Beyond Fentanyl Test Strips: Investigating Other Urine Drug Test Strips for Drug Checking Applications

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ABSTRACT

Use of immunoassay test strips for the detection of fentanyl in drug samples has become increasingly commonplace in harm reduction, law enforcement, public health, customs, and forensic science settings. With the increase of xylazine in the drug supply in recent years, use of xylazine test strips has also begun to take root. As adoption and implementation of this tool continues, a desire to implement test strips for other drugs may emerge. However, since these strips are designed for urine testing, it is important to understand their applicability to testing drugs themselves. In this work, we investigate the utility of seven types of urine immunoassay test strips – amphetamine, benzodiazepine, cocaine, methamphetamine, nitazene, opiate, and xylazine – for drug checking applications. Reproducibility, sensitivity, cross-reactivity, and the effect of prolonged exposure to elevated temperatures were studied. Generally, the tests were found to be reproducible, able to detect trace (µg/mL) levels of the analyte of interest, and minimally affected by prolonged storage at elevated temperatures. Nearly all tests showed cross-reactivity with compounds other than the analyte of interest, highlighting the need to better understand these limitations prior to implementation in a drug checking scenario (that may involve additional chemical analysis on or off site). The viability of expired cocaine, fentanyl, and methamphetamine and test strips was also interrogated, and little to no change in sensitivity was found even though the tests were multiple years expired.

Keywords

Immunoassay; Drug Checking; Harm Reduction

Introduction

There is a continued need for rapid, on-site drug detection technologies to support efforts across the public health, public safety, customs, and emergency medicine disciplines. While advanced analytical techniques, such as Fourier transform infrared spectroscopy (FTIR), Raman spectroscopy, ion mobility, and mass spectrometry, are routinely used by personnel in these fields[1], there is also a need for non-technical, inexpensive tools that do not require electricity, gases, or interpretation of spectra. Color tests and lateral flow immunoassays have long filled this need[1] – providing law enforcement[2], harm reduction personnel[3], and even emergency department nurses and doctors[4] capabilities to rapidly identify drugs in real-time.

The use of lateral flow immunoassays, herein referred to as test strips, has been of particular interest in recent years due to their ability to facilitate detection of low levels of fentanyl in complex street mixtures[5]. They are also inexpensive, relatively durable, and easy to use. Originally designed for urine drug testing, test strips have become commonplace in public health and public safety applications. An increasingly large body of work now exists around off-label use of these test strips for detection of fentanyl in a physical drug sample. Research surrounding both the performance and limitations of these test strips[5–10] as well as informing drug use behavior[3,11–14] is now widely available.

With the increased prevalence of xylazine into the drug supply (often in combination with a controlled substance and often unknown to the consumer), the implementation of test strips designed for xylazine detection is increasing along with initial studies into their performance and limitations[15,16] and societal impact[17].

While fentanyl and xylazine test strips have seen increased adoption, they are not foolproof. Cross-reactivity – where non-target analytes produce a positive result – with other compounds present in the drug supply has been identified for both types of tests[6–10,15,16], creating scenarios where the result may be inaccurate due to interfering compounds. Potential issues with using these tests in environments with elevated temperatures have also been identified[18], which could affect results when people may carry them on their body and or in motor vehicles for extended periods of time. The high sensitivity of these tests also poses the possibility of positive results being attributed to trace contamination of a sample or the environment in which the test is completed.

Regardless of these limitations, it is likely that the utility and acceptance around test strips continues to grow due to their low cost and ease of use. With this increase will likely come the desire to incorporate additional types of test strips (*i.e.*, to detect a wider range of drugs or to address cross-reactivity concerns). For instance, the use of benzodiazepine test strips in combination with fentanyl test strips and FTIR has already been demonstrated[19]. As additional test strips, also designed for urine testing, are considered, it is important to understand if they are viable for the analysis of physical drug specimens. In this work, we investigate the utility of seven types of test strips – amphetamine, benzodiazepines, cocaine, methamphetamine, nitazene, opiate, and xylazine – for drug checking applications. Like previous studies for fentanyl test strips, we investigate reproducibility, sensitivity, and cross-reactivity. Additionally, to better understand potential limitations in real-world field detection scenarios, we investigate the effect of prolonged storage at elevated temperatures and the viability of expired test strips that we had available.

