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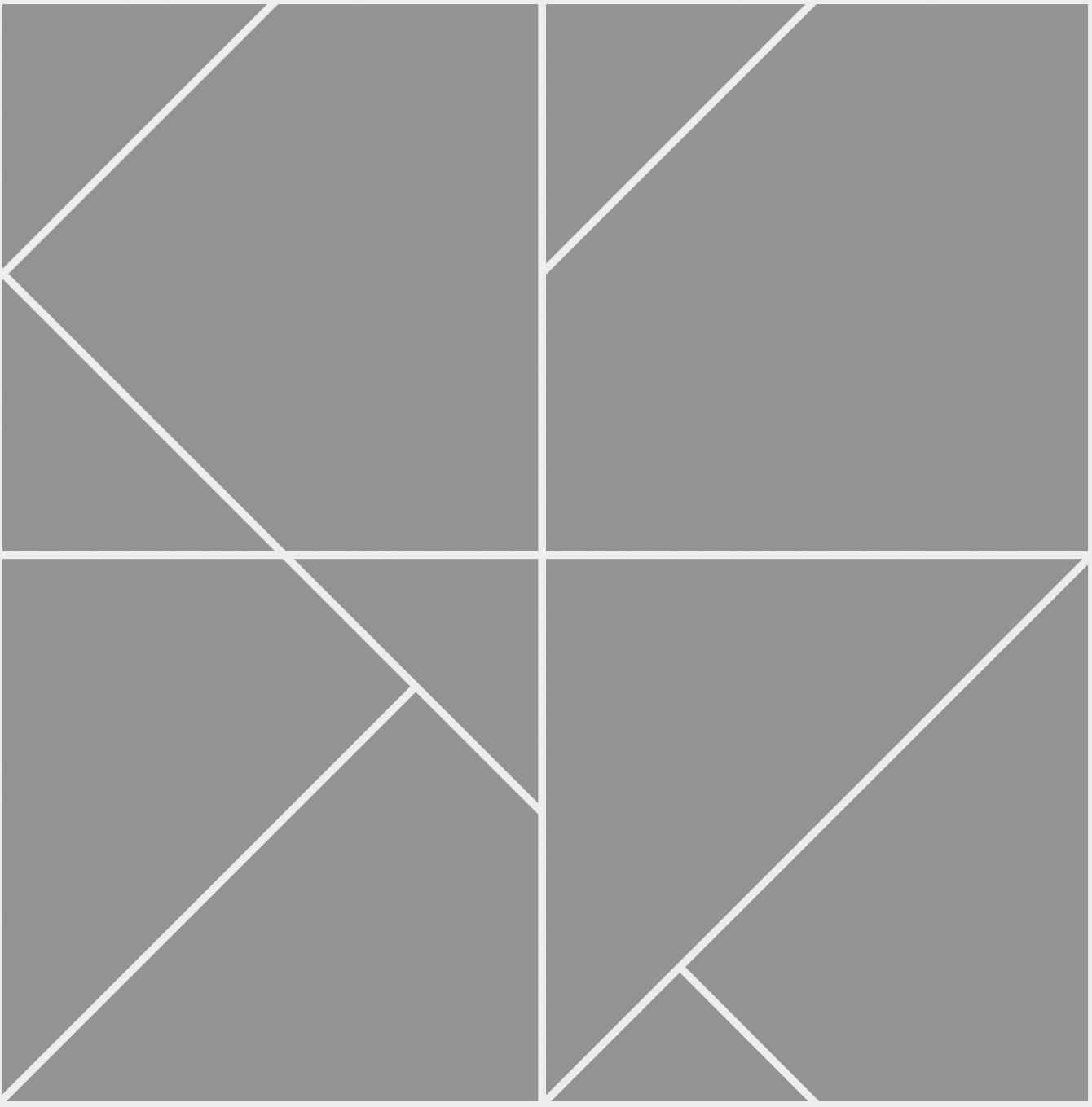

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EDITORIAL

A new journal is born

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In the city of Weimar (Germany), in 1919, Walter Gropius (1883-1969) founded the Bauhaus, a school of art, design and architecture which aimed to combine artistic creativity with industrial production, defying traditional academies. Their motto was 'form follows function' (*Form folgt Funktion*), reflecting the idea that the design of an object or building should be guided by its practical usefulness, therefore getting rid of any unnecessary embellishment. As publishers, we have followed this concept not only in the minimalist aesthetic of our publications, but also as a guideline that directs all our processes.

We are pleased to present a scientific journal that seeks to disseminate information in the field of Clinical Toxicology. We intend to provide a platform where all aspects regarding an intoxicated patient are addressed, as well as breakthroughs in toxicological diagnostics and therapy. Fostering the dissemination of high-quality research allows toxicologists to make informed clinical decisions, so as to improve patient safety and care.

The journal is free to access, edited through peer review process and published in English. It is fully digital, committing to the preservation of natural resources and the environment. It also subscribes to the *Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals* of the International Committee of Medical Journal Editors' (ICMJE) and to the ethical guidelines established by the Committee on Publication Ethics (COPE).

In the last years, especially during the COVID-19 pandemic, we have witnessed an alarming rise in the number of intoxications due to isolation, the increase in mental illness, and economic inequality. The health crisis has made visible the weaknesses and challenges we face not only as individuals, but as a community. This awkward mirror demands we take decisive and collaborative action to safeguard the wellbeing of the population and to foster a society that is safer and more aware of its future.

We thank all the editors, reviewers (both local and foreign) and assistants for all their experience and their commitment when preparing the articles. This allows us to offer a publication of the utmost scientific quality, ensuring that we abide by the international editorial standards. Additionally, we would like to highlight the selfless support of our families, teachers and colleagues, who have trusted this project since its inception.

We will work together to improve how we understand and handle intoxications, therefore protecting and promoting health in our community. A new journal is born to inspire toxicologists. Welcome to The Poison®.

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REVIEW

Novel psychoactive substances (NPS): Update, issues and challenges

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Abstract. Novel psychoactive substances (NPS), which emerged at the beginning of the 2000s have become a serious and internationally complex situation. These emerging drugs are mostly synthetic and analog derivatives of existing controlled drugs. They are sold ubiquitously not only on traditional markets but also through the Deep Web/Dark Web, where their regulation is even harder. The most important groups comprise synthetic cannabimimetics, cathinones and psychodysleptic phenethylamines. The variety and evolution of this kind of substances has given rise to a continuous analytic challenge for their detection and quantification in seizures and biological fluids. Instrumental chromatographic techniques such as HPLC-DAD, GC-MS, LC-MS, UPLC-MS-MS have provided considerable advantages, although there is still a paucity of standards available and lack of awareness of the metabolites that might be generated in vivo. In this review we will provide an overview of each group of substances and their toxicity, as well as of the analytical methods used in seizures and biological matrices.

Key words: *Novel psychoactive substances; NPS; designer substances; synthetic cannabimimetics; cathinones; phenethylamines; recreational benzodiazepines; Opiomimetics.*

The United Nations Office on Drugs and Crime (UNODC) defines *novel psychoactive substances* (NPS) or *emerging drugs* as those substances of ab use, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat.[1] They are known as "designer drugs", "legal substances" or "legal highs" on the illicit market.

Most of these drugs come from tests performed licitly by researchers of the pharmaceutical industry searching for new "drugs" for the treatment of various illnesses. When a multitude of structures are synthesized (with chemical variations), they are screened thus separating a potentially useful set of substances from those which are useless. Nevertheless, some of the former are on the way to be discarded due to technical problems or possible side and/or harmful effects justifying their exclusion from the most advanced pharmacological research. Their therapeutic value is determined through affinity assays by central nervous system (CNS) receptors or by modification of biochemical reactions in the nerve cell to achieve the efficiency pursued.[2]

We may redefine "designer drugs" (within the context of their abuse) as: *"those non-natural compounds, synthesized in laboratories, of highly diverse structures and initially unregulated, of limited or non-existent therapeutic value, administered to humans to modify the normal functioning of specific and central cell receptors, either by direct action on them or by modification of indirect biochemical reactions leading to their activation, in order to achieve psychostimulation with alteration of the perception of reality, thus causing behavioral changes and unusual moods"* (author's own definition).

In Fig. 1 we can observe the beginning of semisynthetic drugs, from heroin to laboratory-made synthetic drugs (nitazenes, fentanyls, among others) and the new semisynthetic substances, such as benzopyran derivatives HHC, HHP and THC derivatives which have recently appeared. The year 2000 was a milestone for NPS, with the publication of the first reports on cannabimimetics.

