BRITISH COLUMBIA CENTRE ON SUBSTANCE USE

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Detection of xylazine by immunoassay test strips in community drug samples: A preliminary report

May 2024

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Land Acknowledgement

The British Columbia Centre on Substance Use would like to respectfully acknowledge that the land on which we work is the unceded territory of the Coast Salish Peoples, including the territories of the xʷməθkwəyəm (Musqueam), Sḵwxwú7mesh (Squamish), and səlílp lwətaɬ (Tsleil-Waututh) Nations.

We recognize that the ongoing criminalization, institutionalization, and discrimination experienced by people who use drugs disproportionately harms Indigenous peoples and that continuous efforts are needed to dismantle colonial systems of oppression. We are committed to the process of reconciliation with Indigenous peoples and recognize that it requires significant and ongoing changes to the health care system.

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Acknowledgements

We thank our partners for their collaboration on this project. We recognize the dedication of the drug checking technicians at Mountainside Harm Reduction Society, Foxglove Housing, Insite, LIFT Community Services, ʔaǰɯmɛt (Tla'amin Nation), and Get Your Drugs Tested for their efforts to collect the presented data and to reduce drug-related harms in their communities. We also thank Health Canada Drug Analysis Service for their assistance as a resource of knowledge and expertise, and for their support in conducting confirmatory analysis. Finally, we thank British Columbia Centre on Disease Control and Cheryl Young, who provided guidance on the research protocol. Without these generous contributions of time and labour, this evaluation would not have been possible.

Background

In Canada, the emergence of xylazine in the unregulated drug supply has become an increasingly pressing concern[1,2]. Among unregulated opioid samples submitted for drug checking in British Columbia (BC), the prevalence of xylazine is relatively low compared to the frequencies observed in the east of the US, but has steadily increased over time.[3] Xylazine is an animal tranquilizer and alpha-2 adrenergic receptor agonist that has not been approved for human consumption. Recent data from Canada and the United States has shown that xylazine can contribute to adverse health effects, including increased risk of drug toxicity when co-consumed with opioids and/or benzodiazepines, and skin lesions that are difficult to heal[4].

Current technologies used at drug checking sites in BC, including Fourier-transform infrared spectroscopy (FTIR), do not consistently detect xylazine in mixtures, particularly when it is present in concentrations near or below the limit of detection [(LOD) approximately 5%].[6] These limitations have prompted interest in the implementation of xylazine-specific lateral flow immunoassay test strips (XTS). Immunoassay test strips currently used by drug checking services to detect fentanyl and benzodiazepines at concentrations below the FTIR LOD do not detect the presence of xylazine. XTS may be a helpful tool to determine the presence of xylazine at low concentrations given their purported sensitivity, but so far very little research has been conducted in real-world settings to validate their use in community drug checking services[9].

Given the lack of evidence on real-world effectiveness of XTS in samples from BC's unregulated drug supply, we conducted a pilot study to evaluate XTS (BTNX, Pickering, ON) in the BC context. The present study sought to 1) evaluate the specificity and sensitivity of XTS to detect xylazine in samples submitted to community drug checking sites; 2) assess the LOD of XTS on samples collected and tested by Health Canada Drug Analysis Service[15]; and 3) evaluate potential cross-reactivity with other substances commonly found in unregulated opioid samples with laboratory reference standards.

Methods

Study design

Between August 1, 2023 and January 31, 2024, community drug checking partner sites in the Vancouver Coastal and Fraser Health regions of BC collected and tested drug samples with FTIR and XTS. All XTS originated from the same lot (#D0AA2306042) to ensure consistency in the antibodies used to manufacture the test strips. As xylazine has been found mostly in unregulated opioid samples, drug checking technicians prioritized testing samples with XTS that service users expected be "down" (a mixture of unregulated opioids and cuts such as sugar and caffeine), or

fentanyl, and unknown samples that were representative of unregulated opioids upon FTIR analysis. Technicians also performed XTS on samples that service users expressed concern about containing xylazine, or suspected the presence of xylazine. These included stimulants (e.g., powder cocaine, crack cocaine, and methamphetamine), benzodiazepines (e.g., bromazolam, unspecified benzodiazepines), and polysubstance samples (e.g., pre-mixed down and methamphetamine).

