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Nitazene test strips: a laboratory evaluation

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Abstract

Background 2-Benzylbenzimidazole 'nitazene' opioids pose a growing threat to public health. Nitazene analogues are increasingly found mixed with or (mis)sold as heroin and in falsified (non-)opioid medications, posing a great risk of intoxication in users (un)knowingly exposed to these potent opioids. Lateral flow immunoassay nitazene test strips (NTS; BTNX Rapid Response™) became commercially available in Q1 2024, with the aim to enable rapid detection of nitazene analogues in drug samples. As only limited independent data is available on the performance of these strips, this lab-based study aimed at evaluating their potential for drug checking applications.

Methods Following dilution of drug standards in water, the NTS readouts were analyzed independently by two individuals and by ImageJ. The limit of detection for isotonitazene was determined using two manufacturing lots of NTS. Cross-reactivity with 32 other nitazene analogues was evaluated. Six sourced drug samples were tested to explore the ability of NTS to detect the presence of a nitazene analogue in authentic samples.

Results The limits of detection for isotonitazene were 2000 or 3000 ng/mL, depending on the lot. Twenty-four of the 33 tested nitazene analogues cross-reacted with the NTS at concentrations ≤ 9000 ng/mL. Structural analysis indicated that either substitution or removal of the 5-nitro group, or lengthening the linker between the two aromatic rings, generally hampered detection. All six authentic drug samples consistently tested positive, with no observed false negatives.

Conclusions This study provides a better understanding of the potential of NTS for drug checking purposes. Our findings indicate that NTS can theoretically alert to the presence of most nitazene analogues that have emerged on recreational drug markets. However, 'desnitazenes' (lacking the 5-nitro group) may yield false negative results due to low cross-reactivity. Although factors like specificity, lot-to-lot variability, nitazene analogue content in drug samples, solubility, and different testing conditions should be considered, our study results indicate that, at least under the conditions evaluated here (using reference standards and sourced powders), NTS are capable of detecting the presence of a wide range of nitazene analogues. Hence, NTS may alert users of the presence of nitazene analogues in drug samples.

Keywords New synthetic opioids (NSO), 2-Benzylbenzimidazole 'nitazene' opioids, Harm reduction, Drug checking, Immunoassay test strips

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Introduction

In recent years, new synthetic opioids (NSO) have become one of the fastest growing groups of new psychoactive substances [1, 2]. In response to legislative measures targeting fentanyl analogues [3–5], opioids belonging to the 2-benzylbenzimidazole 'nitazene' class have emerged on recreational drug markets worldwide and have become the predominant class of non-fentanyl derived NSO in most markets [6–9]. Since the first detection of isotonitazene in 2019 [10, 11], numerous other analogues have emerged, with a total of 18 different nitazene analogues having been notified to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA, now European Union Drugs Agency (EUDA)) Early Warning System (EWS) between Q3 2019 and Q2 2024. Nitazene opioids generally display high levels of opioid activity, with potencies and efficacies often exceeding that of fentanyl [7, 12, 13]. Etonitazene, for example, the prototypical nitazene drug and one of the most potent analogues, was found to be at least 10 times more potent than fentanyl in vivo [14, 15]. The high harm potential of nitazene analogues, combined with the increasing number of reports of severe intoxication and death cases involving these drugs worldwide [13, 16–19], indicates a growing threat to public health and security [20].

While initially, nitazene-associated intoxications were primarily related to individuals purchasing these substances online, nitazene analogues have now clearly entered the 'street level'. Particularly worrying is that nitazene analogue-adulterated street drug samples are increasingly being detected, with nitazene analogues found to be mixed with heroin [19–21] or present as ingredients in other opioid (e.g., oxycodone, buprenorphine, fentanyl) drugs [20, 22, 23], as well as non-opioid (e.g., benzodiazepine, ecstasy, ketamine, cocaine, synthetic cannabinoids) drugs [24–29]. Individuals using nitazene analogue-adulterated drug samples are often unaware of the adulteration, placing them at a high risk of unintentional overdose. A recent report from the United Kingdom exemplifies this danger, as 19 drug users were hospitalized with severe opioid intoxication, each unaware that they had taken a nitazene opioid [17]. Moreover, in January 2024, three individuals in Sydney, Australia, were hospitalized after taking tablets that were sold to contain 3,4-methylenedioxymethamphetamine (MDMA) but actually contained a nitazene analogue instead [26]. This emphasizes that opioid-naïve persons who use drugs (PWUD), such as stimulant users, are particularly at risk, given their lack of tolerance for opioids and since they are not likely to carry the antidote naloxone.

The entry of nitazene analogues at the street level and their presence in a variety of drug preparations highlight that different strategies, such as harm reduction approaches, are needed to mitigate the harm of nitazene analogues in PWUD. While strategies like overdose prevention education and an increased availability of the opioid antagonist naloxone, which can rapidly reverse an opioid overdose, are crucial [30], also approaches involving the use of immunoassay test strips have been put forward. In recent years, fentanyl test strips (FTS) have been successfully deployed as a low-cost, easy-to-use drug checking tool [31]. Various studies have shown that FTS are deemed as acceptable harm reduction and overdose-prevention tools, associated with positive changes in drug use behavior among PWUD [32–37]. BTNX Inc., a Canadian biotechnology company and the manufacturer of the most widely available brand of FTS [38, 39], has recently developed lateral flow immunoassay nitazene test strips (NTS), which became commercially available in Q1 2024. These strips, which are based on a competitive principle (i.e., the absence of a line indicates positivity), aim at allowing users to rapidly determine whether or not a sample contains a nitazene opioid.

Only limited information on the performance of NTS is provided by the manufacturer, particularly regarding their cross-reactivity with different nitazene analogues. BTNX Inc. states that these strips are calibrated using isotonitazene, with a cut-off of 2000 ng/mL, and are able to detect 3 other analogues (etonitazene, protonitazene, and *N*-pyrrolidino etonitazene; listed cut-offs range from 1300 ng/mL to 4500 ng/mL), but are unable to detect 2 different analogues (metodesnitazene and etodesnitazene) at a concentration of 100 µg/mL [40, 41]. Regarding specificity, the manufacturer states that the NTS do not cross-react with various common cutting agents (e.g., acetaminophen, caffeine, diphenhydramine), other opioid (e.g., heroin, methadone, fentanyl), and non-opioid drugs (e.g., xylazine, MDMA, cocaine, ketamine) at concentrations of 100 µg/mL or higher [40, 41]. Several preliminary third-party assessments of BTNX NTS have already been conducted. The Center for Forensic Science Research and Education (CFSRE) screened a series of nitazene analogues at a concentration of 3000 ng/mL and found that 24 of the 29 assessed nitazene analogues cross-reacted [41]. Notably, metodesnitazene and etodesnitazene could be detected, a finding that is in apparent contradiction with that of the brand's report (cfr. *supra*). Also the Chicago Recovery Alliance evaluated NTS performance, using real-world street drug samples with varying nitazene analogue contents. Preliminary data from 12 samples showed that the strips achieved a sensitivity of 100% and 89%, when using dilution factors of 10 mg per 1 mL or 10 mg per 5 mL, respectively, and a specificity of 100% [41]. Last, Sisco et al. [42] evaluated the limit of detection (LOD, i.e., the minimum concentration that consistently yielded a positive result) of the NTS for metonitazene,