Latest reports on the alarming increase of NPS

In 2023, the number of NPS reported to the UNODC increased to 1230, while the number of countries reached 141. This trend is illustrated in Table 1 and Fig. 2. However,

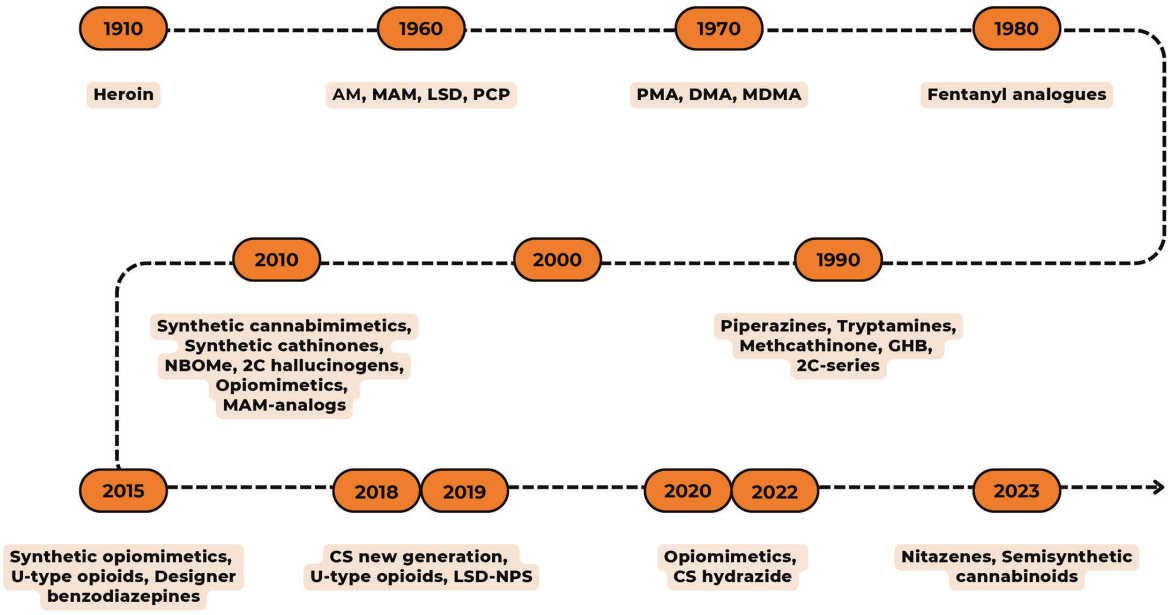

Figure 1. Historical course of the production of semisynthetic and synthetic drugs (NPS and non-NPS).

TABLE 1. Evolution of NPS and number of countries reporting seizures in their territories for the first time.

Year	Number of NPS reported	Total number of countries
2009	166	65
2013	348	90
2014	541	95
2016	644	102
2018	888	118
2022	1,124	134
2023	1,230	141

new structures continue appearing month after month, a clear proof of the globalization of a problem with unforeseeable consequences.[3]

Marketed as "legal drugs" or "designer drugs" and sold openly (including via the internet), they are surpassing the efforts to impose an international control. Criminals hastened to enter this lucrative market. As they are not subject to controls, they are easily introduced in the countries, even by declaring them with names of chemicals customarily used in many industries.[4] Since the toxicity of most of them has not been sufficiently tested, their harm potential is even greater than that of some traditional drugs.

New approaches on the problem of designer drugs

In this section it is worth mentioning the opinion of those who argue that penalization is *per se* a motive for fostering greater drug marketing and consumption. It is paradoxically verified that the use and abuse of these "legal" design drugs, initially non-controlled, continuously and alarmingly increase worldwide.

What is more dramatic is that a wide range of these drugs has been used in sexual assaults or chemical submission. Victims are exposed to substances that cause temporary memory loss and/or distorted sense of time and space, thus being left passively subject to the perpetrator or sex offender, not subsequently retaining any image of the event, as evidenced in the case of GHB (gamma hydroxybutyrate).

"Modus operandi" of design drug dealers

As defined above, an NPS usually appears as a modification of an unregulated substance to prevent authorities from considering it illicit. The spreading of a design drug might be conceived as a cyclical series of events:

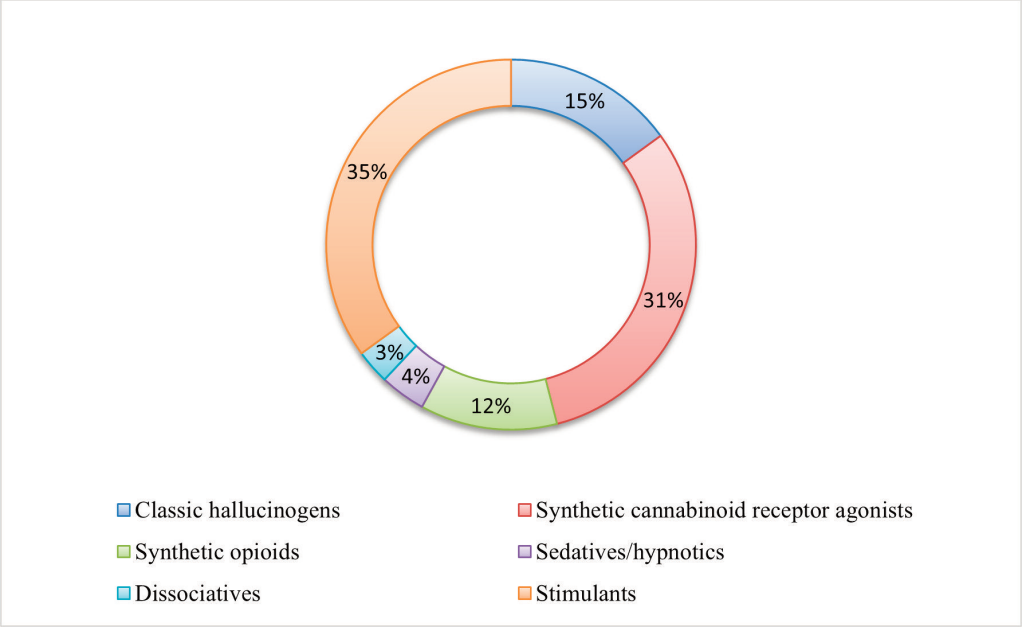

Figure 2. Classification by compound families.

1. Synthesis of a chemical substance which may act similarly to a controlled substance

2. Subsequently the chemical products are marketed as "legal" alternatives to an illicit drug or as "research chemicals, not for human consumption"

3. A small number of users experimenting with the drug report on their experiences via the internet: blogs, forums, videos, among others. If the results are positive, more and more people start using it, thus becoming more popular

4. When laws are updated and this new drug is included on the list of "illegal drugs", the cycle restarts

A new problem arises regarding the control of the sale and distribution of this kind of drugs, since they are chemically modified substances whose structure differs from that of those classified as "illegal substances", thus hindering their identification.[5]

NPS CLASSIFICATION AND DESCRIPTION

Synthetic cannabimimetics (SC)

Overview. A priori the term *cannabimimetic* would seem to be better related to the nature of these compounds, since they do not share the basic, psychoactive structure of cannabis.[2] The term *cannabinoid* would be more in line with those synthetic compounds that have a main chemical nucleus or benzopyran core (e.g. Hu) typical of THC (tetrahydrocannabinol) or CBD (cannabidiol). Most of them are named through combinations of letters and numbers, and the latest as acronyms of structural parts of the molecule (e.g. MB PINACA, where "inaca" corresponds to indazol-carboxamides).[6] Fig. 3 shows the structure of "K2" or JWH-018.[7]

Some compounds appearing on the black market of cannabimimetics do not directly activate the specific cannabinoid receptors CB_1 and CB_2, but they rather inhibit the enzymes that catalyze chemical reactions (e.g. URB447, URB937, which inhibit anandamide degradation) generating toxicity due to the increase in the concentration of endocannabinoid anandamide.

Until the beginning of 2023 SCs amounted to about 381 compounds.[3] Only in Japan, 858 synthetic cannabimimetics have been included on their lists of controlled substances.[8] Most researchers are of the opinion that the international

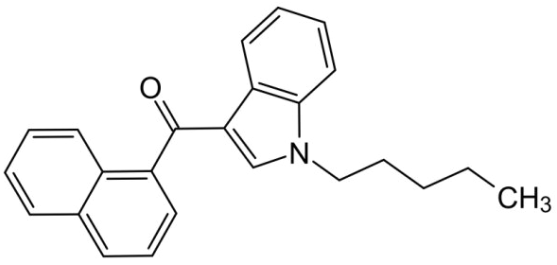

Figure 3. Chemical structure of JWH-018 (K2) (Credits: Mattern R).

situation is critical not only because of the continuous mutation of the active compounds added to the illicit preparations but also due to the scarcity of short and long-term toxicological information. For instance, there is no information regarding the affinity constant for receptor (K_i) for many of these substances. An equally worrying situation are the episodes of acute intoxication they caused among various consumers (even infrequent drug users). Several reports inform about cases of acute psychosis and even acute myocardial infarction among teenagers without previous heart disease.[6,9]

Toxicity. One of the greatest problems of these substances is the lack of information available, particularly regarding their toxicokinetics and toxicodynamics in the human body. Physiological and psychological effects start at minute 10, with a peak at minute 30. Users expound that they experience an effect that is similar or greater to the one presented when smoking cannabis. Nevertheless, many cases of intoxication with severe symptoms have been reported.

