Clinica Chimica Acta 561 (2024) 119826

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Case report

"Tranq-dope": The first fatal intoxication due to xylazine-adulterated heroin in Italy

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ARTICLE INFO

Keywords:
Xylazine
Adulterant
Opioid
Fatal case
Tranq-dope
Liquid chromatography-high-resolution mass spectrometry (LC-HRMS/MS)

ABSTRACT

Background and aims: The number of xylazine-involved overdose deaths tremendously increased from 2019 onwards in the US. This is due to the "tranq-dope" trend consisting in mixing opioids with the sedative to reduce drug manufacturing costs and enhance their effects. In this study, we report the first fatality involving xylazine-adulterated heroin in the EU.

Materials and methods: The subject was a 33-year-old Caucasian male with a documented history of drug abuse who was found dead in a public area with puncture marks at the elbow. Peripheral blood and urine were collected at the autopsy and analyzed by liquid chromatography-high-resolution tandem mass spectrometry (LC-HRMS/MS) after protein precipitation.

Results: 6-Monoacetylmorphine, total/free morphine, and codeine blood concentrations of 20.3, 236/105, and 38.3 ng/mL, respectively, indicated recent heroin consumption. Methadone blood concentration was below 10 ng/mL. Alprazolam, nordiazepam, and flurazepam blood concentrations were 23.9, 61.4, and 55.0 ng/mL, respectively. Benzoylecgonine blood concentration was below 5 ng/mL. Xylazine blood and urine concentrations were 105 and 72.6 ng/mL, respectively.

Conclusion: The combination of central nervous system depressants, i.e., opioids, benzodiazepines, and xylazine, was the principal cause of death by cardiorespiratory failure. The case was promptly reported to the UE Early Warning System on drugs.

1. Introduction

In 2023, the European Monitoring Center on Drugs and Drug Addiction (EMCDDA), soon to be renamed the EU Drugs Agency (EUDA), reported over 6,100 drug-related deaths in Europe, of which 74 % involved opioids [1]. Although the illicit market offers a panel of new synthetic opioids every year, heroin still remains the most abused opioid in most countries, with a purity ranging between 13 and 47 % at retail level [2]. Beside the inherent health threat posed by the use of illicit drugs, opioid adulteration also exposes drug users to the unexpected consumption of a broad spectrum of pharmacologically active substances. The deliberate addition of active ingredients to adulterate the primary drug may interfere with the pharmacological effects and increase its potency, thereby augmenting the profit of drug dealers [3]. Among adulterants, xylazine raised particular concerns due to the ever-increasing number of deaths involving this molecule, especially in North America [4–6].

Xylazine is a nonopioid sedative, analgesic, and muscle relaxant

Abbreviations: AGC, automatic gain control; CNS, central nervous system; ddMS^2, data-dependent tandem mass spectrometry; DEA, drug enforcement administration; EDDP, 2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine; EMA, European medicines agency; EMCDDA, European monitoring center on drugs and drug addiction; EUDA, EU drugs agency; EWS, early warning system; FDA, food and drug administration; FullMS, full-scan mass spectrometry; HRMS, high resolution mass spectrometry; IT, injection time; LC-HRMS/LS, liquid chromatography-high-resolution mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MP, mobile phase; NCE, normalized collision energy; NPS, new psychoactive substance; OSAC, organization of scientific area committees; SNAP, sistema nazionale di allerta precoce.

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https://doi.org/10.1016/j.cca.2024.119826
Received 2 May 2024; Received in revised form 14 June 2024; Accepted 19 June 2024
Available online 21 June 2024

approved for veterinary use by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Structurally related to clonidine, it is an α-2 adrenergic receptor agonist inhibiting norepinephrine and dopamine release at the neuronal synapse; xylazine is also a competitive inhibitor of norepinephrine transport [7]. A secondary mechanism involving the μ-opioid receptor was also demonstrated in a murine model [8]. In humans, xylazine can induce an opioid-like toxidrome including hypotension, bradycardia, and respiratory depression eventually leading to death, and repeated exposure may also cause withdrawal symptoms and severe necrotic cutaneous ulcers leading to amputation [7]. Xylazine toxic doses range from 40 to 2400 mg with an estimated median lethal dose of 15 mg/kg [7,9]. Postmortem blood concentrations vary from "traces" to 16 μg/mL, but there is a significant overlap in blood concentrations in fatal and non-fatal cases, and there is currently no consensus on xylazine toxic and fatal concentrations [7]. It is believed to have a rapid clearance in humans: pharmacokinetic studies in animal models indicate short distribution and elimination half-life, with peak plasma concentrations occurring in 12–14 min [10,11]. Approximately 70 % of xylazine is eliminated unchanged in urine [9].

