Forensic Chemistry 35 (2023) 100512

Contents lists available at ScienceDirect

Forensic Chemistry

journal homepage: www.sciencedirect.com/journal/forensic-chemistry

Development and validation of a liquid chromatography tandem mass spectrometry method for the analysis of 53 benzodiazepines in illicit drug samples

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ARTICLE INFO

Keywords:
LC-MS/MS
Benzodiazepines
Drug checking
Lateral flow immunoassay strip

ABSTRACT

An LC-MS/MS method for the analysis of 53 benzodiazepines, including various designer benzodiazepines, was developed. The developed method was applied to a total of 79 illicit street drug samples collected in Chicago, IL. Of these samples, 68 (84%) had detectable amounts of at least one benzodiazepine. Further, of the 53 benzodiazepines included in the developed method just 14 were measured in samples. Clonazolam, a potent designer benzodiazepine and derivative of clonazepam, was the most frequently measured benzodiazepine in 63% of samples and was measured in the highest concentrations. Other benzodiazepines measured in more than 10% of samples included clonazepam, alprazolam, flualprazolam, and oxazepam. Mixtures of benzodiazepines were frequently measured in samples, with just 24% of samples containing just one benzodiazepine. To determine the response of benzodiazepines on a rapid, point-of-use drug checking tool, all 53 benzodiazepine standards were screened on a lateral flow immunoassay benzodiazepine test strip. Sixty eight percent of standards gave a positive BTS response at a concentration of 20 μg/mL, demonstrating BTS have response to a wide variety of benzodiazepines, including many designer benzodiazepines. A comparison of this data to previous data reported for the same samples demonstrated all samples containing a benzodiazepine also had an opioid present, with fentanyl being present in 94% of benzodiazepine samples. These results highlight high rates of polysubstance drug presence in Chicago, IL illicit drug samples, posing an increased risk of drug overdoses in people who use drugs.

Introduction

Benzodiazepines represent a widely prescribed class of drugs sold under brand names such as Xanax (alprazolam), Valium (diazepam), Ativan (lorazepam), and Klonopin (clonazepam). Chemical structures of benzodiazepines are composed of a benzene ring combined with a 7-membered diazepine ring and commonly contain a phenyl ring attached to the 5′ position of the diazepine ring [1]. Additionally, benzodiazepines modified with various functional groups and fluorinated, brominated, and chlorinated analogs of benzodiazepines are common and are synthesized in both pharmaceutical and clandestine laboratories to generate compounds with varying potency and use [2,3]. As a class of drugs, benzodiazepines are central nervous system depressants that act as sedatives used clinically for the treatment of anxiety and insomnia, and as well as for muscle relaxation among other uses [4].

In 2015 an estimated 30 million adults, approximately 12.5% of the population, in the United States had a benzodiazepine prescription [5–7]. Although benzodiazepines represent one of the most widely prescribed types of medications in the United States, serious side effects and increased rates of mortality have been associated with their use [4]. Side effects related to clinical uses of benzodiazepines include increased drowsiness, psychomotor slowing, and unsteadiness in select populations [1,4,8]. Additional side effects can be observed when the dose or frequency of benzodiazepine use is increased, including confusion, issues with memory and speech, and a slowed respiratory rate [9]. When taken at high enough doses, benzodiazepines can lead to overdose [10].

Notably, benzodiazepines are commonly used in combination with other classes of drugs. In populations of people who use drugs, polysubstance drug or alcohol use has been estimated in 40–60% of those visiting trauma centers [11]. Two common classes of drugs that co-occur

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https://doi.org/10.1016/j.forc.2023.100512
Received 10 March 2023; Received in revised form 6 June 2023; Accepted 12 June 2023
Available online 16 June 2023

are benzodiazepines and opioids [11]. In 2014–2016, the annual rates of physician office visits where a benzodiazepine was prescribed was estimated to be 27 out of 100 visits. Of these visits, one-third also involved an overlapping opioid prescription [12]. In 2020, co-use of benzodiazepines and opioids in the United States was noted, with 16% of opioid overdose deaths showing co-occurrence of opioids and benzodiazepines [13,14]. Importantly, both intentional and unintentional polysubstance drug use increases the risk of harm associated with drug use. In 2017, the United States Food and Drug Administration noted the increased risks observed when benzodiazepines and opioids were taken in combination, issuing Boxed Warnings on the labels of opioids and benzodiazepines [15].

Inherent to implementing approaches to reduce the harm associated with drug use, especially polysubstance drug use, is a need for high-quality data that can inform on the type and scale of illicit drug use across different populations of users [16]. It remains difficult to adequately assess illicit drug use at the population level for several reasons. One reason is that much data collected on illicit drug use arises from circumstances "of consequence" such as drug seizures by law enforcement, autopsy data collected from fatal overdoses, and intake or laboratory data collected by hospitals that are likely to be biased in the types of illicit drugs measured or reported [17–22]. Other types of data, namely survey-based reports, have the benefit of collecting information from a wider variety of drug users but suffer from known issues with the accuracy and transparency of self-reporting. Few studies have measured the occurrences of benzodiazepines through analysis of illicit drug samples. Benzodiazepine report data from the National Forensic Laboratory Information System showed an estimated 263,538 reports between 2015 and 2018, with decreases in the reports of prescription benzodiazepines occurring as reports of designer benzodiazepines increased [23]. Additional reports from Canada highlight benzodiazepine frequency in upwards of 21% of illicit drug samples screened, and further characterize the presence of designer benzodiazepines for which there is limited understanding of their pharmacological or toxicological profiles [24–26].