N-piperidinyl etonitazene, and protonitazene and found that their respective LODs were 1000, 5000, and 5000 ng/mL. Nevertheless, more information on the cross-reactivity with other nitazene analogues and the overall performance of these NTS is needed to allow a better insight into the potential of these NTS for drug checking purposes. The current study is the first to independently assess, in a laboratory context and using drug standards, the potential of the first commercially available BTNX Rapid Response™ NTS for drug checking purposes. Aspects that were evaluated include lot-to-lot variability, cross-reactivity with other nitazene analogues, and the ability of the NTS to detect the presence of a nitazene analogue in authentic drug samples.

Materials and methods

Materials

The reference standards for nitazene citrate (**1**), clonitazene (**2**), 4'-hydroxy nitazene (**3**), metonitazene (**4**), etonitazene (**5**), protonitazene hydrochloride (**6**), isotonitazene (**7**), butonitazene (**8**), *N*-pyrrolidino 4'-hydroxy nitazene citrate (**9**), *N*-pyrrolidino metonitazene citrate (**10**), *N*-pyrrolidino etonitazene (**11**), *N*-pyrrolidino protonitazene (**12**), *N*-pyrrolidino isotonitazene citrate (**13**), *N*-piperidinyl 4'-hydroxy nitazene citrate (**14**), *N*-piperidinyl metonitazene citrate (**15**), *N*-piperidinyl etonitazene citrate (**16**), *N*-piperidinyl protonitazene citrate (**17**), *N*-piperidinyl isotonitazene citrate (**18**), metodesnitazene hydrochloride (**19**), etodesnitazene citrate (**20**), protodesnitazene citrate (**21**), isotodesnitazene citrate (**22**), *N*-pyrrolidino metodesnitazene citrate (**23**), *N*-pyrrolidino etodesnitazene citrate (**24**), 5-methyl etodesnitazene citrate (**25**), 5-aminoisotonitazene (**26**), *N*-desethyl metonitazene hydrochloride (**27**), *N*-desethyl etonitazene (**28**), *N*-desethyl protonitazene hydrochloride (**29**), *N*-desethyl isotonitazene hydrochloride (**30**), ethylene nitazene citrate (**31**), ethylene etonitazene citrate (**32**), ethyleneoxynitazene citrate (**33**), and brorphine hydrochloride were kindly provided by Cayman Chemical (Ann Arbor, MI, United States). Methanol (MeOH), dimethylsulfoxide (DMSO), and acetonitrile (ACN) were purchased from Chem-Lab NV (Zedelgem, Belgium), Merck KGaA (Darmstadt, Germany), and Biosolve (Valkenswaard, Netherlands), respectively. Ultra-high purity water (18.2 $M\Omega cm^{-1}$) was obtained from a Milli-Q Eq 7000 water purification system (Millipore SAS, Molsheim, France). All reference standards were prepared as 1 mg/mL stock solutions in MeOH, ACN, DMSO or MeOH/DMSO. BTNX Rapid Response™ NTS were procured from Exchange Supplies (Dorchester, United Kingdom). Sourced powders containing isotonitazene, metonitazene, protonitazene, butonitazene, or *N*-pyrrolidino etonitazene were obtained in the context of prior routine drug market monitoring by the Belgian Early Warning System on Drugs (BEWSD), part of Sciensano (the Belgian Institute for Public Health) [10, 43].

Methods

Nitazene test strip analysis

Two different manufacturing lots of BTNX Rapid Response™ NTS (lot A: DOAB23120001 and lot B: DOAB24010002) were used in this study. Both lots were employed for LOD experiments to assess lot-to-lot variability in test strip performance. Lot B was used for cross-reactivity experiments and for testing of the sourced drug samples. The test strips were used according to the manufacturer's instructions, as specified in the product insert [40]. Each strip was removed from its sealed pouch and immediately immersed in the solution of interest (cfr. *infra*) for 15 s, making sure that the liquid did not exceed the maximum water line marked on the strip. Next, the strip was placed horizontally on a non-absorbent flat surface to develop. Test strip results were photographed with a smartphone after 5 and 10 min, using a dedicated set-up to ensure consistent picture quality (Supplementary Information S1). As these NTS are competitive lateral flow immunoassays, a negative result is indicated by the presence of a line in both the control and test areas, whereas the absence of a line in the test area (with a line being present in the control area) indicates a positive result (i.e., a nitazene analogue is present in the sample). Strips that produce no lines or only a line in the test area are to be interpreted as invalid tests. Additionally, the manufacturer's instructions state that the intensity of colour in the test region can vary, hence any shade of colour in the test area should be interpreted as a negative result [40].

All images were visually assessed by two independent raters (L.D.V. and M.V.) who were blinded to the identity of the samples and scored the readout as '+', '-', or '?' (Fig. 1). A '+' score indicates that the raters were confident that the readout was positive (Fig. 1g), while a '-' score indicates that the raters were confident that the readout was negative (Fig. 1a-e). A '?' score reflects a lack of confidence by at least one of the raters in assessing the test result (Fig. 1f). No discrepancies (i.e., one rater assigning a '+' while the other assigned a '-' score or vice versa) were found between the raters' scores.

