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Case Report

Isotonitazene: Fatal intoxication in three cases involving this unreported novel psychoactive substance in Switzerland

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ABSTRACT

The paper describes the first three deaths reported in Europe involved in isotonitazene consumption, a potent benzimidazole derivate opioid consumed in the recreational drug scene. Isotonitazene powder and purity determination was performed on the sample collected in the first death scene by NMR, HRMS, GC-FTIR, ATR-FTIR and GC–MS. Isotonitazene purity was determined by GC–MS analysis and proton NMR, and was defined to be above 95 % and 98 %, respectively.

Quantification of isotonitazene in biological samples was performed using a targeted analysis based on SPE extraction and ultra-high performance liquid chromatography tandem mass spectrometry.

The isotonitazene median concentration in femoral whole blood was 1.20 ng/mL. Isotonitazene concentration in hair was similar or even lower compared to that seen in fentanyl abusers. Isotonitazene distribution in tissues converges in the brain, lungs and heart, respectively. Surprisingly, isotonitazene concentration in liver is the lowest measured for all tissues and fluids analyzed.

Based on circumstantial evidence, autopsy findings and the results of the toxicological analysis, the medical examiner concluded that the cause of all three deaths was an acute intoxication with isotonitazene.

Since isotonitazene toxic concentration levels are very low, the consumption of this new psychoactive drug is a real hazard for human health.

1. Introduction

In 2019, the first data about isotonitazene was published in Belgium by Blanckaert et al. [1], with the purpose to determine the structure and the purity of this NPS opioid derivative. Isotonitazene has been subsequently quantified in 18 post-mortem blood samples in the United States (USA) by Krotulski et al. [2]. In the spring 2020, the NPS discovery reported a median of 40 US Americans dying monthly related to isotonitazene consumption [3]. To the authors' knowledge, this paper describes, for the first time in Europe, a series of three deaths involved in isotonitazene abuse.

Isotonitazene is a benzimidazole derived opioid analgesic drug related to etonitazene [4–6]. In vitro assays comparison between isotonitazene and μ-receptor agonist's hydromorphone and

Abbreviations: NPS, new psychoactive substances; GC, gas chromatography; UHPLC, ultra-high pressure liquid chromatography; HRMS, high resolution mass spectrometry; MS, mass spectrometer; MS/MS, triple quadrupole mass spectrometer; ToF, time-of-flight; MRM, multiple reaction monitoring; ESI, electron spray ionization; SLE, solid liquid extraction; SPE, solid phase extraction; CX, cation exchange; LLOQ, lowest limit of quantification; LOQ, limit of quantification; LOD, limit of determination; ULOQ, upper limit of quantification; THC, tetrahydrocannabinol; EDTA, ethylenediaminetetraacetic acid; MOR, mu-opioid receptor; FTIR, Fourier-transform infrared spectroscopy; NMR, nuclear magnetic resonance; APCI, atmospheric pressure chemical ionization; DQF-COSY, double quantum filtered correlation spectroscopy; edHSQC, edited heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation; NOE, nuclear overhauser effect.

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fentanyl, demonstrate the isotonitazene potency: about 20 times higher than hydromorphone and slightly higher than those obtained for fentanyl [1]. Although toxicological and lethal doses have still to be determined by toxicologists, isotonitazene is confirmed to be a very strong opioid derivate. Krotulski et al. [2] determined an average of 2.2 ng/mL isotonitazene in blood from 18 post-mortem specimens, with the highest concentration measured at 9.5 ng/mL.

Since new psychoactive substances have appeared on the market, percentage of neurological symptoms such as coma, fainting and convulsion increased in years in NPS abuser, probably reflecting the increased drugs number [7–9]. It may be difficult, at present, to predict the impact of NPS misuse on the health care system [10]. According to Nutt et al. [11], different dimensions of risks may need to be identified, such as hazards for mental and physical health as a consequence of chronic intake, for instance with the development of addiction. In this dimension, even considering the low scientific knowledge on NPS, new markers on different matrices should be determined to screen and control abusers, to avoid development of NPS addiction. In this study, we present three case reports, which add new objective data on this NPS intoxication.

2. Materials and methods

2.1. Forensic chemistry analysis

In the present study, the structural characterization of isotonitazene followed a previously published procedure, with some slight modifications [12]. The structural characterization procedures include the analyses briefly described hereafter.

2.1.1. Gas chromatography mass spectrometry (GC–MS)

GC–MS analyses were performed with an Agilent 7890A gas chromatograph equipped with an Agilent 7683B autosampler and coupled to an electron ionization (EI) Agilent MSD 5975C mass spectrometer (Agilent Technologies, Basel, Switzerland). Isotonitazene was dissolved in ethyl acetate and 1 μL of the solution was injected at a concentration of about 1 mg/mL into the GC-system. Helium was used as a carrier gas at a constant flow rate (1.3 mL/min). The injector (280 °C) was used in split mode (split rate 1:25). Transfer line and ion source were set at 280 °C and 230 °C, respectively. GC-separations were performed on a 30 m 5% phenyl-/ 95 % dimethylpolysiloxane (DB5) capillary column (internal diameter: 0.25 mm, film thickness: 0.25 μm). The column temperature was programmed as follows: from 80 °C to 320 °C with a heat rate of 15 °C/min; then held for 4 min at 320 °C. Data handling was carried out with the corresponding workstation and Agilent MSD Chem Station software. The purity of the analyzed material was assessed using the peak purity method, by calculating the contribution of the total ion signal at the peak apex (at 18.7 min in the chromatogram) belonging to the isotonitazene peak relative to the contribution of impurities.

2.1.2. Attenuated total reflection – Fourier-transform infrared spectroscopy (ATR-FTIR)

Infrared spectra were obtained on a Varian 660 IR model using a Golden Gate-ATR system (Palo Alto, California, USA) with a scan range 4000–650 cm^{-1}, a resolution 2 cm^{-1} and 16 scans. Data were acquired with the Resolution Pro, KnowItAll software (Agilent).

2.1.3. Gas chromatography Fourier-transform infrared spectroscopy (GC-FTIR)

GC-FTIR analysis was performed by means of the DiscovIR spectrometer from Spectra Analysis (Marlborough, Massachusetts, USA) coupled with an Agilent 7890A gas chromatograph. The chromatographic conditions were identical to the GC–MS analyses described above. Solid phase FTIR spectra of the isolated isotonitazene were obtained for direct comparison with ATR-FTIR spectra from our in-house library. The obtained data are consistent with the ATR-FTIR spectra.

2.1.4. High resolution mass spectrometry (HRMS)

Confirmation of the chemical formula was performed with an atmospheric pressure chemical ionization (APCI) Q Exactive Hybrid Quadrupole-Orbitrap high resolution mass spectrometer (HRMS). 2 μL of the isotonitazene solution was directly injected into the atmospheric pressure chemical ionization (APCI) source, which was run in positive and negative modes with the following conditions: corona discharge current 20 μA, vaporizer temperature 350 °C, and capillary temperature 225 °C. The HRMS mass scan range was *m/z* 80–600 with a resolution (full width at half maximum) min. 70'000.

2.1.5. Nuclear magnetic resonance (NMR)

NMR spectra were recorded in deuterated methanol-d_4 on a Bruker Avance III 500 MHz spectrometer (Billerica, Massachusetts, USA) at 298 K. The spectra were processed with MestReNova 11.0 (Mestrelab Research, S.L., Santiago de Compostela, Spain), and chemical shifts reported in ppm relative to the residual solvent signals of methanol-d_4 ^{1}H δ = 3.33 ppm and ^{13}C δ =47.61 ppm as internal standards. ^{1}H- and ^{13}C-experiments, as well as double quantum filtered correlation spectroscopy (DQF-COSY), edited heteronuclear single quantum coherence (edHSQC) and heteronuclear multiple bond correlation (HMBC) experiments, both on ^{13}C and ^{15}N, as well as two dimensional NOE experiment (NOESY) were also performed for complete signal assignments.