Materials & Methods

The overall study consisted of five sub-studies: reproducibility, sensitivity (approximate limit of detection), cross-reactivity, effect of exposure to elevated temperatures, and sensitivity of expired test strips. A suite of seven different test strips (BTNX, Pickering, ON, Canada) were used for all sub-studies except for the effect of exposure to elevated temperature and the sensitivity of expired test strips where eight and three test strip types were used, respectively (Table 1). Regardless of the sub-study the following procedures were used:

1) Test strip (TS) is removed from foil packaging.
2) TS is placed, vertically, into an aqueous solution containing a known analyte at a known concentration.
3) TS is kept in solution until the liquid is visible in the test and control band area.
4) TS is placed, horizontally, on a clean, sorbent-lined lab bench.
5) After five to ten minutes, the result of the TS is determined, and the TS is photographed.

All the test strips were competitive immunoassays, so the presence of a test band was considered a negative result and the absence of a test band was considered a positive result (the presence of a control band was necessary for the test to be deemed valid). A delineation between a test band with the same intensity as the control band (negative) and a test band that was significantly fainter than the control band (faint negative) was also made, as faint negatives may indicate positive results could be obtained at higher concentrations. An example of a negative versus a faint negative is provided in Figure 1.

Figure 1. Example of test strip showing a faint negative (left), negative (center), and positive (right) result.

Table 1. Test strip types and corresponding positive control solutions used for analyses. All positive control solutions were aqueous. Pure water was used for the negative control regardless of test strip type. Test strip types with a (*) were used only for the expired test sensitivity studies. Test strip types with a (#) were only used for elevated temperature studies.

Test Strip Type	Cutoff (µg/mL)	Expiration Date	Positive Control Compound
Amphetamine	1	05/2024	Amphetamine (1,000 µg/mL)
Benzodiazepine	0.3	07/2025	Diazepam (10 µg/mL)
Cocaine	0.2	09/2024	Cocaine (1,000 µg/mL)
Cocaine - Expired*	0.3	01/2020	Cocaine (1,000 µg/mL)
Fentanyl#	0.2	02/2025	Fentanyl (1,000 µg/mL)
Fentanyl - Expired*	0.02	12/2019	Fentanyl (1,000 µg/mL)
Methamphetamine	1	08/2024	Methamphetamine (1,000 µg/mL)
Methamphetamine - Expired*	1	12/2019	Methamphetamine (1,000 µg/mL)
Nitazene	2	12/2025	Metonitazene (1,000 µg/mL)
Opiate	2	07/2025	Heroin (1,000 µg/mL)
Xylazine†	1	07/2025	Xylazine (1,000 µg/mL)

†The xylazine test strips studied here contained an antibody different than those used in previous studies[15,16] which were shown to cross-react with lidocaine.

Table 2 contains a list of analytes used in this study. All analytes were dissolved in water to a stock solution with a concentration of 1 mg/mL or 0.5 mg/mL. Thirty compounds, denoted in Table 2, were not easily dissolved in water at this concentration, so acetonitrile was added to facilitate dissolution (see Supplemental Table 1 for more information). For analytes that were purchased as a solution, the solvent was allowed to nearly evaporate after which water was added to bring the

solution to the desired concentration. Further dilution of all samples was completed, volumetrically, with water. Both water (OmniSolve HPLC Grade) and acetonitrile (OmniSolv LC-MS Grade) were purchased from MilliporeSigma (Burlington, MA, USA).

Table 2. Analytes (n = 79) used in this study. If the salt form of a compound was used, the salt is provided in parentheses.

Drugs	Drugs (cont'd)	Cutting Agents & Diluents
2C-B (HCl)[1]	Methadone[1]	Acetaminophen[3]
3,4-MDA[3]	Methamphetamine[3]	Aspirin[1a]
3,4-MDMA[3]	Metonitazene[1a]	Benzocaine[3a]
Acetyl Fentanyl (HCl)[1]	Naloxone (HCl)[1]	Caffeine[3]
Alprazolam[1a]	Oxycodone[4†a]	Dextromethorphan[2*†a]
Amphetamine[3]	Pentobarbital[2*†a]	Dimethylsulfone[3]
Bromazolam[1a]	Phencyclidine (HCl)[1]	Diphenhydramine (HCl)[3]
Buprenorphine (HCl)[1]	Phentermine[2*a]	Ephedrine (HCl)[3]
Bupropion (HCl)[3]	N-Piperidinyl Etonitazene[1†]	Guaifenesin[3]
Cannabidiol[2*†a]	Protonitazene[1]	Ibuprofen[1a]
Carfentanil[1*†a]	Remifentanil (HCl)[2*†a]	Lactose[3]
Cocaine[3]	Sufentanil ($C_6H_8O_7$)[1*†a]	Levamisole (HCl)[3]
Codeine[2]	Tramadol (HCl)[2*†a]	Lidocaine[3a]
Deschloroketamine (HCl)[1]	U-47700[2*†a]	Medetomidine (HCl)[1]
Diazepam[1a]	Zolpidem[2*†a]	Melatonin[3a]
N,N-Dimethylpentylone (HCl)[1]	Δ-8-Tetrahydrocannabinol[2*†a]	Metamizole (Na)[3]
Etizolam[1a]	Δ-9-Tetrahydrocannabinol[2*†a]	Methylphenidate (HCl)[1]
Eutylone (HCl)[1]		Noscapine[3a]
Fentanyl[4]		Papaverine (HCl)[3]
p-Fluorofentanyl (HCl)[1†]		Phenacetin[3a]
p-Fluoroisobutyryl fentanyl (HCl)[1]		Phenylephrine[3*a]
Flubromazepam[1a]	**Other Compounds of Interest**	Piracetam[3]
Gabapentin[1]	4-ANPP[1†a]	Procaine[3]
Heroin[3†]	6-Monoacetylmorphine[1a]	Quetiapine (½$C_4H_4O_4$)[1a]
Hydroxyzine[1s]	Aniline (HCl)[1]	Quinine[3a]
Ketamine (HCl)[2*†a]	Benzoylecgonine[1]	Sorbitol[3]
Lisdexamfetamine (2CH_4SO_3)[1]	Nicotine[3]	Trazodone (HCl)[3]
LSD[2*†a]	Phenethyl 4-ANPP[1†s]	Xylazine[3]