Our 2023 report sought to measure the occurrences of 22 illicit drugs in 124 samples of street drugs collected in Chicago, IL. In this work 3 benzodiazepines, alprazolam, diazepam, and lorazepam, plus one metabolite of alprazolam, alpha hydroxyalprazolam, were included in the developed liquid chromatography tandem mass spectrometry (LC-MS/MS) method. Samples were also screened against commercially available benzodiazepine lateral flow immunoassay strips (BTS) that have reported positive responses for 17 benzodiazepine compounds. Results from these analyses showed low occurrences of benzodiazepines in these samples, with just one sample containing alprazolam from LC-MS/MS and a different sample producing a positive BTS response. As part of the collection and analysis of samples, data was collected from participants from which drug samples were collected. This data included their expectations of drug content, as outlined in Swartz *et al.* (2023) [27]. A comparison of participant expectations to the analytical results produced by LC-MS/MS and BTS highlighted a critical disparity, with 46% of participants expecting a benzodiazepine to be present in their sample.

To address this disparity, and to better understand the occurrences of benzodiazepines in street drug samples, we sought to develop a LC-MS/MS method for the analysis of a larger number of benzodiazepines. The goals of this work were to 1) develop a LC-MS/MS method for a total of 53 benzodiazepines, including both commonly prescribed and illicitly produced compounds; 2) screen samples against the developed method to determine benzodiazepine content in samples; and 3) determine and characterize the reactivity of the field-friendly BTS to a larger number of benzodiazepine compounds.

Materials and methods

Reagents, chemicals, and supplies

Analytical standards of all 53 analytes were purchased from Cayman Chemical Company (Ann Arbor, MI) as part of an analytical standard panel (Item # 26260). These standards were purchased as individual vials containing 100 μg of solid drug with residual glycerol. Each standard was then prepared into solution following manufacturer instructions, where 500 μL of LC-MS grade methanol was spiked into the vial and the vial briefly vortexed before it was placed on an orbital shaker set to 100 rpm for 30 min. 10 μL aliquots of each 200 μg/mL solution were combined to create a mixed standard in 100% LC-MS grade methanol containing 100 ng/mL of each analyte. This 100 ng/mL stock solution was then used for subsequent experiments.

Formic acid (98%) was purchased from Sigma-Aldrich (St. Louis, MO). LC-MS grade methanol was purchased from Fisher Scientific (Waltham, MA). LC-MS grade acetonitrile was purchased from VWR (Radnor, PA). 18 MΩ water was used for all standard, sample, and mobile phase preparations. 2 mL polypropylene HPLC vials and polypropylene caps (part No. 5191–8151 and 5191–8150) were purchased from Agilent Technologies (Santa Clara, CA). Rapid Response Benzodiazepine Test Strips (BTS) at a 300 ng/mL cutoff concentration (Lot No. DOA2204108 and No. DOA2206398) were purchased from Lochness Medical Inc. (Buffalo, NY) and were run and analyzed per manufacturer recommendations.

LC-MS/MS analysis

The instrument used to analyze all samples was an Agilent 1290 Infinity II coupled to an Agilent 6460 triple quadrupole MS/MS. For all 53 analytes ion optimization experiments were required to determine the exact ions (precursor and product) as well as the optimal parameters (fragmentor voltage and collision energy) that are required to produce and fragment the ions. The 100 ng/mL stock solution containing all 53 analytes in 100% LC-MS grade methanol was used for ion optimization experiments. At least one, but up to four, product ions and their unique collision energies were identified for each analyte. Following ion optimization, the 53-standard mix was diluted to 20 ng/mL in a mixture of 90:10 mobile phase A to mobile phase B for chromatography development. The parameters for liquid chromatography were adapted from a previous publication focused on the analysis of 22 illicit drugs [28]. The determined retention time of each compound was used to determine the delta retention window which is a segment of time that represents ± 30% of the width of the peak. The retention time and the delta retention time were used to convert the MRM transitions into a dynamic MRM (dMRM) method. All analytes were optimized and analyzed in positive mode electrospray ionization. The ion source parameters were optimized following the finalization of the chromatography with 100% of the flow from the LC sent to the MS/MS (i.e., no splitter was used).

Method validation (linearity, precision & accuracy, LoD/LoQ) and data analysis

A stock solution containing all 53 analytes was prepared at a concentration of 20 ng/mL of each analyte in 90:10 mobile phase A to mobile phase B. Eight dilutions of this stock were then made at a final concentration of each analyte at 0.1, 0.25, 0.5, 0.75, 1, 5, 10, and 20 ng/mL in the initial chromatographic conditions. 500 uL of each standard was transferred to a polypropylene HPLC vial. For determining precision and accuracy, 7 replicate injections at concentrations of 0.1, 0.5, 1, and 10 ng/mL were performed. The average measured peak area and the standard deviation of these peaks were used to calculate the relative standard deviation (RSD). The same 7 replicate injections of a mid-range of these points were used to determine accuracy. The average measured peak area and the equation of fit determined from linearity experiments

was used to calculate the average measured concentration of the replicate injections. The percent difference of this measured concentration from the true concentration was then calculated. Interday precision & accuracy were determined via replicate injections of a 1 ng/mL standard analyzed on a day separate from the initial validation.

To determine the limit of detection for each analyte the process of first determining the limit of blank was utilized. This process is described in more detail elsewhere [28,29]. A total of 7 replicate injections of a blank were run and data from the replicate injections of 4 standards (0.1, 0.5, 1, and 10 ng/mL) were used to estimate an LoD. The Limit of Quantification (LoQ) for most analytes was estimated as LoQ = 3.3*LoD. This value was compared to precision and accuracy data to ensure the RSD and percent difference values fell within ± 30%. For an estimated LoQ whose precision and accuracy data did not fall within ± 30%, the LoQ was set as the lowest concentration where precision and accuracy data did fall within ± 30%. Peak identification, integration, and analysis for all samples and standards was performed on Agilent MassHunter Quantitative Analysis for QQQ software Version 10.1.