2.2. Systematic toxicological analysis

Isotonitazene was diluted in methanol at 1 mg/mL and stored at −20 °C. The reference material methadone-d9 (at 100 μg/mL) in methanol was obtained from Cerilliant (Sigma, Germany). Methanol, dichloromethane, acetonitrile, acetone and hexane were obtained from Carlo Erba (Carlo Erba, Italy). Formic acid 98–100 % LC–MS grade was purchased from Sigma (Sigma, Germany). Ammonium hydroxide was obtained from Sigma (Sigma, Germany). FastPrep was purchased from MP Biomedicals (MP Biomedicals, France). H_2O was obtained from Milli-q system from Millipore. All solvents and inorganic chemicals were of analytical grade. SPE Evolute CX Express 30 mg/3 mL were purchased from Biotage (Biotage, Sweden).

2.2.1. Toxicological screening and drugs quantification

A toxicological screening was performed to investigate for illegal drugs, medical drugs, alcohol, volatile substances and other poisons. Post-mortem samples, cardiac whole blood, peripheral whole blood and urine were screened using LC-QToF-MS (Waters, Switzerland) and GC–MS (Agilent, Switzerland) to identify illicit drugs of abuse, medical drugs and other poisons [13].

Quantitative determination of benzodiazepines, cocaine, amphetamines and opioids in peripheral whole blood, cardiac whole blood, urine, vitreous and pericardial fluid was performed using UHPLC-MS/MS analysis after SPE extraction. Cannabinoids quantification was performed using UHPLC-MS/MS analysis after SLE extraction. Non-steroid antinflammatory substances were quantified using GC–MS analysis after liquid-liquid extraction. Ethanol determination was performed using HS-GC-FID analysis.

2.2.2. Samples preparation and extraction for Isotonitazene quantification

Prior to the SPE extraction, tissues and hair were homogenized. 1 g tissue (lungs, liver, kidneys, heart, brain, spleen, femoral

muscle) was homogenized with 2 inox and 4 ceramic balls in the FastPrep with 4.0 m/s, 30 s, TN 12 × 15 repeated 3 times. 0.5 mL Milli-Q water was added and 1 homogenization cycle was repeated; 1.5 mL Milli-Q water, isotonitazene for the calibration curve and 50 μL methadone-d9 (at concentration of 10 ng/mL) were added. Samples were centrifuged and the supernatant was extracted with Evolute CX Express (Biotage, Sweden). Quantification in tissues was prepared using appropriate bovine matrix at the following calibration levels: 50, 100, 250, 500, 1000, 2500, 5000 and 10000 pg/mg.

Hair was washed by shaking for 5 min in Milli-Q Water, then for 5 min in acetone and for 5 min in hexane [14]. Hair was dried with nitrogen. 50 mg hair (case 1: 3 cm cut scalp hair; case 2: 3 cm thorax hair; case 3: 0–3 cm cut scalp hair and 3–6 cm cut hair) were homogenized 2 times with 2 small inox balls with the Retsch MM 400 (Retsch, Germany) on 30 times per second for 12 min. 1.2 mL methanol were added. At this point, isotonitazene for the calibration curve and 10 μL methadone.d9 (at concentration of 1000 ng/mL) were added to the samples. Hair samples were subsequently mixed with Retsch MM 400 for 90 min at 30 times per second and samples were then centrifuged for 10 min at 8000 rpm before Evolute CX Express (Biotage, Sweden) extraction. Quantification in hair was made using negative human hair matrix with the following calibration levels: 2.5, 10, 50, 100, 250, and 500 pg/mg.

For fluids analysis, isotonitazene used for calibration curve and 25 μL methadone-d9 (at concentration of 10 ng/mL) were added to the tube and evaporated under nitrogen prior to liquid addition (peripheral whole blood, cardiac whole blood, urine, vitreous and pericardial fluid).

Fluids (peripheral whole blood, cardiac whole blood, urine, vitreous and pericardial fluid), homogenized tissues, and hair were extracted with Evolute CX Express (Biotage, Sweden). 0.5 mL fluids, homogenized tissues and hair extract were added to 1.5 mL ammonium acetate 50 mM pH 5.0 buffer and were added to the SPE column. Aqueous wash was performed with 2 mL ammonium acetate 50 mM pH 5.0 buffer and the organic wash with 2 mL methanol. Elution was executed with 2 mL DCM:MeOH:NH_3 (78v:20v:2v). After evaporation, samples were recovered with 100 μL H_2O with 0.1 % formic acid and 7.5 μL were injected into the UHPLC-MS/MS system.

2.2.3. Chromatographic and mass spectrometry parameters for Isotonitazene quantification

The UHPLC-MS/MS analysis was performed using an Acquity separations module coupled to the Acquity TQD mass detector equipped with ESI interface (Waters, Switzerland). UHPLC-MS/MS analysis was performed for isotonitazene quantification in human samples. Chromatographic separation was achieved using an Accucore RP-MS C18 column (Termo Scientific, Switzerland) (50 mm length x2.1 mm i.d., 2.6 μm particle size) with an Accucore RP-MS C18 guard column (Termo Scientific, Switzerland) (10 mm length x 2.1 mm i.d., 2.6 μm particle size) at 30 °C. The mobile phases consisted of 0.1 % formic acid (A) and 0.1 % formic acid in acetonitrile (B). The following gradient elution was used (runtime 6.5 min), starting with 95 % A increased to 98 % B in 5.5 min, held for 5.8 min, changed to 95 % A in 5.81 min and maintained at initial conditions for 6.5 min. The flow rate was 0.5 mL/min. The electrospray source was operated in the positive ionization mode (ESI+). Product ions were obtained by collision-induced fragmentation, which allowed the MS/MS to be operated in the multiple reaction monitoring (MRM) mode. MRM transitions and conditions for measurement of isotonitazene (retention time 1.95 min) are: 411.2 > 100.0 (quantifier), 411.2 > 72.0, 411.2 > 107.0; cone voltage 42 V; collision energy 22 V, 40 V and 50 V, respectively. Methadone-d9 (retention time: 2.10 min) conditions are: 319.2 > 104.9 (quantifier), and 319.2 > 268.1; cone voltage 34 V; collision energy 26 V and 16 V respectively. The source temperature and desolvation gas (nitrogen) temperature were set at 150 °C and 500 °C, respectively. The flow gas was delivered at rate of 1000 L/h. The capillary voltage was 0.6 kV. Waters Mass-Lynx system software Version 4.2 was used for instrument control and quantification.

2.2.4. Calibration and validation

Calibrators and quality controls (LLOQ: 0.05 ng/mL, low: 0.15 ng/mL, medium: 4.0 ng/mL, high: 8.0 ng/mL) were prepared by addition of standard solution to isotonitazene free pooled whole blood prior to extraction. Eight points calibration curves were constructed within the concentration range 0.05 ng/mL – 10.0 ng/mL using whole bovine blood matrix. Calibrators and controls were prepared separately with the same white powder isotonitazene diluted in methanol. Method validation was based on the document: "Guideline on bioanalytical method validation" published by the European Medicines Agency (2016) [15]. The following parameters were assessed: calibration model, accuracy, sensitivity, selectivity, specificity, precision, carry-over, recovery, interferences, ionization suppression/enhancement, limit of detection (LOD) and limit of quantification (LOQ).

Accuracy and precision were determined for each QCs (quality controls) in quintuples in three independent analytical runs. Sensitivity was determined for the LLOQ in six fold in three independent runs. Selectivity and specificity were determined injecting 10 different human blood samples, which were fortified at the QC low and injecting 6 different human blood samples, which were positives to the following drug groups: benzodiazepines, THC, cocaine, opioids, LSD, antidepressant and neuroleptics. Carry-over was evaluated in triplicate following injection of the 3xULOQ (upper level of quantification) calibration standard. Recovery was assessed by comparing pre-spike samples with the post-spike samples in triplicate for three different QCs (low, medium, high). Matrix effect was determined by comparing post-spike samples in matrix with the post-spike samples without matrix in triplicate for three different QCs (low, medium, high). Limit of quantification and the limit of detection were evaluated visually for signal-to-noise ratio S/N = 10 for the LOQ and for S/N = 3 for the LOD.

Although we did not have deuterated isotonitazene, matrix effect and recovery were assessed analogously (low, medium, high concentration) in triplicate in bovine liver matrix to compare results with those obtained in blood matrix.

2.3. Samples

The white powder was found on the site of the first case death. The powder was analyzed by the Zurich Forensic Science Institute and the Institute for Pharmaceutical Science (IPW).