[1]Analyte purchased from Cayman Chemical (Ann Arbor, MI).
[2]Analyte purchased from Cerilliant (Round Rock, TX).
[3]Analyte purchased from Sigma-Aldrich (St. Louis, MO).
[4]Analyte purchased from US Pharmacopeia (Rockville, MD).
[*]Analyte purchased as a 1 mg/mL solution instead of a powder.
[†]A "high concentration", stock solution of 0.5 mg/mL was prepared instead of 1 mg/mL.
[a]Acetonitrile was added to the stock solution to enable dissolution of the analyte. See Supplemental Table 1 for more information.
[s]Stock solution was a cloudy suspension.

Limit of Detection

The approximate limit of detection was defined as the minimum concentration of analyte that consistently produced a positive result. This was determined by creating a multi-point, volumetric, serially prepared calibration curve using the positive control analyte listed in Table 1 for each test strip. Concentration levels between 1,000 µg/mL and 0.05 µg/mL were used, with exact values dependent on the test strip being studied (see Supplemental Table 2). Pure water was used as the 0 µg/mL concentration level. For each concentration level examined, ten test strips were analyzed.

The approximate limit of detection was defined as the minimum concentration where all ten test strips produced a clear, positive result.

Reproducibility

Reproducibility, defined as the ability to consistently produce expected positive and negative responses over an extended period of time, was studied by analyzing the positive control (Table 1) and negative control (pure water) five times over the course of four weeks. At the final (fifth) time point, the solution corresponding to the approximate limit of detection was also analyzed to see if there was a loss in sensitivity. Duplicate tests were run for both the positive and negative controls at each time point.

Cross-Reactivity

The ability for test strips to react with compounds other than those used for the positive control was examined by analyzing a set of 79 drugs, cutting agents, diluents, and other compounds (Table 2) at two concentrations (defined as High and Low). For each analyte / concentration combination, two of each test strip type were analyzed. The High concentration was either 1,000 µg/mL or 500 µg/mL (Table 2) and the Low concentration was 10 µg/mL.

Effect of Elevated Temperatures

The effect of elevated temperatures on the test strips was studied by placing unopened test strips in a 55 ºC (131 ºF) oven for two weeks. After that time, the positive control solution (Table 1), negative control (pure water), and solution corresponding to the approximate limit of detection were analyzed in triplicate. If the limit of detection solution did not consistently produce a positive response, solutions with increasing concentration were analyzed to determine the difference in sensitivity between heated and unheated strips. All analytes which showed cross-reactivity for a particular test strip were also analyzed, in duplicate, to determine if positive results would still be obtained after exposure to heat. For analytes where both the high and low cross reactivity concentrations produced a positive result, only the low concentration was analyzed.

Sensitivity Past Expiration

Where available, test strips that were multiple years past the manufacturer's listed expiration date (see Table 1) were used to see if a positive result for the control analyte could still be obtained. To establish the sensitivity of the expired tests, the positive control (Table 1), negative control (pure water), and solution corresponding to the approximate limit of detection were analyzed in triplicate. If the solution corresponding to the approximate limit of detection produced a negative or faint negative result, solutions with a gradually increasing concentration were analyzed until clear positive results were produced for all three replicates. This was completed for expired tests that had been stored under typical laboratory conditions and expired tests that were exposed to elevated temperatures for two weeks before use (identical to the previous section).