Technicians followed the XTS protocol provided by BTNX Inc.[13] First, 5-10 mg of sample was dissolved in approximately 5 ml water. For each sample, one XTS was dipped into the solution for 10-15 seconds, removed, and then left to develop for at least one minute before determining the result. The presence of a pink control band indicated the test had been performed properly. The presence of a second pink band (test band) indicated a negative result. The absence of the test band indicated a positive result. Technicians then logged the result in the BCCSU drug checking database.[14]

Figure 1. Example of a positive XTS result with only the control band present (Image A) and a negative XTS result with both the test and control bands present (Image B)

A. Positive XTS

B. Negative XTS

Sensitivity and specificity of XTS

A non-random subset of samples was saved for confirmatory analysis and sent to the Health Canada Drug Analysis Service (DAS)[15]. The technologies used to confirm the presence or absence of xylazine included quantitative nuclear magnetic resonance (qNMR) and/or gas chromatography-mass spectrometry (GCMS). When possible, qNMR was used to determine xylazine concentrations. We then reported descriptive statistics, as well as preliminary measures of XTS sensitivity and specificity. Sensitivity was calculated as the proportion of true XTS-positive samples out of all the samples confirmed to contain xylazine by confirmatory analysis. Specificity was calculated as the proportion of true XTS-negative samples out of all the samples confirmed to not contain xylazine by confirmatory analysis.

Limit of detection of XTS

To address the second objective of our study, we used XTS on 26 samples collected by DAS. The collected samples were selected to reflect common drug compositions and adulterants found in the unregulated opioid supply. All 26 samples included xylazine at varying concentrations. The samples also contained different combinations of caffeine, fentanyl, para-fluorofentanyl, bromazolam, desalkylgidazepam, etizolam, diphenhydramine, and alpha-pyrrolidinoiso-hexanophenone (a synthetic stimulant), although not all of the additional substances were contained in each sample. We extrapolated xylazine quantifications measured by qNMR to determine the lowest concentration at which XTS detected xylazine within these samples.

Cross-reactivity of XTS

To address the final objective of our study, we evaluated potential cross-reactivity with substances that had been previously found to cause false-positive results in XTS (e.g., lidocaine, levamisole, ketamine),[8-10,13] as well as a number of benzodiazepines (e.g., alprazolam, bromazolam, flubromazepam, flualprazolam, etizolam). Furthermore, xylazine laboratory standards were used to confirm the LOD of XTS advertised by BTNX Inc. which is currently 1,000 ng/ml (0.001 mg/ml).[13] XTS were used on concentrations of 0.1, 0.01, and 0.001 mg/ml for each of the substances listed above and replicated two additional times.

Results

The results are divided into three parts: 1) results of XTS performed on community samples, 2) results of XTS performed on prepared mixtures, and 3) results evaluating XTS cross-reactivity.

Community samples

Between August 1, 2023 and January 31, 2024, community drug checking partner sites tested a total of 256 samples with XTS. Most samples tested with XTS were expected to be unregulated opioids: 194 samples were submitted as expected-down, and 33 as expected-fentanyl. The remaining samples tested with XTS included 33 unknown samples, 5 expected-stimulants, 4 expected-benzodiazepines, and 1 polysubstance sample. Of all the samples tested with XTS, 42 (16.4%) yielded positive results and 214 (83.6%) yielded negative results. Xylazine was detected by XTS in 37 (19.1%) expected-down samples, 2 (6.1%) expected-fentanyl samples, and in 3 (16.7%) samples where the expected drug was unknown. No expected-stimulant or expected-benzodiazepine samples tested positive for xylazine with XTS. **Table 1** shows the types of drug samples tested, split by the proportion of samples that yielded xylazine-positive and negative results.