QA/QC

Alongside the samples, a set of standards and blanks were prepared to perform QA/QC. Continuing calibration checks at a mid-range calibration point (1 ng/mL) were prepared to ensure instrumental performance was within the expected tolerances. Double blanks of the diluent (90:10 mobile phase A to mobile phase B) were prepared to measure any background levels of analyte present in the solvents or tools used during sample preparation. Each instrumental analysis batch began with a double blank injection, followed by a continuing calibration check to ensure the retention times & peak areas of each analyte were as expected. During instrumental analysis, a double blank was injected every 5 samples. A continuing calibration check was injected every 10 samples and was immediately proceeded and succeeded by a double blank to prevent carryover from the standard to samples. Additionally, every 10th sample was injected in triplicate to get a measure of intraday injection variability for the samples themselves.

Continuing calibration checks were found to have precision values within a tolerance of ± 30% (RSD values ranged from 6 to 28%) and accuracy values within a tolerance of ± 30% (absolute percent difference values ranged from 0 to 30%) except for four analytes, 4-hydroxyalprazolam, n-desmethylclobazam, 3-hydroxy phenazepam, and delorazepam which had RSD and/or percent difference values outside of the ± 30% tolerance. For samples that were injected in triplicate during instrumental analysis to measure intraday injection variability in samples the RSD of measured concentrations ranged from 1 to 24% (median of 5%). Additionally, all replicate injections showed matched detection of analytes (i.e., all three replicates had exact overlap of analytes that were classified as non-detect, non-quantifiable, or quantifiable).

Sample collection, storage, and preparation

Samples were pulled from those collected as described in Whitehead *et al.* (2023) [28]. Briefly, samples were originally collected in the Fall of 2021 through early Spring of 2022 in the Chicago, IL neighborhoods of Austin and Humboldt Park. Samples were collected from participants who were recruited from among those seeking syringe exchange and health care services at one of two street outreach facilities located within these neighborhoods. After collecting participant data solid drug samples were collected on modified paper test cards that have previously been employed for rapid screening of illicit drugs [30]. For this initial study a small section of the card was cut away and used for extraction and LC-MS/MS analysis of 22 analytes. These samples were extracted into 5 mL of a 90/10 methanol to water solution. Following extraction both the liquid extracts and the paper test cards containing additional solid drug were stored at 4 °C. A total of 124 samples were prepared and analyzed in this original work.

For the measurement of benzodiazepines here, a total of 79 of these original samples were selected for analysis. These 79 samples were a mix of those with and without suspected benzodiazepine presence and, importantly, all contained sufficient sample mass (at least 1–2 mg of solid sample remaining) on the test card to perform a second extraction and analysis.

These 79 samples were extracted using a modified extraction protocol. Briefly, 15 mL centrifuge tubes were labeled with the unique sample IDs. Sample cards were removed from their bags and placed on a fresh paper towel. Approximately 1–2 mg of solid drug was removed from the test card, though, individual input mass for each sample was not recorded. Solid drug was removed either by transferring a segment of the paper test card containing solid drug using an X-acto knife to cut the segment away, or by directly transferring solid drug from the card to the centrifuge tube using a fresh bamboo stick. After each sample the X-acto knife was rinsed with acetone and the paper towel was disposed. To get a measure of any potential carry-over between samples, a piece of card with no drug deposited on it was prepared in the same manner after every 5 samples to generate a method blank. In total, 16 method blanks were generated. After transferring each sample to the centrifuge tubes 5 mL of the extraction solvent (90% H_2O, 10% MeOH) was added. Samples were briefly vortexed before being sonicated for 30 min. After sonication the samples were stored, with the paper deposition region remaining in the tube, at 4 °C until preparation for LC-MS/MS on the day of instrumental analysis. Samples were prepared into labeled 2 mL polypropylene HPLC vials. 500 μL of the diluent (90:10 mobile phase A to mobile phase B) was added to the vial. Next, 500 μL of the sample was transferred to the vial. The vial was then sealed with a polypropylene cap and vortexed before analysis.

In addition to the new 79 extracts, extracts for the same 79 samples that were extracted and analyzed as part of Whitehead *et al.* (2023) were analyzed on the developed method. An additional 13 samples included in the original work, but not re-extracted with these new extracts were also selected for analysis on the developed benzodiazepine method. These extracts were prepared in 2021 and 2022 and were analyzed to look for differences in measured benzodiazepine analytes or concentrations based on the age of the extract. These extracts were kept at 4 °C after their original extractions in 2021 and 2022. For their analysis on the developed benzodiazepine method the extracts were removed from the refrigerator, vortexed for approximately 15 s before 500 μL was transferred to a labeled 2 mL polypropylene HPLC vial to which 500 μL of the diluent (90:10 mobile phase A to mobile phase B) was added. In total, 79 new extracts were analyzed on the developed method. An additional 79 paired extracts, those originally extracted in 2021 and 2022 for Whitehead *et al.* (2023), were also analyzed. Finally, 13 extracts prepared in 2021 and 2022 for Whitehead *et al.* (2023) were also analyzed giving a total number of 171 analyzed samples.

Lateral flow immunoassay analysis

Rapid Response Benzodiazepine Test Strips (BTS) at a 300 ng/mL cutoff concentration were used. Standards of benzodiazepines were analyzed on BTS by dilution from their 200 μg/mL stock solution into 100% water to a final concentration of 20 μg/mL and 0.5 μg/mL. Sample extracts were analyzed by dilution of 100 μL of extract into 900 μL of 100% H_2O. Images of each BTS were collected approximately 5 min after samples were run. These saved images were used to determine the presence or absence of benzodiazepines in each sample. To ensure accuracy of the BTS a positive control (300 ng/mL alprazolam in 100% H_2O) and a negative control (no benzodiazepine present) were screened on BTS at the start of each batch.