Nonetheless, xylazine has been used for recreational purposes for decades, and sporadic poisonings were reported from 1979 onwards [12]. In the early 2000s, it was first reported as a drug adulterant in Puerto Rico by the DEA and laboratory analysis. In the last few years, however, xylazine detection by forensic laboratories tremendously increased in North America, following the "tranq-dope" new trend: xylazine ("tranq" as in "tranquillizer") is mostly found mixed with other opioids ("dope") like heroin or fentanyl as an adulterant to reduce manufacturing costs of the illicit drugs or to enhance their effects [4]. However, combining central nervous system (CNS) depressants may increase the risks of intoxication and fatal overdose. The situation is concerning in the US, where the opioid crisis already reshaped the landscape of drug-related fatalities at the dawn of the millennium [6]. Xylazine-involved overdose death rates increased 567 % from 2018 to 2019, 135 % from 2019 to 2020, and 126 % from 2020 to 2021 in the US, with many seizures and fatalities reported in the North Eastern States [4]. According to the US Drug Enforcement Administration (DEA) laboratory system, approximately 23 % of fentanyl powder and 7 % of fentanyl pills seized by the DEA in 2022 contained xylazine [13]. In Canada, the number of identifications in exhibits submitted by law enforcement agencies increased 4000 % from 2018 to 2019, was stable from 2019 to 2020 (−3.4 %), and increased again 171 % from 2020 to 2021 and 152 % from 2021 to 2022 [14]. In the EU also, xylazine has been detected in several product seizures by the police and customs since 2019, with an anecdotal case reported in 2006 in Belgium [15]; the substance was found either alone or in combination with heroin or benzimidazole opioids, which recently replaced fentanyl analogues on the illicit drug market due to strengthened legislation [3,16]. In the EU however, the number of cases appears to be limited, and no overdose involving xylazine was reported through the European Early Warning System (EWS) to date [17]. Noteworthy, xylazine true prevalence is currently unclear as it is not strictly monitored.

We hereby report the first fatal intoxication related to xylazine-adulterated heroin in the EU, detailing the toxicological findings and highlighting the particular importance of national EWS in individuating such cases.

1.1. Case description

A 33-year-old Caucasian male with a documented history of drug abuse was found dead in a public area in the central Italian region in summer. The external cadaveric examination revealed traces of sero-hematic fluid effusion from the nasal cavity and labial corners, puncture marks in the left antecubital region, abrasions on the ventral surface of the left arm, on the dorsal-medial surface of the left forearm, on the anterior surface of the knees and legs. Furthermore, an electro-patch was attached to the chest, consistent with a defibrillator electrode, suggesting a tentative cardiopulmonary resuscitation in the rescue phase. The autoptic exam revealed significant subcutaneous blood infiltrate at the puncture mark level at the crease of the left elbow and intense polyvisceral congestion. Moreover, a condition of hyperemia of the gastric mucosa was observed with 100 mL of blood inside the relative lumen, from an hemorrhagic gastritis likely related to the agonal phase, in the absence of other macroscopic causes.

During the autopsy, peripheral blood and urine samples were collected in polyethylene tubes without preservative for toxicological investigation; the samples were stored at −20 °C until analysis. No histological tests were conducted. The preliminary screening revealed the presence of opioids, corroborating the hypothesis of a drug-related death. Since a series of suspected fatal intoxication occurred in the same geographical area in a short period, the Italian EWS, "Sistema Nazionale di Allerta Precoce" (SNAP), on New Psychoactive Substances (NPS) was informed to further investigate the possible presence of unusual psychoactive substances in the biological matrices.

