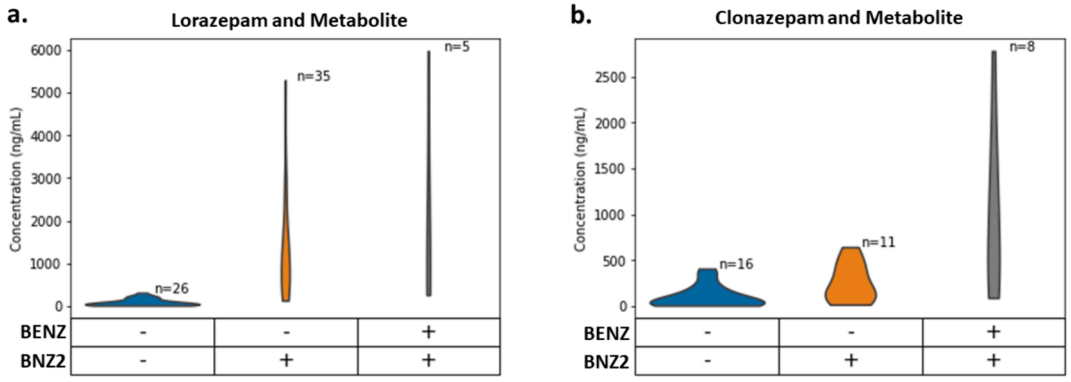

Fig. 2. The distribution of concentrations in both ED and compliance monitoring specimens positive for a) lorazepam and/or metabolite by LC-MS/MS and b) clonazepam and/or metabolite by LC-MS/MS are shown for those negative by both immunoassays (blue), positive by BNZ2 and negative by BENZ (orange) and positive by both BNZ2 and BENZ (gray). The number of specimens in each group is shown above the bars. BENZ = Roche Benzodiazepines Plus; BNZ2 = Roche Benzodiazepines II; LC-MS/MS = Liquid chromatography-tandem mass spectrometry.