Results & discussion

Method development

Method development was performed for all 53 compounds. Precursor ions, fragmentor voltage, and at least one, but up to four, product ions and their unique collision energies were determined for each analyte. Of the product ions, the ion generated in the greatest abundance was used as the quantifying ion with any remaining product ions used as qualifying ions. For all 53 analytes their precusor and product ions, fragmentor voltage, collision energies, and cell accelerator voltages are given in Table 1. The retention time and the delta retention time of each compound used to generate the dMRM method are also given in Table 1. The developed dMRM method had a cycle time of 1500 ms, with a minimum dwell time of 10 ms for any individual transition. The developed chromatographic parameters gave a total run time (including post-time) of 8.1 min, as shown in Table 2. The gradient allows all analytes to be eluted between 1.2 and 6.0 min.The ion source parameters used are also shown in Table 2.

Linearity, precision & accuracy, LoD and LoQ

The 8 prepared solutions ranging from 0.10 to 20 ng/mL of each analyte were injected to generate a calibration curve. Of these 8 points, 4 were injected in triplicate with the remaining being injected 7 times. The average measured peak area across the replicates of each concentration were used to examine a plot of concentration versus measured peak area. Across all 53 compounds, the generated calibration curves were best described by a linear curve fit with no weighting and a forced intercept with R^2 greater than 0.994 as demonstrated in **Table S1**. Most analytes had a linear fit applied in the range of 0.1–20 ng/mL. Two analytes, 4-hydroxy alprazolam and lorazepam, were fit from 0.2 to 20 ng/mL and one analyte, 3-hydroxy phenazepam, was fit from 0.5 to 20 ng/mL as these compounds did not produce peaks at lower concentrations.

Seven replicate injections of a mid-range calibration concentration at 1 ng/mL were used to measure precision & accuracy. Precision was measured by determining the RSD of the average measured peak area across these replicate injections. For all analytes the RSD values ranged from 3 to 25% (median of 11%) except for n-desmethylclobazam and 3-hydroxy phenazepam which had RSD values of 31 and 32% respectively. For these two compounds, the RSD of 7 replicates of 10 ng/mL were within ± 30, at 11and 18% respectively. The same 7 replicate injections at 1 ng/mL were used to determine accuracy through the percent difference of this measured concentration from the true concentration. The absolute percent difference values ranged from 0.2 to 21% (median of 7%). The results of precision & accuracy for each compound are given in **Table S2**. The LoD and LoQ for each analyte were determined as described above and in the Supporting Information. The LoD for all analytes ranged from 0.01 to 0.36 ng/mL, except for 3-hydroxy phenazepam which was at 2.94 ng/mL. **Table S3** gives the calculated LoD and LoQ for all analytes.

Selected samples

As previously described, samples chosen for analysis were those previously collected in Whitehead *et al.* (2023) for the analysis of 22 illicit drugs using LC-MS/MS. Comparison of analytical results generated in Whitehead *et al.* (2023) to participant surveys in Swartz *et al.* (2023) and highlighted a disparity in the number of participants who suspected benzodiazepines as compared to the number of samples for which a benzodiazepine was measured on either LC-MS/MS or BTS. Using participant data collected and described further in Swartz *et al.* (2023), the suspected drug composition given by the participant was combined with sample ID to identify the samples for which a benzodiazepine was suspected [27].

Of the 138 samples collected, 56 had suspected benzodiazepine presence by participants. Of these 56 samples, 4 samples were excluded from analysis in Whitehead *et al.* (2023) due to either the growth of mold during shipping or due to insufficient drug deposition on the cards. All remaining 52 cards were removed from the refrigerator and visually inspected to determine if sufficient drug (i.e. at least 1–2 mg of visible solid drug) remained on the card to perform a second round of extractions. Of the remaining 52 samples, 39 had sufficient drug deposited to perform this second extraction. From there, the remaining cards that were included in Whitehead *et al.* (2023) were also visually inspected to determine if sufficient drug was present to be extracted from the card. An additional 40 samples were found to have sufficient drug deposition. This gave a total of 79 samples, including 39 with suspected benzodiazepine presence. Of the 40 samples that did not have suspected benzodiazepine presence, 8 samples did not have corresponding participant survey data that was collected and therefore had no suspected drugs listed.

These 79 samples were then prepared for LC-MS/MS analysis by extraction of solid drug using the procedure described above. Additionally, all 79 samples had extracts generated in Whitehead *et al.* (2023) that were also prepared for analysis on the developed benzodiazepine method. Additionally, the original extracts for the 13 samples for which benzodiazepine presence was suspected but insufficient drug was on the card for a second extraction were also analyzed. This gave a total of 171 sample extracts that were analyzed on the developed method.

LC-MS/MS results

Of the 79 new extracts, 68 samples (84%) had detectable concentrations of at least one benzodiazepine. The number of samples with detectable concentrations of at least one analyte did not vary significantly between the samples that had suspected benzodiazepine presence based on participant data versus those without suspected benzodiazepine presence at 84 and 88% respectively. Of the 53 analytes included in the developed method, just 14 were found above their detection limit in at least one sample as shown in **Table S4**. Further, only 6 analytes were found in more than 10% of samples, with clonazolam (63%) and its metabolite 8-aminoclonazolam (43%), oxazepam (39%), and clonazepam (38%) having the highest detection frequencies across samples. A comparison of the analytes detected in those with and without suspected benzodiazepine presence is given in **Figure S1** highlighting no major differences in the detection frequency of analytes in samples with and without suspected benzodiazepine presence. The presence of 8-aminoclonazolam in samples containing clonazolam has been previously noted and is expected to be due to the poor long-term stability of clonazolam [31].

Fig. 1 displays an upset plot relating the intersection of analytes that were measured in at least 10% of samples. From these results, several patterns emerged. These trends are described in detail in the Supporting Information, and briefly described below.

