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Drug Checking

Detection of etizolam, flualprazolam, and flubromazolam by benzodiazepine-specific lateral flow immunoassay test strips

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Summary

In British Columbia, there have been reported cases of unusual presentations following overdose at supervised consumption sites. Drug samples collected following these overdoses and confirmatory drug checking results reveal the presence of etizolam in expected opioid samples. Etizolam is a sedative drug that closely resembles benzodiazepines such as diazepam (Valium), alprazolam (Xanax), and lorazepam (Ativan) but is not approved for therapeutic use in Canada. It was recently discovered that etizolam is has being added to unregulated opioids to enhance and prolong the desired sedating effects. The purpose of this study was to evaluate the use of benzodiazepine test strips, designed to detect alprazolam, for their ability to detect the presence of etizolam and other benzodiazepines in drug samples. Solutions of etizolam, flubromazolam, flualprazolam, and alprazolam were prepared at different concentrations and tested by the BTNX benzodiazepine test strips. Fentanyl was also used at a very high concentration to evaluate whether or not it can interfere with the test. Results indicate that flualprazolam and flubromazolam have similar detection limits to alprazolam on the test strips. The minimum concentration of etizolam required for detection was higher than the other drugs but still very much within the range to be utilizable for drug checking. Fentanyl at extreme concentrations did not interfere with the test. Benzodiazepines do not dissolve well in water so vigorous mixing and concentrated solutions must be used to minimize the chance of not detecting the drug when it is present in a sample. Nevertheless, benzodiazepine test strips are a useful tool for drug checking as they are an inexpensive, rapid, and simple method to monitor drug trends in an everchanging drug market.

Background

Deaths associated with unregulated opioid use have increased dramatically in the last 5 years in British Columbia (BC). However, a significant decline in illicit opioid deaths was observed in 2019 when compared to 2018.[1] This has been largely attributed to a wide array of harm reduction efforts, including the distribution of naloxone to first responders and community members[2]. Naloxone is an opioid receptor antagonist that has been effective in the reversal of opioid toxicity. However, this drug is ineffective at reversing the effects of central nervous system depressant drugs that act on non-opioid receptors.

There have been reported cases of unusual presentations following opioid overdoses at supervised consumption sites in BC in recent years. In such cases, some individuals remained unresponsive following administration of naloxone. Drug samples collected at overdose prevention sites in Vancouver have tested positive for etizolam in suspected heroin.[3] Consistent with Health Canada's Drug Analysis Service data, drug samples that contain both heroin and etizolam have been identified since at least April 2018 (unpublished data). More recently, novel benzodiazepines, including flualprazolam and flubromazolam, have been identified in the illicit drug supply in BC by the Provincial Toxicology Centre (unpublished data). In order to better inform people who use drugs about the presence of benzodiazepines in the unregulated supply, drug checking services at supervised consumption sites have started testing using lateral flow immunoassay test strips that target benzodiazepines. However, the cross-reactivity of the benzodiazepine test strips with etizolam, flualprazolam, and flubromazolam is not known.

Etizolam is a thienotriazolodiazepine derivative that has similar pharmacological effects and potency to alprazolam.[4] While the drug has not been approved for therapeutic use in Canada, it is not currently listed on the Controlled Drugs and Substances Act (CDSA) as a scheduled substance.[5] Therefore, the drug can be produced and distributed in limited quantities without legal ramifications so long as it is not sold for the purpose of human consumption. Flualprazolam and flubromazolam are benzodiazepine derivatives that are expected to have similar pharmacological effects to other high potency short acting benzodiazepines.[6] While the latter two are not legal under the CDSA, few laboratories possess the capability to detect these substances, particularly in biological fluids.

The purpose of this study was to evaluate the use of benzodiazepine lateral flow immunoassay test strips that were designed to detect alprazolam for the identification of etizolam, flubromazolam, and flualprazolam. Given the high concentrations of fentanyl that may be present in some drug samples, the potential for interference from fentanyl was also evaluated.

Methodology

Chemicals and reagents

Reference standards for etizolam, flubromazolam, and flualprazolam were purchased from Cayman Chemical Company (Ann Arbor, MI). Fentanyl and alprazolam samples were obtained by Dr. Stuart Huckin and verified in house. Powdered fentanyl, etizolam, flubromazolam, flualprazolam, and alprazolam were weighed out and dissolved in ultrapure water. Stock solutions were sonicated and vortexed to ensure complete dissolution and serial diluted to the final concentrations listed in Table 1. Lateral flow immunoassay test strips that target alprazolam (BZO-1S27-100 and BZO-1S7-100) with cut-off concentrations of 100 and 200 ng/mL were purchased from BTNX Inc. (Markham, ON).