Table 1. Community samples tested with xylazine test strips between August 1, 2023 and January 31, 2024 at drug checking sites in British Columbia (N = 256)

Expected Substance	Total *N* = 256	Xylazine Test Strip Result, *n* (%)	
		Positive *n* = 42 (16.4)	Negative *n* = 214 (83.6)
Benzodiazepine (Unknown)	3	0 (0.0)	3 (100.0)
Cocaine	1	0 (0.0)	1 (100.0)
Crack Cocaine	2	0 (0.0)	2 (100.0)
Down (Unknown Opioid)	194	37 (19.1)	157 (80.9)
Down and Methamphetamine	2	0 (0.0)	2 (100.0)
Fentanyl	33	2 (6.1)	31 (93.9)
Flualprazolam	1	0 (0.0)	1 (100.0)
Methamphetamine	2	0 (0.0)	2 (100.0)
Unknown	18	3 (16.7)	15 (83.3)

Of all the samples tested with XTS, 47 were sent to DAS for confirmatory analysis. Of that subset, 19 samples (40.4%) had tested positive for xylazine with XTS, and 28 samples (59.6%) had tested negative for xylazine with XTS. **Appendix A** provides a breakdown of the samples sent for confirmatory analysis by the expected drug type, stratified by XTS result.

Confirmatory analysis found that XTS correctly detected xylazine (true positive) in 16 samples, and incorrectly detected xylazine (false positive) in 3 expected-down samples. Of the XTS-negative samples, confirmatory analysis determined the absence of xylazine (true negative) in 28 samples, and found no false-negative XTS results. **Table 2** provides a breakdown of XTS-positive and negative samples sent to confirmatory analysis, stratified by whether or not xylazine was detected during confirmatory analysis (yes vs. no). From this, we calculated the sensitivity of XTS to detect xylazine as 100.0%, and the specificity as 90.3%. The concentration of xylazine was measured by qNMR in 13 xylazine-containing samples, and ranged from 0.5% and 11.1% (w/w) of the whole sample.

Table 2. Community samples tested with xylazine test strips stratified by confirmatory analysis results (n=47)

	Xylazine detected by confirmatory analysis (*N* = 47)		
XTS Result	Yes (%)	No (%)	Total
Positive	16 (84.2)	3 (15.8)	19
Negative	0 (0.0)	28 (100.0)	28

DAS collected samples

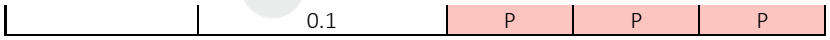
All 26 (100.0%) xylazine-containing samples collected by DAS yielded positive XTS results. When these samples were included into our measurement of diagnostic accuracy, both sensitivity and specificity remained the same. In the 15 samples where xylazine was quantified by qNMR, xylazine concentrations ranged from 1.12% to 23.2% (w/w). Converting this range into mg, XTS detected xylazine in concentrations as low as 0.015 mg/ml, and as high as 0.325 mg/ml, respectively. We note that xylazine concentrations were not determined by qNMR for all samples. See **Appendix B** for details of each individual sample tested.

Cross-reactivity

Of the substances selected for cross-reactivity evaluation, only lidocaine produced a false positive result at concentrations of 0.1 and 0.01 mg/ml, but not at 0.001 mg/ml (**Table 3**). Levamisole, alprazolam, bromazolam, flubromazepam, flualprazolam, etizolam, and ketamine did not produce false positive results at any of the concentrations evaluated. Xylazine produced a true positive result on the XTS when prepared at 0.1 and 0.01 mg/ml concentrations, but was not detected at a concentration of 0.001 mg/ml.

Table 3. Xylazine test strip results by concentration on selected substances

Standard	Standard Concentration (mg/ml)	XTS Result 1	XTS Result 2	XTS Result 3
Lidocaine	0.001	N	N	N
	0.01	P	P	P
	0.1	P	P	P
Levamisole	0.001	N	N	N
	0.01	N	N	N
	0.1	N	N	N
Alprazolam	0.001	N	N	N
	0.01	N	N	N
	0.1	N	N	N
Bromazolam	0.001	N	N	N
	0.01	N	N	N
	0.1	N	N	N
Flubromazepam	0.001	N	N	N
	0.01	N	N	N
	0.1	N	N	N
Flualprazolam	0.001	N	N	N
	0.01	N	N	N
	0.1	N	N	N
Etizolam	0.001	N	N	N
	0.01	N	N	N
	0.1	N	N	N
Ketamine	0.001	N	N	N
	0.01	N	N	N
	0.1	N	N	N
Xylazine	0.001	N	N	N
	0.01	P	P	P