In 2011, Dr Colin Kane (pediatric cardiologist in UT Southwestern & Children's Medical Center in Dallas) and his team published an alarming report on three cases of intoxication in 16-year-old teenagers who had used K2 herbal blend.[9] Chest pain, acute myocardial infarction, convulsions, anxiety attacks, increased heart rate and blood pressure, vomiting and disorientation were described. Indazol-carboxamides can also cause hypothermia.

Analytical methods used in seized materials and biological matrices. UNODC's Manual[1,10,11], Namera et al[12] and Worob & Wenthu's reviews[6] feature interesting references. Among them we find the following:

a. Colorimetric assays: the Duquenois–Levine color test, which is widely used in field tests to identify classical cannabinoids such as Δ9-tetrahydrocannabinol, is negative for the synthetic cannabimimetics. The van Urk color test, which is used to identify indole-containing structures, is also negative for these compounds. The use of 2,4-dinitrophenylhydrazine, which reacts with a keto moiety, is capable of reacting with synthetic cannabimimetics, such as the naphthoylindole, phenylacetylindole, benzoylindole, and cyclopropylindole classes, either in powder form or adsorbed onto plant material, and a positive test solution turns from yellow to orange. Nevertheless, the limit of detection has not been stated.[12]

b. Chromatographic and spectrometric methods: mass spectra in GC-MS analysis reflect acceptably the structures of SCs. Naphthoylindole fragmentation pathways have been well studied by gas chromatography-mass spectrometry (GC-MS). Therefore, the identification of these compounds is facilitated by the comparison of the spectra with commercial and open databases. UNODC has published an online open-access manual, with a description of instrumental analytical techniques: gas chromatography-flame ionization detector (GC-FID), GC-MS, liquid-chromatography-mass spectrometry (LC-MS-MS) and a detailed procedure for the identification and quantification of SCs.[10,11] Regarding spectroscopic techniques, such as Fourier-transform infrared spectroscopy (FTIR), they present the analytic difficulty of the herbal base to which SCs are added. Nevertheless, provided there is a previous extraction, i.e. separated from the vegetable matrix, they may be analyzed quite easily. Even so, the spectrum of these extracts differs from that of the one obtained from the pure substance. In general, attenuated total reflectance-Fourier-transform infrared (ATR-FTIR) techniques are suitable for the analysis of isomers in those solids lawfully seized.

Cathinone-related synthetic drugs (psychoactive khat derivatives)

Overview. This is the group of drugs that has had the most continuous use until now. They are synthetic compounds with a chemical structure related to *cathinone,* an alkaloid found in the khat plant (Fig. 4 and 5)[13,14,15], quite similar to amphetamines. According to the report of the National Institute on Drug Abuse-National Institute of Health (NIDA-NIH), the name *"*bath salts*"* given to this kind of substances (due to their macroscopic appearance) may refer to a single substance or a mix of them.

"Bath salts" generally appear as a crystalline white or brown powder (Fig. 6)[16] and are sold in plastic bags or alu-

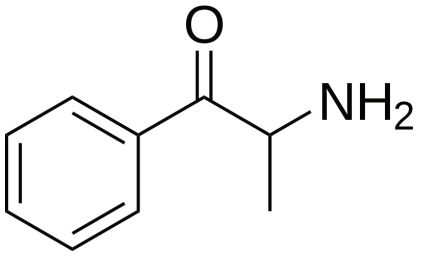

Figure 4. Basic structure of cathinone (Credits: Harbin).

(a) (b)

Figure 5. Carrying "Khat" in a Bangladeshi market (a) and a man dividing the plant into bunches (b) (Credits: Galib E and Frodesiak A respectively).

minum foil packages labelled as "not for human consumption". They are sold on the internet (Deep Web/ Dark Web) and in drug paraphernalia stores under a variety of names such as "Ivory Wave", "Vanilla Sky", "Cloud Nine", among others.

Synthetic cathinones commonly found in the "bath salts" include 3,4-methylenedioxypyrovalerone (MDPV), butylone and eutylone, but there are many others. How these substances affect human brain and their properties, which may vary between one cathinone and the other, is still to be known. They are chemically similar to amphetamines, such as methamphetamine (MA) and ecstasy (MDMA). It is accepted that the methylenedioxy substitution on the aromatic ring gives the drug its entantogenic or empathogenic characteristic. *First-generation* synthetic cathinones derive from substitutions in cathinone structure. However, *second-generation* cathinones have recently appeared, characterized mainly by halogen substitution in the structures of the primary compounds.

Toxicity. "Bath salts" have been marketed as cheap and, not until long, legal substitutes for stimulants, such as amphetamines and cocaine. A study found that MDPV increases dopamine levels in the brain just as cocaine, but it is at least 10 times more powerful.[17] Psychomotor agitation is common since they increase the dopamine (DA) levels in the brain circuits that regulate reward and motion. DA surges in these circuits result in feelings of euphoria and a rise in activity thus increasing heart rate and blood pressure. Incidentally, patients may suffer from dehydration, rhabdomyolysis and renal failure. They are frequently abused and highly addictive, and may lead to death.

Their hallucinatory effects frequently reported are similar to those of the other drugs such as lysergic acid diethylamide (LSD) or MDMA, which increase serotonin levels (5-HT). This can result in serious psychiatric symptoms such as paranoia and panic attacks. Self-harm episodes are not uncommon, as a result of their psychiatric effects and a reduced sensitivity to pain.

The dangers involved in "bath salts" are aggravated by the fact that these products usually contain other ingredients of unknown chemical nature, which may have their own toxic effects. Besides, drug users who believe are buying other substances such as MDMA, may be in danger of receiving synthetic cathinones instead. For example, it has been frequently found that MDMA has been substituted with mephedrone in the tablets sold as ecstasy in the Netherlands.

Analytical methods used in seized materials and biological matrices. Nowadays there are various methods to analyze this kind of substances:

Figure 6. Macroscopic characteristics of mephedrone (Credits: DMTrott).

a. *Colorimetric assays:* the Marquis reagent, which reacts with all nitrogen-containing drugs, is negative for cathinone and mephedrone, but is positive for cathinone analogs that have a methylenedioxy moiety in each molecule, such as MDPV. The cathinone analogs with a methylenedioxy moiety also react with the Chen reagent, which changes to orange in positive tests. However, the limit of detection (LOD) has not been reported. We[18] have modified a method[19] based on the neocuproine reaction with Cu^{+2} salts, thus obtaining an orange color with cathinones, with very few interferences, even of amphetamine derivatives.

b. Instrumental methods: the methods published for the cathinone tests in biological materials generally use a basic extraction (LLE) for cathinones of biological materials. Chromatographic conditions are also simple and do not usually require a special technique. Contributions using LC-MS-MS have been published.[20] Cathinone-detection strategy by LC-MS-MS is almost similar to that of the synthetic cannabinoids; almost all the methods use multiple reaction monitoring (MRM) or selected reaction monitoring (SRM) for sensitive determination. Likewise, by GC-MS or else by time-of-flight-mass spectrometer (ToF-MS) or tandem mass spectrometry (MS/MS) to study the molecular structure. Finally, NMR spectroscopy is used to elucidate molecular structures.

Psychodysleptic phenethylamines

Overview. A little more than ten years ago, a new group of designer substances derived from phenethylamine called NBOMe, "N-bomb" or "Smile" were introduced on the illicit market. They are a powerful family of 5HT2A serotonin receptor agonists, therefore they increase 5-HT activity.

The name "NBOMe" derives from the initials of the chemical groups comprising the structure of this family of drugs: **N**: nitrogen; **B:** benzyl and **OME**: oxymethyl. 2,5-Dimethoxy-N-(N-methoxybenzyl)phenethylamine (NBOMe) was discovered in 2003 and was synthesized as a radioactive tracer for positron emission tomography (PET) in Copenhagen (Denmark). Since it was the first full agonist radioligand for the 5-HT2A receptor, it was promising as a more functional marker of these receptors, which might be involved in mental disorders such as depression and schizophrenia. They are N-(2-methoxy)benzyl derivatives of the "2C compounds" (2,5-dimethoxyphenethylamines with various substituents at C-4), with 33 variations depending on the substitutions occurring on the molecule. Fig 7. shows the chemical structure of 2C-C-NBOMe, a special type of psychodysleptic phenethylamine.[21] In recent years many demethylated derivatives known as "NBOHs" have been detected.

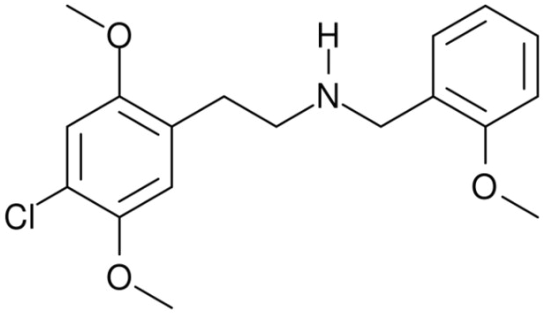

Figure 7. Structure of a special type of psychodysleptic phenethylamine: 2C-C-NBOMe (Credits: C6541).

Formulation and administration. Only active if taken sublingually (also on lips or gums) or intranasally (sniffing). No psychoactivity is present if taken orally. Sold in 0.5-1 mg blotter papers (Fig. 8)[22] which are slightly bigger than those used in the illicit marketing of LSD. They have also been seized as white and crystalline powders.