Clonazolam, the analyte with the highest detection frequency, was most commonly measured with its metabolite 8-aminoclonazolam and clonazepam in 22% (n = 11) of the 50 samples where clonazolam was detected. Clonazolam was first synthesized in 1971 as a triazolo analogue of clonazepam but has never been approved for therapeutic use in the United States. Clonazolam has high potency compared to clonazepam and alprazolam, with typical doses in the range of 0.2–0.4 mg [32]. The third most detected analyte, oxazepam, was detected alone in 14 (45%) of the 31 samples in which it was detected in. Oxazepam is an FDA-approved benzodiazepine sold under the brand name Serax that is typically prescribed in doses up to 120 mg per day; little data on its occurrences in illicit drugs is available [33]. In general, results shown in Fig. 1 highlight that most samples show complex mixtures of benzodiazepines, including combinations of prescription benzodiazepines and designer benzodiazepines that are not approved for therapeutic use in the United States. **Figures S2 and S3** displays an upset plot for samples

Table 1
dMRM parameters from ion and chromatographic optimization experiments. Quantitative ions are given in bold.

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor voltage	Collision Energy	Cell Accelerator Voltage
1-demethyl Phenazolam	373	**345.0**	4.51	1.5	172	32	4
		294.1				28	
		283.0				40	
		181.9				40	
1-hydroxy Midazolam	342.1	**324.2**	3.117	1.5	174	24	4
		203.2				28	
		176.0				40	
		168.0				40	
3-hydroxy Phenazepam	365	347.0	4.5	1.5	134	16	4
		319.0				24	
		273.1				36	
4-hydroxy alprazolam	325.1	**307.1**	4.049	1.5	104	20	4
		280.3				24	
		239.1				40	
		77.2				40	
7-Aminoflunitrazepam	284.1	227.2	1.573	1.5	146	28	4
		226.0				36	
		135.1				32	
7-Aminometazepam	266.1	238.1	1.201	1.5	104	28	4
		209.1				28	
		208.1				36	
		135.1				28	
8-Aminoclonazolam	324.1	296.1	1.49	1.5	162	32	4
		256.0				28	
		220.0				40	
		213.1				36	
Adinazolam	352.1	**58.4**	1.6	1.5	74	20	4
alpha-hydroxy alprazolam	325.1	**297.1**	4.117	1.5	178	28	4
		176.0				32	
Alprazolam	309.1	**281.0**	4.553	1.5	132	32	4
		205.0				40	
		192.1				36	
Bromazepam	316.0	261.0	3.204	1.5	142	28	4
		209.2				32	
		208.0				40	
		181.9				32	
Bromazolam	353.0	**325.0**	4.67	1.5	138	32	4
		274.1				32	
		205.1				40	
		171.0				28	
Cinolazepam	358.1	340.1	4.4	1.5	138	16	4
		312.1				24	
		272.1				40	
		245.0				40	
Clonazepam	316.1	**270.1**	4.33	1.5	162	28	4
		241.0				40	
		214.1				40	
		207.2				40	
Clonazolam	354.1	**308.1**	4	1.5	200	28	4
Cloniprazepam	370.1	**316.2**	5.7	1.5	162	24	4
		270.1				40	
		241.1				40	
		55.2				40	
Delorazepam	305.0	241.9	4.7	2	114	36	4
		190.0				40	
		165.0				36	
		140.0				40	
Desalkylflurazepam	289.1	**140.0**	4.69	1.5	88	36	4
Desmethylclotiazepam	305.1	277.1	4.359	1.5	168	24	4
		218.0				28	
		213.0				40	
		140.0				32	
Diazepam	285.1	257.0	5.273	1.5	58	24	4
		222.0				28	
		193.1				40	
		154.0				32	
Diclazepam	319	**154.1**	5.366	1.5	140	36	4
Difludiazepam	321.1	**229.1**	5.11	1.5	138	36	4
		201.1				40	
		154.0				36	
Estazolam	295.1	**267.1**	4.331	1.5	192	28	4
		205.0				40	
		164.3				40	
Ethyl Loflazepate	361.1	289.1	5.4	1.5	114	20	4

(*continued on next page*)

Table 1 (*continued*)

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor voltage	Collision Energy	Cell Accelerator Voltage
		287.1				20	
		259.1				40	
		166.0				36	
Etizolam	343.1	**314.0**	4.776	1.5	200	28	4
		211.1				40	
Flualprazolam	327.1	299.1	4.352	1.5	130	32	4
		292.3				28	
		223.0				40	
		165.1				36	
Flubromazepam	333	226.2	4.804	1.5	166	32	4
		209.0				40	
		205.9				40	
		184.0				36	
Flubromazolam	371	343.2	4.49	1.5	160	36	4
		292.2				32	
		237.1				40	
		223.1				40	
Fluclotizolam	333	**298.1**	4.374	1.5	164	24	4
		243.1				36	
		229.1				40	
		227.9				40	
Fludiazepam	303.1	**211.0**	5.142	1.5	172	36	4
		192.9				40	
		177.0				40	
		154.0				36	
Flunitrazepam	314.1	**268.1**	4.558	1.5	170	32	4
		239.5				32	
		239.1				40	
		211.0				40	
Flunitrazolam	338.1	**292.1**	3.665	1.5	148	28	4
		264.1				40	
		207.1				40	
		183.4				40	
Flurazepam	388.2	317.3	2.23	1.5	70	24	4
		315.1				28	
		288.1				28	
		100.3				36	
Flutoprazepam	343.1	**289.1**	5.888	1.5	178	24	4
		205.7				40	
		140.0				40	
Halazepam	353.1	**241.0**	5.9	1.5	58	40	4
		222.1				36	
		212.3				40	
		201.9				40	
Ketazolam	369.1	**285.2**	5.55	1.5	134	24	4
Lorazepam	321	**275.1**	4.1	2.5	138	28	4
Meclonazepam	330.1	**284.1**	4.805	1.5	142	32	4
		239.1				40	
		214.0				40	
		204.0				40	
Methylclonazepam	330.1	284.1	4.885	1.5	164	28	4
		256.1				32	
		255.1				40	
		221.1				40	
Midazolam	326.1	**291.3**	2.29	1.5	134	32	4
		249.1				40	
		244.1				28	
		209.0				40	
N-desmethylclobazam	287.1	**245.0**	4.287	1.5	88	20	4
N-desmethylflunitrazepam	300.1	**254.1**	4.032	1.5	142	28	4
		225.1				40	
		198.0				40	
Nimetazepam	296.1	**250.1**	4.639	1.5	112	24	4
		179.2				40	
Nitrazepam	282.1	**236.2**	4.162	1.5	142	24	4
		180.0				40	
Nitrazolam	320.1	292.1	3.8	1.5	112	28	4
		274.1				36	
		246.1				40	
		198.1				40	
Nordiazepam	271.1	208.1	4.768	1.5	118	36	4
		164.9				32	
		140.0				32	
		91.1				40	
Oxazepam	287.1	**241.0**	4.338	1.5	136	24	4
Phenazepam	349	207.3	5.028	1.5	190	40	4