P = positive for xylazine on XTS
N = negative for xylazine on XTS

Discussion

As the unregulated drug supply in Canada continues to evolve, it is necessary to evaluate potential technologies that can accompany existing drug checking methods[5,6]. Our pilot study was designed based on the recommendations suggested by Bunting and Crepeault (2023), which synthesized findings from the few existing studies evaluating XTS, including those manufactured by BTNX Inc.[7] The present study is one of the first to evaluate the efficacy of BTNX Inc. XTS to detect xylazine in real-world samples submitted for drug checking.

We observed that XTS detect xylazine with high sensitivity. Among community samples, we found the sensitivity of BTNX Inc. XTS to be 100.0%, which is consistent with previous findings by Krotulski *et al.* (2023)[9]. However, this result differed slightly from Sisco *et al.* (2023), who reported a sensitivity of 97.4%. Specificity calculations for XTS have varied significantly across existing evaluation studies. While Sisco *et al.* (2023)[8] reported 100.0% specificity, Krotulski *et al.* (2023)[9] reported 85.0% specificity, and we determined the specificity of BTNX XTS to be 90.3% when tested on community samples. At this stage of our pilot study, it is unclear what may have contributed to the differences in sensitivity and specificity observed between these studies.

One possible reason for these differences is that test strips are susceptible to user error, as results depend on the subjective interpretation of the technician. Negative XTS results may have been interpreted differently by technicians depending on how clear the test band developed. Another potential reason is that XTS may take a variable amount of time to produce a reliable result if a cross-reacting substance is present. For example, Lieberman (2023)[10] found that a small amount of lidocaine will initially yield a positive result on the XTS unless the test strip is left to develop for five minutes, at which point it displays a clearer negative result. While lidocaine was not present in the community samples that produced false-positive XTS results, it is possible another substance could be cross-reacting with the XTS that we did not evaluate.

During the time of our study, ortho-methylfentanyl, a fentanyl analogue, began to be detected in the drug supply. While not included in the initial design of this study, there was some theory that ortho-methylfentanyl may be cross-reacting with the XTS as it was detected through confirmatory analysis in all three samples that produced false-positive results (**Appendix C**). Upon observing this, we asked DAS to perform an ad hoc evaluation of XTS on a sample of ortho-methylfentanyl (**Appendix D**). When diluted into a solution, ortho-methylfentanyl produced negative XTS results at concentrations of 0.1, 0.01, and 0.001 mg/ml, replicated three times each. DAS then performed XTS on ortho-methylfentanyl at concentrations of 1 mg/ml and 2 mg/ml, alone and with a low concentration of xylazine (0.0025 mg/ml). Notably, when XTS was performed on ortho-

methylfentanyl alone at a concentration of 2 mg/ml, the XTS produced a very faint test band, which should still be interpreted as negative for xylazine.

These results speak to the limitations of subjective interpretation of XTS results and how variability by technician in interpreting the faintness of a negative test band could have affected our measurement of specificity. We also note that at this time, however, DAS did not have a laboratory standard of ortho-methylfentanyl, and that the sample contained ortho-methylfentanyl at 95.4% concentration. While the concentration of ortho-Methylfentanyl is high in this sample, we cannot rule out that another substance may be present that could be causing interference with the XTS. Appendix E provides a comparison of XTS results tested on high concentrations of ortho-methylfentanyl with and without xylazine, as well as on water which acted as the control.

Upon evaluating the XTS LOD, we found that XTS are capable of detecting xylazine at concentrations as low as 0.01 mg/ml, as assessed on samples collected by DAS. We also found XTS detected xylazine when present at concentrations as low as 0.015 mg/ml in samples collected by DAS. Notably, we confirmed that XTS will not detect xylazine at the LOD advertised by BTNX Inc. of 1,000 ng/ml (0.001 mg/ml) when we assessed the test strips on a laboratory reference standard of xylazine. Previous evaluations conducted by Krotulski *et al.* (2023)[9] and Lieberman (2023)[10] suggest that the true LOD of BTNX XTS is closer to 0.002 mg/ml concentration, however we did not assess this level of concentration. It is also important to note that although the XTS did not detect xylazine at 0.001 mg/ml concentration, we do not know the amount of xylazine needed to produce sedative effects upon consumption, or the effects of repeated exposure to low levels of xylazine on morbidity.