Toxicity. Among their most relevant effects we should mention sweating, tachycardia, arterial hypertension, psychedelic syndrome, affective lability, distortions in time and sensory perceptions, illusions of movement and impaired judgement. Their clinical effects can be divided into three successive categories from the moment they enter the body[23]:

a. Positive effects ("High"):

- Visual illusions and hallucinations with the open or closed eyes (seeing paths, color changes, fractals, brightness)
- Euphoria, mood boosting, good humor, laughter
- Mental and physical stimulation; associative and creative thoughts; greater awareness and appreciation
- Spiritual experiences: inner peace, introspection, ecstasy
- Sexual thoughts: feelings of love and empathy (entantogenic or empathogenic effect)

b. Neutral effects:

- Generalized change in the state of awareness
- Mydriasis
- Difficulty concentrating
- Unusual body sensations (blushing, piloerection, body energy)
- Changes in time perception
- Slight increase in heart rate
- Yawning

c. Negative effects ("Down", "Comedown" or "Bad trip"). This effect is heightened when the dose is increased. It encompasses:

- Confusion
- Looping
- Nausea
- Insomnia
- Repetitive, recursive and uncontrolled thinking
- Paranoia: fear and panic

Customary doses are: threshold (50-250 μg), low (200-600 μg), median (500-800 μg) and high (700-1500 μg). Taking into account that the doses are in micrograms, measuring the dose-response becomes highly difficult when formulated in powder form. Their effects last between 6 and 10 hours if taken sublingually, or between 4-6 hours if sniffed. The duration of the effects also depends on the dose. The "comedown" takes place between 1 - 4 hours, and the residual effects may last 1 to 7 days. Since the "high" may take 2 hours, the user is subject to the temptation of redosing due to the feeling of lack of effects. Redosing is not recommended, due to the higher risk of toxicity and death. They are highly addictive. It has been proven that in a few months the individual may increase their nightly 1-tablet dose to even 6 to 8, leading to intoxication and even death.

This kind of substance is metabolized mainly by monoamine oxidase enzymes (MAO-A and MAO-B), just like 5-HT. Their long-term effects are still unknown, but it is known that they generate a rapid cross-tolerance with other drugs, such as LSD.

Figure 8. "NBOMe" blotter papers (Credits: Heisenbug).

Analytical methods used in seized materials and biological matrices. Chromatographic methods are the most widely used for this purpose: high-performance liquid chromatography (HPLC) or ultra performance liquid chromatography (UPLC) coupled with several detection systems: MS-MS or time-of-flight mass spectrometry (HRToF-MS). LLE system and solid phase extraction (SPE) are widely use as extraction procedure. Some authors developed techniques for the identification and quantification of 25I-NBOMe and 25C-NBOMe in serum from intoxicated subjects. The samples were collected using SPE technique and 25H NBOMe as internal standard. On the other hand, GC-MS has been successfully used in our country for the identification of 25-I-NBOMe. ATR-FTIR techniques have also been described for the analyses of seized blotter papers.[24]

National and international current legal status. Although their use spread in Europe in 2010, nowadays they are present worldwide. Various cases of deaths have been reported among young people, mainly in Europe, Australia and the United States.[25] In 2013, in Chile, they were seized as blotter papers and since the preliminary tests for banned substances did not detect LSD in their analyses (but a substance that did not appear on the list) the product and the dealers were released. In Argentina, the 33 variations of NBOMe were included in the Presidential Decree No 772 of 2015, therefore nowadays it is a family of substances under control. Initially, seizures coming from Argentine Northwest entered the country as 25-I-NBOMe, a short time after being detected in Chile. Finally, 5 years ago NBOHs started to be identified. They are a group of substances prevailing nowadays in our country as well as in the rest of South America.

Designer benzodiazepines

Overview. Designer benzodiazedpines have increased dramatically in the last two years.[27,28] Although benzodiazepines have always had a long history of abuse, the emergence of pharmacologically potent structures for recreational use may be the beginning of a new surge of these sedatives.[29] The chemical structure of diclazepam, brotizolam and quazepam[30,31,32] can be seen in Fig. 9 and 10, as examples of this kind of substances.

The first designer benzodiazepines available on the internet were: diclazepam, flubromazepam and pyrazolam, none of which have been approved for medicinal use in any country.[33] Since 2020 they have been detected together with other NPS, particularly fentanyl derivatives.

Almost all these compounds have been synthesized as drug candidates by different pharmaceutical companies. Their synthesis, as well as the data from animal testing, are

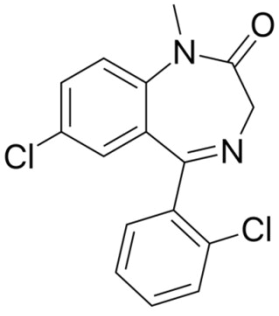

Figure 9. Diclazepam as an example of designer benzodiazepines (Credits: Vaccinationist).

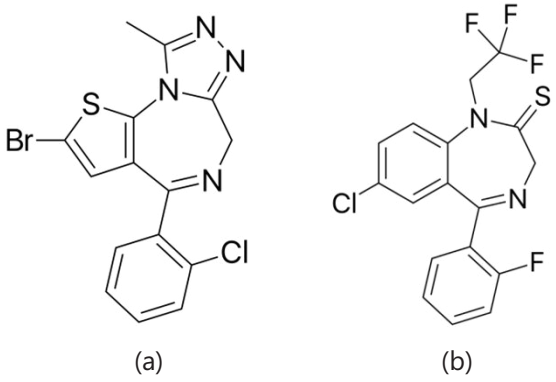

Figure 10. Designer benzodiazepines: some structures detected in recent months. In order: brotizolam (a) and quazepam (b) (Credits: Calvero).

described in the literature, together with many other potentially viable compounds. Their formulations are marketed as pills, scored tablets or blotter papers in various doses, generally attractively colored. Besides, these drugs are also offered as pure powder at prices as low as US$ 0,05-0,10 per dose.

Toxicity. They are highly active and toxic substances. These design psychoactive substances usually have a long half-life, hence, their remarkable residual effects or hangover, which involve an impairment of the higher cognitive and coordination functions. Their widespread availability through internet suppliers and their low price may facilitate the development of addiction among consumers.

Analytical methods used in seized materials and biological matrices. Immunochemical tests applied in clinical cases and in drug seizures are sensitive enough to detect most designer benzodiazepines. However, some of these substances show a high score of cross-reactions with other congeners, e.g. flubromazolam and diclazepam in serum. MS is necessary for the confirmation, particularly due to the fact that the lack of reference standards would not allow to cover the latest designer benzodiazepines that may continue appearing on the recreational market.

Synthetic opiomimetics

Overview. These substances, featuring a highly varied chemical structure, are opioid receptor agonists. Since the beginning of 2010 they have generated an epidemic in the USA, which has for the first time exceeded the heroin overdose deaths, leading the NIH to declare a national emergency. As reference, of the 68,630 opioid-related deaths registered in 2020, 56,516 were synthetic opioids.[34, 35]

(b)

Toxicity. Some effects of synthetic opioids include relaxation, euphoria, pain relief, sedation, confusion, drowsiness, dizziness, nausea, vomiting, urinary retention, miosis, and respiratory depression. Due to the potency of some of them, death is a possible outcome.[36]

Argentinian massive intoxication. In Argentina, at the beginning of 2022, there was a massive intoxication due to a fentanyl derivative. Through the Ministry of Public Safety of the Province of Buenos Aires samples of the substance used were obtained and subsequently sent for testing, confirming it was a mix of cocaine adulterated with carfentanil.[37] The intoxication caused 24 deaths and more than 100 hospitalized victims.[38,39] This massive event, the first worldwide, allowed us to become aware of the analytic difficulties we are facing due to NPS. In this case, the weight ratio of the cocaine in relation to the agent found after deep tests (carfentanil) was very high, therefore the chances of detecting and quantifying it were not promising. We learned that the interaction between emergency physicians, toxicologists and analytical chemists is essential.

On 2nd February at 6 a.m., six male and one female individuals were admitted in the emergency room of a hospital in the west of the province of Buenos Aires. The clinical assessment showed the following signs: miosis, shock, sensory depression, respiratory distress (bradypnea), psychomotor excitement, seizures, and cardiorespiratory arrest. Some even showed certain rigidity like "wooden" chest, typical of opiomimetics such as fentanyl or its derivatives. As the hours passed, the number of cases increased dramatically. At 2 p.m. the epidemiological situation was: 10 deaths and 59 people in intensive care units. All those admitted to hospital had sniffed "a line of cocaine" bought in the suburbs of the province. The photograph (Fig. 11) shows one of the packages found in the clothes of one of those people hospitalized. The victims were treated with naloxone at high doses and with a continuous infusion drip, in some cases, at higher doses than those used for classical opioids.

Figure 11. Photograph of one of the packages seized.