Analysis of solutions using benzodiazepine test strips

Ten 1 mL aliquots of each drug at the concentrations listed in Table 1 were prepared in plastic 1.5 mL microcentrifuge tubes. Each test strip was placed in a distinct aliquot of drug solution to avoid contamination from repeated sampling. Test strips were dipped in solution for approximately 15 seconds then placed on a flat surface for 5 minutes in accordance with the manufacturer's instructions. The presence of a pink band at the control line is an indication that the test strip performed properly. The presence of a pink band at the target line is an indicator that no drugs were detected (see figure 1) while the absence of a band at the target line indicates detection of a benzodiazepine. The presence of a band at the target line in the absence of a band at the control line is deemed to be an invalid result.

DRUGS	C1 (µg/mL)	C2 (µg/mL)	C3 (µg/mL)	C4 (µg/mL)	C5 (µg/mL)	X1 (µg/mL)	X2 (µg/mL)	X3 (µg/mL)	X4 (µg/mL)	X5 (µg/mL)
ALPRAZOLAM	0.125	0.5	1	5	50	X	X	X	X	X
FLUALPRAZOLAM	0.125	0.5	1	10	100	X	X	X	X	X
FLUBROMAZOLAM	0.125	0.5	1	10	100	X	X	X	X	X
ETIZOLAM	5	10	50	100	500	2.5	1.25	0.625	0.5	0.125
FENTANYL	100	200	500	1000	5000	X	X	X	X	X

Table 1: Concentrations of compounds used for the determination of cross-reactivity of the Benzodiazepine BTNX immunoassay test strips.

Results

Analysis of positive and negative controls

Figure 1 displays test strips that were placed in water to serve as negative controls. Figure 2 is a photograph of test strips tested with various concentrations of alprazolam in water. Alprazolam was detected at a concentration of 0.500 μg/mL; however, the test strips challenged against the lowest concentration (0.125 μg/mL) did not detect the presence of benzodiazepines. The reported limit of detection for these test strips is 0.100 μg/mL of alprazolam.

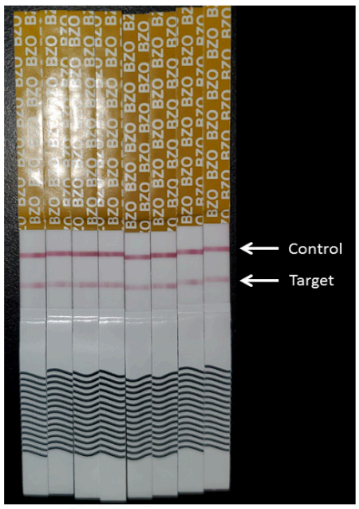


Figure 1: Negative Controls. All drug-free water samples were negative for benzodiazepines according to the test strips.

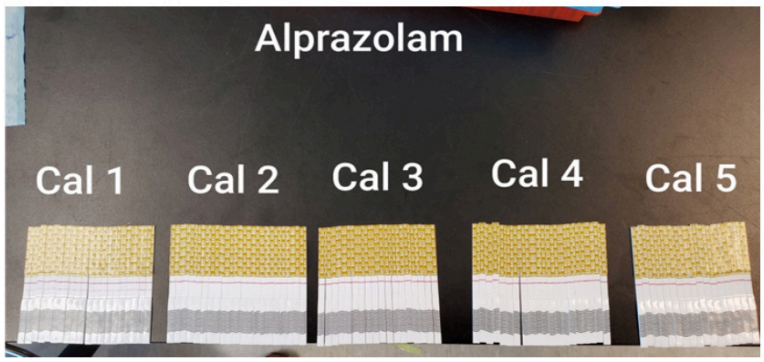


Figure 2: Alprazolam. Positive results were observed in Cal 5, 4, 3 and 2. Negative result was observed in Cal 1. Lowest detectable concentration was 0.5 μg/mL.

Determination of cross-reactivity with novel benzodiazepines

Figures 3 and 4 are photographs of the benzodiazepine test strips challenged with flualprazolam and flubromazolam, respectively. Similar to those tested with alprazolam, test strips detected benzodiazepines as low as 0.500 μg/mL but not at the lowest concentration (0.125 μg/mL).