2. Materials and methods

2.1. Chemicals and reagents

Morphine-d3, 6-monoacetylmorphine-d3, benzoylecgonine-d3, methadone-d3, and ketamine-d4 reference standards were purchased from LGC Standards (Teddington, England). Xylazine and xylazine-d6 reference standards were obtained from Cayman Chemical (Ann Harbor, MI, US). Standards were solubilized to 1 mg/mL in methanol, or acetonitrile for 6-monoacetylmorphine-d3, and stored at −20 °C before analysis. LC-MS grade water, methanol, acetonitrile, and formic acid were purchased from Carlo Erba (Cornaredo, Italy). LC-MS grade ammonium formate was obtained from Honeywell FlukaTM (Morristown, NJ, USA) and reagent grade ammonium acetate from Levanchimica (Bari, Italy). Acetic acid was supplied by Sigma Aldrich (St. Louis, MO, US), and hydrochloric acid was obtained from Honeywell FlukaTM (Morristown, NJ, US). β-Glucuronidase (50 units/μL) from limpets (P. vulgata) was purchased from Sigma Aldrich.

2.2. Sample preparation for qualitative toxicological analysis

A 5 μL volume of 6-monoacetylmorphine-d3, benzoylecgonine-d3, methadone-d3, and ketamine-d4 at 1 μg/mL in acetonitrile (internal standards) was added to 100 μL whole blood. The sample was then mixed with 250 μL acetonitrile with 0.2 % hydrochloric acid and centrifuged for 5 min, 15,000g, at room temperature. The supernatant was evaporated to dryness under nitrogen in a conical glass tube and the residue was reconstituted with 100 μL mobile phases A:B 90:10 (v/v). After centrifugation for 5 min, 15,000g, at room temperature, the supernatant was transferred to an autosampler vial with a glass insert.

A different protocol was applied to urine. A 50 μL aliquot was fortified with 5 μL of 6-monoacetylmorphine-d3, benzoylecgonine-d3, methadone-d3, and ketamine-d4 at 1 μg/mL in acetonitrile (internal standards). The sample was then mixed with 10 μL 10 mol/L ammonium acetate at pH 5.0 and 10 μL β-glucuronidase. After incubation for 2 h at 50 °C, the sample was mixed with 125 μL acetonitrile with 0.2 % hydrochloric acid and centrifuged for 5 min, 15,000g, at room temperature. The supernatant was evaporated to dryness under nitrogen in a conical glass tube and the residue was reconstituted with 100 μL of mobile phases A:B 90:10 (v/v). After centrifugation for 5 min, 15,000g, at room temperature, the supernatant was transferred to an autosampler vial with a glass insert.

2.3. Instrumental conditions for qualitative analysis

Biological samples were screened with a previously described liquid chromatography-high-resolution tandem mass spectrometry (LC-

HRMS/MS) method used for the identification of over 1400 substances, including common drugs of abuse, NPS, and other psychoactive substances such as antidepressants, benzodiazepines, cannabinoids, cathinones, hallucinogens, ketamine derivatives, neuroleptics, opioids, phenethylamines, and metabolites [18]. The analysis was performed with an UltiMate 3000 chromatographic system from Dionex coupled with a Q Exactive mass spectrometer from Thermo Scientific (Waltham, MA, US) equipped with a heated electrospray ionization source.

Chromatographic separation was performed with a Phenyl-Hexyl column (100 x 2.1 cm, 2.6 μm particle size) from Phenomenex (Castel Maggiore, Italy) maintained at 40 ± 1 °C using a mobile phase gradient composed of 2 mmol/L ammonium formate in water with 0.1 % formic acid (mobile phase A) and 2 mmol/L ammonium formate in methanol: acetonitrile:water 49.5:49.5:1 (v/v/v) with 0.1 % formic acid (mobile phase B). The gradient started with 1 % B for 1 min and increased to 99 % within 9 min; 99 % B was maintained for 1.5 min, before returning to initial conditions within 0.01 min; re-equilibration time was 3.99 min. Total run time was 15.5 min with a 0.5 mL/min flow rate.