Subsequent tests allowed to prove the presence of carfentanil in doses of 30-60 micrograms per 200 mg of powder sold as cocaine.[39] The first tests consisted in basic analyses through color reactions, high-performance thin layer chromatography (HPTLC), and ultra-performance liquid

Figure 12. Four powder samples seized, and cocaine standard inserted on the plate. We can see at 254 nm a spot at Rf: 0.64 coincident with the cocaine standard. When we applied eosin Y, we couldn´t see any spot attributable to fentanyl or other derivative as carfentanil.

chromatography method with diode array detection (UPLC-DAD), testing positive for cocaine and negative for fentanyl-like structures. Subsequently and due to the lack of positive indicators for fentanyl derivatives, we proceeded to apply NMR and LC-ToF-MS through MRM analysis. In Fig. 12 we can see the detection of cocaine and related compounds through NMR and absence of piperidine derivatives typical of fentanyl compounds.

The workflow is described in detail below:

1. Preliminary analysis by TLC and UPLC-DAD: initially we decided to apply the basic method by TLC.

- Silica stationary phase (Silicagel GF254)
- Mobile phase: methanol-ammonia (100:1.5)
- Standard: Cocaine - LGC©USA
- Plate development and subsequent sequential revealed:
  a) UV 254 nm
  b) Sprayed with eosin Y (recommended by some authors to reveal fentanyl since opiomimetics are suspected, according to the details of clinical toxicological interventions)

2. Samples analysis by UPLC-DAD:

- Column: Acquity UPLC HSS T3 1.8 µm; 2.1 x 100 mm
- Mobil Phase A: water + 0.1 % formic acid
- Mobil Phase B: Acetonitrile

TABLE 2. Gradient.

Time program (min)	Flow rate (ml/min)	%A	%B
Initial	0,4	90	10
0,5	0,4	90	10
9,0	0,4	10	90
9,1	0,4	90	10
12	0,4	90	10

- Column Temp: 35 °C
- Sample Temperature: 15 °C
- Gradient: see Table 2
- Diode Array Detector: Scan 200 to 400 nm, Spectral and Optical Bandwidth 1.2 nm]

3. Validation of method for cocaine determination: we built the calibration curve with the standard at six concentration and calculated the LOD and LOQ, recovery and robustness of the method. Calibration curve was carried out by plotting peak area vs concentration. The least squares linear regression analysis of the data gave us the equation: $y= 11900x + 11.95$ ($r^2 >0.9997$).

LOD = 3 µg/g; LOQ = 10 µg/g; η = 96.8 %

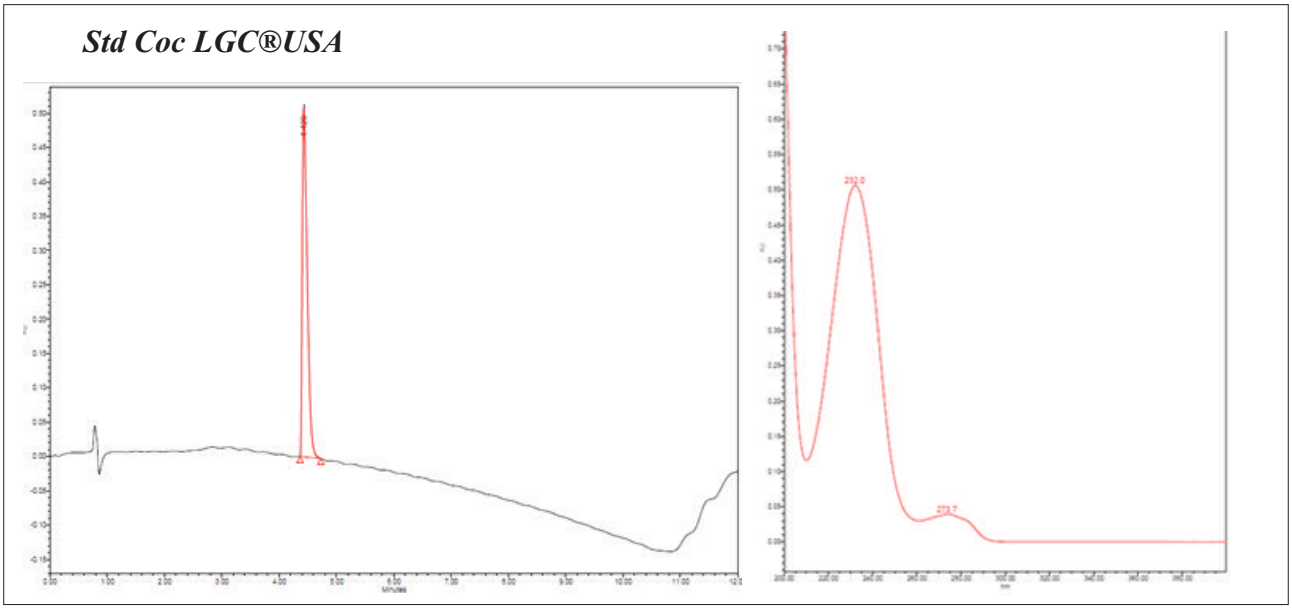

Figure 13. Chromatogram and UV spectra of cocaine standard.

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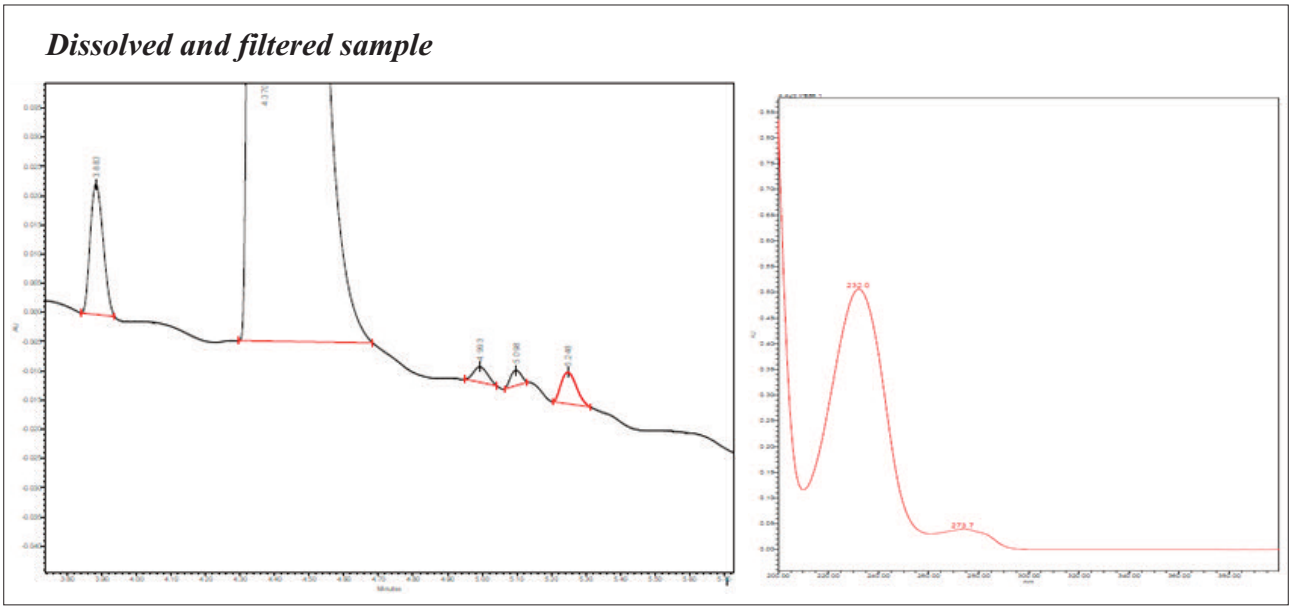

Figure 14. Chromatogram and UV spectra of one powder sample seized.

The conclusions that we were able to draw from what was done in the UHPLC are the following:

a) The samples identified as victim and Lab seized are equivalent, their chromatographic profile is identical
b) In all analyzed samples a high concentration of cocaine and the presence of four unidentifiable chromatographic peaks were observed. The peak retention time 3.88 minutes by its spectrum behaves like a metabolite or substance related to cocaine
c) The quantitative data showed a 51.6% cocaine content with respect to the total weight of the sample

4. Analysis by NMR:

4.1 Sample preparation: the sample was suspended in 1 ml of chloroform-d, shaken, and centrifuged. The solid was subjected to a second wash with chloroform, obtaining the insoluble fraction in chloroform (Fraction I). The chloroform solution was taken to dryness obtaining the soluble fraction (Fraction S).

4.2 Preliminary analysis: it was performed by nuclear magnetic resonance of ^{1}H and ^{13}C to identify major components. Spectra were measured on a Bruker Avance Neo 500 spectrometer (^{1}H 500 MHz, ^{13}C 125 MHz). Directed by Prof. Dr. G. Burton, UMYMFOR-CONICET of the University of Buenos Aires and his team). Fraction S: the spectra were measured in deuterochloroform. NMR spectra showed the characteristic signals of *cocaine hydrochloride* and small amounts of *cis and trans cinnamoyl ecgonine methyl ester hydrochloride*, a natural product that usually accompanies cocaine. Fraction I: spectra were measured in dimethylsulfoxide-d_6 solution. The main component was identified as mannitol. A small remnant of *cocaine and cinnamyl ecgonine methyl ester* is also observed.

