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Article

Urine Drug Screening in the Era of Designer Benzodiazepines: Comparison of Three Immunoassay Platforms, LC–QTOF–MS and LC–MS/MS

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Abstract

This study investigated the presence of designer benzodiazepines in 35 urine specimens obtained from emergency department patients undergoing urine drug screening. All specimens showed apparent false-positive benzodiazepine screening results (i.e., confirmatory testing using a 19-component liquid chromatography–tandem mass spectrometry (LC–MS/MS) panel showed no prescribed benzodiazepines at detectable levels). The primary aims were to identify the possible presence of designer benzodiazepines, characterize the reactivity of commercially available screening immunoassays with designer benzodiazepines and evaluate the risk of inappropriately ruling out designer benzodiazepine use when utilizing common urine drug screening and confirmatory tests. Specimens were obtained from emergency departments of a single US Health system. Following clinically ordered drug screening using Abbott ARCHITECT c assays and laboratory-developed LC–MS/MS confirmatory testing, additional characterization was performed for investigative purposes. Specifically, urine specimens were screened using two additional assays (Roche cobas c502 and Siemens Dimension Vista) and LC–quadrupole time-of-flight mass spectrometry (LC–QTOF–MS) to identify presumptively positive species, including benzodiazepines and non-benzodiazepines. Finally, targeted, qualitative LC–MS/MS was performed to confirm the presence of 12 designer benzodiazepines. Following benzodiazepine detection using the Abbott ARCHITECT, benzodiazepines were subsequently detected in 28/35 and 35/35 urine specimens using Siemens and Roche assays, respectively. LC–QTOF–MS showed the presumptive presence of at least one non-Food and Drug Administration (FDA)-approved benzodiazepine in 30/35 specimens: flubromazolam (12/35), flualprazolam (11/35), flubromazepam (2/35), clonazolam (4/35), etizolam (9/35), metizolam (5/35), nitrazepam (1/35) and pyrazolam (1/35). Two or three designer benzodiazepines were detected concurrently in 13/35 specimens. Qualitative LC–MS/MS confirmed the presence of at least one designer benzodiazepine or metabolite in 23/35 specimens, with three specimens unavailable for confirmatory testing. Urine benzodiazepine screening assays from three manufacturers were cross-reactive with multiple non-US FDA-approved benzodiazepines. Clinical and forensic toxicology laboratories using traditionally designed LC–MS/MS panels may fail to confirm the presence of non-US FDA-approved benzodiazepines detected by screening assays, risking inappropriate interpretation of screening results as false positives.

Introduction

For ∼60 years, benzodiazepines have been commonly used for the treatment of various psychiatric disorders like anxiety and insomnia. Benzodiazepines are now omnipresent, with their use having quickly outpaced the more toxic barbiturates. Among US ambulatory patient–physician visits, the fraction involving benzodiazepine prescription approximately doubled from 3.8% to 7.4% from 2003 through 2015; during the same period, rates of benzodiazepine co-prescription with opioids and other sedating medications increased from 0.5% to 2.0% and from 0.7% to 1.5%, respectively (1).

As benzodiazepine use has increased, overprescribing and overuse have become matters of concern (2). Additionally, benzodiazepines can be readily and inexpensively purchased online without a prescription, creating a serious challenge for the public health system (3–5). Among the benzodiazepines available on the unregulated online market, many have not been licensed as legal benzodiazepines but are structurally derived from medically used compounds.

Benzodiazepines can be divided into several groups based on their chemical structure, including triazolobenzodiazepines, 1,4-benzodiazepines and thienotriazolodiazepines (6). In the last 5–7 years, numerous novel psychoactive substances (NPSs, also referred to as new psychoactive substances), including benzodiazepines of the above structural classes, have been developed. NPSs are structurally modified variants of prototypical drugs or pharmaceuticals developed for medical use but lacking widespread approval. 'Designer

drugs', a subset of NPSs intended to structurally deviate from scheduled substances such that sellers may avoid regulatory control and law enforcement, have increasingly included benzodiazepines (5, 7).