In addition to the visual assessment and to objectify the results, the test strip result images were evaluated using ImageJ, a free open-source image analysis program from the National Institute of Health (NIH, Bethesda, MD, USA). The ImageJ data analysis method was developed to allow an alternative and objective (non human eye-based) scoring of the readouts. The methodology of the ImageJ analysis method was optimized based on a preliminary experiment with NTS lot A, where isotonitazene (7) was tested at 4 concentrations of 500, 1000, 2000, and

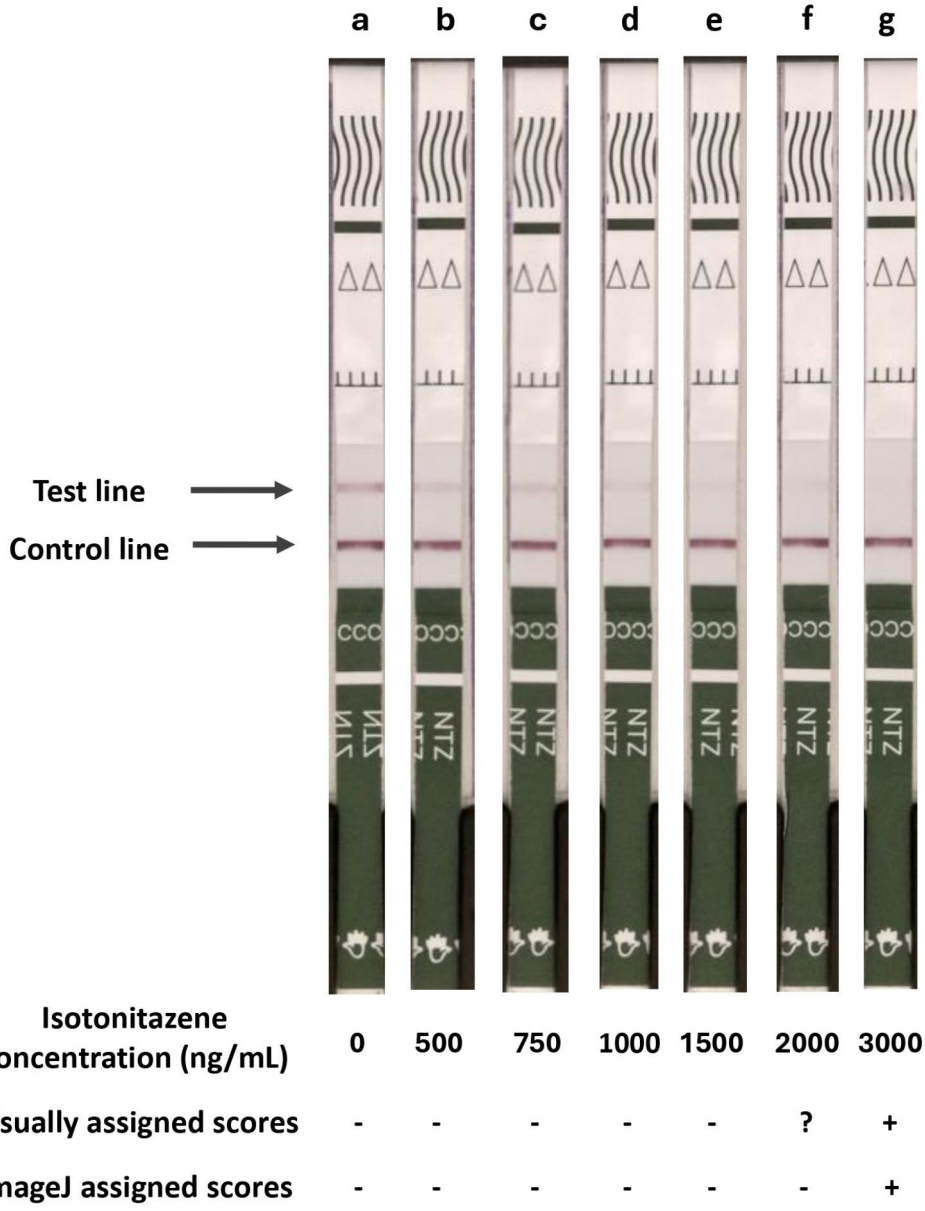

Fig. 1 Example of nitazene test strip images obtained from the limit of detection experiments, along with their visually assigned scores by two independent raters and their ImageJ scores. If both raters were confident that a test line was present or absent, the test was scored as negative ('-') (**a-e**) or positive ('+') (**g**), respectively. The test was indeterminate ('?') (**f**) if at least one of the raters had a lack of confidence in assessing the readout. The images depict one representative readout for each tested concentration of isotonitazene from NTS lot A, selected from a total of 6 replicate measurements conducted on three different days, with duplicates run each day (Fig. 2)

4000 ng/mL, along with a suitable negative control (1.8% MeOH in Milli-Q water). Also with ImageJ, the readouts were scored either '+', '-', or '?' (detailed information on the exact methodology can be found in the Supplementary Information S2).

Limit of detection

Solutions of isotonitazene (7), used as a reference, with concentrations of 500, 750, 1000, 1500, 2000, and 3000 ng/mL, were prepared in Milli-Q water, yielding 1.8% solutions of MeOH in water. A solution consisting of 1.8% MeOH in Milli-Q water (0 ng/mL isotonitazene) was prepared and included as a negative control. Each concentration was tested on three different days with both NTS lots, each in duplicate. In line with other reports [42, 44–46], this study defined the LOD as the lowest concentration at which all replicate measurements produced a positive readout. Note that the manufacturer only specifies a cut-off value for isotonitazene (2000 ng/mL) and reports that samples with nitazene analogue

concentrations at 50% and 200% of the cut-off were determined to be all negative or positive, respectively [40].

Cross-reactivity

To determine which nitazene analogues are detectable with the NTS, a panel of 32 different nitazene reference standards was evaluated alongside isotonitazene (**7**). Solutions of the test compounds at 3000 ng/mL, as well as at 9000 and/or 1000 ng/mL were prepared in Milli-Q water from the stock solutions, resulting in solutions of 1.8% solvent (MeOH, ACN, DMSO, or MeOH/DMSO) in water. Each compound was initially tested at 3000 ng/mL (corresponding to 150% of the cut-off for isotonitazene listed by BTNX Inc.) ($n=1$). Compounds that tested positive ('+') at 3000 ng/mL were subsequently tested at 1000 ng/mL, while those that tested negative ('-') at 3000 ng/mL were then tested at 9000 ng/mL. Compounds that gave at least one indeterminate ('?') or inconsistent result (i.e., a different score was obtained with the 5 and 10 min readouts) with either the visual or ImageJ assessment at 3000 ng/mL, were additionally tested at both 1000 and 9000 ng/mL. Every compound was tested once at each specified concentration and was therefore tested at least two times. All above-mentioned concentrations for the prepared solutions are expressed as free base concentrations. Appropriate negative controls were taken along (100% Milli-Q water and 1.8% MeOH, ACN, DMSO, or MeOH/DMSO in Milli-Q water) and were tested at least once.