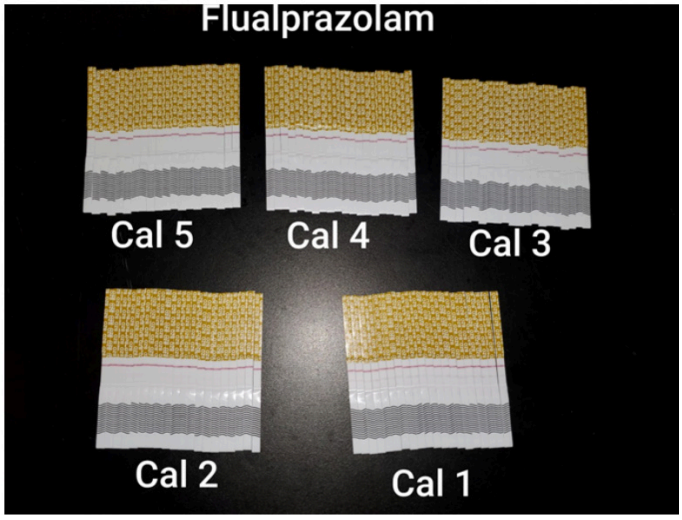


Figure 3: Flualprazolam. Positive results were observed in Cal 5, 4, 3 and 2. Negative result was observed in Cal 1. Lowest detectable concentration was 0.5 μg/mL.

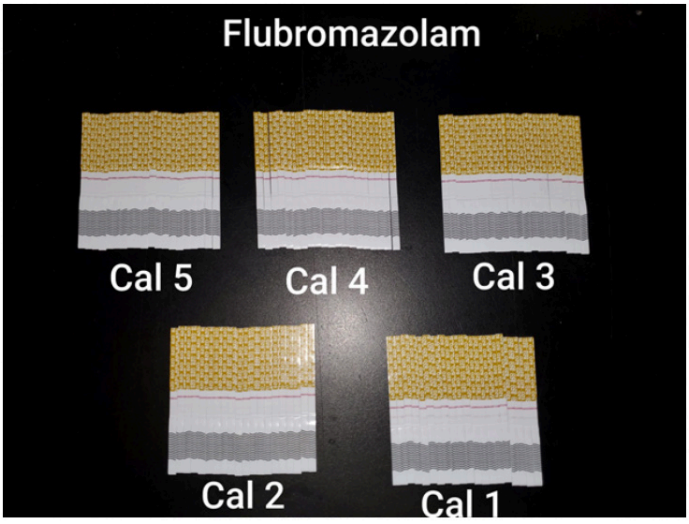


Figure 4: Flubromazolam
Positive results were observed in Cal 5, 4, 3 and 2. Negative result was observed in Cal 1. Lowest detectable concentration was 0.5 μg/mL.

Determination of cross-reactivity with etizolam

Given the lack of cross-reactivity with fentanyl, it was postulated that the benzodiazepine antibodies were specific to the benzotriazolodiazepine component of alprazolam, which differs from the thienotriazolodiazepine structure of etizolam. Accordingly, initial studies (Figure 5) were performed using concentrations of etizolam ranging from 5 to 500 μg/mL (see Table 1). The lowest concentration was then diluted serially down to 0.125 μg/mL. As shown in Figure 6, the limit of detection for etizolam was 1.25 μg/mL.

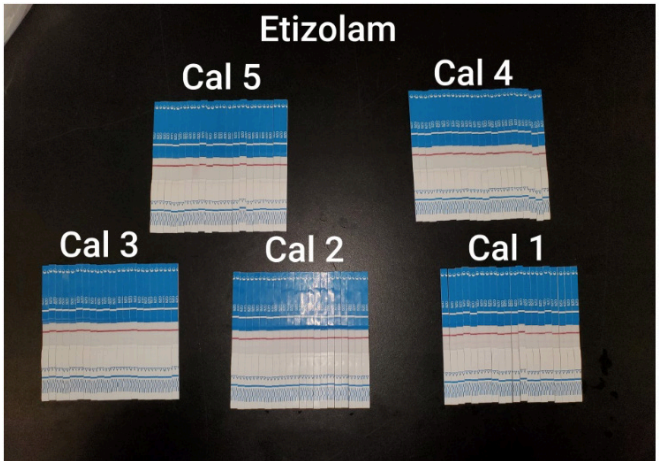


Figure 5: Etizolam (Cal 1, 2, 3, 4, 5). Positive results were observed in Cal 5, 4, 3, 2 and 1. Further tests were conducted (X1, X2, X3, X4 and X5) to obtain the estimated cut-off limit. Reported cut-off for blue test strips is 0.200 μg/mL of alprazolam. Reported cut-off for brown test strips is 0.100 μg/mL of alprazolam.