Some NPSs of the benzodiazepine class, such as phenazepam and etizolam, have been described in specialized medical literature and are even legally used in some countries (e.g., Japan and Russia) (5). Others, including pyrazolam and flubromazepam, are true designer drugs in the sense that they are not used medically anywhere and have been developed solely to evade regulatory control (5). Among the ~30 uncontrolled and recreationally used benzodiazepines identified to date, commonly referred to as designer benzodiazepines, little is known about their pharmacodynamics and possible toxicity. Morbidity and mortality associated with benzodiazepine overdosage occur more often when they are combined with other sedative substances, like opioids and alcohol (8), although medical professionals and health regulatory organizations should be aware of the heterogeneity of designer benzodiazepines with unknown and possibly different pharmacokinetics and pharmacodynamics from traditional drugs that may increase risk (9).

Although designer benzodiazepines are similar to prototypic agents, they have different chemical structures, and their intake may go undetected if detection methods target only legally prescribed benzodiazepines. Laboratories seeking to add designer benzodiazepines to, e.g., liquid chromatography–tandem mass spectrometry (LC–MS/MS) screening or confirmatory methods are challenged by the lack of both a systematic database for designer drugs and reference standards as well as the high rate of turnover and frequent introduction of new drugs to the recreational market. This creates a situation where analytical methods, like targeted LC–MS/MS, must be updated continuously to address rapid changes in drug development and use (10).

An additional challenge facing toxicology laboratories arises from the fact that immunoassays targeting legally prescribed benzodiazepines often detect designer benzodiazepines due to structural similarity and subsequent antibody cross-reactivity (11–13). Some designer benzodiazepines are metabolized to traditional benzodiazepines (e.g., diclazepam to lorazepam) (5, 14), further increasing the likelihood of reactivity with immunoassay screens. If confirmatory testing fails to detect designer benzodiazepines or detects only a subset of those present on the market, screening results may be inappropriately interpreted as false positive when designer benzodiazepines are present, complicating interpretation and clinical management.

The aims of the present study were to identify the possible presence of designer benzodiazepines in urine specimens with apparent false-positive benzodiazepine screening results, characterize the reactivity of multiple commercially available screening immunoassays with designer benzodiazepines and evaluate the risk of inappropriately ruling out designer benzodiazepine use when utilizing common urine drug screening and confirmatory tests. This work specifically studied 35 emergency department patient specimens that screened positive for benzodiazepines, while confirmatory testing using a 19-component LC–MS/MS panel showed no commonly prescribed benzodiazepines at detectable levels, suggesting either false-positive screening results (i.e., cross-reactivity with non-benzodiazepine compounds) or false-negative confirmatory results (i.e., the presence of benzodiazepines not included in the confirmatory panel).

Methods

Urine specimens obtained from patients undergoing urine drug screening in emergency departments of a single US Health system (Michigan) with discrepant screening and confirmatory benzodiazepine results were included in this study. Drug screening using the Abbott ARCHITECT c benzodiazepine assay showed the presumptive presence of benzodiazepines in all specimens, while no benzodiazepines were detected by lab-developed LC–MS/MS confirmatory testing targeting 19 prescribed benzodiazepines and metabolites (diazepam, nordiazepam, temazepam, oxazepam, chlordiazepoxide, alprazolam, α-hydroxyalprazolam, clonazepam, 7-aminoclonazepam, lorazepam, midazolam, α-hydroxymidazolam, flunitrazepam, 7-aminoflunitrazepam, desalkylflurazepam, 2-hydroxyethylflurazepam, α-hydroxytriazolam, estazolam and clobazam). Cutoff concentrations were 5 ng/mL for clonazepam, 7-aminoclonazepam, alprazolam, alpha-hydroxyalprazolam and midazolam and 20 ng/mL for all others. The Abbott ARCHITECT c benzodiazepine assay utilizes polyclonal antibodies reactive to oxazepam to semiquantitatively detect multiple benzodiazepines in urine. The assay is calibrated using oxazepam and relies on competition between native benzodiazepines in a urine specimen and glucose-6-phosphate dehydrogenase (G6PD)-labeled oxazepam for antibody binding sites. G6PD enzymatic activity is monitored spectrophotometrically and is directly proportional to the cumulative benzodiazepine concentration in urine. The local laboratory applied a benzodiazepine cutoff concentration of 300 ng/mL to distinguish detected and non-detected results. This cutoff was validated locally and differs from the ARCHITECT c package insert cutoff of 200 ng/mL. Abbott ARCHITECT c assays were also used to screen for amphetamines, barbiturates, cocaine, methadone, opiates and tetrahydrocannabinol (THC).