Testing of authentic powder samples

To gain insight into the applicability of NTS to detect a nitazene analogue in authentic powders, 6 different drug samples were tested with the NTS. These samples contained either metonitazene, protonitazene, isotonitazene, butonitazene, or *N*-pyrrolidino etonitazene (for the latter, two independently sourced powders were available). Liquid chromatography coupled to time-of-flight mass spectrometry (LC-QTOF-MS) analysis of the powder samples was performed as previously described [47]. The 1/10 powder sample dilutions in tap water (obtained after testing with the NTS – cfr. *infra*) were diluted 100-fold in diluent (12.5% 50/50 ACN/MeOH in water) and 10 µL of the resulting 1 µg/mL solutions were injected. The LC-QTOF-MS analysis revealed that in all instances, the nitazene analogue was the major compound present in the sample, with an estimated purity of >90%. This purity estimate was defined as the ratio of the peak intensity of the nitazene analogue in question to the overall peak intensities of all substances detected in a given sample, using the HighResNPS library [48] with a cut-off set at 50 (data not shown). It should be noted that a limitation of this procedure is the difficulty in directly comparing the relative abundance of drugs within a sample due to the varying ionization efficiencies of each compound [49]. The CFSRE [40] and the New Zealand Drug Foundation [50] recommend testing a drug sample with the NTS by adding 5–10 mg of drug sample to 5 mL of water so that a 1–2 mg/mL solution is obtained, assuming complete dissolution of the sample. Following these recommendations, and to ensure that the employed testing methodology resembles real-world use of the NTS as much as possible, 1 mg of neat powder of every sample was weighed in a 50 mL glass container using an analytical balance and 1 mL of tap water was added volumetrically using a pipette. The mixtures were briefly (10 s) stirred with a clean spoon to facilitate dissolution and were then immediately tested with one test strip. Next, a secondary 10-fold dilution of the mixtures was performed. This involved adding 9 mL of tap water to each 1 mL mixture, a strategy previously employed for testing authentic samples with xylazine test strips by the CFSRE [51]. The diluted mixtures were once again briefly mixed and subsequently tested once with the NTS. A negative control consisting of 100% tap water was included.

Results

None of the NTS used in this study produced an invalid result, meaning there were no NTS in which the control line did not develop. All negative controls were consistently scored as negative with both the visual assessment and the ImageJ analysis. Notably, in all cases where a line appeared in the test region, it was visibly less bright than the control line (Fig. 1). Furthermore, fewer indeterminate results were obtained when evaluating the NTS at 10 min compared to 5 min (i.e., more results were scored as '?' with the 5 min readouts than with the 10 min readouts). This may be attributed to the NTS having more time to dry at 10 min than at 5 min, resulting in a more homogeneous white background and allowing a better distinction of the test line from the background. Hence, the results of the 10 min readouts are reported in the following sections. The results following a 5 min readout are provided in Supplementary Information S3.

Limit of detection

Six replicate measurements of six different concentrations of isotonitazene (**7**) (500 to 3000 ng/mL) were conducted with two lots of NTS to determine the LOD and to assess potential lot-to-lot variability. A representative series of images of the readout for each concentration, together with the visually and ImageJ assigned scores, is presented in Fig. 1. Figure 2 provides a summary of all results obtained with both lots of test strips, determined by either the visual assessment or the ImageJ analysis. Based on both the visual assessment and the ImageJ analysis of the strip readouts, the LOD for isotonitazene was

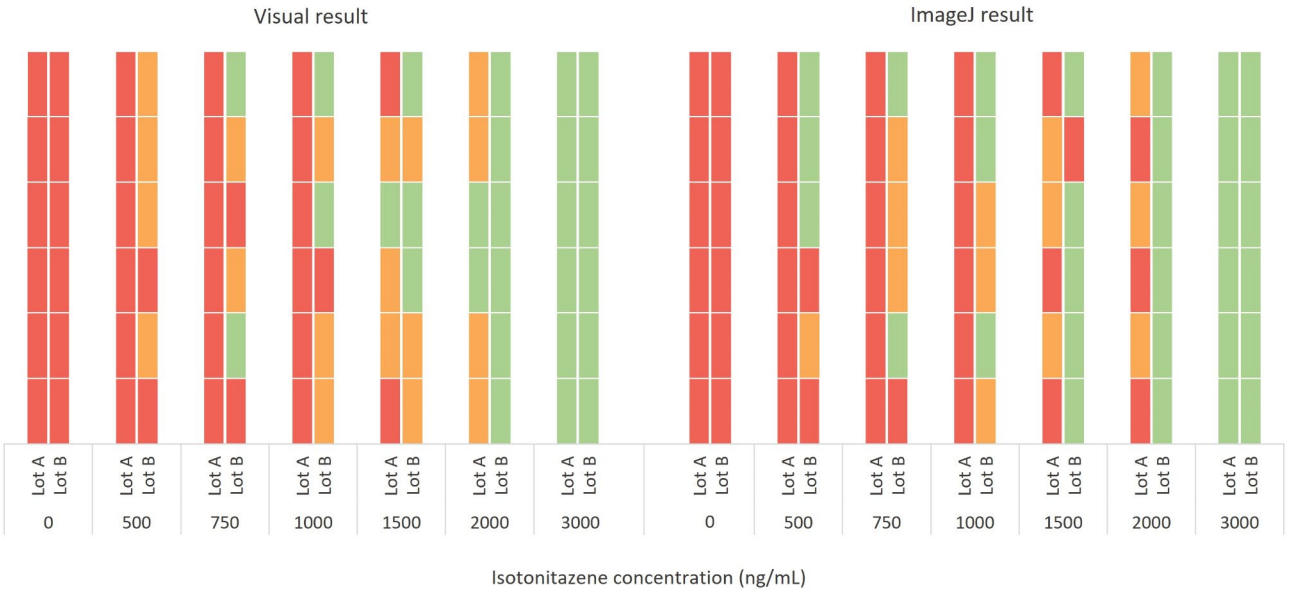

Fig. 2 Overview of limit of detection results for BTNX Rapid Response™ nitazene test strips for isotonitazene from two different manufacturing lots, as obtained by visual assessment (left) and ImageJ analysis (right) ($n = 6$). Negative results ('-') are indicated by a red rectangle, positive results ('+') by a green rectangle, and indeterminate results ('?') by an orange rectangle

determined to be 3000 ng/mL for lot A, and 2000 ng/mL for lot B.