Results and Discussion

Effect of Organic Solvents on Test Strip Response

An important observation the came to light in this study was the effect the presence of an organic solvent in the test solution had on the results. To overcome solubility issues for some of the test analytes, while minimizing the risk of degradation, acetonitrile was added to some solutions (Supplemental Table 1). During the analysis of some of these solutions, abnormalities in test strip

wicking and test strip results were observed. A small, side study was completed to investigate the effect of organic solvent on test strip performance. The distance the solution wicked up the test strip and the presence or absence of a control band when mixtures with different ratios of water to organic solvent were used was studied. While acetonitrile was used for creating stock solutions here, methanol was also studied due to its common use in laboratory settings.

The presence of any substantial (>10 % v/v) amount of organic solvent was shown to slow the speed at which a solution was wicked up the test strip. While slower, a 1:4 organic solvent:water mixture was still able to wick completely up the test strip and the control band was readily observed, irrespective of whether the organic solvent was methanol or acetonitrile. Beyond this ratio, methanol was shown to be less detrimental to test strip performance than acetonitrile. Mixtures up to 3:2 methanol:water were able to wick completely and produce control bands. Conversely, incomplete wicking was observed with a 2:3 acetonitrile:water mixture, leading to a lack of control band and test band development. Figure 2 summarizes these results. Given that the test strips are designed for testing urine (an aqueous solution) and require wicking solution up an adsorbent material, it is not unexpected that volatile organic solvents would negatively impact performance.

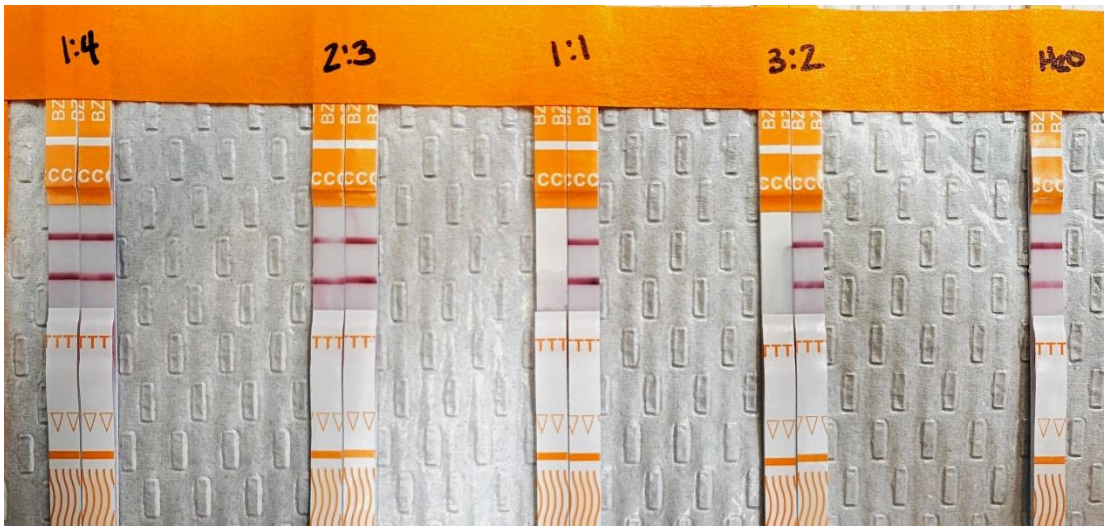

Figure 2. Effect of increasing amount of organic solvent on test strip results. The ratio of organic solvent:water increases from left to right, with results from pure water on the right hand side for reference. For each ratio, the acetonitrile:water mixture is on the left and the methanol:water mixture is on the right. Note the lack of development of the control band (top band) and the test band (lower band) at high (1:1 and 3:2) acetonitrile:water ratios. No analyte was added to solution so that the test band would also be developed.

An additional observation of note is that any significant (>10 % v/v) level of acetonitrile in the solvent was found to negatively affect the performance of benzodiazepine test strips. The presence of acetonitrile at or above this level resulted in a negative test result even when a positive result should have been elicited. These initial observations highlight that a more in-depth study into the effect of organic solvents may be fruitful. Understanding potential complications from the use of organic solvents is necessary, especially if these solvents may be used to increase solubility of target analytes. Use of different acidic or basic conditions should also be explored.

Limit of Detection

Understanding the sensitivity of the test strips is useful for establishing the necessary amount of a drug powder needed to elicit a positive response and for determining whether test strips can be used for bulk and/or trace analysis. The approximate limit of detection was determined for all seven test strips studied (excluding fentanyl and the expired cocaine, fentanyl, and methamphetamine strips).