5. Analysis by LC ToF-MS (MRM): considering the clinical history, it was decided to use in the first instance a chromatographic method capable of detecting fentanyl and its analogs in the Fraction I, enriched in said components, thus reducing interference. The sample was analyzed in a Bruker MicroToF-Q II mass spectrometer coupled with an Agilent 1200 liquid chromatograph.

Chromatographic conditions:

- Column: Phenomenex Luna 3 µm C18(2) 100 A 100 x 2.0 mm
- Solution A: ammonium formate 5mM adjusted pH 3 with formic cid
- Solution B: 0.1% formic acid in acetonitrile
- Detection: ESI +
- Flow: 0.15 ml/min
- Inyection vol: 20 µl
- Temperature: 25°C
- Running time: 15 min
- Elution program (see Table 3)

Table 3. Elution program.

Time (min)	Disolution A	Disolution B
0,00	87	13
0,50	87	13
10,00	50	50
10,75	5	95
12,25	5	95
12,50	87	13
15,00	87	13

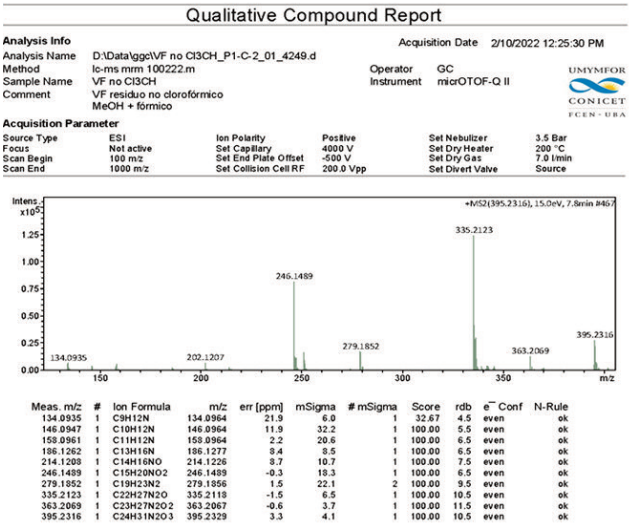

Figure 16. MS spectra (MRM) of signal at Rt 7.8 min.

We obtained the following chromatogram:

It was performed by MRM and MS/MS fragmentation analysis. The carfentanil characteristics fragments are shown in Fig. 16. The structure of each observed ion is presented,

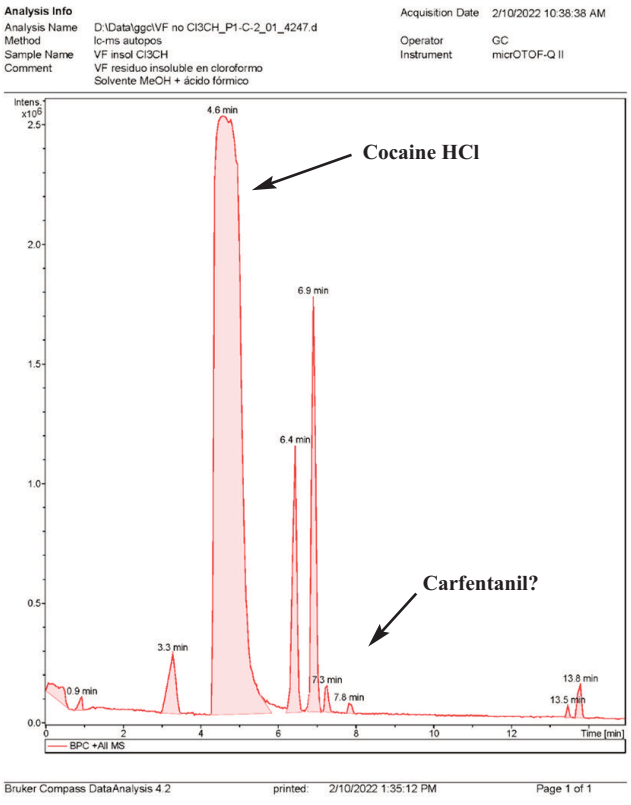

Figure 15. Chromatogram obtained in LC-MS system.

the exact mass measured, and the mass calculated for the formula. In all cases, the isotopic distribution according to the elements present in the formula is observed. In the following spectrum obtained from one of the analyzed samples we can observe the m/z ions obtained as exact mass, compared with the *Waters Corp*. database and the analysis of the ions, with their exact masses. Note the coincidence to the third decimal place of the mass.

6.1 Analysis in biological samples

In the urine of a fatal victim who consumed a line of more than 400 mg, only a signal could be detected by GC-MS that could correspond to carfentanil in non-quantifiable

Table 4. Molecular formulas assigned based on the exact mass value and the isotopic value compared to the calculated values.

RT	m/z	Fórmula (M+H)	Compound
3,3	290,1380	$C_{16}H_{20}NO_4$ (290,1387)	Benzoylecgonine
4,6	304,1538	$C_{17}H_{22}NO_4$ (304,1543)	Cocaine
6,4	330,1644	$C_{19}H_{29}NO_4$ (330,1700)	Cinnamylcocoine (cis/trans)
6,9	330,1693	$C_{19}H_{19}NO_4$ (330,1700)	
7,3	330,1705(+2)	$C_{38}H_{48}N_2O_8$ (330,1700)$[M+2H]^{+2}$	Truxilline
7,8	395,2332	$C_{24}H_{31}N_2O_3$ (395,2329)	**Carfentanil?**

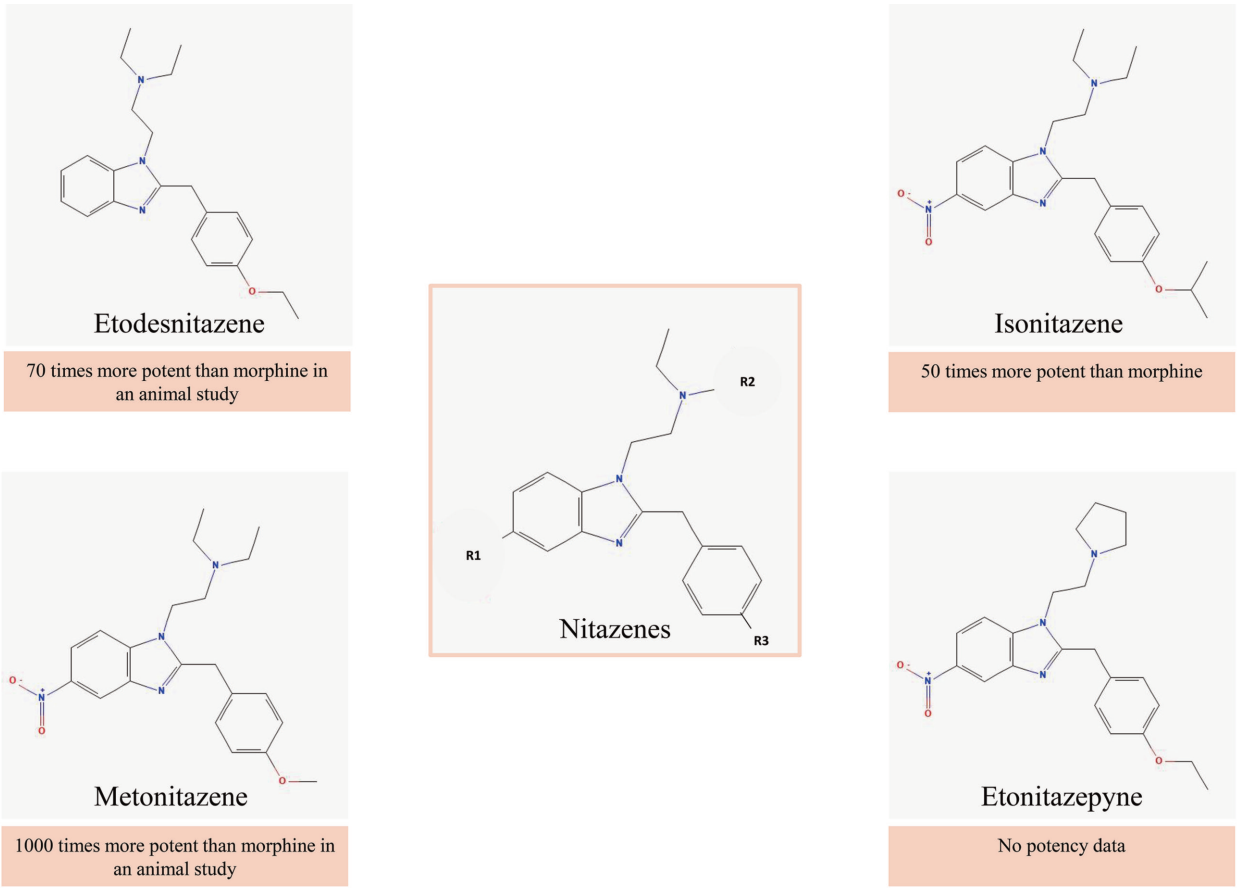

Figure 17. Basic structures of nitazenes and some derivatives that have recently appeared on the illicit market. Please notice that metonitazene is ten times more potent than fentanyl (1000 times higher than morphine) (Source: Pubchem).

values. In other cases, it could not be detected with this methodology, for the reasons already explained in the introduction. It was not possible to analyze the blood and urine of the victims by the methods listed in item 5: LC-ToF-MS/MS.