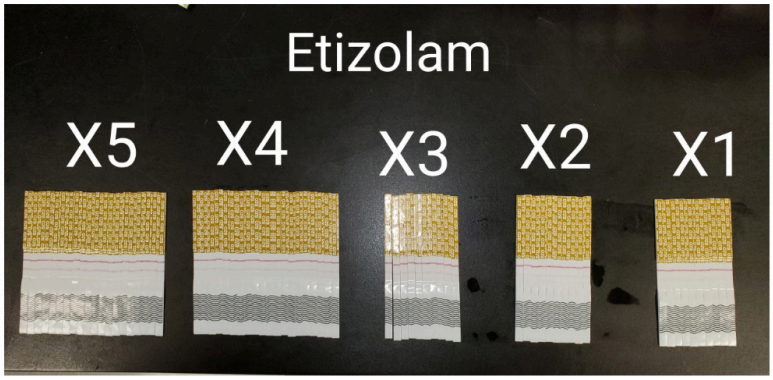


Figure 6: Etizolam (X1, X2, X3, X4, X5). The positive and negative results showed very vague lines. X5, X4, an X3 were shown to be negative results while X1 and X2 were shown to be positive results. Lowest detectable concentration was 1.25 μg/mL.

Determination of interference from fentanyl

Drug checking services at supervised consumption sites has identified the presence of fentanyl co-occurring with etizolam and novel benzodiazepines. Accordingly, it was necessary to demonstrate that fentanyl does not cross-react with the benzodiazepine test strips. Figure 7 displays benzodiazepine test strips that were challenged with fentanyl. The highest concentration of fentanyl (5000 μg/mL) was not detected by the benzodiazepine test strips, indicating that cross-reactivity with fentanyl is unlikely. Analysis of lower concentrations of fentanyl was not performed.

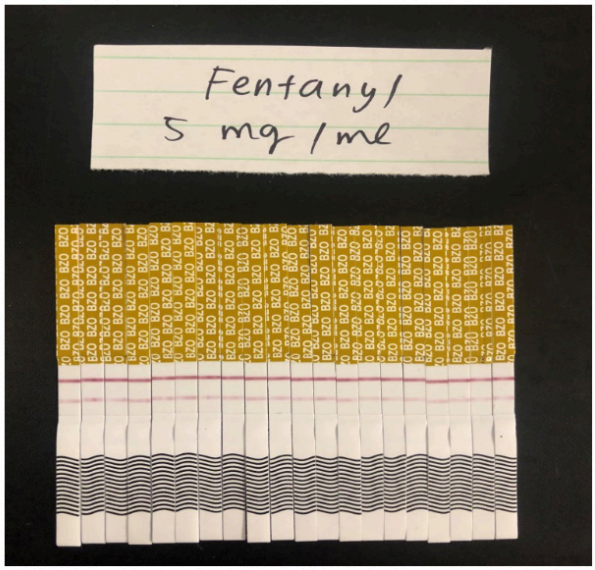


Figure 7 Fentanyl. Negative results were observed in Cal 5 (5 mg/mL). The remaining tests were not performed.

Discussion

The use of novel psychoactive substances (NPS) has increased dramatically in recent years in an effort for illicit drug manufacturers to evade detection and prosecution.7 NPS are either derived from pharmaceuticals that are currently in use or can be derived from drugs that were never brought to market.8 The identification of etizolam and benzodiazepines in the illicit opioid supply has prompted the use of benzodiazepine test strips at supervised consumption sites. This study has demonstrated that the BTNX Inc. benzodiazepine test strips utilized at supervised consumption sites in BC are capable of detecting flualprazolam, flubromazolam, and etizolam at concentrations as low as 0.5, 0.5, and 1.25 μg/mL, respectively. Fentanyl, even at a concentration of 5000 μg/mL was not detected by the benzodiazepine test strips, indicating that the antibody does not cross-react with fentanyl. The high sensitivity of the benzodiazepine test strips to etizolam and novel benzodiazepines and the lack of interference from fentanyl provide support for use when testing illicit drugs in a harm reduction environment.