Approximate limits of detection for the test strips ranged from 0.1 µg/mL (benzodiazepine test strip, diazepam) to 2.5 µg/mL (cocaine test strips). An approximate limit of detection of 0.5 µg/mL was obtained for the amphetamine test strips and 1 µg/mL was obtained for the methamphetamine, nitazene (metonitazene), opiate (heroin), and xylazine test strips (Supplemental Table 2).

Since the benzodiazepine test strips reacted for multiple benzodiazepines in the test panel, the limit of detection was approximated for alprazolam, bromazolam, and flubromazepam. The approximate limit of detection of alprazolam was 0.1 µg/mL and was 0.25 µg/mL for bromazolam and flubromazepam. Similarly, the limit of detection was approximated for N-piperidinyl etonitazene and protonitazene using the nitazene test strips, and found to be 5 µg/mL.

The high sensitivity of test strips is not unexpected, given they are designed for toxicological testing. In a harm reduction or law enforcement drug checking scenario, this level of sensitivity has its strengths and weaknesses. Given that standard practice for using fentanyl test strips in a drug checking scenario involves dissolving a few milligrams of powder into one or two milliliters of water[6], single to sub-µg/mL detection limits means that low levels of a drug (<1 %) in a mixture should be readily detected. The low-level sensitivity also means that visible amounts of powder are not necessarily needed to elicit a positive test result, and collection of a residue could represent a sufficient amount of material for testing – something that has been demonstrated in prior work[15].

The high sensitivity of these tests also means that trace level contamination of a powder may be sufficient to produce a positive result. If a container (*i.e.*, a pill bottle) that contains a powder was previously used to store pills or different types of powders, the trace residue left behind from the initial material could mix in and create a misleading, positive result. For perspective, it has been reported that the typical U.S. banknote contains 28.75 µg of cocaine[20] – roughly ten times more than the approximate limit of detection for the cocaine test strip, assuming 1 mL of water is used to create a solution.

Reproducibility

The reproducibility of the test strips was studied (for all except fentanyl and expired cocaine, fentanyl, and methamphetamine) to see if they consistently produced expected positive and negative results over a one-month period using multiple tests on the same solution. All test strip types were found to produce positive results for the positive controls and negative results for the negative controls over the period studied. At the end of the one-month study, the test strips also produced positive results at their approximate limit of detection, indicating no significant loss in sensitivity over this time frame.

Cross-Reactivity

Immunoassays have been found to be susceptible to cross-reactivity with non-target analytes due to structural similarities between compounds. Cross-reactivity of fentanyl test strips have been studied in the past – where certain fentanyl analogs[7–10], methamphetamine, and diphenhydramine[6,9] have been shown to produce positive results. Certain xylazine test strips have also demonstrated cross-reactivity with lidocaine[15,16]. An important consideration when examining cross-reactivity is that there may be a concentration dependence (*i.e.*, cross-reactivity may only be observed at concentrations significantly higher than the limit of detection of the target analyte). Because of this, we examined cross-reactivity at two concentrations – High (0.5 mg/mL or 1 mg/mL) and Low (0.01 mg/mL). The High concentration was examined to see if a positive result would be produced when a compound is a major component while the Low concentration represented an instance where a compound would be a minor component in a drug mixture or a contaminant. It should be noted that the differences in High concentrations were due to solubility limitations for certain compounds.

Cross-reactivity was studied for a suite of 79 analytes (Table 2), chosen to represent common drugs, cutting agents, diluents, and other compounds found in illicit drug samples. The list is not exhaustive, and there are likely additional compounds that should be included based on the drug landscape in a particular geographical region.

Cross-reactivity from at least one analyte in the panel was observed for all test strip types except xylazine (Table 3). Amphetamine test strips reacted with other amphetamines including 3,4-MDA, 3,4-MDMA, methamphetamine, and phentermine. 3,4-MDMA and methamphetamine only produced a positive result at the High concentration. The High concentration procaine solution elicited a faint negative result. Similar cross-reactivities were observed for the methamphetamine test strips, where 3,4-MDMA (both concentrations) and amphetamine (High concentration) elicited positive results. High concentrations of phenylephrine were also shown to cross-react.