Source settings were: spray voltage, + 3.5 kV; sheath gas flow rate, 40 a.u.; auxiliary gas flow rate, 10 a.u.; capillary temperature, 350 °C; S-lens radio frequency level, 50 a.u. Data were acquired in full-scan HRMS/data-dependent HRMS/MS modes (FullMS/ddMS^2). FullMS settings were: resolution, 70,000 (full width at half maximum at *m/z* 200), mass range, *m/z* 100–1000, automatic gain control (AGC) target, 3×10^6; max injection time (IT), 256 ms. ddMS^2 settings were: resolution, 17,500; topN, 5 (pick others if idle); intensity threshold, 5×10^4; dynamic exclusion, 2 s; isolation window, *m/z* 1.2; normalized collision energy, 17.5, 35, and 52.5 %; AGC target, $1x10^5$; max IT, 64 ms. An inclusion list was used to prioritize the fragmentation of the analytes with a specific time window (analyte's retention time ± 0.2 or 0.5 min). The orbitrap was calibrated before analysis and a lock mass list with previously identified background ions was used throughout the analysis for better accuracy [19].

LC-HRMS/MS data were processed with TraceFinder (v. 4.1, Thermo Scientific). LC-HRMS peaks were detected based on their theoretical mass (tolerance, ± 5 ppm) and retention time (tolerance, ± 0.5 min) with an intensity threshold of 10^5; the detection of at least two HRMS/MS fragments (tolerance, ± 5 ppm) was necessary for identification; isotopic distribution (fit threshold, 70 %; mass deviation tolerance, 5 ppm; intensity deviation tolerance, 30 %) was used for confirmation but was not mandatory for identification. The results were manually verified for final identification.

The validation parameters were measured to confirm the suitability of the method, as recommended for quality assurance in comprehensive screening methods involving LC-HRMS. These parameters included sensitivity, selectivity, matrix effect, and carryover [20].

2.4. Quantitative toxicological analysis

The quantification of morphine and xylazine in whole blood and urine samples was performed by LC-HRMS/MS. Sample preparation was the same as described for the qualitative analysis, with xylazine-d6 and morphine-d3 at 20 ng/mL in methanol as internal standards. Chromatographic and spectrometric parameters were the same as described for the qualitative analysis, although the processing method and the inclusion list contained only the analytes of interest and their internal standard: xylazine, xylazine-d6, morphine, and morphine-d3. The area ratio between the analytes and their corresponding deuterated internal standard was used for quantification.

The analytical method was validated and analytical bias, limits of detection (LOD) and quantification (LOQ), linearity, and carryover were evaluated according to the Organization of Scientific Area Committees (OSAC) for Forensic Science. Moreover, matrix effect and recovery were assessed according to Matuszewski et al. [21]. The validation parameters satisfied the OSAC requirements. LOD and LOQ were 0.2 and 1.0 ng/mL, respectively, for both analytes in both matrices. Working range was 10–200 ng/mL for xylazine in whole blood and urine, and 25–500 ng/mL for morphine in both matrices. Analytical bias, in terms of precision and accuracy, was within ±20 % for the two analytes. No carryover was observed for the two analytes, and there were no interfering peaks. Matrix effect and recovery rate were ±20 % for xylazine and morphine in whole blood and urine.

3. Results

3.1. Qualitative toxicological analysis

The blood sample was positive for xylazine; heroin metabolites (6-monoacetylmorphine, morphine, codeine), methadone, cocaine metabolite (benzoylecgonine), and benzodiazepines (alprazolam, flurazepam, norflurazepam, nordiazepam) were also detected. The extracted-ion chromatogram for xylazine in blood and the HRMS fragmentation pattern of xylazine in comparison to the reference standard are displayed in Fig. 1 and Fig. 2, respectively. Xylazine main fragment at *m/z* 90.0372 was produced by the thiazine cycle after contraction; sulfur loss further produced a minor fragment at *m/z* 58.0653. Two other minor fragments at *m/z* 147.0917 and 164.0529 were produced through the cleavage of the thiazine cycle.

The urine sample was positive for xylazine; heroin metabolites (6-monoacetylmorphine, morphine, 6-acetylcodeine, codeine, papaverin), methadone metabolite (2-ethylidene-1,5-dimethyl-3,3-diphenyl pyrrolidine, EDDP), dextromethorphan, cocaine and metabolites (benzoylecgonine and ecgonine methyl ester), and benzodiazepines (nordiazepam, flurazepam, norflurazepam) were also detected. A chromatographic peak was detected at 3.30 min of the gradient produced an ion at *m/z* 237.1050 (elemental composition with a 5-ppm mass tolerance, $C_{12}H_{17}N_2OS^+$), which was fragmented following a pattern similar to that of xylazine: fragments at *m/z* 58.0653 and 90.0372 indicated a thiazine, and minor fragments at *m/z* 163.0864 and 180.0474, corresponding to xylazine's fragments at *m/z* 147.0917 + 15.9945 and 164.0529 + 15.9947, suggested the addition of an oxygen atom. The spectrum was similar to that of hydroxy-xylazine isomers 1 and 2, produced by hydroxylation of xylazine dimethylphenyl ring and identified by Meyer et al. in vivo metabolite identification studies with rat urine and an authentic case report of xylazine overdose [22].