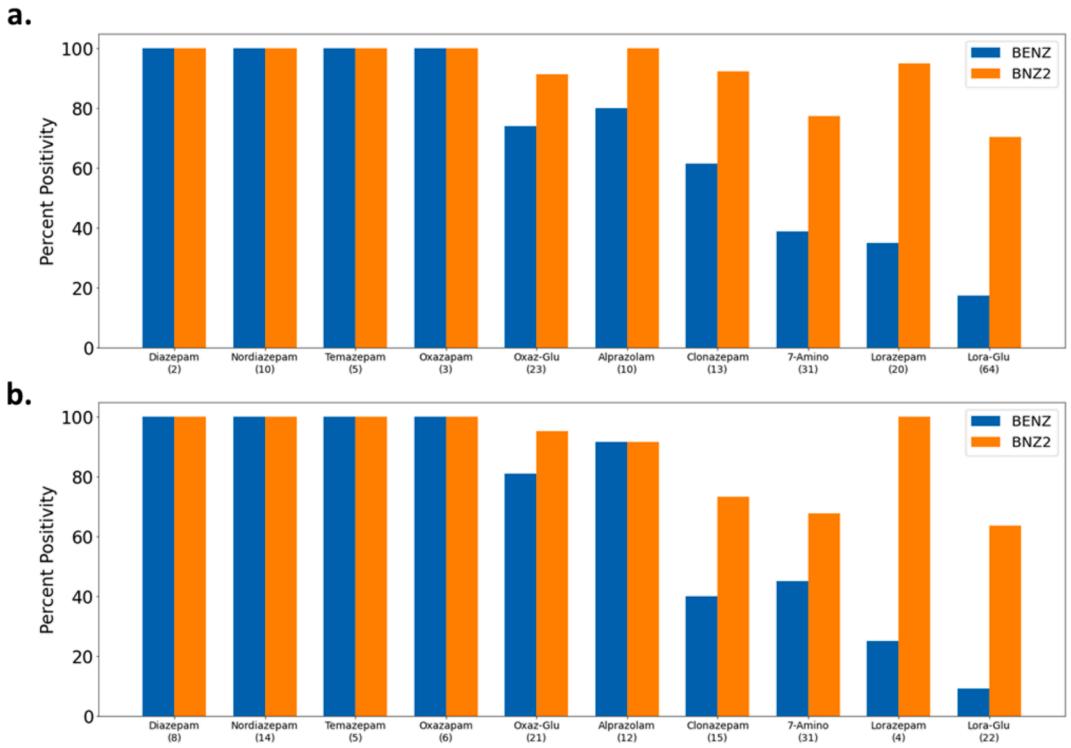

Fig. 3. The percent positivity of BENZ immunoassay (blue bars) and BNZ2 immunoassay (orange bars) compared to BWH LC-MS/MS is shown for the **a)** all LC-MS/MS positive UDS ED specimens (n = 107), **b)** all LC-MS/MS positive compliance monitoring specimens (n = 70). The number of specimens positive for each drug is shown below the drug name. Note: Specimens may contain more than one benzodiazepine. BENZ = Roche Benzodiazepines Plus; BNZ2 = Roche Benzodiazepines II; UDS = urine drug screening; ED = emergency department; LC-MS/MS = Liquid chromatography-tandem mass spectrometry.

specimens with detectable oxazepam glucuronide (n = 4) and 7-aminoclonazepam (n = 11). For the majority of specimens with detectable lorazepam glucuronide (34 out of 36; 94 %), the BENZ assay was also negative. However, the BENZ assay was positive for the majority of specimens with detectable alprazolam (3 out of 4; 75 %).

In contrast, the BNZ2 assay was positive for all specimens with detectable alprazolam (n = 4) and 50 %, 45 % and 50 % of specimens with detectable oxazepam glucuronide (n = 4), 7-aminoclonazepam (n = 11) and lorazepam glucuronide (n = 36); respectively. Both the BNZ2 and BENZ assays were positive for the one specimen with nordiazepam.

Of the six specimens in group 3 that tested positive by BNZ2 and BENZ, but negative by BWH LC-MS/MS, five had detectable benzodiazepines on the extended LC-MS/MS panel. These five specimens included four with alpha-hydroxymidazolam at concentrations ranging from 143 to > 1600 ng/mL (median 1225 ng/mL), and one with bromazolam at a concentration of 18 ng/mL. As a result, these specimens were considered true positive.

However, there was one specimen in group 3 that had no detectable benzodiazepines on the extended LC-MS/MS panel and no known cross-reacting medications. This specimen was likely a false positive or a true positive due to the presence of another synthetic benzodiazepine not detected by either LC-MS/MS method.

Regarding the two specimens in group 4 that tested positive on BNZ2 and negative on both BENZ and LC-MS/MS, neither had detectable benzodiazepines on the Luxor extended LC-MS/MS panel and no known cross-reacting medications. These specimens were also likely false

positive or true positive due to the presence of another synthetic benzodiazepine not detected by either LC-MS/MS method (Fig. 1).

LC-MS/MS compliance monitoring population

In patients who were being monitored for compliance (n = 191), the sensitivity was 72.9 % and 45.7 %, specificity was 94.2 % and 98.3 %, with a 5.8 % and 1.7 % FPR and 27.1 % and 54.3 % FNR for BNZ2 and BENZ, respectively (Table 1).

There were 45 discrepant specimens that fell into the same groups and patterns as the ED population (Fig. 1, Supplemental Table 2):

Similar to the ED population, the BNZ2 and BENZ assays were positive for all specimens that contained detectable levels of diazepam and/or its metabolites (nordiazepam, temazepam, oxazepam) as determined by LC-MS/MS. Fig. 3b demonstrates that the BNZ2 assay had higher positivity rates in specimens with detectable levels of oxazepam glucuronide, clonazepam, 7-aminoclonazepam, lorazepam, and/or lorazepam glucuronide. However, except for lorazepam, the BNZ2 positivity rates did not reach 100 %.

When considering specimens with only one benzodiazepine or metabolite detected by LC-MS/MS, it was observed that the BENZ assay was negative for all specimens with detectable lorazepam glucuronide (n = 14), as well as for the majority of specimens with detectable oxazepam glucuronide (4 out of 5; 80 %). However, the BNZ2 assay was positive for 50 % of specimens with detectable lorazepam glucuronide (n = 14) and 80 % of specimens with detectable oxazepam glucuronide (n = 5). Both the BENZ and BNZ2 assays detected 90 % of specimens with detectable alprazolam (n = 9) and 33 % of specimens with detectable 7-aminoclonazepam (n = 3).