The benzodiazepine test strips produced a positive result for four of the five benzodiazepines investigated – diazepam (target analyte), alprazolam, bromazolam, and flubromazepam (the first two are prescription benzodiazepines while the latter two are not). As discussed previously, however, the benzodiazepine test strips were shown to be negatively affected by the presence of organic solvents in a solution – resulting in negative results for the High concentration solutions of these compounds, but positive results for the Low concentration (where the solution was significantly diluted with water). Interestingly, etizolam did not produce a positive result at either concentration, possibly due to the presence of the thiazole ring. It is, technically, a thienodiazepine though it has a high affinity for the benzodiazepine site of GABA receptors[21]. This is important to note because etizolam is observed in seized drug samples submitted to forensic laboratories[22]. Several other etizolam concentration levels between 0.01 mg/mL and 0.5 mg/mL were analyzed to see if a positive result could be obtained, but these efforts were unsuccessful. Several other compounds – 3,4-MDMA, amphetamine, and metamizole – produced faint negative (barely visible test band) results at the High concentration.

Cocaine test strips had an expected cross-reactivity with benzoylecgonine, a structurally similar impurity of cocaine[23]. High concentration solutions of 3,4-MDA and N,N-dimethylpentylone were also shown to be cross-reactive while eutylone and nicotine produced faint negative results.

Nitazene test strips produced positive results for all three nitazenes in the panel of compounds tested (N-piperidinyl etonitazene and protonitazene in addition to metonitazene).

For opiate test strips, opiates beyond heroin were shown to elicit positive results, including 6-monoacetylmorphine (a heroin impurity), codeine, and oxycodone (High concentration only). High concentrations of ibuprofen and phenacetin were shown to produce faint negative results.

Xylazine test strips were not shown to cross-react with any of the other analytes examined. For these strips specifically, a suite of an additional set of 14 analytes (Supplemental Table 3) were tested to provide complementary cross-reactivity results to previous work[15]. Of the additional analytes, four α_2-agonists or structurally similar compounds – brimonidine, clonidine, romifidine, and tizanidine – were found to cross-react at High concentrations.

Understanding cross-reactivities is critical to establishing limitations of test strips for inferring what is in a drug sample and identifying what combination(s) of test strips could answer the question at hand. For instance, fentanyl test strips have been previously shown to cross-react in the presence of high levels of methamphetamine[6,9]. Because the methamphetamine test strip was not shown to cross-react to fentanyl, it might be feasible to use the combination of these two strips to increase the confidence in a fentanyl identification; specifically in the instance of x, testing with y and z, would suggest the situation is a. For example, knowing that a cocaine sample tested positive on a fentanyl test strip but tested negative on a methamphetamine test strip would increase the confidence that the fentanyl detection was a true positive. As cross-reactivities of different test strip types continue to be understood, there is potential for the development of best practices for test strip use.

Table 3. Instances where a positive or faint negative result was obtained when analyzing the cross-reactivity analytes (excluding the analyte used as a positive control). An asterisk (*) indicates that only the High concentration solution of that analyte elicited the noted result. A dagger (†) indicates that only the Low concentration solution of that analyte elicited the noted result.

Test Strip Type	Cross-Reactive Analyte(s)
Amphetamine	*Positive:* 3,4-MDA, 3,4-MDMA*, Methamphetamine*, Phentermine *Faint Negative:* Procaine*
Benzodiazepine	*Positive:* Alprazolam, Bromazolam, Flubromazepam *Faint Negative:* 3,4-MDMA*, Amphetamine*, Metamizole*
Cocaine	*Positive:* 3,4-MDA*, Benzoylecgonine, N,N-Dimethylpentylone* *Faint Negative:* Eutylone*, Nicotine*
Methamphetamine	*Positive:* 3,4-MDMA, Amphetamine*, Phenylephrine* *Faint Negative:* None
Nitazene	*Positive:* N-Piperidinyl Etonitazene, Protonitazene *Faint Negative:* None
Opiate	*Positive:* Codeine, 6-Monoacetylmorphine, Oxycodone* *Faint Negative:* Ibuprofen*, Phenacetin*
Xylazine	*Positive:* None (see Supplemental Table 3 for cross-reactivities beyond those in the main panel) *Faint Negative:* None

Effect of Elevated Temperature

In a harm reduction, law enforcement, or customs setting, test strips may be handed out to individuals who may maintain possession of them for some time before use or test strips maybe

be stored in vans or patrol vehicles for prolonged periods of time. In these situations, it is possible that test strips would be exposed to elevated temperatures for an extended period, which could alter the efficacy of these tests.

To better understand the effects of elevated temperatures on test strips for all analyte types, unopened test strips were stored in a 55 ºC oven for two weeks prior to being used. Strips were then tested using the positive control (Table 1) and negative control (pure water) to determine if they still elicited the desired responses. The strips were also tested using a solution of the desired analyte at the approximate limit of detection, and increasingly more concentrated solutions if needed, to determine if there was any loss in sensitivity due to the strips being heated. It should be noted that unlike previous work investigating the effect of temperature by Hauck *et al*.[18], test strips were only stored at elevated temperatures, the test strips were not unpackaged and used at elevated temperatures.