Following clinically ordered testing, residual urine specimens underwent repeat screening using assays from two additional manufacturers (Roche, performed on a cobas c502 instrument; Siemens, performed on a Dimension Vista 1500 instrument). The Roche cobas c502 benzodiazepine assay utilizes nordiazepam–microparticle conjugates to semiquantitatively detect multiple benzodiazepines in urine. In the absence of native benzodiazepines in a urine specimen, antibodies bind to nordiazepam–microparticle conjugates, creating particle aggregates detected by changes in light transmission. Native benzodiazepines compete with nordiazepam–microparticle conjugates for free antibody, causing inhibition of particle aggregation and increased light transmission. Cumulative benzodiazepine concentration is proportional to light transmission. The assay is calibrated using nordiazepam, and the local laboratory applied a cutoff concentration of 100 ng/mL. The Siemens Dimension Vista 1500 benzodiazepine assay utilizes polyclonal antibodies reactive to lormetazepam to semiquantitatively detect multiple benzodiazepines in urine. The assay is calibrated using lormetazepam and relies on competition between native benzodiazepines in a urine specimen and G6PD-labeled lormetazepam for antibody-binding

sites. G6PD enzymatic activity is monitored spectrophotometrically and is directly proportional to the cumulative benzodiazepine concentration in urine. The local laboratory applied a cutoff concentration of 200 ng/mL. Both Roche and Siemens assays were also used to repeat screening for amphetamines, barbiturates, cocaine, methadone, opiates and THC.

Specimens were also screened on a Waters Xevo G2-XS QTof LC–quadrupole time-of-flight mass spectrometer (LC–QTOF–MS). Fragmentation and high-resolution mass accuracy *m/z* data for precursors and fragments, as well as retention time, were queried to identify presumptively positive benzodiazepines and other compounds. Mass spectral data for all designer benzodiazepines queried were confirmed by infusing authentic standards. Metabolites of designer benzodiazepines were not included in the benzodiazepine library, which consisted of the following: 4-chlorodiazepam, 4-hydroxytriazolam, adinazolam, alprazolam, alpha-hydroxyalprazolam, alpha-hydroxyalprazolam-glucuronide, bromazepam, bromazolam, brotizolam, chlordiazepoxide, desmethyl chlordiazepoxide, clobazam, desmethyl clobazam, clonazepam, 7-acetamido-clonazepam, 7-amino clonazepam, clonazolam, clotiazepam, delorazepam, demoxepam, desalkylflurazepam, deschloroetizolam, diazepam, diclazepam, estazolam, etizolam, flualprazolam, flubromazepam, flubromazolam, flunitrazepam, 7-amino flunitrazepam, desmethyl flunitrazepam, flurazepam, 2-hydroxy ethyl flurazepam, halazepam, loprazolam, lorazepam, lorazepam glucuronide, lormetazepam, meclonazepam, medazepam, methylclonazepam, metizolam, midazolam, alpha-hydroxy midazolam, nimetazepam, nifoxipam, nitrazepam, 7-amino nitrazepam, nitrazolam, nordiazepam, oxazepam, oxazepam glucuronide, phenazepam, prazepam, pyrazolam, quazepam, temazepam, temazepam glucuronide, tetrazepam, triazolam, alpha-hydroxy triazolam and zolazepam.

After completion of the above testing, specimens were stored at 4°C for 10 months. Confirmatory testing for 12 designer benzodiazepines was then performed at NMS Labs using a qualitative LC–MS/MS panel detecting the following compounds, with reporting limits shown in parentheses: alpha-hydroxyetizolam (2.0 ng/mL), bromazepam (5.0 ng/mL), clonazolam (5.0 ng/mL), delorazepam (5.0 ng/mL), diclazepam (5.0 ng/mL), etizolam (2.0 ng/mL), flualprazolam (2.0 ng/mL), flubromazepam (20 ng/mL), flubromazolam (2.0 ng/mL), flunitrazolam (2.0 ng/mL), nitrazolam (5.0 ng/mL) and phenazepam (20 ng/mL). Three specimens had insufficient volume for confirmatory LC–MS/MS testing.

This project was approved by the local institutional review board and was not considered human subject research.