Nowadays, the twenty fentanyl derivatives are being slowly substituted by a new family of NPS with benzimidazole structure: *nitazenes*.

Nitazenes. Metonitazene and isotonitazene belong to this new kind of synthetic opioids whose structural core is benzimidazole (Fig. 17)[40-44]. They were developed in the 1950s by Ciba-Geigy as painkillers. These agents have been object of recent research as synthetic opioids have become more frequent. However, to date, the data about the prevalence of designer opiomimetics is incomplete, since the traditional post-mortem toxicology tests may not be sensitive to detect these compounds in such low concentrations, particularly when the blood is drawn from a peripheral site instead of central tissues such as the brain, the lungs or the heart.

As with all opioids, respiratory depression is a major risk factor in overdose and even a "wooden" chest may occur. A possible treatment for respiratory depression is the use of naloxone and for muscle rigidity benzodiazepines such as diazepam.[40]

CONCLUSIONS

The varied chemical nature of the different families of NPS constitute an enormous obstacle when it comes to basic and instrumental analysis. The scientific information regarding the level of toxicity of each family and even between the compounds of each group is scarce. Nevertheless, the challenge is not over: new structures, a variety of mixes and assorted adulterants which will make toxicology and analytic clinical assessment even harder are on the horizon.

Declaration of interest
The author declares no conflicts of interest.

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ORIGINAL ARTICLE

Accidental ingestion of coumarin rodenticides: A retrospective study of 139 patients in a pediatric hospital

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Abstract. Coumarin-derived rodenticides are currently the most popular pesticides to control rodents. The accidental ingestion (AI) of these compounds in pediatric patients is frequently a reason for consultations. This is a descriptive retrospective study based on data gathered from the medical records (MRs) of patients seen on-site at the "Superiora Sor María Ludovica" Interzonal Acute Hospital Specialized in Pediatrics in the course of 6 years and 9 months. For this study, 139 MRs of patients between 0 and 14 years of age seen in the Emergency Service and in outpatient care were considered. None of the patients showed at the time of admission signs or symptoms compatible with hemorrhage or irregularities in their coagulation tests, except for two patients. In line with the bibliography consulted, most AI in pediatrics do not have any relevant clinical effect. There is no formal prescription for complementary biochemical studies for patients under 6 years of age with AI of coumarin rodenticides. By means of a thorough anamnesis, the physician will be able to detect those patients that require special care, coagulograms and clinical monitoring with alarm instructions aimed at the appearance of bleedings.

Key words: *Accidental ingestion; Pediatrics; Dicoumarin rodenticides; Superwarfarin; Vitamin K.*

Most consultations in Toxicology are related to the accidental ingestion (AI) of drugs or household products, and have low or no toxicity, either due to the low dosage ingested or to the lack of toxicity of the substance. Among the products available in the household, coumarin rodenticides (both warfarin and superwarfarin-type) are the most common compounds used in rodent control and are rarely related to medically relevant poisoning.[1]

In our field there is no up-to-date epidemiologic data. Therefore, provided below is the detail of the main characteristics of patients seen on-site at the "Superiora Sor María Ludovica" Interzonal Acute Hospital Specialized in Pediatrics (SSMLSP), in the city of La Plata (Buenos Aires, Argentina), regarding AI of coumarin rodenticide in the course of 6 years and 9 months.

MATERIALS AND METHODS

Descriptive retrospective study based on data gathered from the medical records (MRs) of patients seen on-site at the SSMLSP between January 1, 2017 and September 30, 2023. The information was collected from the MRs taken by clinical toxicologists at the Toxicology Service of the aforementioned hospital. 139 MRs of patients presenting AI of coumarin rodenticides between the ages of 0 and 14 who made on-site consultations, whether through the Emergency Service (ES) or outpatient care (OC). A search of available bibliography was performed in virtual databases such as PubMed, TripDataBase, Cochrane, Google Scholar y SciELO, scientific dissemination books, and consensus documents. The keywords used (both in English and Spanish) were: "rodenticides", "anticoagulants", "antithrombotics", "superwarfarin", "warfarin", "poisonings", "accidental inges-

tion", and "pediatrics". The results in both languages were arranged in order of importance and 20 scientific articles were selected including case reports, systematic reviews, consensus and clinical practice guidelines.

RESULTS

The qualitative variables are presented in absolute numbers and in percentages. Out of the 139 patients, 85 (61.1%) were male, 52 (37.4%) were female and 2 patients (1.4%) no data. The age average was 26.7 months, the youngest being 8 months old and the oldest 8 years of age. 124 patients (89%) were seen in the ES and 15 (10.7%) patients in OC. Out of those who visited the ES, 71 patients (58.2%) did it within 2 hours of the ingestion, 8 (6.4%) between 2 and 6 hours, and 6 (4.8%) after 12 hours of the ingestion. There is no data for this variable for 38 patients (30.6%).

Regarding medical treatment, a decontamination procedure with activated charcoal (AC) was performed on 76 patients (61.2%). Tests were performed on 81 patients (58.2%) upon their admission, including a hemogram and coagulogram with prothrombin time (PT) and activated partial thromboplastin clotting time (KPTT). The normality of the PT was tested using the Kolmogorov-Smirnov test. The median was 98 and the interquartile range was between 86-100. The PT median was 91.87 with a standard deviation (SD) of 11.57. Out of this percentage, 50 patients (35.9%) were monitored with a coagulogram after 48 hours of the ingestion, with an average PT of 86.5 and SD of 10.9. 29 patients (20.8%) continued with the examinations after one week, with an average PT of 87 and SD of 7.6. Of these patients, 15 were examined after one month with an average PT of 88.2 and SD of 10.6. Only two patients (1.5%) showed irregularities in their PT upon their admission, successfully treated with the prescription of a dose of vitamin K (VK).

None of the patients showed upon their admission or afterwards any sign or symptoms compatible with hemorrhage. Out of all the patients, 90 (65%) attended the postliminary examinations. These examinations were performed 48 hours, one week and one month after the first consultation. The remaining 49 patients (35%) did not attend the appointments or contact was lost.

DISCUSSION

General aspects

Rodenticides are pesticides made for killing rodents. Three species are considered worldwide plagues: 1) the "brown rat", "sewer rat" or *Rattus norvegicus*, 2) the "black rat" or *Rattus rattus*, and 3) the "house mouse" or *Mus musculu*. They have adapted very well to the urban environment, being related to numerous sanitary issues.[2] A wide variety of rodenticides is used to control them, which pose a potential health risk. Humans can also suffer their effects, being that they are chemicals specifically designed to kill mammals, and both rodents and humans live in the same environment. Rodents have developed a resistance against classic rodenticides, which is why new substances have been created which boast more power, effectiveness and toxicity.[3]

Rodenticides are classified into three groups: gaseous, mineral, and organic. Examples of *gaseous* rodenticides are hydrogen cyanide and methyl bromide. *Mineral* rodenticides include arsenic, phosphorus, thallium, barium and fluorine. Finally, *organic* rodenticides include strychnine and coumarin-based compounds, both warfarin and superwarfarin-type. The latter are VK antagonists, widely used for rodent control worldwide.[4] In Argentina there are 48 formulas available for professional use and 6 sold over-the-counter of warfarin and superwarfarin rodenticides. They are registered in the National Administration of Drugs, Food and Medical Devices (ANMAT, by the Spanish acronym). In professional-grade formulas, 46.2% contain 0.005% brodifacoum, while 40.7% contain bromadiolone at an equal concentration. The rest contain flocoumafen, difetialone, difenacuom or warfarin. Most of those available over-the-counter (66.6%) contain 0.005% Bromadiolone. These rodenticides are sold as paraffin-coated baits, pellets and small seeds.[5] A liquid formula sold in the informal market has been identified, which had hydroxycoumarin as an active ingredient. It was banned by ANMAT in 2018.[6]

The origin of rodenticides can be traced back to 1939 with the identification of bis-hydroxywarfarin or dicoumarol, after hemorrhagic disorders (with a decrease in plasma prothrombin levels) were observed in bovines that had ingested *Melilotus albus* or "sweet clover". This in turn gave way to the later synthesis of warfarin, commercially available from 1955.[7,8] Superwarfarins, a group comprised of 4-hydroxycoumarins and indandiones, were produced as a result of the resistance to the warfarin developed by rodents. The 4-hydroxycoumarins used today are difenacoum, brodifacoum, bromadiolone, and coumatetralyl. Indandiones comprise chlorophacinone, diphenadione, and pindone. All of them have a two-ring structure similar to warfarin.