After storage at elevated temperatures, all test strips produced clear positive results for their positive controls. All test strips also produced clear negative results for pure water, indicating that the strips were still working properly (Supplemental Table 4). Apart from the xylazine test strips, all test strips produced clear positive results with solutions corresponding to their approximate limits of detection, indicating little to no loss in sensitivity. Heated xylazine test strips produced faint negative responses at the approximate limit of detection, 1 µg/mL, and were found to require a xylazine concentration of 10 µg/mL to consistently elicit a positive response. Given the high sensitivity of these tests, and the format of sample preparation used for drug checking, this minor loss of sensitivity would likely not decrease the ability to detect xylazine in a drug sample. Nearly all drug samples that have been quantitated to date contain greater than 1 % by weight xylazine[24], equating to a solution concentration in excess of 10 µg/mL (assuming greater than 1 mg of powder is dissolved in greater than 1 mL of water).

In addition to understanding changes in sensitivity, changes in cross-reactivity were also studied. All analytes that elicited a positive or faint negative response in the cross-reactivity study were reanalyzed using heated strips. For instances where only the High concentration of analyte produced a positive or faint negative response in the cross-reactivity study, only the High concentration level was examined here. If both the High and Low concentrations of an analyte produced a positive response, the Low concentration level was examined.

All analyte / test strip combinations that produced a positive result in the cross-reactivity study also elicited a positive result when heated test strips were used. Similarly, all analytes that originally produced a faint negative also produced a faint negative when analyzed using heated strips. It is important to note that this study only looked at whether positive cross-reactivity was maintained, it did not investigate whether prolonged heating caused new cross-reactivities.

Fentanyl test strips were also examined in this sub-study and were found to be unaffected by exposure to elevated temperatures with respective to sensitive (approximate limit of detection of 0.5 µg/mL obtained for both strips that were and were not exposed to higher temperatures). Cross-reactivity of fentanyl test strips was not studied.

Sensitivity Beyond Expiration

It is possible that test strip users may have test strips that are not used before their expiration date. To save resources, they may desire to still use the strips, making it important to understand whether sensitivity wanes beyond expiration. To test this, three different test strip types – cocaine, fentanyl, and methamphetamine – that were over four years expired were examined. Similar to the heated test strip study, the positive control (Table 1), negative control (pure water), and solution corresponding to the approximate limit of detection (or increasingly more concentrated solutions until a consistent positive was obtained) were analyzed, in triplicate. A subset of the expired tests was also exposed to elevated temperatures for two weeks before analysis.

The expired tests were found to produce clear positive results for the positive controls and clear negative results for the negative controls (Supplemental Table 5). The approximate limit of detection for expired cocaine test strip was the same as the non-expired test strips (2.5 µg/mL), even though the listed cutoff of the expired test strip was slightly higher than the non-expired test strip. Expired methamphetamine test strips were found to be slightly less sensitive than non-expired strips (5 µg/mL versus 1 µg/mL); however, concentration of this minor decrease in sensitivity would, generally, not affect the ability to detect methamphetamine in a sample, as it is often very high and so should be readily detectable when present at clinically relevant levels. As with the non-expired test strips, prolonged elevated temperatures were not found to impact sensitivity. For this sub-study expired fentanyl test strips were also examined and found to have an approximate limit of detection higher than the reported value (0.25 µg/mL identified versus 0.02 µg/mL). However, like methamphetamine, this decreased sensitivity would still not preclude detection of samples with fentanyl present at 1 % by weight. Elevated temperatures were found to affect the sensitivity of the expired test strips.

The ability for expired tests to still react to target analytes multiple years after expiration presents the opportunity for organizations to be able to continue to use expired tests and save on resources. However, it should be noted that these tests were stored in normal laboratory conditions up until this study and were not subject to harsh environmental conditions for prolonged periods. The development of a quick and easy method to validate that the expired tests are still working, and working with appropriate sensitivity, should be explored to provide confidence in use of expired tests before distributing.

Conclusions

The results of these studies demonstrate that test strips beyond the commonly employed fentanyl test strips may be viable tools for drug checking in law enforcement, public health, forensics, or customs scenarios provided the limitations presented by cross-reactivity are well documented. Test strips, regardless of analyte, were found to be reproducible, sensitive, and unaffected by storage in elevated temperatures for short periods of time. Cross-reactivities remain a concern and present limitations when specific compound identification is required. Barring a few exceptions, cross-reactivity was largely limited to compounds of a similar class to the analyte of interest, which could provide utility in some situations. The use of expired tests to obtain consistent results also seems generally appropriate, though the sample set studied was limited and tests well beyond their expiration date were not studied.