The three post-mortem cases belonged to a small region of around 350′000 people located in the south of Switzerland.

2.3.1. Case 1

Approximately 72 h after death, an autopsy was carried out, which revealed a blood congestion of the organs, the presence of gastric material in the upper and lower respiratory tract, with the right lung weighing 840 g and the left lung 640 g.

Multiple samples were taken for toxicological investigation: femoral whole blood, cardiac whole blood, urine, vitreous, pericardial fluid, lungs, liver, kidneys, heart, brain, spleen, femoral muscle and hair.

The deceased was suffering from a depressive syndrome that arose about 3 years earlier, following a love disappointment, which was followed by drug abuse, including fentanyl. The young man

was recovered for a cardiac arrest one year before death, after an abuse of fentanyl. He was known as a fentanyl consumer. The individual was a computer technician and was well integrated socially. The white powder identified subsequently to be isotonitazene has been collected on the scene of the first death.

2.3.2. Case 2

Approximately 48 h after death, an autopsy was carried out, which revealed a blood congestion of the organs, the presence of gastric material in the upper and lower respiratory tract, with the right lung weighing 1260 g and the left lung 900 g.

Multiple samples were taken for toxicological investigation: femoral whole blood, cardiac whole blood, urine, vitreous, pericardial fluid, cerebrospinal fluid, lungs, liver, kidneys, heart, brain, spleen, femoral muscle and hair.

About 2 months prior to the death, the young man had been hospitalized voluntarily for an isotonitazene detoxification. The individual explained that his addiction developed rapidly after about 1 g of isotonitazene consumed in 1 month. He started to take isotonitazene for recreational purpose, because of the relaxant and euphoric effects in the first period. Afterward the initial period, he explained he began to need the drug in greater quantities and more frequently, with increasing withdrawal symptoms, such as: transpiration, nausea, jaw tension and intense psychic stress. During the short hospitalization, interrupted after seven days by the patient, the main initial symptoms were psycho-motor agitation, insomnia and episodes of cold sweating likely correlated with isotonitazene withdrawal. He was described in a general state of euphoria with preserved judgment skills and absence of craving. Before de-hospitalization, the individual no longer presented the initial addiction symptoms. Following the patient's request to return home, a medical examiner carried out a psychoeducational interview and concluded he could return home.

The young man was a bank accountant and was well integrated socially.

At the site of the second case, a white powder of isotonitazene and a pipe (see Image 1) were collected. The pipe was for an aerial vaporization administration containing a solution determined by HRMS to be isotonitazene. The night of his death, the individual had taken a lorazepam (*Temesta*) before going out. When he came back home he prepared a meal and he took an isotonitazene dose, probably using the vaporizing pipe. During his hospitalization for isotonitazene detoxification, he explained to the hospital collaborators that isotonitazene was taken with a vaporization pipe (see Image 1).

Image 1. Pipe discovered on the site of the second case. The pipe presents a broken side on the ampoule front.

2.3.3. Case 3

Approximately 96 h after death, an autopsy was carried out which revealed a blood congestion of the organs, the presence of gastric material in the upper and lower respiratory tract, with the right lung weighing 610 g and the left lung 580 g.

Multiple samples were taken for toxicological investigation: femoral whole blood, cardiac whole blood, urine, vitreous, pericardial fluid, lungs, liver, kidneys, brain, spleen, femoral muscle.

In the third case the deceased had been suffering from a reactive depressive syndrome for approximately one year due to the death of his brother. He was followed by a psychologist, which reported an improvement in his mood. The histological analysis confirmed an alveolar edema as well as observed in the macro autopsy, with several alveolar blood emoragies. The young man was a social educator and was well integrated socially. Fluids and tissues samples of all cases were stored at −20 °C for 1–3 days before screening analysis and for 1–15 months before isotonitazene quantification. Hair was stored at room temperature.

3. Results

3.1. Forensic chemistry analysis

The identification of the seized material based on GC–MS, HRMS, FTIR, and NMR has been performed analogously by Blanckaert et al. [1]. Here, we present the identification in more details, to enable its application in practice.

3.1.1. Gas chromatography – mass spectrometry identification of isotonitazene

The molecular ion (M^+) of isotonitazene was observed in the mass spectra, although weak at *m/z* 410. The parent compound can be ionized at different positions leading to different fragmentation pathways (see Fig. 1). On the one hand, alpha cleavage eliminating fragments at *m/z* 338, followed by *m/z* 296, and *m/z* 250. Further, fragments at *m/z* 282 followed by *m/z* 236 were observed. Additionally, a McLafferty rearrangement leading to *m/z* 58 was observed. Furthermore, peaks at *m/z* 149, 107, 86 base peak, 72, and 58 could be attributed to the parent compound. As several consecutive steps can lead to these fragments, it was not possible to prioritize one of the possible pathways. The purity of the isotonitazene peak was above 95 %.

3.1.2. High resolution mass spectrometry (HRMS)

In positive ionization mode, the protonated molecule of isotonitazene ($M+H^+$) was found at *m/z* 411.237430, which corresponds to a mass error of -3.9807 ppm compared to the theoretical value of 411.239067. In negative ionization mode, the deprotonated molecule of isotonitazene ($M-H^-$) was observed at *m/z* 409.223940, which corresponds to a mass error of -1.4027 ppm compared to the theoretical value of 409.224514. These two results allowed to confirm the chemical formula $C_{23}H_{30}N_4O_3$.

3.1.3. Fourier-transform infrared spectroscopy (FTIR)

The ATR-FTIR spectrum (see Fig. 2) showed bands of a tertiary amine and/or an aryl-nitro function at 1339 cm^{-1}. The latter function was also indicated by the band at 1508 cm^{-1}. The band at 1235 cm^{-1} corresponds to the aryl-ether group of isotonitazene. Finally, the bands at 2975 and 2937 cm^{-1} correspond to the CH_3- and CH_2- valance, respectively. The FTIR spectrum was confirmed with the GC-FTIR analysis, providing in addition the chromatographic separation of the isotonitazene compound (Fig. 3).

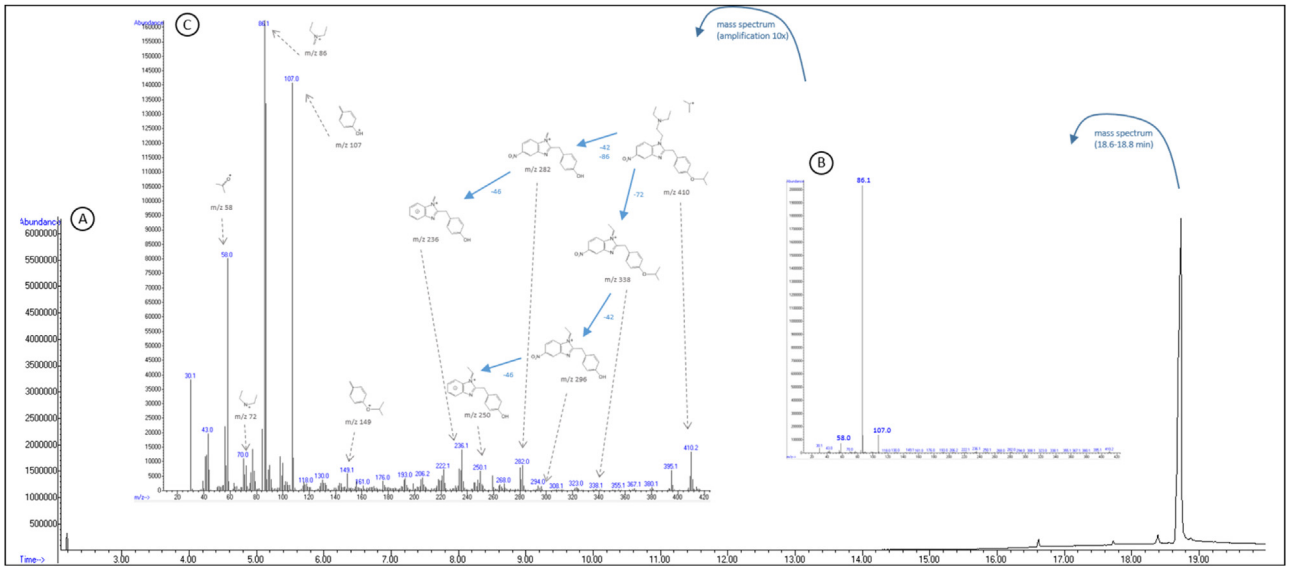

Fig. 1. (A) GC Chromatogram with the peak of isotonitazene (total ion current), (B) corresponding EI-MS spectrum of the isotonitazene peak, and (C) EI-MS spectrum amplified ten times with the proposed fragments of the parent compound.