Results

This study evaluated 35 residual urine specimens in which benzodiazepines were presumptively identified by the Abbott ARCHITECT c benzodiazepine assay, while confirmatory testing by LC–MS/MS showed no detectable benzodiazepines (cutoff concentrations 5–20 ng/mL). Residual urine specimens were re-screened by Roche and Siemens assays and showed detectable benzodiazepines in 35/35 and 28/35 specimens, respectively (Tables I and II). Abbott, Roche and Siemens benzodiazepine screening assays are calibrated using oxazepam, nordiazepam and lormetazepam, respectively (Table II). Local laboratory cutoff concentrations used to identify presumptively positive results ranged from 100 to 300 ng/mL (Table II).

Additional screening of residual urine specimens by LC–QTOF–MS showed the presumptive presence of at least one non-FDA-approved benzodiazepine (including those used outside the USA and true designer benzodiazepines with no approved medical use) in 30/35 specimens (Tables I and III): flubromazolam (12/35), flualprazolam (11/35), flubromazepam (2/35), clonazolam (4/35), etizolam (10/35), metizolam (5/35), nitrazepam (1/35) and pyrazolam (1/35). Two or three designer benzodiazepines were detected concurrently in 13/35 specimens. Medazepam, rarely prescribed in the USA, was presumptively identified in another specimen, resulting in 31/35 specimens with at least one benzodiazepine identified by LC–QTOF–MS. Among the four specimens that showed no benzodiazepines by clinically ordered LC–MS/MS or LC–QTOF–MS, one patient stated use of Xanax (alprazolam); no evidence of benzodiazepine use was present in the medical record for the other patients.

Qualitative, targeted LC–MS/MS identified at least one designer benzodiazepine in 23/32 specimens; 3 specimens were unavailable for confirmatory testing due to insufficient volume. Among the 36 individual designer benzodiazepines identified by LC–QTOF–MS across all samples, 25 were confirmed by LC–MS/MS, excluding cases in which insufficient volume remained for testing (specimens 20, 22 and 29, Table I) or presumptively identified benzodiazepines were not included in the LC–MS/MS panel (specifically metizolam, nitrazepam, medazepam and pyrazolam). Qualitative LC–MS/MS showed no detectable designer benzodiazepines in the four specimens for which LC–QTOF–MS also detected no benzodiazepines. The presence of flubromazolam, flualprazolam, flubromazepam, etizolam and clonazolam was confirmed by qualitative LC–MS/MS in 9/11, 7/10, 0/2, 9/10 and 0/3 specimens, respectively. Alpha-hydroxyetizolam, a metabolite of etizolam not included in LC–QTOF–MS screening, was detected by LC–MS/MS in 12 specimens. Among these, etizolam was not detected by either LC–QTOF–MS or LC–MS/MS in three specimens; LC–MS/MS showed no other detectable benzodiazepines, while LC–QTOF–MS showed the presence of flubromazepam (specimen 5), medazepam (specimen 31), and flubromazolam and clonazolam (specimen 33). Clonazolam was detected by LC–MS/MS but not LC–QTOF–MS in one specimen (specimen 23).

Among the 30 specimens in which non-FDA-approved benzodiazepine(s) were presumptively identified by LC–QTOF–MS, all showed positive screening results using Roche (cutoff 100 ng/mL) and Abbott (cutoff 300 ng/mL) assays. Siemens results (cutoff 200 ng/mL) were positive in 26 of these 30 specimens. Among the four negative Siemens screens, LC–QTOF–MS presumptively identified clonazolam (specimens 20 and 32), etizolam (specimen 35), and etizolam and metizolam (specimen 28). LC–MS/MS confirmed the presence of etizolam in specimens 28 and 35, while clonazolam was not detected in specimen 32; confirmation of clonazolam in specimen 20 could not be obtained due to insufficient volume, while metizolam could not be confirmed because it was not targeted by the method. Qualitative disagreement between Siemens and Roche/Abbott screening results (i.e., negative and positive, respectively) was observed in three additional specimens. LC–QTOF–MS identified no

Table I. Qualitative Benzodiazepine (BZ) Detection by Three Immunoassays, Presumptive Identification by Liquid Chromatography–Quadrupole Time-of-Flight–Mass Spectrometry (LC–QTOF–MS) and Confirmatory Identification by Liquid Chromatography Tandem Mass Spectrometry (LC–MS/MS) in 35 Residual Urine Specimens