Among the specimens in group 1 (n = 19) that were negative on both BNZ2 and BENZ assays but positive on LC-MS/MS, it was found that nine patients were prescribed either lorazepam or clonazepam, eight patients were using either lorazepam or clonazepam without a prescription, one patient had both prescribed and not prescribed use, and one patient had an unknown status with clonazepam prescribed on the day of specimen collection (Supplemental Table 2).

In group 2 (n = 19), which consisted of specimens that were negative on BENZ, but positive on both BNZ2 and LC-MS/MS, it was observed that 17 patients were prescribed either lorazepam or clonazepam. One patient was using diazepam or oxazepam (based on oxazepam glucuronide of 113 ng/mL by LC-MS/MS) without a prescription, and one patient had both prescribed and unsuspected use.

In group 3, there were two specimens that tested positive on both BNZ2 and BENZ assays, but were negative on LC-MS/MS. One of these specimens had alpha-hydroxyalprazolam detected (135 ng/mL) by the extended LC-MS/MS panel. The other specimen had alpha-hydroxymidazolam detected at a concentration exceeding the upper limit of quantification (>1600 ng/mL) by the Luxor extended LC-MS/MS panel (Supplemental Table 2).

In group 4, there were five specimens that tested positive on BNZ2 and negative on both BENZ and LC-MS/MS. One of these specimens had alpha-hydroxyalprazolam detected (44 ng/mL) by the Luxor extended LC-MS/MS panel. The remaining four specimens did not have any benzodiazepines detected, and medication review did not reveal a known cross-reactant. Therefore, it is likely that these specimens were falsely positive or represented true positives due to the presence of another synthetic benzodiazepine that was not detected by either LC-MS/MS method (Fig. 1, Supplemental Table 2).

Discussion

Our verification analysis of the BNZ2 assay has demonstrated that the assay performed in a manner consistent with the manufacturer's claims in terms of precision, sensitivity, and specificity. Notably, the sensitivity was higher, and the FNR was lower, particularly in specimens where the manufacturer indicated improved cross-reactivity, specifically lorazepam, clonazepam, and their metabolites.

In both the ED and compliance monitoring populations, both the BENZ and BNZ2 assays were able to detect specimens containing diazepam and its metabolites. This is expected, as most immunoassays were developed when diazepam was the most commonly prescribed benzodiazepine.

The improved clinical sensitivity of the BNZ2 assay was primarily attributed to its improved cross-reactivity with newer benzodiazepines and their metabolites, specifically lorazepam-glucuronide, oxazepam-glucuronide, clonazepam, 7-aminoclonazepam, and alprazolam.

The results from the consecutive ED and compliance monitoring populations further highlight the improved clinical sensitivity and lower FNR of the BNZ2 assay when compared to the BENZ assay. These findings are particularly significant as the BNZ2 assay was able to detect samples with relatively high concentrations of lorazepam and/or its metabolites that were missed by the BENZ assay. This suggests that patients presenting with a lorazepam overdose would only be identified by the BNZ2 assay and not by the BENZ assay.

In general, the BNZ2 and BENZ assays tended to produce negative results when the sample contained lower concentrations of benzodiazepines and/or their metabolites. Although the BNZ2 assay had a lower FNR, more than a quarter of the specimens in both the ED and compliance monitoring populations were falsely negative. In the ED, where rapid turnaround time is crucial, implementing an immunoassay with higher clinical sensitivity, like BNZ2, is recommended. However, a FNR of 27 % may still be considered too high for compliance monitoring purposes. In the compliance monitoring population, it was observed that nine out of 19 (47 %) specimens that were falsely negative by BNZ2 were from patients using a benzodiazepine without a prescription. These cases may have been missed if LC-MS/MS testing was not performed.