In our evaluation of cross-reactivity, we found that only lidocaine produced false-positive XTS results. Our findings of XTS cross-reactivity with lidocaine is consistent with what is advertised by BTNX, as well as findings from other independent studies[8,10]. While Lieberman (2023)[10] observed XTS cross-reactivity with lidocaine at a very high concentration (10 mg/ml), we determined cross-reactivity occurs at concentrations as low as 0.01 mg/ml, but not as low as 0.001 mg/ml. For this reason, BTNX Inc. does not recommend using XTS on expected-cocaine samples because lidocaine is a common cocaine adulterant in some settings.[13] Additionally, while Lieberman (2023) found cross-reactivity of XTS with levamisole, we did not. This is likely due to the aforementioned evaluation testing high concentrations of levamisole (1 mg/ml and 10 mg/ml), whereas we determined no cross-reactivity at much lower concentrations (0.1, 0.01, and 0.001 mg/ml), which is consistent with what was reported by Sisco *et al.* (2023).[8] Similarly, we did not find cross-reactivity with ketamine at low concentrations, whereas a previous evaluation found cross-reactivity occurred at 10 mg/ml.[10] Testing cocaine or ketamine with XTS may not be of concern at present given that xylazine has been predominantly found in unregulated opioid samples.

We note that there has been recent concern about medetomidine being increasingly detected in the unregulated opioid supply in Canada.[11,12] Like xylazine, medetomidine is approved for use as

an animal tranquilizer and is considered to be more potent than xylazine. In the present study, two community samples that tested positive for xylazine on the XTS and were determined to contain xylazine through confirmatory analysis also contained medetomidine. One sample that tested negative for xylazine on the XTS, and was found to be absent of xylazine through confirmatory analysis, was also determined to contain medetomidine. While findings from the cross-reactivity evaluation conducted by Sisco *et al.* (2023) determined no cross-reactivity with medetomidine, we cannot comment on potential cross-reactivity from the single xylazine-negative and medetomidine-containing sample we tested. The second phase of our study will include an evaluation of XTS cross-reactivity with medetomidine.

Limitations

This phase of our study has some limitations to consider when interpreting these preliminary results. Importantly, while many community samples were tested with XTS, we were only able to obtain a small number of samples for confirmatory analysis. This is due to the amount of sample necessary to perform the XTS at the community drug checking site (5-10 mg), as well as the additional amount of sample needed for confirmatory analysis (5-10 mg). This was a major barrier to data collection as XTS are destructive to drug samples, meaning that once the sample is dissolved in solution to perform the XTS, we could not send it for confirmatory analysis. Many people who engage with drug checking services and participated in this study experience socio-economic and substance use-related barriers that may have prevented them from, or influenced their decision not to provide an additional sample for confirmatory analysis. The small sample size we obtained limited the statistical power of our measurements of sensitivity and specificity. As our study is ongoing, we aim to collect a larger number of samples to obtain a more robust measurement of diagnostic accuracy, which will be included in future updates. We also note that because our study employed convenience sampling to obtain data from community samples, we cannot comment on the prevalence of xylazine from these results.

Conclusion

The emergence of xylazine in the unregulated drug supply has warranted calls for the evaluation of new methods that could increase the capacity for detection by point-of-care drug checking services in Canada. Our findings highlight that XTS are a highly sensitive tool for drug checking technicians to determine the presence of xylazine, especially in cases when it presents at concentrations below the LOD of FTIR spectrometry. However, as the specificity of BTNX XTS has varied across studies, more research is needed to determine how reliable XTS are in correctly producing negative results in complex mixtures of drugs amidst the increasing unpredictability of the unregulated drug supply.