3.2. Quantitative toxicological analysis

Common drugs of abuse and pharmaceutics were quantified in blood with routine laboratory techniques including LC-MS/MS and gas chromatography (GC)-MS/MS. 6-Monoacetylmorphine, total/free morphine, and codeine concentrations were 20.3, 236/105, and 38.3 ng/mL, respectively; the percentage of free morphine was 44.5 %. Alprazolam, nordiazepam, and flurazepam concentrations were 23.9, 61.4, and 55.0 ng/mL, respectively; norflurazepam was not quantified. Methadone and benzoylecgonine concentrations were below their respective limit of quantification, i.e., 10 and 5 ng/mL.

Xylazine concentrations were 72.6 ng/ml in urine and 105 ng/mL in blood. Hydroxy-xylazine was not confirmed due to the lack of commercially available reference standards at the time of the analysis. The coroner concluded that death occurred from acute cardiorespiratory failure due to CNS depression resulting from polydrug intoxication with the synergistic action of opiates and xylazine, although free morphine blood concentration alone is potentially, but not unequivocally, lethal, especially if the consumption followed a withdrawal period [23,24].

4. Discussion

To the best of our knowledge, this is the first fatality involving xylazine-adulterated heroin in the EU. In the last years, xylazine garnered a lot of attention from mainstream media and toxicologists in the US as the main fentanyl adulterant, provoking an alarming increase

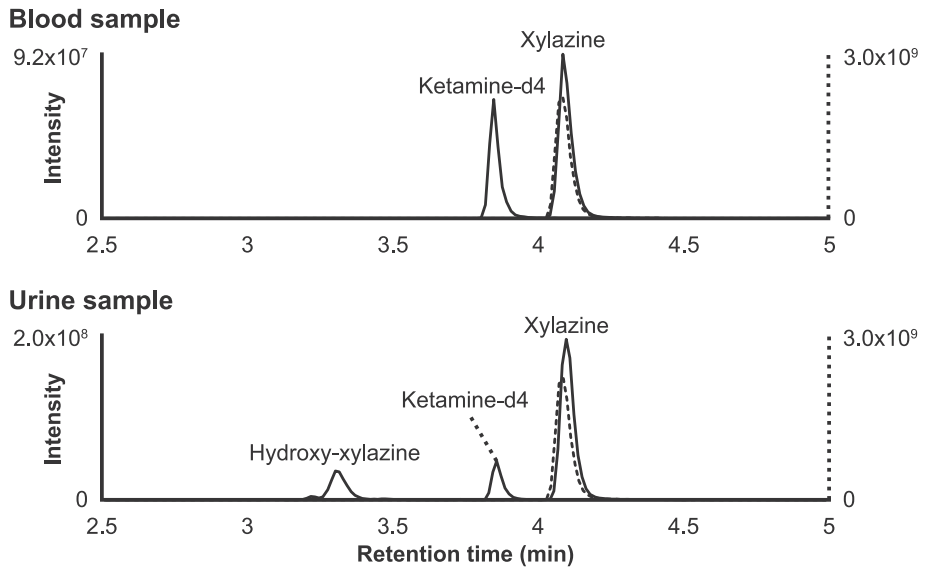

Fig. 1. Screening results: Liquid chromatography-high resolution mass spectrometry trace of xylazine (*m/z* 221.1107), hydroxy-xylazine (*m/z* 237.1056), and ketamine-d4 (internal standard, *m/z* 242.1244) in the blood and urine from a xylazine overdose case (plain line) versus xylazine reference standard at 1 μg/mL in mobile phases A:B 90:10 (v/v) (dotted line). Mass tolerance, 5 ppm.