Specimen ID	Immunoassay BZ detection			LC–QTOF–MS BZ identified	LC–MS/MS designer BZ confirmation
	Abbott	Roche	Siemens		
1	D	D	D	ND	ND
2	D	D	D	Flubromazolam	D
3	D	D	D	Flualprazolam	D
4	D	D	D	Flualprazolam	D
5	D	D	D	Flubromazepam	ND[a]
6	D	D	ND	ND	ND
7	D	D	D	Flualprazolam	ND
8	D	D	D	Nitrazepam, 7-amino nitrazepam	n/a
9	D	D	D	Flubromazolam	D
10	D	D	D	Flubromazolam, clonazolam	D, ND
11	D	D	D	ND	ND
12	D	D	D	Flualprazolam, etizolam	D, D[a]
13	D	D	D	Flualprazolam, etizolam	D, ND
14	D	D	D	Etizolam, metizolam	D[a], n/a
15	D	D	D	Flubromazolam, etizolam, metizolam	D, D[a], n/a
16	D	D	D	Flualprazolam, etizolam	ND, D[a]
17	D	D	D	Flualprazolam, pyrazolam	D, n/a
18	D	D	D	Flubromazolam	D
19	D	D	D	Flubromazolam	ND
20	D	D	ND	Clonazolam	n/a
21	D	D	D	Flualprazolam	D
22	D	D	D	Flubromazolam	n/a
23	D	D	D	Flualprazolam, flubromazolam	D, D[b]
24	D	D	ND	ND	ND
25	D	D	D	Flubromazepam, etizolam, metizolam	ND, D[a], n/a
26	D	D	D	Flualprazolam	ND
27	D	D	D	Flubromazolam, etizolam	D, D[a]
28	D	D	ND	Etizolam, metizolam	D[a], n/a
29	D	D	D	Flualprazolam	n/a
30	D	D	D	Flubromazolam, etizolam, metizolam	D, D[a], n/a
31	D	D	ND	Medazepam	n/a[a]
32	D	D	ND	Clonazolam	ND
33	D	D	D	Flubromazolam, clonazolam	ND[a]
34	D	D	D	Flubromazolam	D
35	D	D	ND	Etizolam	D[a]

[a]Alpha-hydroxyetizolam also detected.
[b]Clonazolam also detected.
D, detected; ND, non-detected; n/a, testing not performed. LC–MS/MS reporting limits: 2.0 ng/mL (alpha-hydroxyetizolam, etizolam, flualprazolam and flubromazolam), 5.0 ng/mL (clonazolam) and 20 ng/mL (flubromazepam).

Table II. Characteristics of the Immunoassays Used and Rate of Benzodiazepine (BZ) Detection in 35 Residual Urine Specimens

Immunoassay	Method	BZ calibrator identity	BZ cutoff (ng/mL)	Rate of BZ detection
Roche	KIMS[a]	Nordiazepam	100	35/35
Abbott	HEIA[b]	Oxazepam	300	35/35
Siemens	EMIT[c]	Lormetazepam	200	28/35

[a]Kinetic interaction of microparticles in solution.
[b]Homogeneous enzyme immunoassay.
[c]Enzyme-multiplied immunoassay technique.

Table III. Rate of Presumptive non-FDA-Approved Benzodiazepine (BZ) Detection by Liquid Chromatography–Quadrupole Time-of-Flight–Mass Spectrometry (LC–QTOF–MS) in 35 Residual Urine Specimens

Designer BZ	Rate of detection by LC–QTOF–MS
Flubromazolam	12/35
Flualprazolam	11/35
Flubromazepam	2/35
Clonazolam	4/35
Etizolam	10/35
Metizolam	5/35
Nitrazepam	1/35
Pyrazolam	1/35

designer benzodiazepines in two of these specimens (6 and 24) and presumptively identified medazepam in the third (31); LC–MS/MS also detected no designer benzodiazepines in specimens 6 and 24 but detected alpha-hydroxyetizolam in specimen 31.

Concurrently detected species, presumptively identified using Abbott screening assays, included THC (26/35), cocaine (10/35), opiates (7/35), amphetamines (6/35) and methadone (2/35). Roche and Siemens assays failed to identify amphetamines in one specimen, while Roche assays identified amphetamines and methadone individually in two additional specimens. All other non-benzodiazepine screening results showed a qualitative agreement between the three assays.