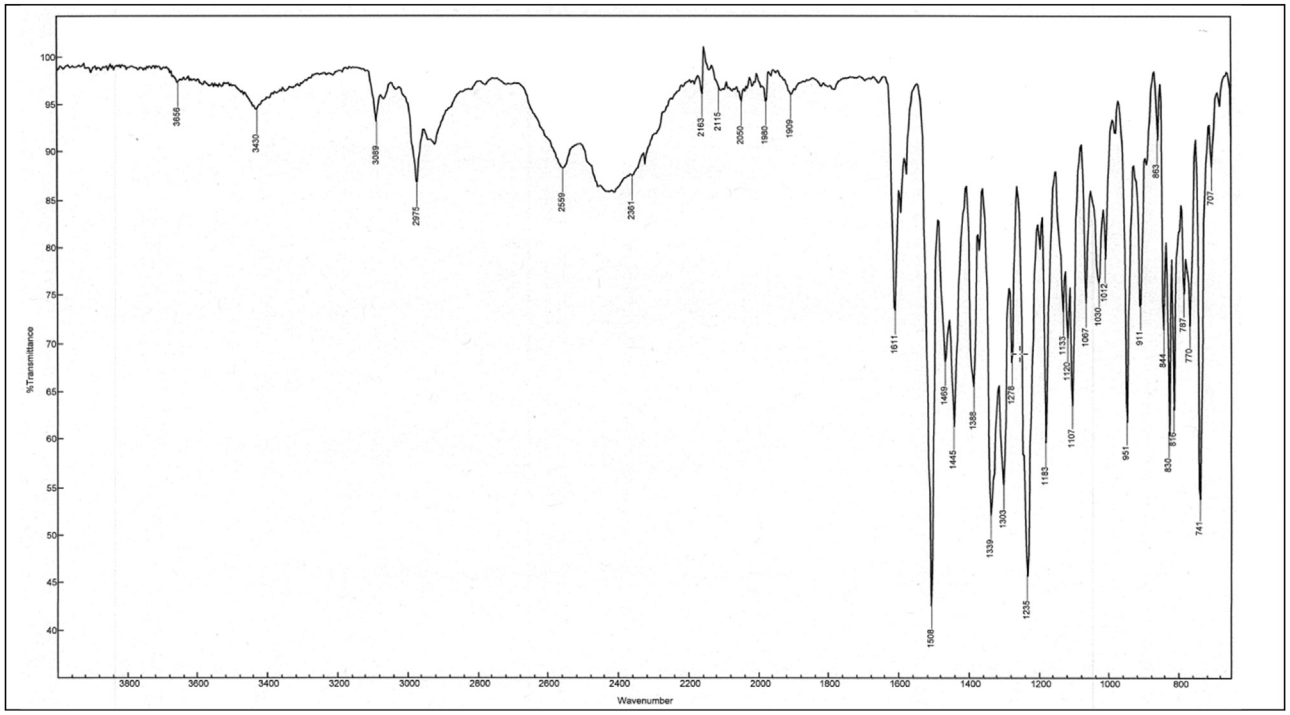

Fig. 2. ATR-FTIR spectrum of isotonitazene as HCl salt.

3.1.4. Nuclear magnetic resonance (NMR)

For a full characterization and to confirm the structure, a number of NMR experiments were conducted including 2D experiments (see annexes) such as double quantum filtered correlation spectroscopy (DQF-COSY), edited heteronuclear single quantum coherence (edHSQC) and ^{13}C and ^{15}N heteronuclear multiple bond correlation (HMBC) and NOESY. ^{1}H- and ^{13}C-chemical shifts and proton coupling constant, including multiplicity (in case of ^{13}C deduced from edHSQC), as well as ^{1}H–^{1}H-correlation from COSY and ^{1}H–^{13}C and ^{1}H–^{15}N correlation from HMBC and NOEs from NOESY are shown in Table 1. ^{13}C- and ^{1}H-signals indicated the presence of several aromatic systems with at least two six membered aromatic rings and some aliphatic groups. The two multiplet signals at 7.29 – 7.22 ppm and 6.97 – 6.91 ppm are typical for a para substituted phenyl ring while the two doublets at 8.56 and 7.83 ppm together with the doublet of doublet at 8.28 ppm indicate a 1,2,4-trisubstituted phenyl ring. All of these aromatic protons also show the expected COSY correlations and coupling constants for such systems. The ^{13}C-signal at 158.1 ppm reveals another quaternary aromatic carbon to be present which does not belong to either of the two phenyl groups. The doublet at 1.31 ppm and the triplet at 1.29 ppm correspond to a pair of identical methyl groups each. COSY shows

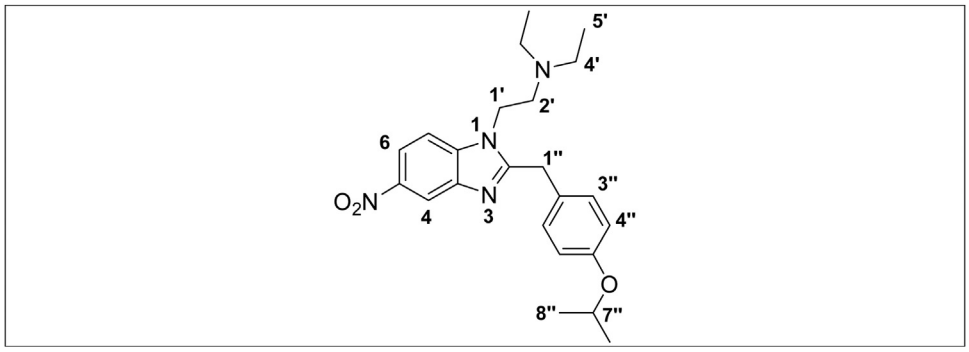

Fig. 3. Numbered chemical structure of isotonitazene.

Table 1
^{13}C NMR, ^{1}H NMR, COSY, NOESY, ^{13}C-HMBC and ^{15}N-HMBC spectral data for isotonitazene.

Position	δ ^{13}C (multiplicity)	δ ^{1}H[a]	COSY (^{1}Hx→^{1}H)	NOESY (^{1}Hx→^{1}H)	HMBC (^{1}H→^{13}C)	^{15}N-HMBC (^{1}H→^{15}N)
2	158.1 (s)					
3a	141.1 (s)					
4	114.6 (d)	8.56 (d, 2.1)	H-6		C-3a (w)C-5 C-6, C-7a	
5	144.1 (s)					
6	118.5 (d)	8.28 (dd, 8.9, 2.2)	H-4, H-7	H-7	C-4, C-5, C-7a,	
7	110.1 (d)	7.83 (d, 8.9)	H-6	H-6, H-1',H-2', H-4', H-5'	C-3a, C-4(w), C-5	N-1
7a	138.9 (s)					
1'	38.5 (t)	4.76 (m)	H-2'	H-2', H-4', H-1''	C-2, C-2', C-7a	N-1
2'	49.0 (t)	3.10 (m)	H-1'	H-4', H-5'	C-1', C-4'	N-1
4'	47.3 (t)	3.26 (m)	H-5'	H-5'		
5'	7.6 (q)	1.29 (t, 7.3)	H-4'		C-4'	N-3'
1''	32.4 (t)		H-3''	H-1'	C-2, C-2'', C-3''	N-1, N-3
2''	126.7 (s)					
3''	129.6 (d)	7.26 (m)	H-1'' H-4''	H-1', H-2', H-4', H-5', H-1'' H-4''	C-1'', C-4'', C-5''	
4''	116.2 (d)	6.94 (m)	H-3''	H-3'', H-7'', H-8''	C-2'', C-5''	
5''	157.5 (s)					
7''	69.7 (d)	4.59 (hept, 6.1)	H-8''		C-5'', C-8''	
8''	20.9 (q)	1.31 (d, 6.0)	H-7''		C-7''	

Spectrum recorded in Methanol-d_4 at 298 K; 500.13 MHz for ^{1}H, 125.76 MHz for ^{13}C. 50.697 Hz for ^{15}N, ^{13}C-HMBC ($J_{long\ range}$ =8 Hz), ^{15}N-HMBC (J =90 Hz, $J_{long\ range}$ = 3 Hz), multiplicity-edited HSQC (J =145 Hz) NOESY (500 ms) and DQF-COSY. w = weak.