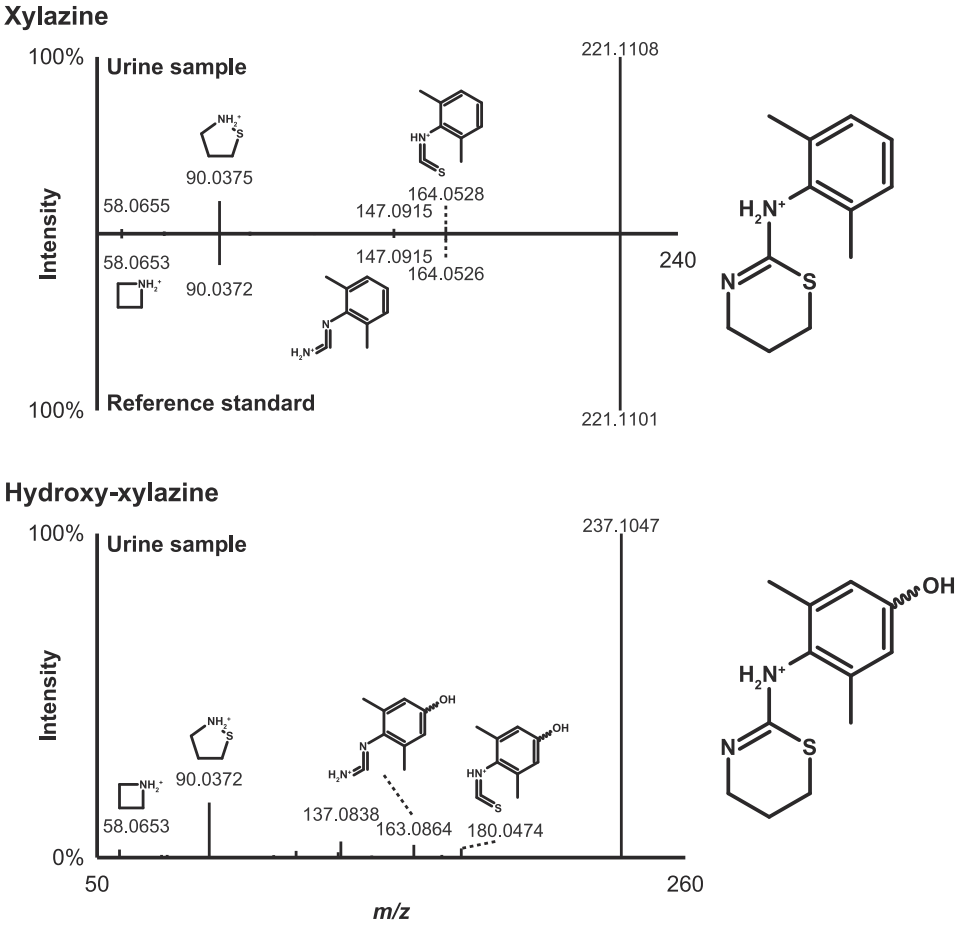

Fig. 2. Screening results: Positive electrospray ionization-high-resolution tandem mass spectrometry spectra of xylazine, with comparison to reference standard, and hydroxy-xylazine in the urine from a xylazine overdose case. Wavy line indicates unclear position at the dimethylphenyl ring.

in opioid fatal overdoses that are non-responsive to naloxone [25–27]. In 2023, the first xylazine-related overdose in association with fentanyl and morphine was reported in the UK, suggesting that the "tranq-dope" trend reached the European continent [17]. In the present case, fentanyl or metabolites were not detected, but xylazine was taken with heroin, as suggested by the presence of 6-monoacetylmorphine in the biological