Flualprazolam and flubromazolam showed similar cross-reactivity to alprazolam. Cross-reactivity with etizolam, however, was lower than that of the benzodiazepines used in this study. The structures of the compounds used in this study are shown in Table 2. While the benzodiazepines share a common core structure, etizolam has a thiophene ring in place of benzene. The results presented herein suggest that the antibody used in the BTNX benzodiazepine test strips targets the benzotriazolodiazepine core structure but that thiophenetriazolodiazepines can be detected, albeit with decreased cross-reactivity. Further characterization of these test strips may be required if the emergence of novel benzodiazepines and thiophenetriazolodiazepines continues.

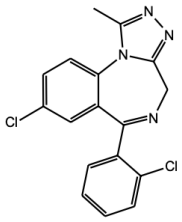
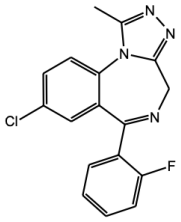
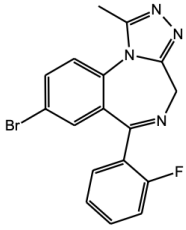
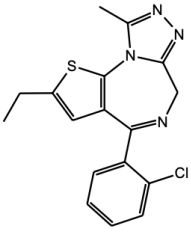
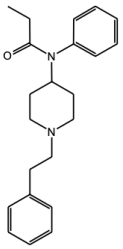
Structure					
Drug Name	Alprazolam	Flualprazolam	Flubromazolam	Etizolam	Fentanyl
Molar mass	308.77 g/mol	326.76 g/mol	371.21 g/mol	342.07 g/mol	336.47 g/mol
LOD (µg/mL)	0.500	0.500	0.500	1.25	---
LOD (µmol/L)	1.62	1.53	1.35	3.65	---

Table 2: Chemical structures of alprazolam, flualprazolam, flubromazolam, etizolam, and fentanyl. Experimentally derived limits of detection (LOD) are provided in μg/mL and μmol/L.

The benzodiapine test strips used in this study contain an antibody that was designed to target alprazolam at concentrations of 0.100 μg/mL and greater. However, in our study, alprazolam was detected at 0.500 μg/mL but not at 0.125 μg/mL. This discrepancy in detection limits may be due to solubility of benzodiazepines in water at neutral pH. Alprazolam is relatively insoluble in water at neutral pH but dissolves well under acidic conditions.9 While the solubility of flualprazolam, flubromazolam, and etizolam are not well characterized, they are assumed to have similar properties. While solubility may increase under acidic conditions, the use of acids in a supervised consumption site environment is not recommended for safety reasons.

This study provides evidence that etizolam and the novel benzodiazepines, flualprazolam and flubromazolam, can be detected using the BTNX benzodiazepine test strips. However, there are some limitations to consider. Firstly, this study involved small sample sizes. While a larger sample size would provide greater confidence in the results, no variability was seen between test strips with any of the drugs nor was there variability at any concentration. Secondly, detection of a drug relied upon the subjective visualization of a faint band. The presence and absence of bands on the target line was more pronounced on extreme ends of the calibration ranges. However, concentrations near the limit of detection were less clear. Thirdly, drug concentrations were calculated as mass per unit volume rather than equimolar to alprazolam for ease of interpretation. Given that the test molecules do not vary substantially in molecular weight, the use of mass rather than number of mols had little impact on the reported limits of detection. Finally, while this study did evaluate the potential for cross-reactivity with fentanyl, other adulterants have been detected in samples that contain these novel compounds, including lidocaine, methamphetamine, mannitol, and synthetic cannabinoids. Future studies could evaluate the cross-reactivity of these substances.

Herein, we evaluated a benzodiazepine test strip that can identify the presence of novel benzodiazepines and etizolam with no observable cross-reactivity with fentanyl. Solubility under conditions available at supervised consumption sites may pose challenges. It is therefore recommended that when dissolving drug in water, the prepared solution should be highly concentrated and mixed vigorously. The use of lateral immunoassay test strips may continue to gain popularity as the illicit drug market evolves as they are an inexpensive, rapid, and relatively simple method to check drugs.

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