[a] Signal multiplicity and coupling constants (Hz) are shown in parentheses.

the doublet at 1.29 ppm correlates to the heptet at 4.59 ppm indicating an isopropoxy functionality. The triplet on the other hand shows a COSY correlation to a large multiplet at 3.34 – 3.18 ppm belonging to two similar methylene groups (HSQC) connected to a nitrogen (however only the ^{15}N correlation from the methyl groups at 1.29 ppm was observed in the ^{15}N-HMBC – 54.7 ppm (N-3')) proving the presence of a diethylamino. Further three more aliphatic methylene groups can be observed with the multiplets at 4.79 – 4.72 ppm and 3.14 – 3.07 ppm being adjacent to each other forming a 1,2-substituted ethyl group. The singlet at 4.45 ppm on the other hand indicates an isolated methylene group. From ^{13}C-HMBC and COSY correlations, it is evident that the isopropoxy group and the singlet methylene at 4.45 ppm are the two substituents of the para substituted phenyl ring therefore forming a 4-isopropoxybenzyl substituent. ^{13}C-HMBC correlation from the 3.14 – 3.07 ppm methylene group to the methylene groups of the diethylamino function reveal the *N,N*-diethyl-2-aminoethyl substituent. The substitution pattern on the benzimidazole itself can be elucidated by ^{13}C-HMBC. The pattern on the six-membered ring is evident from the 1H signals (vide supra) with one substituent at the C-5 position. The bridge carbons can be identified as 141.1 ppm being C-3a and 138.9 ppm being C-7a as from H-6 sees a strong correlation to C-7a while none is observed to C-3a. From H-7 and H-4 the ^{3}J coupling to C-3a and C-7a, respectively, is more prominent than the ^{2}J correlation (if detected at all) to the other bridge atom as the HMBC experiment was optimized as such. The substitution position of the 4-isopropoxybenzyl and *N,N*-diethyl-2-aminoethyl substituent on the benzimidazole was also deduced from the ^{15}N- ^{13}C-HMBC. H-7 shows a correlation to the nitrogen at 147.5 ppm, which therefore must be N-1. While both methylene groups (H-1' and H-1'') from the two substituents connected to benzimidazole show a correlation to N-1, only the 4-isopropoxybenzyl methylene group (H-1'') shows a correlation to the nitrogen at 239.9 ppm (N-3). Also, the H-1' of the *N,N*-diethyl-2-aminoethyl substituent shows a correlation to C-7a and C-2 while H-1'' only shows a correlation to C-2. Observed NOEs show a proximity of H-7 to the *N,N*-diethyl-2-aminoethyl substituent as do the two substituents amongst each other. No NOE was observed from H-4 to any of the two substituents. No correlation was detected to the substituent at C-5 indicating the probable absence of carbon and hydrogen in that substituent. However, the nitrogen of the nitro group was also not observed in the ^{15}N-HMBC, which is not unusual in that experiment. From all spectral evidence the NMR assignment is in line with all observed signal shifts, coupling constants and correlation for the structure of isotonitazene.

Regarding the purity of the NMR sample the proton experiment was found to contain the described compound with a purity higher than 98 % regarding NMR active impurities.

3.2. Forensic toxicology analysis

An overview of the assessed validation parameters and validation data of isotonitazene quantification in the biological

Table 2
Validation parameters and validation data.

Validation parameter	Validation data
Calibration model	Weighted linear curve 1/x, methadone-d9 as internal standard. Mean correlation coefficient (R^2): 0.9976 Eight point calibration curves with levels: 0.05 ng/mL, 0.10 ng/mL, 0.25 ng/mL, 0.50 ng/mL, 1.00 ng/mL, 2.50 ng/mL, 5.00 ng/mL, 10.0 ng/mL
Bias	Measured using four separate samples in quintuples per concentration over three different runs: At concentration LLOQ (0.05 ng/mL): 11.9 % At concentration low (0.15 ng/mL): 1.9 % At concentration med (4.0 ng/mL): 6.0 % At concentration high (8.0 ng/mL): 7.1 %
Precision	Measured using four separate samples in quintuples per concentration over three different runs. Within-run CV: At concentration LLOQ (0.05 ng/mL): 9.6 % At concentration low (0.15 ng/mL): 8.7 % At concentration med (4.0 ng/mL): 9.0 % At concentration high (8.0 ng/mL): 5.6 % Between-run CV: At concentration LLOQ (0.05 ng/mL): 12.1 % At concentration low (0.15 ng/mL): 10.6 % At concentration med (4.0 ng/mL): 11.6 % At concentration high (8.0 ng/mL): 6.4 %
Carry over	No carryover was observed after 3xULOQ (30 ng/mL) after a three injection repetition.
Interference studies	No interfering signal from matrix, internal standard, common drugs of abuse and prescription medications from 10 samples taken from 10 human sources.
Recovery	50–65%
Limit of quantification (LOQ)/ limit of detection (LOD)	LOQ: 0.015 ng/mL LOD: 0.010 ng/mL
Matrix effect	Matrix effect: 105–114%
Selectivity, specificity	No interferences in selectivity and specificity

samples are shown in Table 2. An isotonitazene chromatogram at the LLOQ concentration (0.05 ng/mL) in matrix is shown in Fig. 4B. The distribution of isotonitazene in the post-mortem fluids, tissues and hair is summarized in Table 3.

In liver, isotonitazene recovery was measured and was between 10 % and 15 %. Matrix effect assessment revealed an isotonitazene ion enhancement of 155–175 %. Consequently, isotonitazene quantification in all post-mortem non-blood fluids and tissues was measured using the same appropriate bovine matrix used for the calibration curve preparation.

3.2.1. Case 1

In the first case, isotonitazene concentration in the whole blood was 2.28 ng/mL and the concentrations of the others drugs were: diazepam (29 ng/mL), nordiazepam (71 ng/mL), oxazepam (4.8 ng/mL), mefenamic acid (under 5.0 μg/mL), domperidone (6.0 ng/mL) and acetaminophen (4.8 (μg/mL). All the drugs concentrations were found in or under their respective therapeutic range [16,17].

3.2.2. Case 2

In the second case, isotonitazene concentration in the whole blood was 0.59 ng/mL and the concentrations of the other drugs