Cross-reactivity

Cross-reactivity for the BTNX Rapid Response™ NTS beyond isotonitazene was evaluated for a series of 32 different nitazene analogues and the results are shown in Fig. 3. Irrespective of whether the readouts were visually scored or objectively analyzed with ImageJ, 24 nitazene analogues were detectable at or below 9000 ng/mL (compounds **1–18**, **25**, **27–30**, and **33**), whereas 9 nitazene analogues were not detectable at 9000 ng/mL (compounds **19–24**, **26**, and **31–32**). Based on the visual assessment of the NTS readouts, 16 of the 24 detectable nitazene analogues gave a positive result at both 1000 and 3000 ng/mL (compounds **1**, **3–7**, **9–11**, **13–15**, and **27–30**), and 7 of the 24 detectable nitazene analogues gave a positive result only at 3000 ng/mL (compounds **2**, **8**, **12**, **17–18**, **25**, and **33**). *N*-Piperidinyl etonitazene (**16**) gave a positive result only at 9000 ng/mL. Largely similar findings were obtained with the objective ImageJ analysis of the NTS readouts, with the main differences being that three compounds (**2**, **16**, and **33**) were scored positive with ImageJ at 1000 ng/mL, while they were rated as indeterminate at that concentration when assessed visually. Specifically, 19 of the 24 detectable nitazene analogues gave a positive readout at 1000 and 3000 ng/mL (compounds **1–7**, **9–11**, **13–16**, **27–30**, and **33**), and 5 of the 24 detectable nitazene analogues gave a positive readout at 3000 ng/mL (compounds **8**, **12**, **17–18**, and **25**). It should be noted that for *N*-pyrrolidino 4'-hydroxy nitazene (**9**) and *N*-piperidinyl etonitazene (**16**) a retesting was performed, as the initial testing yielded discrepant results with ImageJ (a negative result was obtained at 3000 ng/mL, while a positive result was obtained at 1000 ng/mL). This discrepancy was no longer present upon retesting, with ImageJ analysis resulting in a positive scoring of both substances at the 3 evaluated concentrations. The latter scoring was considered to make the above-mentioned categorization (Fig. 3). To verify the potential influence of using varying sample volumes, isotonitazene (**7**) and isotodesnitazene (**22**) were tested at a concentration of 3000 ng/mL using three different volumes of test solutions. These volumes corresponded to conditions where the test strips were immersed in test solutions at the minimum, middle and maximum water level markings on the strips. Isotonitazene and isotodesnitazene consistently yielded positive and negative results, respectively, indicating no influence on the results when varying the sample volume within the dedicated (i.e., marked) area. Brorphine, a benzimidazolone-containing NSO that is structurally somewhat similar to 2-benzylbenzimidazole nitazene opioids, showed no degree of cross-reactivity or interference at a concentration of 1 mg/mL (data not shown).

Figure 4 shows the structures of the evaluated nitazene analogues, alongside whether they were detectable at least at one concentration or remained undetectable across the different tested concentrations. A structural analysis of the tested nitazene analogues indicates that modifications at the 5-position of the benzimidazole ring or at the linker between the aromatic groups jeopardizes

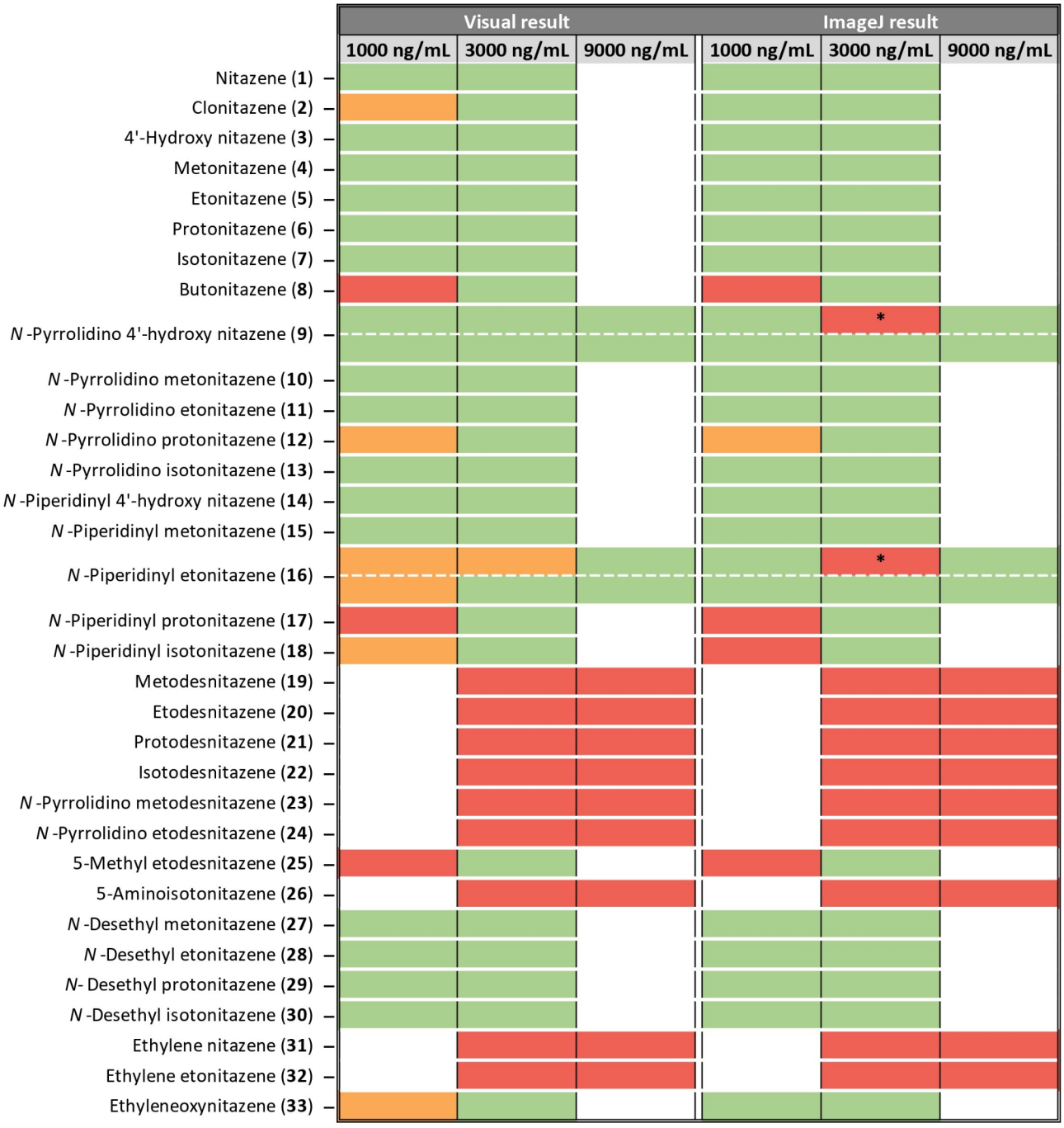

Fig. 3 Cross-reactivity of BTNX NTS for 33 screened nitazene analogues at 3000 ng/mL and at 1000 and/or 9000 ng/mL. A negative result ('-'), positive result ('+'), or indeterminate result ('?') is represented by a red, green, or orange rectangle, respectively. The asterisks denote that for *N*-pyrrolidino 4'-hydroxy nitazene (**9**) and for *N*-piperidinyl etonitazene (**16**) the results from two independent testings are shown, as the first experiment yielded a discrepant result: ImageJ indicated a negative result at 3000 ng/mL and a positive result at 1000 ng/mL. For both substances, the retesting did not yield this discrepancy

detection by the NTS (Fig. 4). Specifically, as can be observed with the ethylene nitazene (**31**) - ethylene etonitazene (**32**) couple, lengthening of the methylene bridge resulted in non-detection. Furthermore, removal of the 5-nitro group, which generates so-called 'desnitazenes' (compounds **19**–**24**), or substitution of the 5-nitro moiety for an amine (compound **26**), also resulted in compounds that could not be detected in our experimental set-up. Notably, as seen with 5-methyl etodesnitazene (**25**), the only tolerated modification at this position to