(*continued on next page*)

Table 1 (*continued*)

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor voltage	Collision Energy	Cell Accelerator Voltage
		206.0				40	
Phenazolam	387	359.2	4.69	1.5	172	36	4
		308.1				32	
Prazepam	325.1	**271.0**	5.944	1.5	88	28	4
		208.1				40	
		165.0				36	
		140.0				36	
Pyrazolam	354	206.1	2.5	1.5	162	40	4
		167.0				36	
Temazepam	301.1	**255.1**	4.814	1.5	88	24	4
Triazolam	343.1	315.2	4.6	1.5	164	32	4
		308.1				28	
		239.0				40	
		165.0				36	

Table 2
Chromatographic and ion source parameters of the developed method.

Sample solvent phase:	90/10 Mobile phase A/Mobile phase B		
Mobile phase A:	90/10 H2O/MeOH + 0.1% Formic acid		
Mobile phase B:	100% ACN		
Column:	Waters ACQUITY UPLC BEH C18, 100x2.1 mm, 1.7 uM		
Column temperature (°C)	40		
Injection volume (uL)	5		
Time	**% A**	**% B**	**Flow (mL/min)**
0	75	25	0.4
2	75	25	0.4
4	50	50	0.4
6	10	90	0.4
8	20	80	0.4
8.1	75	25	0.4
Post-time (min):	2		
Ion source	AJS ESI		
Polarity	Positive		
Gas temp (°C)	350		
Gas flow (L/min)	10		
Nebulizer (psi)	40		
Sheath gas temp (°C)	400		
Sheath gas flow (L/min)	10		
Capillary (V)	3400		
Nozzle voltage (V)	0		

both with and without suspected benzodiazepine content and demonstrate similar trends.

For the same 79 samples, extracts generated in Whitehead *et al.* (2023) were also analyzed to compare the number and concentrations of benzodiazepines measured in the samples based on extract age. Of these 79 old extracts, 52 samples (66%) had detectable concentrations of at least one analyte. A detailed description of the analytes detected in the old extracts is given in the Supporting Information and a comparison of the analytes detected in the new and old extracts for the same 79 samples reveals several interesting trends with detection frequencies of analytes measured in both the old and new extracts compared in **Table S6.** The detection frequency of two analytes with high detection frequencies, clonazolam and flualprazolam, were relatively consistent between new and old extracts. The metabolite of clonazolam, 8-aminoclonazolam, was detected less frequently in the old extracts (28%) than in the new extracts (43%). The same is true of clonazepam which was detected in 38% of new and just 16% of old extracts and of oxazepam which was detected in 39% of new and 0% of old extracts. These results may signal poor stability of both clonazepam and oxazepam in solution for extended, more than 1 year, time periods. Further research is needed to confirm this result.

Response of BTS with standards and samples

All 53 compounds were analyzed on two separate lots of BTS at a concentration of 20 μg/mL in 100% H_2O. Of the 53 compounds, 36 compounds (68%) produced a positive response at this concentration as shown in **Table S7**, with no differences observed in the response of the BTS based on lot number. Compounds that produced a positive result at 20 μg/mL were then diluted to 0.5 μg/mL in 100% water and ran on a fresh BTS from each lot number. Of the 36 analyzed at 0.5 μg/mL, 27 compounds (75%) produced a positive response. Just one compound, clonazepam, produced a positive response on one lot number and negative response on the second. The BTS manufacturer reports information on the response of 22 benzodiazepines, including 16 compounds that overlap with those measured here. A comparison of the response of these 16 compounds to the lowest concentrations that produced a positive response from the manufacturer's testing is given in **Table S8**. A detailed discussion between the results generated here and those of the manufacturer is given in the Supporting Information. Broadly, the comparison of data generated here to that of the BTS manufacturer suggests strong similarities at elevated concentrations of benzodiazepines, with greater differences observed at lower concentrations. In Whitehead *et al.* (2023) only 1 sample of 124 screened with BTS gave a positive response. Results of the 80 samples re-extracted and analyzed again on BTS showed no changes to the BTS results published in Whitehead *et al.* (2023) with just one sample, AU027, producing a positive result on BTS. These results of samples on BTS show low response rates compared to LC-MS/MS results for the samples.

Comparison of BTS and LC-MS/MS data based on measured concentrations

The low response rates of BTS compared to LC-MS/MS for these 79 samples is important to contextualize given the use of BTS for rapid screening of benzodiazepine presence in drug checking protocols. For the 6 benzodiazepine compounds reported in more than 10% of samples, only one, 8-aminoclonazolam, gave a negative BTS result at either of the concentration tested in standards. The remaining 5 compounds all produced a positive BTS result at the tested concentrations. Of these, 3 compounds- clonazepam, alprazolam, and oxazepam- have limits of detection defined in the manufacturer's literature as shown in Table S8. Of the 2 remaining analytes, the response of flulaprazolam on BTS has previously been analyzed and was found to produce a response down to a concentration of 0.5 μg/mL [34]. The response of clonazolam on a BTS has not been previously described.