samples and the high percentage of free morphine in blood. Currently, xylazine toxic and fatal blood concentrations in humans are not clearly established [7,28]. In the present case, xylazine blood concentration was substantially lower than the concentrations reported in fatalities associated with xylazine alone (2.3 to 16 μg/mL) and some non-fatal cases (up to 4.6 μg/mL) [28]. Considering these limited data, xylazine alone was not sufficient to cause death in the present case. However, xylazine concentration in both matrices was higher than in the British case (38 and 135 ng/mL in blood and urine, respectively), in which xylazine was a contributing cause of death, although fentanyl blood concentration was high (73.9 ng/mL) [17]. Xylazine blood concentration also was in the typical range reported in cases of co-exposure to CNS depressants such as alcohol, opioids, and/or benzodiazepines [7,28]. Recently, Smith et al. showed that fentanyl and xylazine produce a synergistic interaction, substantially increasing the lethality of one another in an animal model [29]. Although the precise mechanism is still unclear, xylazine seems to enhance opioid toxicity, inducing CNS depression and central cardiorespiratory failure. A recent study demonstrated that α-2 adrenergic receptor agonists, such as xylazine, prevent fentanyl tolerance in rats administered with a combination of xylazine and fentanyl, without inducing overexpression of the μ-opioid receptor in the CNS [30]. In the present case, the combination of CNS depressants at recreational to toxic (heroin and xylazine) or subtherapeutic to therapeutic (benzodiazepines) blood concentrations likely caused the death. The detection of nordiazepam in the biological samples may be due do a previous consumption and not a co-consumption of diazepam, although direct nordiazepam use cannot be excluded in the absence of temazepam, a specific diazepam metabolite, in the urine.

Toxicological analyses were performed as part of the SNAP on NPS, which aims at monitoring the NPS market and new trends of drug abuse in Italy [31]. The intricate capillary network established for ongoing surveillance of the dynamic NPS market represents the strength of the entire system, which allowed the prompt identification of this particular fatality. In particular, the collaboration between the institutions and the collaborative units in performing the required toxicological analyses allowed the successful detection of the new threat that arrived in Italy. Since xylazine is available as a veterinary pharmaceutical drug, it is not usually considered in routine toxicological screening for drug-related overdoses, resulting in a possible underreporting of the cases. Furthermore, the opioid-like effects and its association with illicit opioids substantially contribute to possible misinterpretation of fatal intoxications. Similar to NPS, the emerging adulteration practices pose new challenges to the medical staff and analysts in the toxicological laboratories, due to the unexpected toxicity as a result of drug-drug interaction and the problematic analytical detection of unexpected substances [3]. Additionally, the drug is potent and is rapidly eliminated, with a short half-life of 23–50 min, making the analytical detection therefore challenging in clinical and forensic casework [7].

In this concern, comprehensive toxicological analytical methods and last-generation laboratory instrumentation are crucial to identify the actual cause of toxicity. In particular, HRMS/MS provides high selectivity and sensitivity, allowing the accurate identification of unknown molecules through high-resolution fragmentation pattern identification [32]. Although xylazine is not considered as an NPS, the case presented relevant features due to the international alarm raised by the US and the recent detection of xylazine-adulterated fentanyl in the UK. As a consequence, a III-grade Alert was rapidly issued by SNAP to inform the collaborative centers in Italy of the emerging threat. Furthermore, the information on xylazine was shared with the EMCDDA since the SNAP is part of the international network of the EU EWS.

5. Conclusions

Drug of abuse adulteration is a common practice of the manufacturing process, concerning a wide range of active principles. Due to unexpected pharmacological interaction, these mixtures may exert unexpected effects on unaware consumers, which are difficult to interpret by the medical staff of emergency departments. Furthermore, adulterants are often underreported since toxicological analyses usually focus on well-known substances, such as common drugs of abuse or even NPS. Spread from the US, the “tranq-dope” threat raised attention on drug adulterants, which may be as harmful as common drugs. The reported fatal intoxication due to xylazine-adulterated heroin suggests that drug adulteration is a fluctuant phenomenon of global interest, posing similar challenges to those of NPS from clinical, toxicological, and analytical points of view. To this concern, a crucial role is played by the international capillary network of the regional and interregional EWS, which should strengthen their efforts in preventing all drug-related problems, since widespread information is the first step for an effective early warning on drugs. At the laboratory level, analytical toxicologists should be aware of the effects of the deadly combination of xylazine and opioids and include xylazine in their routine toxicology screening.

Declaration of sources of funding

This work was supported by the Department of Anti-Drug policies of the Italian Presidency of Ministries Council, as part of the project “SNAP – Sistema Nazionale di Allerta Precoce”. The funding source had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

CRediT authorship contribution statement

Annagiulia Di Trana: Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Alessandro Di Giorgi:** Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Jeremy Carlier:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Francesco Serra:** Writing – review & editing, Investigation. **Francesco Paolo Busardò:** Writing – review & editing, Investigation. **Simona Pichini:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgement

The authors would like to thank Simonetta Di Carlo, Antonella Bacosi and Michele Sciotti for their technical support.

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