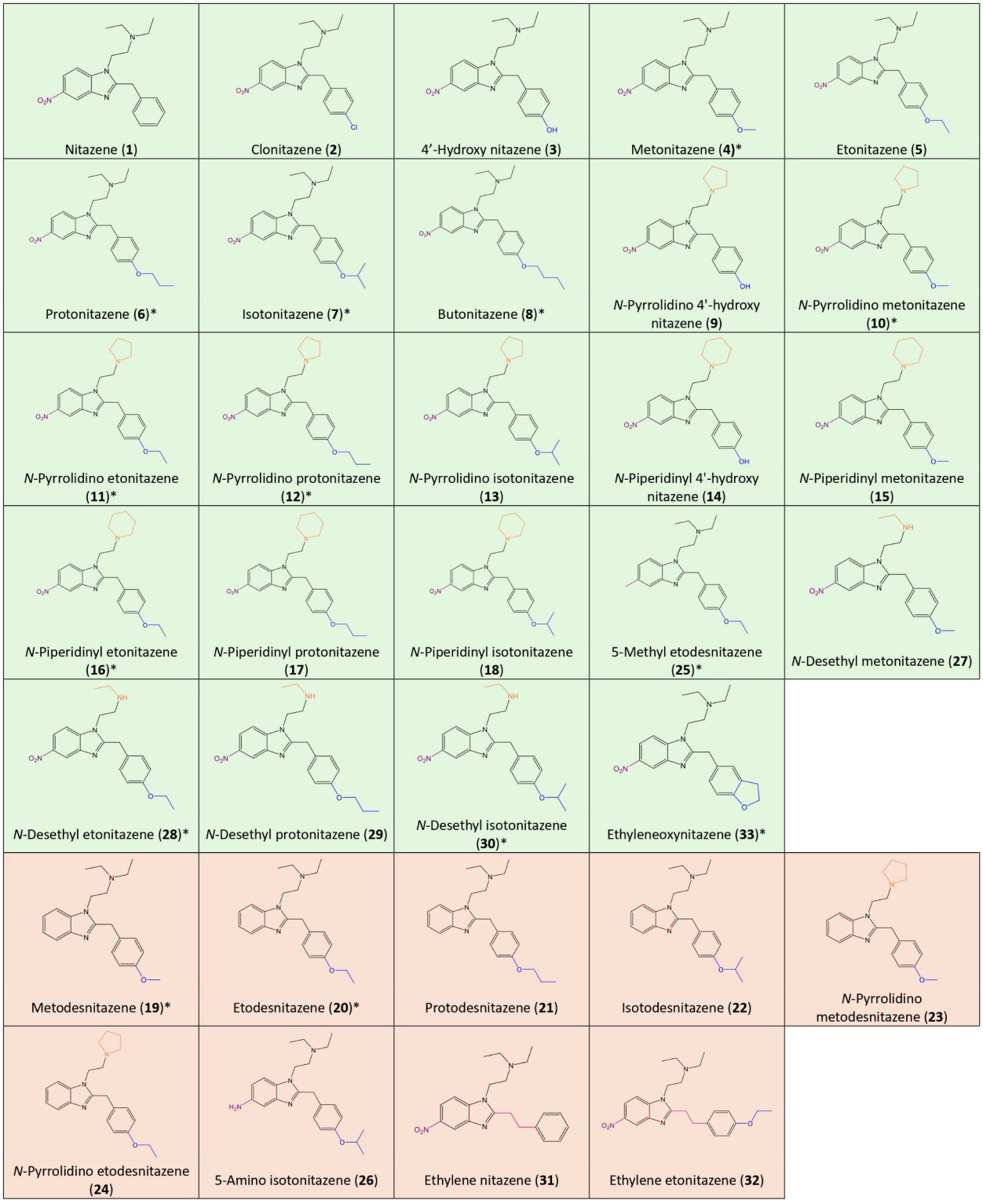

Fig. 4 Structures of the 33 evaluated nitazene analogues that were detected (green background) or not (red background) by the BTNX NTS, with the employed experimental set-up. The highlighted groups correspond to modifications that occur at four distinct positions of the 2-benzylbenzimidazole core structure: (**a**) the *para*-benzyl position (blue), (**b**) the 5-position of the benzimidazole ring (purple), (**c**) the substituted ethyl amino side chain linked to the benzimidazole ring (orange), and (**d**) the linker between the two aromatic rings (pink). * Tested nitazene analogues that have been identified on the recreational drug market in Europe between 2019 and 2023 (14 in total) (EUDA)

still result in detection was the substitution of the 5-nitro group for a methyl group. Modifications at the *para*-benzyl position or to the substituted amine side chain did not result in non-reactivity with BTNX NTS.

Testing of authentic powder samples

Six powder samples, containing either metonitazene (**4**), protonitazene (**6**), isotonitazene (**7**), butonitazene (**8**), or *N*-pyrrolidino etonitazene (**11**) (two samples), were tested with the NTS to get an understanding of the applicability of these test strips to test authentic powder samples. Prior to testing, we noticed that all obtained mixtures, including the secondary 1/10 dilutions, contained some undissolved powder. We did not attempt a full dissolution, as also in real-life circumstances a powder may not fully dissolve. Nevertheless, all readouts (both at 5 and 10 min) consistently yielded positive results, whether assessed visually or objectively with ImageJ. Hence, the BTNX NTS accurately identified the presence of a nitazene analogue in all tested authentic samples, with no observed false negatives.