Importantly, the concentrations of each benzodiazepine in solution will define whether or not a positive response using a BTS would be expected based on the lowest detectable concentrations reported across the literature on these analytes. For the 6 analytes that were detected in more than 10% of the 79 new extracts, a summary of the concentrations measured in samples is given in **Table S9**. To summarize, clonazolam was measured in the highest concentrations of all benzodiazepines followed by flualprazolam. In 68% of samples where clonazolam was detected, the measured concentration exceeded that of the maximal

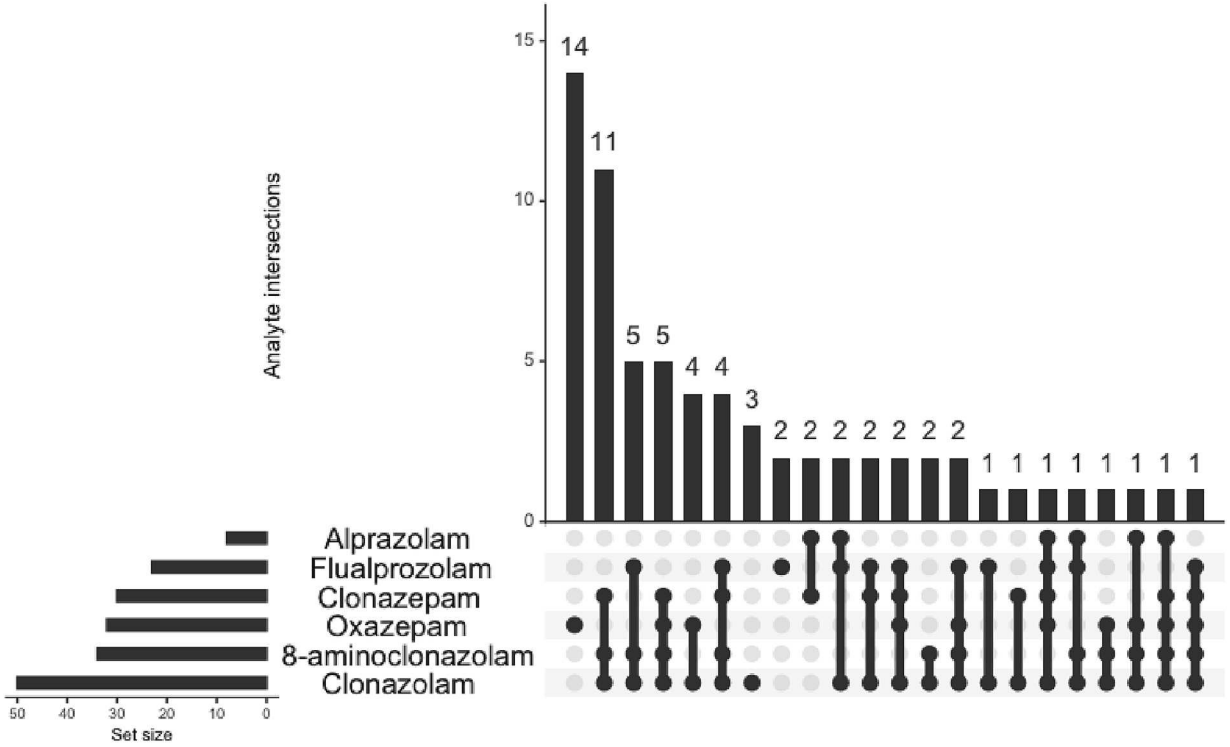

Fig. 1. Upset plot displaying the intersection of the 6 benzodiazepines measured in more than 10% of samples. Horizontal bars on the lower left indicate the set size, or number of times each of the 6 drugs listed were detected in samples. The dots to the right of the drug names indicate the mixture (i.e. a single dot is for a single drug, multiple dots are a combination of those drugs). Vertical bars above these dots indicate the number of times that particular mixture was detected, demonstrating the frequency of the mixtures across all samples.

point of the calibration curve (20 ng/mL). The same is true of flualprazolam where 43% of samples with flualprazolam detected had sample concentrations exceeding this value. For these samples, the measured concentration was replaced with the maximal value of 20 ng/mL for all calculations. Median concentrations for clonazolam and flualprazolam were 20 and 4 ng/mL respectively. Median concentrations of other analytes were typically lower, all less than 1 ng/mL.

These median concentrations can then be related to the median concentrations in the solution before dilution, and then further related to an estimated concentration of the analytes in solutions ran on BTS. Using this approach, the median concentration of oxazepam, flualprazolam, clonazepam, and alprazolam in the solutions ran on BTS ranged from 0.04 to 0.8 ng/mL. These concentrations are significantly lower than the lowest detectable concentrations reported in literature, and explain the low response on BTS for these samples. The only sample which gave a positive response on BTS, AU027, also gave the highest measured signal on LC-MS/MS for a single analyte, flualprazolam. This was the largest measured response of all benzodiazepines across all samples.

Altogether, these results highlight that the preparation method employed here, extraction of 1–10 mg of solid into 5 mL of solvent followed by a 10-fold dilution for BTS analysis does a poor job of screening for benzodiazepine presence in these samples. Existing guidance on the use of BTS for drug checking purposes recommend 1 mg of solid dissolved for every 1 mL of water, allowing for a maximal upper concentration of 1 mg/mL [34]. This concentration is significantly higher than those used here for screening on BTS following preparation for LC-MS/MS and previous reports have highlighted the efficacy of BTS when used following protocols designed for drug checking purposes.

These results suggest that of the benzodiazepines measured here in these select samples collected in Chicago, IL, excluding clonozolam, show relatively low concentrations present. The lower concentrations of these analytes may be due to trace levels introduced during production, distribution, or storage of these street drugs or due to drug heterogeneity in samples as 1–10 mg amounts of sample were sampled from larger quantities of street drugs. Due to the small quantities of reference materials available, extraction efficiency of solid benzodiazepines using the developed extraction method was not determined, and differences in the extraction efficiency of these benzodiazepines may also contribute to differences in the measured concentrations.