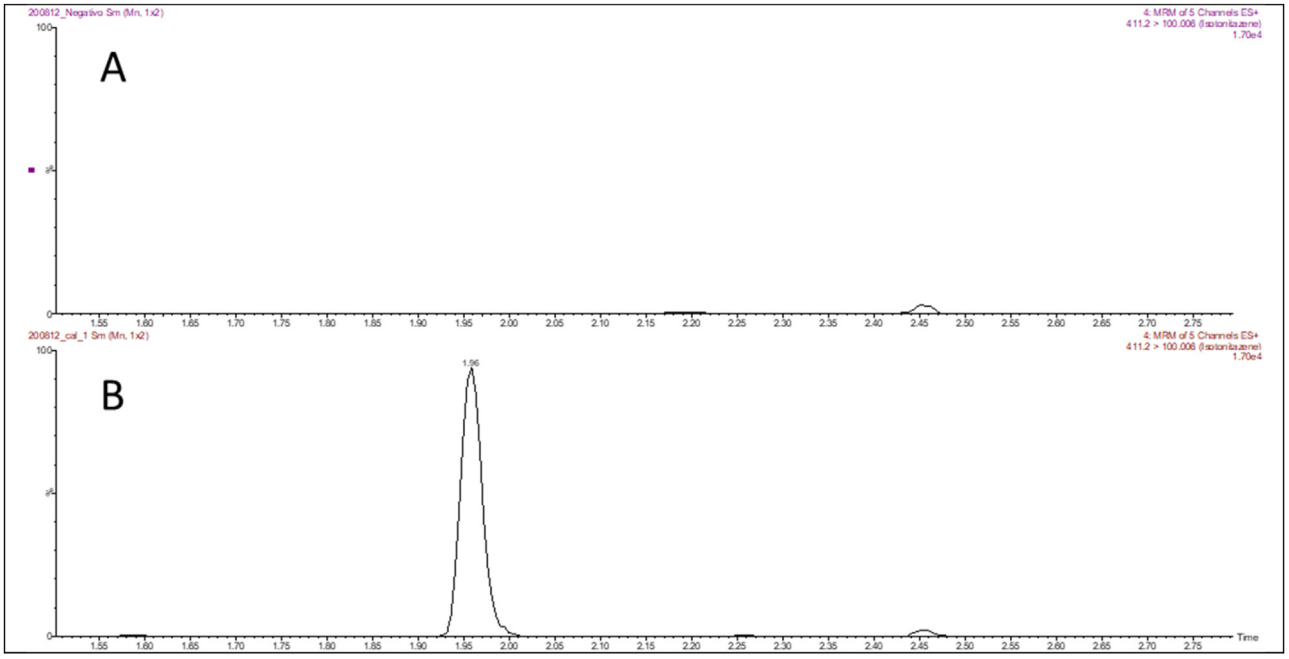

Fig. 4. UHPLC-MS/MS chromatograms. A: negative human blood. B: 0.05 ng/mL (LLOQ) isotonitazene concentration in blood matrix.

Table 3
Distribution of isotonitazene in the post-mortem biological samples.

Biological sample	Isotonitazene concentration [ng/mL] or [ng/g]		
	Case 1	Case 2	Case 3
Femoral whole blood	2.28	0.59	0.74
Cardiac whole blood	1.70	1.13	0.70
Urine	1.88	3.37	0.19
Humor vitreous	0.36	0.12	0.65
Pericardiac fluid	6.70	5.01	2.66
Lungs	0.52	17.9	2.39
Liver	< 0.05 (0.04)	< 0.05 (0.04)	< 0.05 (0.02)
Kidney	1.61	1.02	0.67
Heart	7.74	2.17	–
Brain	18.6	2.72	4.45
Spleen	4.40	3.44	2.62
Muscle	1.15	2.08	1.00
Cerebrospinal fluid	–	0.88[a]	–
Hair	75	182	32 (0–3 cm); 35 (3–6 cm)

[a] Calibration curve for isotonitazene quantification in cerebrospinal fluid prepared using bovine humor vitreous.

were: lorazepam (12 ng/mL), THC (56 ng/mL), THC-OH (1.8 ng/mL), THC-COOH (6.5 ng/mL) and CBN (2.9 ng/mL). Lorazepam, THC and THC-OH concentrations were found in the therapeutic and non-toxic range, respectively [16,17].

3.2.3. Case 3

In the third case, isotonitazene concentration in the whole blood was 0.74 ng/mL and the concentration of ethanol was 0.57 g/kg. The ethanol concentration was found in the non-toxic range [16,17].

4. Discussion

Isotonitazene is a benzimidazole derived opioid analgesic drug related to the analog structure etonitazene [4]. The structure differs from etonitazene by an additional methyl group in position 26. Its potency is assessed to be a strong and effective opioid, even stronger than the known opioid fentanyl [1,18]. Recently, others nitrazenes have been discovered and characterized in Europe and the USA. For instance, Siczek et al. [19] fully characterized etazene, a new psychoactive substance appeared in the Polish market. Structurally similar to the isotonitazene and etonitazene, Krotulski et al. [20] characterized metonitazene discovered in the USA. Krotulski et al. [2], determined several possible isotonitazene metabolites, with the *O*-desalkylation biotransformation products hypothesized to be a common metabolites among the benzimidazole analogues to isotonitazene, etonitazene and metonitazene.

The toxicological analysis revealed the presence of: benzodiazepines, mefenamic acid, domperidone and acetaminophen in the first case; lorazepam, THC, THC-OH, THC-COOH and CBN in the second case and ethanol in the third case report. For all these drugs, the concentrations are located in the therapeutic or non-toxic range [16]. Without the identification of isotonitazene powder and a targeted analysis of post-mortem samples with hyphenated techniques, the presence of isotonitazene would have been probably overlooked due to the very low toxic concentration.

The median isotonitazene concentration in blood was 1.20 ng/mL, with the highest and lowest blood concentrations being 2.28 ng/mL and 0.74 ng/mL, respectively. These values are in agreement with the isotonitazene median concentration of 2.20 ng/mL found by Krotulski et al. [2]. Case report 1 had a history of fentanyl consumption, which could have led the individual to develop a tolerance at the μ-receptor. For this reason isotonitazene concentration in blood could be higher compared to case 2 and 3. Further research should be done on isotonitazene tolerance and correlation between other μ-receptor agonist's drugs.

Isotonitazene redistribution in post-mortem tissues seems to converge in brain (median: 8.59 ng/g), lungs (median: 6.93 ng/g), heart (median: 4.96 ng/g), and spleen (median: 3.49 ng/g). Interestingly, the lowest isotonitazene concentration is measured in the liver of each of the three post-mortem samples (median: 0.04 ng/mL). The very high isotonitazene concentration in lungs in case 2 (17.9 ng/mL) could be influenced by the isotonitazene consumption by the respiratory tract shortly before dying and consequently isotonitazene accumulation in this tissue. The broken pipe (see Image 1) containing isotonitazene collected on the site of the second case could support the thesis of the individual having smoked the drug just before dying, whereby nobody has tampered with the death scene. Assuming that the death in case 2 occurred after an isotonitazene dose, and that the relative distribution is influenced by this event, isotonitazene seems to accumulate in the brain in case 1 and case 3. If a larger number of individual cases are studied and the higher levels of isotonitazene accumulation are confirmed in the brain, then this characteristic should be evaluated, because of the isotonitazene activity in the human brain μ-receptors, which may suggest a lethal impact with a low isotonitazene blood concentration. Moreover, considering the liver properties in drug metabolism, the very low isotonitazene concentration measured in liver can be interpreted in two ways: first, with a normal drug distribution in the body; and second, that isotonitazene in the liver is rapidly metabolized.

The medico-legal analysis revealed a pulmonary edema in the three cases. The mean weight of the three right lungs was 903 g and of the three left lungs was 707 g. Both average weights are higher compared to human healthy male weight [21].

Isotonitazene median concentration in scalp hair (case 1 and 3) was 55 pg/mg. In case 2, the thoracic hair concentration was 182 pg/mg. Hair analysis confirmed that isotonitazene had been consumed for at least 1–2 months for case 1, and at least 4–5 months for case 3. Since case 2 hair was torn from the thorax, we can just confirm that the individual had already consumed isotonitazene days to months before death. Isotonitazene in hair was found in a very low concentration, comparable with low fentanyl levels found in different forensic cases [22,23]. Only case 2 was confirmed to be an isotonitazene addict and, considering the time passed between first hospitalization and death, isotonitazene taken before was probably present in the cut hair sample of 3 cm. In 2015, Madry et al. [24], revealed the presence of MDMA in hair after a single intake, explaining how a single drug administration can be detected and quantified.

Concerning fluids, isotonitazene seems to accumulate especially in the pericardial fluid compared to the other fluids, femoral blood, cardiac blood, urine, humor vitreous. Several studies revealed that pericardial fluids offer is a quiet and isolated compartment for different substances, similar to humor vitreous [25–27]. In the present three cases, isotonitazene concentration in pericardial fluids (median: 4.79 ng/mL) is very high. Considering the interactions of several drugs with the heart [28–30], the possible interactions of isotonitazene with the normal heart physiological functioning should be further studied.

Concentrations measured in full blood were very low and comparable with those reported in the first article concerning isotonitazene consumption in post-mortem forensic cases [2]. Because of the very low isotonitazene concentration in blood and considering isotonitazene as a member of the new psychoactive substances group, forensic toxicological laboratories should think about qualitative screening analytical methods, in order to determine the presence or absence of these compounds. For instance, Trana et al. [31], proposed a comprehensive targeted screening for 77 NPS using a sensitive LC—MS/MS instrument.

Considering that, in December 2019, more than 950 molecules from different chemical classes were reported as NPS to the United Nation Office on Drugs and Crime (UNODC) Early Warning Advisory (EWA), toxicological laboratories should develop a growing number of targeted screening methods to determine the presence or absence of these substances [31].