Discussion

The increasing presence of 2-benzylbenzimidazole 'nitazene' opioids on the recreational drug market has resulted in numerous nitazene-associated cases of severe intoxication and death [13, 16–19], with their presence in drug samples often unknown to PWUD. Similar to the competitive lateral flow immunoassay test strips frequently used to detect fentanyl, BTNX Inc. has recently developed the first commercially available NTS. At the time of writing (Q3 2024), limited third-party evaluations of these NTS had been conducted [41, 42], providing some insight into the sensitivity (LOD), cross-reactivity with other nitazene analogues, and performance with authentic drug samples. This study aimed at further expanding this knowledge base and help determining whether these NTS have the potential to reliably assess the presence of a variety of nitazene analogues in a laboratory setting as well as in authentic drug preparations, the latter in the context of drug checking applications. Specifically, the LOD for isotonitazene for two manufacturing lots of test strips, cross-reactivity for 33 nitazene analogues, and the ability to detect a nitazene analogue in six real-world drug samples were investigated.

Evaluating the readout of test strips by visually assessing whether a line is present or absent is inherently subjective, providing a real-world limitation of the use of test strips. Since NTS are competitive lateral flow immunoassays, the interpretation of results is exactly opposite to that of many pregnancy test strips or e.g. COVID-19 antigen tests. Hence, without proper instruction, or upon misinterpretation of the instruction, the absence of a test line could be misinterpreted as a negative result – and vice versa. Additionally, the intensity of the test and control lines should not be compared, as test lines were consistently fainter than control lines. To decrease subjectivity in this study, all NTS readouts were visually and independently scored by 2 investigators and ambiguous results were scored as indeterminate ('?'). Furthermore, the images of the readouts were also analyzed with ImageJ to have an alternative and objective (i.e., not relying on the human eye) scoring of the test strip results. By scoring the obtained NTS readouts both visually and with ImageJ, we aimed to strengthen the validity of our findings.

Understanding the sensitivity (LOD) of the test strips allows to estimate the amount of (pure) drug powder required to trigger a positive result, thereby allowing to predict whether the test strips are not only suitable for bulk analysis but are also able to detect trace amounts [42]. Moreover, it is important to consider lot-to-lot variability of test strip sensitivity, as it has been previously shown for FTS that certain lots exhibited LODs up to 10 times higher than the manufacturer's specified cut-off, leading to a reduced performance of specific lots [39]. The experimental LOD for isotonitazene with NTS lot B (2000 ng/mL) aligned with the manufacturer's listed cut-off of 2000 ng/mL, whereas NTS lot A yielded a slightly higher LOD of 3000 ng/mL. Note that the manufacturer only specifies a cut-off for isotonitazene and does not report a LOD (cfr. Methods). While our findings show that both lots had largely similar reactivities for isotonitazene, it should be noted that both lots were obtained in a relatively short time span of approximately four months, making it less likely that major alterations to the manufacturing process would have occurred [52]. As previously recommended for FTS [39, 52], manufacturers of NTS should be transparent when implementing changes in their manufacturing processes to ensure that users and researchers are promptly notified about any potential impact on test strip sensitivity and/or cross-reactivity with other drugs.

A panel of 33 structurally distinct nitazene analogues (including isotonitazene) was evaluated to assess which nitazene analogues could be detected and to determine if certain structural modifications might preclude detectability with the NTS. All evaluated nitazene analogues display structural modifications that are confined to four specific regions of the 2-benzylbenzimidazole core structure: (a) the *para*-benzyl position; (b) the 5-position of the benzimidazole ring, typically carrying a nitro group; (c) the amine side chain attached to the benzimidazole ring, which typically contains a tertiary amine with an *N*,*N*-diethyl moiety; and (d) the linker connecting the two aromatic groups. Modifications may occur alone or concurrently with modifications at other positions of the 2-benzylbenzimidazole backbone. Structural analysis of

the tested nitazene analogues suggests that only modifications at the 5-position of the benzimidazole ring (b), or to the methylene linker between the aromatic groups (d), hampered detection by the NTS (Fig. 4). Specifically, all 'desnitazenes' (analogues lacking the 5-nitro group), except for 5-methyl etodesnitazene, and the two tested compounds with an ethylene linker (compounds **31**–**32**) could not be detected in this study. Modifications at the other two positions (a and c) did not result in non-reactivity with BTNX NTS. These findings are generally in line with the limited information available from the manufacturer [40, 41], the CFSRE [41], and Sisco et al. [42]. In our hands, the BTNX NTS were unable to detect metodesnitazene (**19**) and etodesnitazene (**20**) at 9000 ng/mL, which is in agreement with the manufacturer's report but does not align with the results of the CFSRE, who were able to detect both compounds at 3000 ng/mL [41]. The only other discrepancies between our findings and those from the CFSRE were with butonitazene (**8**) and 5-aminoisotonitazene (**26**). Butonitazene (**8**) was detected at 3000 ng/mL in this study, whereas the CFSRE reported it as undetectable at that concentration. Conversely, 5-aminoisotonitazene (**26**) could not be detected at 9000 ng/mL in this study, whereas the CFSRE reported detectability at 3000 ng/mL. These discrepancies might be attributed to e.g. differences in sample preparation methods and/or the specific NTS lot used.

Out of the 18 different nitazene analogues that have been identified on the recreational drug market in Europe between Q3 2019 and Q2 2024, 14 were evaluated in this study for their ability to cross-react with the NTS (Fig. 4.). Flunitazene, an analogue where the *para*-alkoxy tail is replaced by a fluorine halogen, was not tested since another halogenated analogue, clonitazene (**2**), was already included in the investigated panel. In line with our findings that substitutions at the *para*-benzyl position were tolerated, the CFSRE reported that flunitazene could be detected at 3000 ng/mL [41]. *N*,*N*-Dimethyl etonitazene, 6-methyl etodesnitazene, and fluetonitazene, three analogues that were reported to the European EWS around the time when this study was being conducted, were not tested since reference standards were not yet available at our laboratory. While for these nitazene analogues the degree of cross-reactivity is unknown at this point, it can be anticipated, based on our cross-reactivity data, that *N*,*N*-dimethyl etonitazene and fluetonitazene would likely cross-react with the NTS, since these analogues do not have structural modifications at positions found to hamper detection by the NTS. Although 6-methyl etodesnitazene is a positional isomer of 5-methyl etodesnitazene (**25**), the only 'desnitazene' analogue found to cross-react, testing with the NTS is required to make any statements on its potential detectability with the NTS. As the NPS market is highly dynamic and constantly evolving, other nitazene analogues are likely to emerge in the future. If future analogues display similar structural modifications to those that have been reported to the European EWS between Q3 2019 and Q2 2024 (with the exception of 'desnitazenes'), our data indicate that these would likely be detectable by the currently available NTS. However, our data suggest that this may not be the case for analogues with an extended linker between the two aromatic groups. Interestingly, one compound containing an ethylene bridge between the two aromatic groups, ethylene etonitazene, has already been identified by the Ohio Bureau of Criminal Investigation in early 2024 (personal communication). However, as current (Q3 2024) generic legislations attempting to cover nitazene analogues typically do not consider an ethylene bridge [53–56], it can be envisaged that more compounds with extended linkers may emerge in the future, aiming at evading these legislations. Despite some loss of opioid activity (compared to the methylene linker-containing compounds), some of these substances may still have a potency and efficacy comparable to or exceeding that of fentanyl (Vandeputte et al., manuscript submitted).