Discussion

The European Monitoring Centre for Drugs and Drug Addiction monitors 30 designer benzodiazepines that are commonly observed in cases of polydrug use, 21 of which were first detected in the past 5–6 years (6). The rapid pace of designer benzodiazepine development and entry into the recreational drug market contrasts with the relatively slow pace of method development and validation for tests detecting novel drugs, challenging clinical laboratorians who seek to support medical management of patients with suspected recreational benzodiazepine ingestion. Developing solutions to identify designer benzodiazepines is particularly relevant, given the widespread use and abuse of both classic and designer benzodiazepines, the former being present in approximately one-third of opioid deaths in the USA in 2017–2018 (15).

A growing number of publications report LC–MS/MS methods to detect designer benzodiazepines in blood, plasma and urine (11, 16). Despite these advances, clinical laboratories continue to be challenged by the fast pace of drug entry to the recreational market relative to LC–MS/MS method development and validation. Additional challenges preventing broader implementation of LC–MS/MS as a screening or confirmatory method for designer benzodiazepines in blood or urine include the high instrument cost, lower throughput compared to automated immunoassays and, most significantly, the need for qualified personnel to develop, perform and interpret results. While immunoassay screening methods have well-known limitations relative to chromatography–mass spectrometry methods, including lower analytical sensitivity and specificity, they continue to be utilized in most clinical laboratories due to their ease of use and high throughput.

Counterintuitively, immunoassays may have a greater sensitivity for non-classical drugs than targeted LC–MS/MS, depending on the scope of LC–MS/MS testing. This is not a consequence of a true improvement in analytical sensitivity but rather due to antibody cross-reactivity with untargeted or unknown drugs with a similar structure to the target. A study conducted in Sweden in 2014–2015 found a 40% prevalence of designer benzodiazepines in specimens that were immunoassay positive but negative by mass spectrometry confirmation methods, suggesting that reflexive interpretation of such results as false positives could falsely rule out the presence of designer benzodiazepines (17). Correlation with clinical context is important for appropriate interpretation of discrepant screening and confirmatory results, given the ongoing possibility of false-positive immunoassay screening results.

Information about the detection of designer benzodiazepines with commercially available immunoassays is limited to a handful of precedent studies; these have demonstrated generally high designer benzodiazepine cross-reactivity in both urine and blood, although with significant inter-assay variability for some drugs (11–13, 18). Cross-reactivity is possible because previously characterized and novel designer benzodiazepines are often structurally similar to classic drugs and because antibodies used in these immunoassays are not selective for a single benzodiazepine target. Also, prescribed benzodiazepines may be metabolized into designer ones (e.g., flunitrazepam to fonazepam and nifoxipam), while the reverse is also true (e.g., diclazepam to lorazepam) (14, 19); the latter phenomenon may further increase the likelihood of designer benzodiazepine detection by immunoassays, while the former may complicate interpretation of screening or confirmatory results that provide structural identification.

The apparent false-positive benzodiazepine screening rate for the clinical laboratory performing the present study was 2.6% over the year period concurrent with study enrollment. Among the apparent false-positive screens available for additional characterization, this study observed a high rate of presumptive designer or non-FDA-approved benzodiazepine detection by LC–QTOF–MS (86%, 30/35 specimens). Designer benzodiazepines were detected exclusively in the absence of any prescribed benzodiazepines, suggesting screening assays were cross-reactive with the former. Among the designer benzodiazepines presumptively identified by LC–QTOF–MS, including both triazolobenzodiazepines and thienotriazolodiazepines, multiple benzodiazepines have not been characterized by immunoassay manufacturers for cross-reactivity, namely flualprazolam (Roche) and flubromazolam, flualprazolam, flubromazepam, clonazolam, etizolam, metizolam and pyrazolam (Siemens and Abbott). A majority of designer or non-FDA-approved benzodiazepines detected by LC–QTOF–MS were confirmed by qualitative LC–MS/MS, although 11/36 remained unconfirmed. Among the 30 specimens in which LC–QTOF–MS detected at least one designer benzodiazepine, only four showed no detectable designer benzodiazepines by qualitative LC–MS/MS. The primary limitation of the qualitative LC–MS/MS testing used here is the longer interval between specimen collection and testing (10 months longer than LC–QTOF–MS testing). Reporting limits for the five designer benzodiazepines detected by LC–MS/MS range from 2.0 to

20 ng/mL; designer benzodiazepines for which reporting limits were highest (flubromazepam and clonazolam, 20 and 5.0 ng/mL, respectively) were most likely to show discrepancies between LC–QTOF–MS and LC–MS/MS. Due to these limitations, the presence of designer benzodiazepines cannot be definitively ruled out in cases where LC–QTOF–MS and LC–MS/MS disagree, although false-positive LC–QTOF–MS results are possible. Three instances were noted in which alpha-hydroxyetizolam, a major metabolite of etizolam, was detected in the absence of etizolam by the qualitative LC–MS/MS method.