There are several limitations of this study that should be explicitly stated. First, the use of organic solvents to induce dissolution of compounds may have an impact for some results. The use of

organic solvents in the field is not currently commonplace, and therefore should not present a challenge, but the deleterious effect of acetonitrile on the benzodiazepine test strips is noteworthy. Second, the panel used for cross-reactivity is not comprehensive. While efforts were made to ensure a diverse group of analytes was studied, the list of compounds is by no means complete and additional cross-reactive compounds very well may exist. Third, the study did not investigate real world samples or mixtures to identify potential issues that may arise from analyzing complex samples. Prior work on fentanyl and xylazine test strips that has utilized real world samples or mixtures have not uncovered any known issues for immunoassay tests, but that does not mean none exist. Finally, the elevated temperature study only spanned two weeks. Additional studies looking at longer timepoints would likely be useful, especially for applications where test strips might be left in a vehicle for prolonged periods of times.

Perhaps most importantly, this work, and the existing body of research on fentanyl and xylazine test strips, highlights the need for a standard process to investigate and evaluate immunoassay test strips for drug checking applications. Given the wide range of test strip types available – different manufacturers, analytes, antibodies, and cutoff values – it can be difficult for end users to understand which ones will be best suited for their application. Having a standard practice to objectively compare test strips will enable informed decision making and help drive toward best practices for in-field use. Given that cross-reactivity will always be a concern, it is likely inevitable that a combination of test strips will be needed to provide accurate results, coupled with additional important information provided by more advanced chemical analysis completed on or off site. Having a defined way to objectively identify the limitations of different test strips would be critical to determining the best combination of test strips for use.

Given the relatively inexpensive cost of immunoassay test strips compared to portable spectrometer[25] and the speed of result availability compared to laboratory-based methods, the finding that test strips originally designed for urine analysis generally work for identification of drug products has important implications for resource-constrained drug checking. Moreover, these attributes may make qualitative, test strip-based drug checking scalable in a way that more intensive methods have not yet been. Notably, several novel applications could arise from this work. For example, harm reduction or community health organizations may include other test strips to meet client needs for take-home qualitative drug checking kits that can complement existing fentanyl test strip distribution without introducing additional complications. Events or festivals could offer more efficient point-of-service drug checking to large numbers of people. Strips could facilitate presumptive identification of illicit drugs other than fentanyl in a customs or forensic setting. Future studies using real-world samples can further inform these and other applications across disciplines.

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Supplemental Information for:

Beyond Fentanyl Test Strips: Investigating Other Urine Drug Test Strips for Drug Checking Applications

Supplemental Table 1 – Excel "Cross Reactivity (SI1)". Results for the cross-reactivity portion of the study showing the analytes tested, the concentration of the analyte tested, and the amount of acetonitrile, if any, in the solution. All "Low" levels were 10 µg/mL. "P" indicates a positive result was obtained, "N" indicates a negative result was obtained, "FN" indicates a faint negative result was obtained, and "N(A)" indicates a result where the presence of a high amount of acetonitrile in the solution was believed to have impacted the result.

Supplemental Table 2 – Excel "Target LOD (SI2)". Results for the approximate limit of detection study. Greyed out cells indicate that concentration was not tested for the given test strip type. "P" indicates a positive result was obtained, "N" indicates a negative result was obtained, and "FN" indicates a faint negative result was obtained. The cells outlined in bolded yellow indicate the approximate limit of detection determined for the test strip type.

Supplemental Table 3 – Excel "XTS Additions (SI3)". Results for the additional cross-reactivity studies completed for only the xylazine test strips. "P" indicates a positive result was obtained, "N" indicates a negative result was obtained, and "FN" indicates a faint negative result was obtained.

Supplemental Table 4 – Excel "Elevated Temperature (SI4)". Results for the exposure to elevated temperatures study. Greyed out cells indicate that concentration was not tested for the given test strip type. "P" indicates a positive result was obtained, "N" indicates a negative result was obtained, and "FN" indicates a faint negative result was obtained. The cells outlined in bolded yellow indicate the approximate limit of detection determined for the test strip type.

Supplemental Table 5 – Excel "Expired Test Strips (SI5)". Results for the expired test strip study, with expired test strips that were also exposure to elevated temperature shown on the right-hand side (listed as "-Heated"). Greyed out cells indicate that concentration was not tested for the given test strip type. "P" indicates a positive result was obtained, "N" indicates a negative result was obtained, and "FN" indicates a faint negative result was obtained. The cells outlined in bolded yellow indicate the approximate limit of detection determined for the test strip type.