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Appendices

Appendix A. Types of community samples sent to confirmatory analysis stratified by XTS result (n = 47)

Expected Drug Type	XTS Result		Total
	Positive (%)	Negative (%)	
Down (Unknown Opioid)	15 (31.9)	16 (34.0)	31 (66.0)
Fentanyl	2 (4.3)	9 (19.2)	11 (23.4)
Methamphetamine	0 (0.0)	1 (2.1)	1 (2.1)
Unknown	2 (4.3)	2 (4.3)	4 (8.5)
Total	19 (40.4)	28 (59.6)	47 (100)

Appendix B. Xylazine test strip results of samples collected by DAS

Sample	Sample Composition	Sample Concentration (mg/ml)	NMR Quantification of Xylazine (w/w%)	Xylazine Test Strip Result
1	FENTANYL, BROMAZOLAM, XYLAZINE, CAFFEINE	1.334	N/A	Positive
2	FENTANYL, BROMAZOLAM, PARA-FLUOROFENTANYL, XYLAZINE, CAFFEINE	1.436	4.76	Positive
3	FENTANYL, BROMAZOLAM, XYLAZINE, CAFFEINE	1.486	N/A	Positive
4	FENTANYL, BROMAZOLAM, XYLAZINE, CAFFEINE, DIPHENHYDRAMINE	1.646	12.3	Positive
5	FENTANYL, BROMAZOLAM, XYLAZINE, CAFFEINE, DIPHENHYDRAMINE	1.472	9.79	Positive
6	FENTANYL, XYLAZINE, CAFFEINE	1.504	13.0	Positive
7	FENTANYL, BROMAZOLAM, XYLAZINE, CAFFEINE, DIPHENHYDRAMINE	1.482	9.78	Positive
8	FENTANYL, BROMAZOLAM, XYLAZINE, CAFFEINE	1.392	4.27	Positive
9	FENTANYL, BROMAZOLAM, XYLAZINE, CAFFEINE	1.634	8.88	Positive
10	FENTANYL, BROMAZOLAM, XYLAZINE, CAFFEINE	1.588	4.41	Positive
11	FENTANYL, XYLAZINE, CAFFEINE	1.368	N/A	Positive
12	FENTANYL, FLUALPRAZOLAM, XYLAZINE, CAFFEINE	1.388	N/A	Positive
13	FENTANYL, XYLAZINE, CAFFEINE	1.424	N/A	Positive
14	FENTANYL, ETIZOLAM, XYLAZINE, CAFFEINE	1.696	12.5	Positive
15	FENTANYL, FLUBROMAZEPAM, XYLAZINE, CAFFEINE	1.716	N/A	Positive
16	FENTANYL, XYLAZINE, DIPHENHYDRAMINE, CAFFEINE	1.366	1.12	Positive

Appendix B continued. Xylazine test strip results of samples collected by DAS

Sample	Sample Composition	Sample Concentration (mg/ml)	NMR Quantification of Xylazine (w/w%)	Xylazine Test Strip Result
17	FENTANYL, PARA-FLUOROFENTANYL, BROMAZOLAM, CAFFEINE, XYLAZINE	1.766	2.09	Positive
18	FENTANYL, XYLAZINE, DIPHENHYDRAMINE, CAFFEINE	1.63	N/A	Positive
19	FENTANYL, XYLAZINE	1.474	N/A	Positive
20	a-PYRROLIDINOISOHEXANOPHENONE, XYLAZINE, BENZOCAINE, CAFFEINE	1.558	N/A	Positive
21	FENTANYL, DESALKYLGIDAZEPAM, XYLAZINE, CAFFEINE	1.654	N/A	Positive
22	FENTANYL, DESALKYLGIDAZEPAM, ETIZOLAM, XYLAZINE, CAFFEINE	1.68	14.6	Positive
23	FENTANYL, ETIZOLAM, XYLAZINE, CAFFEINE	1.404	23.2	Positive
24	FENTANYL, PARA-FLUOROFENTANYL, BROMAZOLAM, XYLAZINE, CAFFEINE	1.524	2.01	Positive
25	FENTANYL, PARA-FLUOROFENTANYL, BROMAZOLAM, CAFFEINE, XYLAZINE	1.384	5.88	Positive
26	FENTANYL, XYLAZINE, CAFFEINE	1.406	N/A	Positive

Appendix C. Substances detected through confirmatory analysis in xylazine false-positive samples