Comparison to previous studies

The presence of clonazolam in illicit drug samples has been reported in various studies of biological samples and limited studies of street drugs [35–46]. Comparisons of the benzodiazepines measured here to previous reports highlight greater frequency of clonazolam in these samples collected in Chicago, IL. The Center for Forensic Science Research and Education reported clonazolam in approximately 5–15% of the toxicology results containing benzodiazepines in 2019–2022, with a notable rise in 2021 [47].The 2021 Annual Drug Report from the National Forensic Laboratory Information System reported approximately 6,800 cases of clonazolam in laboratory drug reports, or 0.5% of all reports [48]. Reports of clonazolam in the Midwest were elevated at 0.81% compared to 0.28–0.42% in all other geographic regions, suggesting clonazolam presence might be greater in the regions where these samples were collected. In this same report clonazolam was the second-most commonly reported benzodiazepine, behind alprazolam which was present in approximately 2% of reports. Though, reports of clonazolam increased in all regions in 2021 data relative to 2019 data, suggesting a rise in clonazolam presence across the entire U.S.

Outside of the U.S., Laing *et al.* (2021) measured a total of 24 samples on both rapid BTS and on confirmatory analysis using GC–MS for a total of 41 benzodiazepines in British Columbia [25]. Their results showed high occurrences of etizolam in 75% of samples, with lower occurrences (less than 15%) of other benzodiazepines such as flubromazolam and

flualprazolam. The analyte most frequently detected here, clonazolam, was not detected in any of the 24 samples using the confirmatory analysis method. A 2022 report out of British Columbia highlighted that in 555 samples analyzed using LC-MS/MS and GC–MS, 21% percent had detectable amounts of at least one benzodiazepine, with etizolam and flualprazolam being the most commonly detected benzodiazepines. These reports of samples collected in Canada demonstrated higher occurrences of etizolam than what was measured in these samples, highlighting a potential difference in the designer benzodiazepines commonly introduced into illicit drug samples across these locations. Further, neither study reported the occurrence of clonazolam in the analyzed samples.

One important aspect to consider with these data is how the benzodiazepines measured here relate to the analytes measured for the same samples as reported in Whitehead *et al.* (2023) [28]. Benzodiazepine presence in samples from Whitehead *et al.* (2023) was low, but 13 of the 14 benzodiazepines measured above their detection limit here were not included in the previous targeted analysis method. Alprazolam was measured here in 10% of samples, higher than the 1% of samples it was measured in the previous report. One probable explanation is that the median concentration of alprazolam measured was just 0.22 ng/mL, and extracts measured here were run at 100-fold higher concentrations than in Whitehead *et al.* (2023). This increase in sample concentration explains why alprazolam was measured more frequently here. Of the samples measured here with at least one benzodiazepine (n = 68) all showed quantifiable amounts of at least one opioid in Whitehead *et al.* (2023), with 64 (94%) including fentanyl. The co-occurrence of benzodiazepines and opioids, especially fentanyl, is alarming due to the increased risk of overdose when benzodiazepines and opioids are taken together. Data for benzodiazepines measured here can also be compared to data from Chicago, IL overdose deaths. In 2021, benzodiazepine-involved opioid overdose deaths represented 5.6% of all opioid-related overdoses in Chicago, IL, up 33% from the previous year [49]. This compares to 2019 data from Chicago, IL that reported 6 benzodiazepine-only overdoses and 65 overdoses involving both benzodiazepines and opioids. Further, of the overdoses involving fentanyl, 7.1% also involved a benzodiazepine with or without other drugs present [50]. Altogether, the high rate of co-occurrence of benzodiazepines and opioids measured in these samples is alarming and should be studied further both in Chicago, IL and elsewhere.

Conclusions

A LC-MS/MS method for the analysis of 53 benzodiazepines was developed and applied to a total of 79 samples of illicit street drugs collected in Chicago, IL. Results demonstrated high frequency of benzodiazepines (84%) with clonazolam (57%) and its metabolite 8-aminoclonazolam (28%), flualprazolam (22%), and clonazepam (16%) being the most frequently measured analytes. These results, combined with those described in Whitehead *et al.* (2023), demonstrate a high frequency of benzodiazepine and opioid co-occurrence in samples, posing an elevated risk for overdose deaths for users. To better describe the extent to which the rapid, point-of-care benzodiazepine test strips respond to various benzodiazepines, all 53 standards were screened on BTS. Sixty eight percent of standards produced a positive response on a BTS at 20 μg/mL, demonstrating that a variety of benzodiazepines can be measured using BTS. When applied to samples, BTS produced a low response rate compared to LC-MS/MS results likely due to insufficient concentration of analytes in the prepared samples. Altogether, these results highlight a critical need for benzodiazepine testing, both through rapid techniques like BTS and secondary analysis tools like LC-MS/MS, to be included in drug checking protocols. The use of additional techniques, such as high-resolution mass spectrometry, may also prove useful for identification of new or emerging benzodiazepines for which analytical standards are not readily available.

Funding

This work was funded by the National Science Foundation (NSF) PFI TT IIP-2016516 award and by the University of Illinois Chicago Center for Clinical and Translational Science (CCTS) award UL1TR002003.This project was also funded with support from the Indiana Clinical and Translational Sciences Institute and in part by grant UL1TR002529 from the National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

CRediT authorship contribution statement

Heather D. Whitehead: Methodology, Validation, Formal analysis, Investigation. **Kathleen L. Hayes:** Methodology, Validation. **James A. Swartz:** Conceptualization, Funding acquisition. **Marya Lieberman:** Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

The authors thank William Boggess, Director of the Mass Spectrometry and Proteomics facility at the University of Notre Dame (Notre Dame, IN) for his support of LC-MS/MS method development.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.forc.2023.100512.

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