Overall, there were 10 specimens initially classified as falsely positive by both the BENZ and BNZ2 assays. However, further analysis using the extended LC-MS/MS panel detected alpha-hydroxymidazolam in six specimens, alpha-hydroxyalprazolam in one specimen, and bromazolam in one specimen. These findings indicate that these specimens were true positives, which align with the known cross-reactivity of BENZ and BNZ2 for these metabolites, as well as the frequent use of midazolam for sedation. Importantly, this highlights a limitation of the targeted LC-MS/MS method used for compliance monitoring, as analytes not included in the panel or synthetic benzodiazepine use may go undetected.

There were seven specimens that were potentially falsely positive only by the BNZ2 assay, and among these, only 14 % had a benzodiazepine (alpha-hydroxyalprazolam) detected by the extended LC-MS/MS panel. This observation suggests that the BNZ2 assay may be cross-reacting with a designer benzodiazepine that is not detected by either LC-MS/MS panel, such as etizolam, flualprazolam, flubromazepam, flurazepam, flunitrazepam, or desalkylgidazepam [8,17]. The cross-reactivity of immunoassays for newer designer benzodiazepines is not well studied. After analyzing the results, we further conducted testing on eleven specimens with known bromazolam concentrations using the extended LC-MS/MS panel and the BNZ2 assay. Among these specimens, five (45 %) were positive by BNZ2 with concentrations ranging from 33 to > 1600 ng/mL, while five (55 %) were negative by BNZ2, with concentrations ranging from 41 to 88 ng/mL. This suggests that specimens with bromazolam concentrations greater than 33 ng/mL may yield positive results on the BNZ2 assay.

Our study is subject to several limitations. First, the patient populations (i.e., ED, compliance monitoring) were determined based on testing ordered and patient location. We assumed that if the LC-MS/MS panel was ordered, the patient was being monitored for compliance. Secondly, some samples had multiple benzodiazepines and/or their metabolites detected by LC-MS/MS, which complicated the data analysis. However, we included these samples to be representative of real clinical samples sent to the ED and for compliance monitoring. Furthermore, the impact of minor metabolites not detected by LC-MS/

MS and their influence on immunoassay results could not be determined. We conducted medical record reviews based on medications documented in the electronic health record, and the compounds listed by the manufacturer as cross-reactants were considered when assessing possible interference. Lastly, although all methods were not performed on the same day, stability studies suggest that the results would still be within acceptable limits, and our conclusions would not have been affected. The one exception is 7-aminoclonazepam, where concentrations may have decreased by 50 % after four weeks, potentially leading to additional immunoassay results in the ED population being erroneously categorized as falsely positive due to possible 7-aminoclonazepam degradation before LC-MS/MS testing.

Conclusions

Compared to the BENZ assay, the BNZ2 assay has demonstrated improved clinical sensitivity and a lower FNR rate, primarily due to its improved cross-reactivity for clonazepam, 7-aminoclonazepam, and glucuronidated metabolites. However, the BNZ2 assay did miss some specimens that tested positive by LC-MS/MS, particularly those with lower concentrations of benzodiazepines and/or their metabolites. Therefore, while the BNZ2 assay is suitable for UDS in the ED, it cannot replace LC-MS/MS testing for compliance monitoring, as it may not detect instances of unsuspected drug use. Additionally, further investigation into the BNZ2 assay cross-reactivity for newer designer benzodiazepines is warranted.

Ethics statement

This study was conducted with the approval of the Mass General Brigham (MGB) Institutional Review Board.

CRediT authorship contribution statement

Nicole V. Tolan: Writing – review & editing, Visualization, Validation, Supervision, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Sacha Uljon:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **M. Lauren Donnelly-Morell:** Formal analysis, Data curation. **Melissa Zhao:** Data curation. **Grace K. Mahowald:** Validation, Formal analysis, Data curation. **Marion L. Snyder:** Writing – review & editing, Validation, Formal analysis, Data curation, Conceptualization. **Lindsey Contella:** Writing – review & editing, Validation, Data curation. **Elizabeth D. Urwiller:** Data curation. **Maria Daluz Fernandes:** Data curation. **Phillip Kang:** Writing – review & editing, Visualization, Software, Formal analysis, Data curation. **Stacy E.F. Melanson:** Writing – review & editing, Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmsacl.2024.06.002.

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