Expected Substance	XTS Result	Substances Detected by Confirmatory Analysis	
		qNMR	GCMS
Down (Unknown Opioid)	Positive	Caffeine (18.0%) Ortho-Methylfentanyl (16.8%)	Erythritol MAMDPA* (suspected)
Down (Unknown Opioid)	Positive	Desalkylgidazepam (9.4%) Ortho-Methylfentanyl (8.6%) Caffeine (8.1%)	Erythritol Ortho-Methyl 4-APP* (suspected)
Down (Unknown Opioid)	Positive	Caffeine (24.7%) Desalkylgidazepam (7.8%) Para-Fluorofentanyl (3.9%) Methamphetamine (3.7%) Bromazolam (0.1%)	Ortho-Methylfentanyl Fentanyl Xylitol

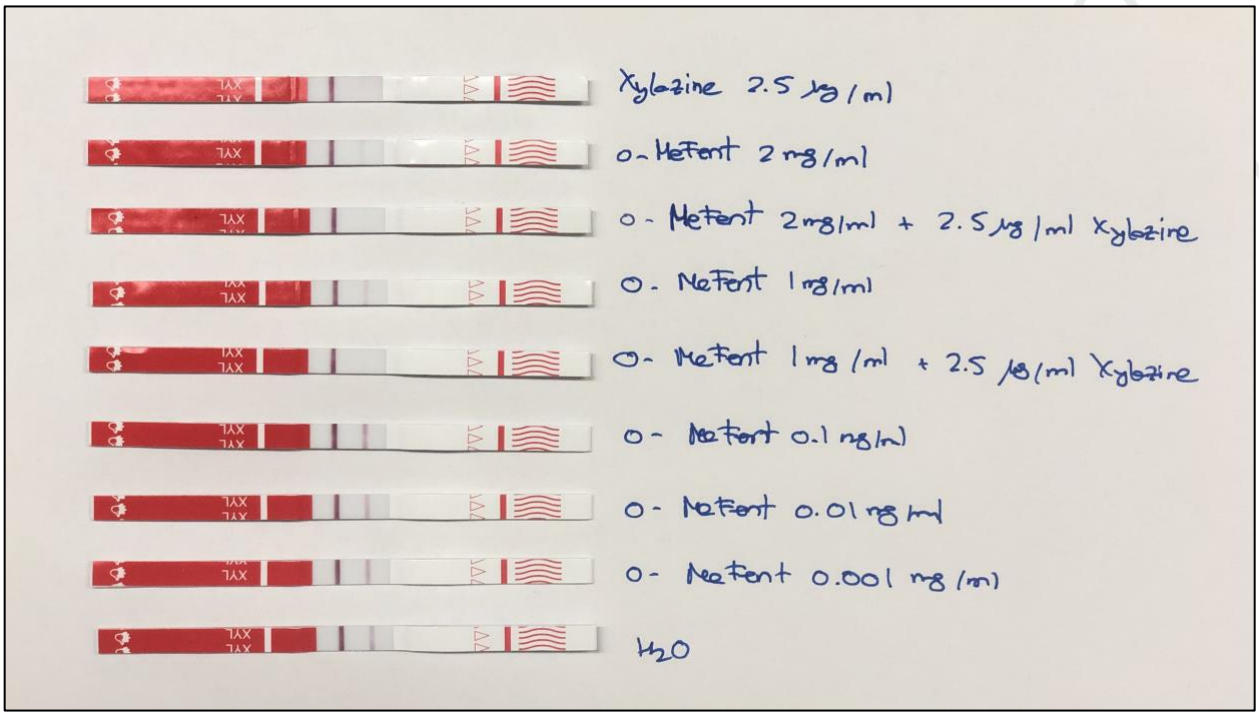
*Precursor used in the synthesis of drugs

Appendix D. XTS results performed on sample of ortho-Methylfentanyl (95.4% concentration)

Sample	Concentration (mg/ml)	Result 1	Result 2	Result 3
95.4% o-Methylfentanyl HCl	0.1	N	N	N
	0.01	N	N	N
	0.001	N	N	N

N = Negative for xylazine on XTS

Appendix E. XTS results performed on samples of ortho-Methylfentanyl, with or without xylazine present



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