Flubromazolam was the most frequently detected designer benzodiazepine (12/35 specimens). Use of flubromazolam is associated with strong and long-lasting sedation, and at least one case report has demonstrated the risk of life-threatening complications of use, including hypotension, rhabdomyolysis, hypoxia and coma (20, 21). While the results of this study suggest common benzodiazepine immunoassays detect flubromazolam, positive screening results could be interpreted as false positives in either the absence of a confirmatory method targeting flubromazolam or additional screening methods enabling qualitative, presumptive structural identification (e.g., LC–QTOF–MS). To avoid inappropriately ruling out designer benzodiazepine use, discrepant screening and confirmatory results should raise suspicion of designer benzodiazepines in the appropriate clinical context.

The results of this and other studies (12) demonstrate a high percentage of detection of multiple designer benzodiazepines by commonly used immunoassays. In our study, qualitative cutoff concentrations varied between assays according to local laboratory practices. Abbott and Roche assays, employing cutoffs of 300 and 100 ng/mL, respectively, showed the highest apparent cross-reactivity with designer benzodiazepines. The Siemens assay, employing a cutoff of 200 ng/mL, showed a lower apparent cross-reactivity, with presumptively positive results obtained in 26 of the 30 specimens that were positive by Roche and Abbott assays and showed non-FDA-approved benzodiazepines using LC–QTOF–MS.

The primary limitation of this study is that no information was obtained regarding the clinical presentations of patients included in the study cohort. In addition, the study did not evaluate the possible presence of designer benzodiazepines in combination with licit or prescribed benzodiazepines and so cannot comment on the rate of designer benzodiazepine use in the local patient population. Confirmatory testing for designer benzodiazepines was delayed by 10 months relative to immunoassay and LC–QTOF–MS screening; given the poorly characterized *in vitro* stability of designer benzodiazepines, the possibility that concentrations decreased over time, resulting in false-negative confirmatory results, cannot be ruled out. Future studies are needed to quantitatively characterize the cross-reactivity of commercially available benzodiazepine immunoassays with designer benzodiazepines.

In summary, a majority of urine specimens obtained from emergency department patients with apparent false-positive benzodiazepine immunoassay screening results showed evidence of designer benzodiazepines when additional characterization techniques (i.e., LC–QTOF–MS and expanded LC–MS/MS) were used. Although between-manufacturer differences in cross-reactivity were observed, urine benzodiazepine screening assays from three manufacturers were generally cross-reactive with multiple non-US FDA-approved (i.e., designer) benzodiazepines, suggesting the utility of screening methods for presumptively detecting designer benzodiazepine use. Clinical and forensic toxicology laboratories using confirmatory LC–MS/MS panels targeting prescribed benzodiazepines may fail to confirm the presence of designer benzodiazepines detected by screening assays, risking inappropriate interpretation of screening results as false positives. As prescribed and unprescribed benzodiazepine use grows, laboratories should consider expanding their confirmatory test panels to include designer benzodiazepines and/or performing untargeted screening with structural identification (e.g., LC–QTOF–MS) to ensure appropriate interpretation of drug screening results. Even in the absence of changes to test offerings, laboratories may reduce the risk of inappropriately ruling out designer benzodiazepine use by educating clinicians on the scope and limitations of confirmatory tests. In the appropriate clinical context, clinicians may alert the laboratory of the need for additional characterization, likely utilizing reference laboratory services, when immunoassay screens provide presumptive evidence of benzodiazepines.

The results of the present study are consistent with previous observations that designer benzodiazepines are generally recognized by immunoassays (11–13, 18). However, the risk of falsely ruling out designer benzodiazepine use based on traditional screening and confirmatory testing paradigms will remain as long as new drug development outpaces LC–MS/MS method development and validation and while adoption of screening methods enabling qualitative identification, like LC–QTOF–MS, remains limited.

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Data availability

All data are incorporated into the article.

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