Understanding cross-reactivity with non-target analytes is crucial to determine the overall performance of test strips, as unwanted cross-reactivity leads to false positive results. For instance, it has been demonstrated that high concentrations (≥1 mg/mL) of MDMA, methamphetamine, levamisole, and diphenhydramine produce false positive results with FTS [39, 45, 57]. In addition, xylazine test strips have been previously shown to cross-react with lidocaine [46, 51]. The manufacturer readily reported non-cross-reactivity with non-nitazene compounds such as frequently used cutting agents (e.g., acetaminophen, caffeine, diphenhydramine), selected other opioids (e.g., heroin, methadone, fentanyl), and common non-opioid drugs (e.g., xylazine, MDMA, cocaine, ketamine) at concentrations of 100 µg/mL or higher [40, 41]. We did not reiterate these selectivity experiments, but did evaluate brorphine, a synthetic opioid that shows some structural similarity with the 2-benzylbenzimidazole scaffold found in nitazene analogues. At 1 mg/mL, brorphine did not cross-react. While currently no interferences with non-nitazene drugs have been observed or reported, additional cross-reacting compounds may exist, and it is warranted that more studies, assessing authentic street samples, look into this aspect. Testing on authentic street drug samples is also relevant to ensure that no false negatives (owing to compounds interfering with the test) occur.

Testing the NTS with six authentic drug samples consistently yielded a positive result. LC-QTOF-MS analysis confirmed that the nitazene analogues were the major compounds in these samples. Further in-depth analytical

characterization, such as nuclear magnetic resonance (NMR) analysis or identification of salt forms, was not conducted for this study. Previous chemical characterization of the isotonitazene [10] and of one the two *N*-pyrrolidino etonitazene [43] powder samples used here, indicated that these powders are pure and were probably sold undiluted. While our results related to assessing the real-life applicability of NTS for drug checking purposes are promising, LC-QTOF-MS analysis of the tested powders indicated that they are likely highly pure, which may not be representative of other real-world samples. Therefore, further testing on authentic mixtures is warranted. Based on the recommendation that 5 mL of water should be added to 5–10 mg of drug sample when the NTS are used for drug checking [40, 50], combined with our findings that most nitazene analogues are detectable in the low µg/mL concentration range, detection of a nitazene analogue down to a level of 0.9–0.1% by weight may be feasible. While this is lower than the 11% *N*-pyrrolidino protonitazene content (by weight) reported by Killoran et al. [19] in a powder mis-sold as heroin, the intrinsic unregulated nature of drug preparations essentially implies that any content is possible. Especially when considering the very high observed in vitro and in vivo opioid activity of many nitazene analogues [7, 12–15], the presence of a nitazene analogue even at trace amounts, which could be below the cut-off for detection with NTS, may readily give rise to dangerous opioid effects in (unknowing) users. Furthermore, our cross-reactivity data indicate that the potential presence of 'desnitazenes' like metodesnitazene (**19**) or etodesnitazene (**20**) (two analogues which have previously been identified on recreational drug markets [58–60]) in even highly pure drug samples, are unlikely to be picked up by the NTS due to limited cross-reactivity. These limitations of the BTNX NTS are highly relevant, as we found e.g. etodesnitazene to have a similar in vitro potency and efficacy as fentanyl [7, 13] and as it may potentially give PWUD a false sense of safety. In addition, the detectability of nitazene analogues in drug samples may be impacted by solubility issues, as highlighted by the incomplete dissolution of the drug samples tested in this study.

As previously observed with FTS [31], NTS could serve as inexpensive, relatively easy-to-use drug checking tools. Currently, the New Zealand Drug Foundation has started distributing NTS, which can be ordered online for free through the Foundation, providing at-home drug checking for PWUD [50]. Various factors related to the intrinsic properties of the NTS (e.g., cross-reactivity, specificity, and lot-to-lot variability in performance), combined with factors that arise when translating the use of NTS from a laboratory setting to the real-life setting (e.g., nitazene analogue content in drug samples, sample type, sample preparation and dilution, solubility of different analogues, testing conditions, and different interpretation of results) should be taken into account when assessing the real-world applicability of these NTS. Despite these limitations and while further research is warranted, this lab-based study and the available reports indicate that BTNX NTS cross-react with most current nitazene analogues that have emerged on recreational drug markets, although they may not be able to detect nitazene analogues present in trace amounts. Nevertheless, these commercial NTS could serve as important overdose prevention tools for PWUD, with positive results 'flagging' drug samples that can be suspected to contain one or more nitazene analogues. However, a negative result obviously does not imply that a sample is 'safe', as it may still contain non-cross-reactive nitazene analogues, other synthetic opioids or other drugs, as well as adulterants. In addition, the authors recommend interpreting the presence of a faint line in the test region as a positive result as a safety precaution when these NTS are used in the context of harm reduction. Finally, PWUD should be aware that, when a positive result is obtained, NTS do not provide any information on the identity, quantity or purity of the nitazene analogue(s) present in the preparation.

Conclusion

This study presents an independent, laboratory-based assessment of the potential of the first commercially available NTS for drug checking applications. The NTS displayed limited lot-to-lot variability, with an experimental limit of detection for isotonitazene of 2000 or 3000 ng/mL. Twenty-four of the 33 evaluated nitazene analogues cross-reacted with the NTS at concentrations at or below 9000 ng/mL. The test strips consistently detected the presence of a nitazene analogue in 6 authentic drug samples. Based on our cross-reactivity data, most of the currently circulating nitazene analogues, except for 'desnitazenes', are likely detectable with the BTNX NTS, while analogues with a lengthened linker between the aromatic groups may not be detectable. Altogether, taking into account limitations that hold true for test strip-based testing in general, and taking into account the cross-reactivity data presented here, the findings from this study indicate that the BTNX nitazene immunoassay test strips show potential to recognize the presence of nitazene analogues in drug preparations in real-life settings.

Supplementary Information

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Supplementary Material 1

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Author contributions
L.D.V.: Conceptualization, Resources, Investigation, Formal analysis, Writing – Original Draft. M.V.: Conceptualization, Supervision, Formal analysis, Writing – Review & Editing. C.S.: Conceptualization, Resources, Supervision, Writing – Review & Editing.

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Data availability
Data can be made available upon request.

Declarations

Conflict of interest
The authors declare that they have no conflict of interest.

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