5. Conclusion

Despite the first isotonitazene discovering publication in Europe was published in Belgium from an isotonitazene sample obtained online in June 2019, our isotonitazene determination in Switzerland was done previously in March 2019 with the first case death.

To the best of our knowledge, we report the first three confirmed deaths on European continent involving in isotonitazene consumption and the first research on the post-mortem redistribution of isotonitazene in fluids, tissues and hair.

Substances that are non-fentanyl related are increasingly (re) appearing on the illicit market [32] and the 3 case deaths in just one year involving isotonitazene in a very small region serve as a wake-up call for the Swiss and European drug control market.

Noteworthy is that the seemingly healthy social and employment status of the three deceased individuals might suggest that, isotonitazene consumption could become a real danger and threat to all people.

CRediT authorship contribution statement

F. Mueller: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **C. Bogdal:** Data curation, Formal analysis, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **B. Pfeiffer:** Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **L. Andrello:** Data curation, Investigation. **A. Ceschi:** Validation, Visualization, Writing - review & editing. **A. Thomas:** Supervision, Validation, Visualization, Writing - review & editing. **E. Grata:** Conceptualization, Investigation, Methodology, Resources, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

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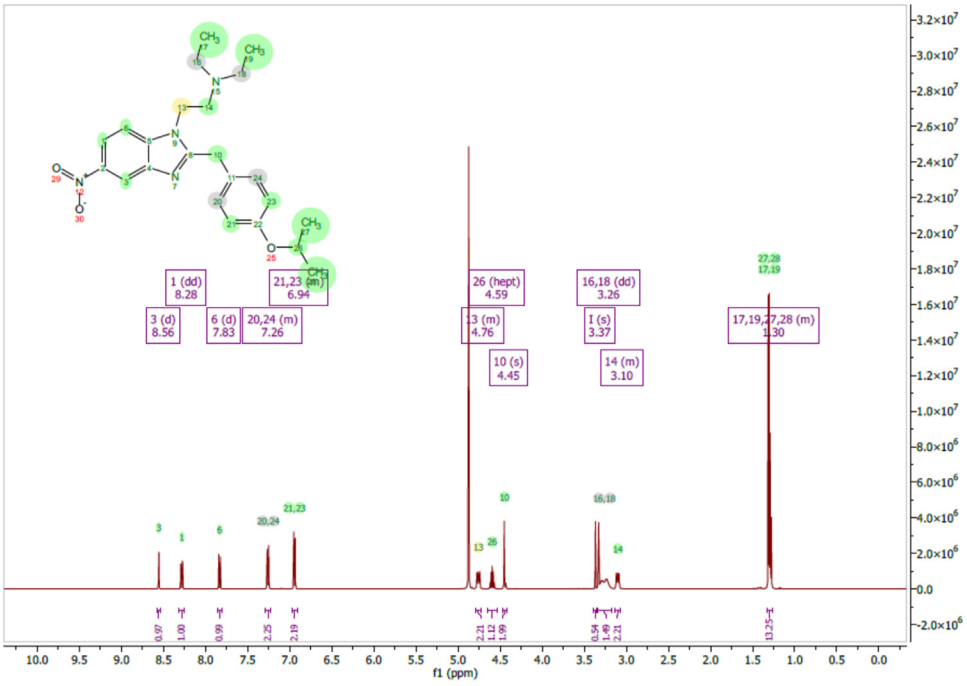
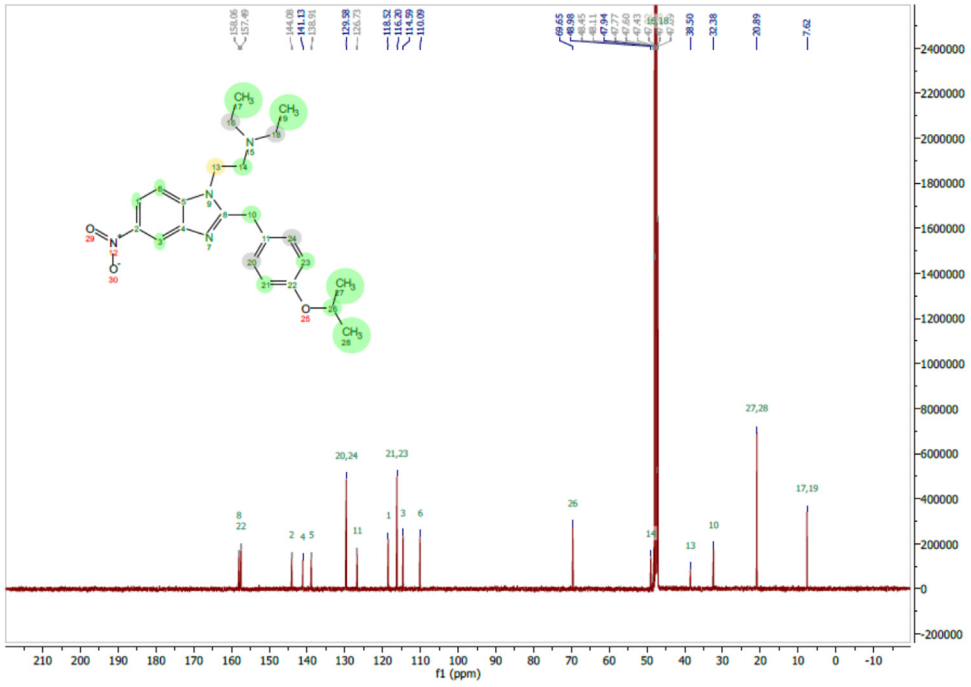
Appendix A

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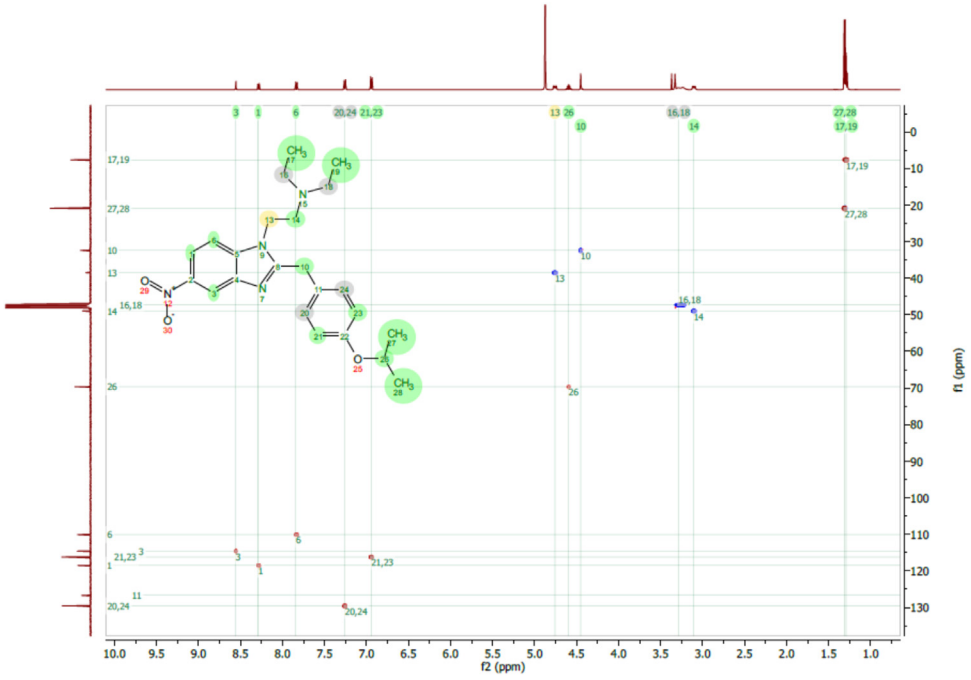
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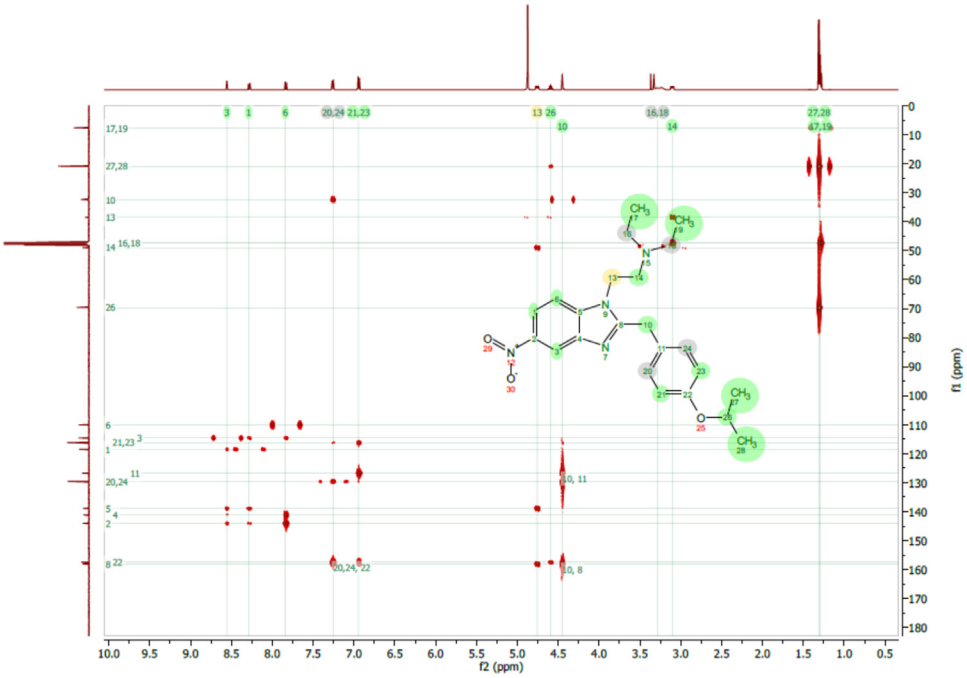
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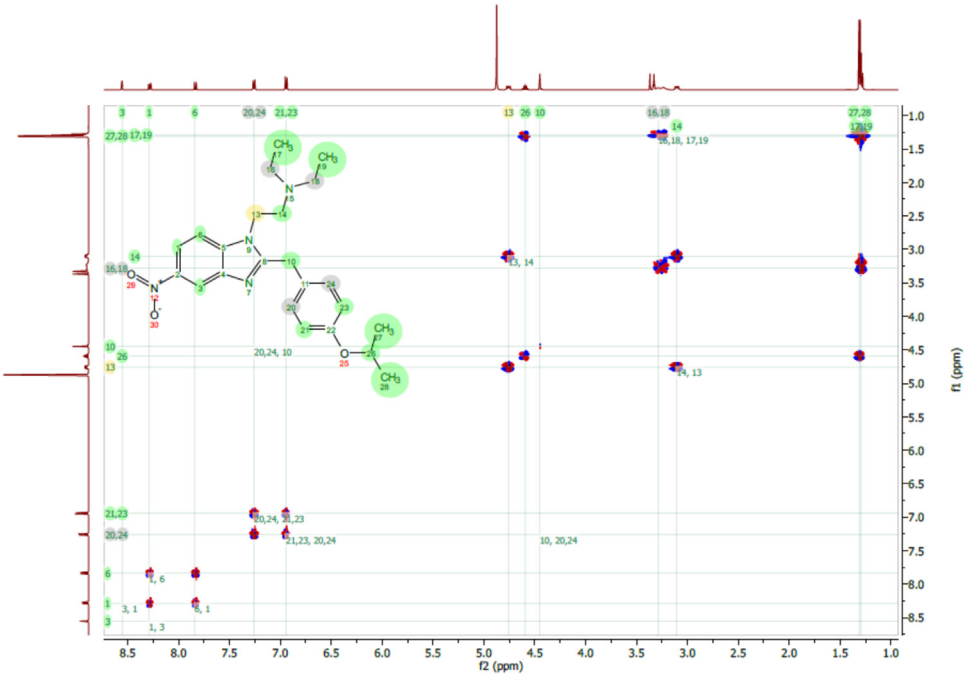
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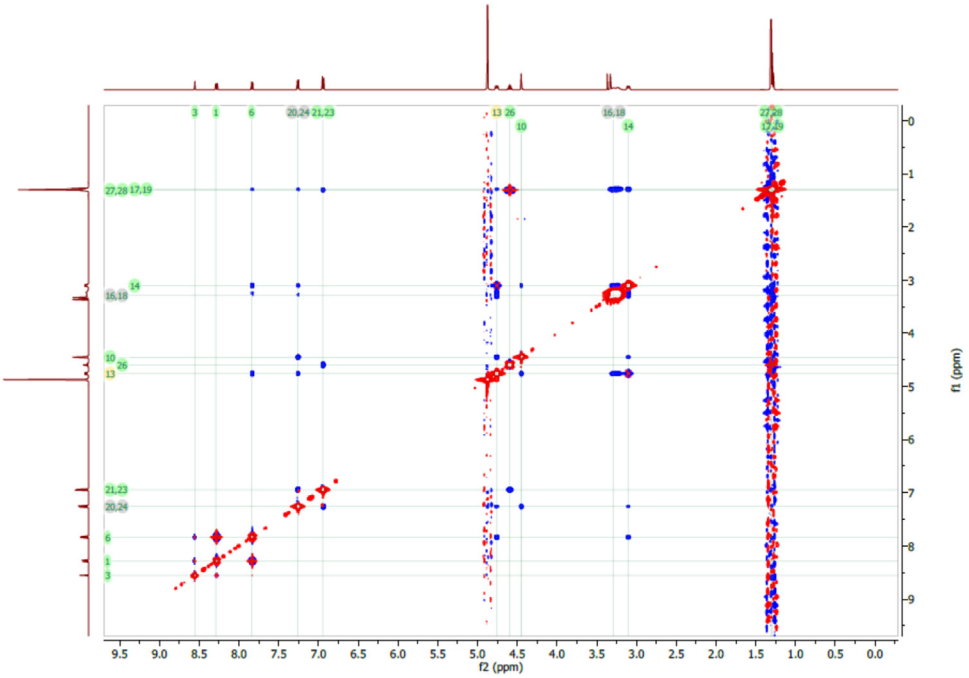
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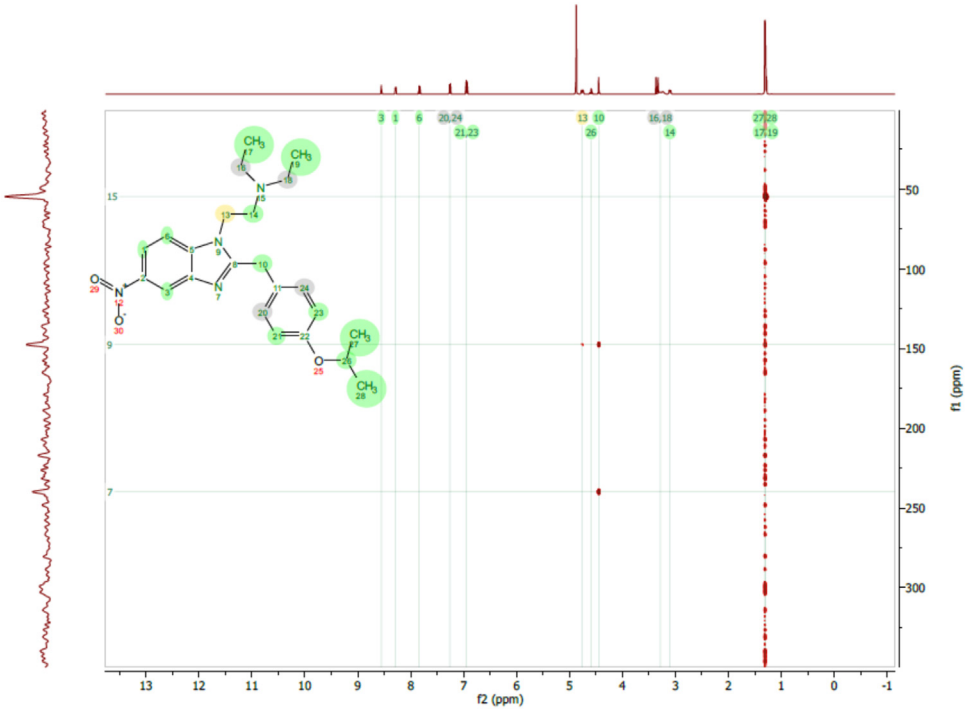
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