Toxicokinetics

The oral bioavailability of warfarin is close to 100%, it permeates the blood–brain barrier and it is distributed in the liver, spleen, lungs, and kidneys, with a half-life of 20 to 60 hours. Its duration of action is 2 to 5 days and 99% circulates bound to proteins, with a low volume of distribution. It is metabolized in the liver and excreted in the urine.[10]

Although superwarfarin is mainly absorbed orally, absorption through the skin and via inhalation has also been observed. It is highly fat-soluble (it accumulates in the liver and adipose tissue) and it undergoes enterohepatic circulation. It is excreted in the urine and fecal matter. Superwarfarin is 100 times more potent than warfarin, with a more prolonged action (even after just one dose). The toxic dose of brodifacoum is 0.1 to 0.27 mg/kg, with a half-life of 114 days; for bromadiolone it is 0.17 mg/kg, with a half-life of 28 to 318 days.[7] It has been observed that 1 mg of the active ingredient of brodifacoum could potentially be fatal in adults.[10] As it is not able to act upon already active factors causes the full anticoagulation effects to occur after several hours and even days.[1] Therefore, after being exposed to superwarfarin the onset of coagulopathy (measurable with PT) depends on the half-life of VK-dependent coagulation factors, which range from 6 hours for factor VII to 60 hours for prothrombin.[7] Table 1 shows the half-life (t1/2) of VK1 and VK-dependent coagulation factors.[8]

TABLE 1. Half-life (t1/2) of VK1 and VK-dependent coagulation factors (adapted from Haddad LM et al., 1998).

	Half-life time (t1/2)
VK1	1.7 hrs.
Factor II	50-80 hrs.
Factor VII	6 hrs.
Factor IX	24 hrs.
Factor X	25-60 hrs.

Toxicodynamics

The action mechanism of these pesticides is determined by their disruption of the coagulation cascade. This chain of events begins with the activation of proteases known as *coagulation factors* and ends with the activation of thrombin, which is key for platelet activity and to convert fibrinogen into fibrin. Factors II -or prothrombin-, VII, IX, and X, and proteins C, S, and Z all depend on γ-carboxylase, a VK-dependent liver enzyme.[8,9]

VK is a fat-soluble vitamin which can be synthesized or found in nature. There are currently 3 subtypes of VK, all of them have a two-ring structure with differing carbon side chains.[7,8] Vitamin K1 (VK1), phytomenadione or phytonadione is synthesized by plants and algae; vitamin K2 (VK2), natural or menaquinone is produced by bacteria; and vitamin K3 (VK3) or menadione is synthetic and is converted into active K2 *in vivo*. VK is necessary for γ-carboxylation of glutamate residue in order to activate factors II, VII, IX, and X, and proteins C, S, and Z, all of which have pro-coagulant effect. Through γ-carboxylase, it is responsible for the conversion of glutamate into γ-carboxyglutamate, which is present in active coagulation factors. This compound chelates Ca^{++}, which allows for the bonding of these factors to phospholipid membranes during the activation of the coagulation cascade. A decrease or absence of VK (which has a half-life of 1.7 hours) renders factors II, VII, IX, and X, and proteins C, S y Z inactive, which affects this process and creates an anticoagulable state.[8]

Coumarin rodenticides act by inhibiting the enzyme VK 2,3-epoxide reductase and prevents the organism from recycling VK. Additionally, there is evidence that suggests that these pesticides could also inhibit the enzyme VK quinone reductase.[9] Fig. 1 exhibits the VK cycle and the enzyme inhibition that is produced by superwarfarin rodenticides. The exact mechanism by which these substances inhibit the aforementioned enzymes is still unclear.[7] After the drop in plasma levels of VK, clinically relevant changes can only be witnessed after 24 hours.[7,8]

Medical approach

Anamnesis. It is essential to specify the latency of the ingestion, the amount and the format of the product. Normally, the pesticide is sold in solid rodenticides similar to grains of rice or paraffin-coated baits of various shapes and sizes. The liquid format is less frequent but it can be found, albeit it is more strictly regulated. Fig. 2 pictures different rodenticides we have confiscated in our institution over time.

Decontamination. In cases where the ingested dose is unknown and within one hour of said ingestion, the preferred gastrointestinal decontamination technique is the

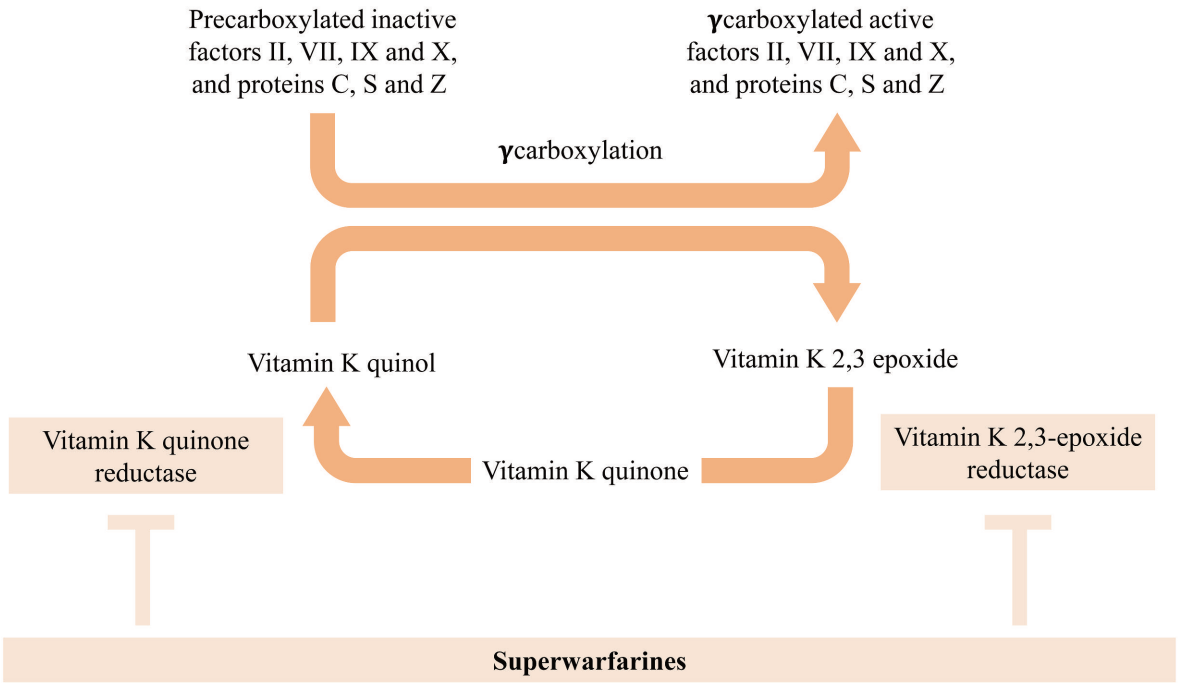

Figure 1. The VK cycle and enzyme inhibition produced by superwarfarin rodenticides (adapted from Nelson L et al., 2019).

administration of AC.[1] Prior nasogastric intubation in order to perform a gastric lavage depends on the rodenticide dose being potentially lethal, which could be possible if the ingestion was deliberate or if the patient suffers from any cognitive disability.

Clinical presentation. In line with our findings and the bibliography that was consulted, most AI in pediatrics do not have clinical effects. None of the patients showed hemorrhage upon admission or in subsequent examinations. Only 2 patients presented irregularities in their PT, which were succesfully treated with a single dose of VK (this could have been caused by a preexisting deficit and not by coumarin rodenticide poisoning). The low risk of poisoning described in the bibliography is linked to the small amounts that are ingested (because of, for example, bittering substances that are added to the rodenticides) and the fact that the dose of active ingredients is usually low in household preparations. In cases where larger amounts are ingested, mainly in adults or children over 6 years of age, these agents begin to have clinical effects 24 to 48 hours after ingestion, due to the half-life of coagulation factors. Signs and symptoms of hemorrhage are the most distinctive clinical characteristics of this poisoning.

The first signs include gingival bleeding, epistaxis, macroscopic hematuria, gastrointestinal bleeding (melena or hematochezia), metrorrhagia, hemoptysis, ecchymosis, and peri-articular hematomas, which in some cases could be severe. Patients can also show signs of anemia or hypovolemia such as tachycardia, arterial hypotension, fatigue and dyspnea on exertion, headache, abdominal or chest pain. In cases of severe poisoning the patient can suffer a hypovolemic shock and subsequent death.[3,7] It has also been observed that VK antagonists could have a procoagulant effect, some cases being reported where patients exposed to superwarfarin suffered from thrombosis as well as bleeding, which is likely to be related with the inhibition of proteins C and S.[12]

Diagnosis. The gold standard is the determination of superwarfarin in serum by a high-performance liquid chromatography (HPLC)[7], which in our context is performed in few laboratories and with limited access. The PT and the international normalized ratio (INR) are widely available biomarkers which can be used to identify the coagulopathy caused by coumarin rodenticides.[7] Alterations in these parameters are normally seen 24 to 48 hours post ingestion, after VK-dependent coagulation factors decay. The PT reference range varies from 10 to 14 seconds and activity over 70%. Results may vary significantly depending on the technique used, which is why INR was developed in an attempt to standardize said result.[12,13]

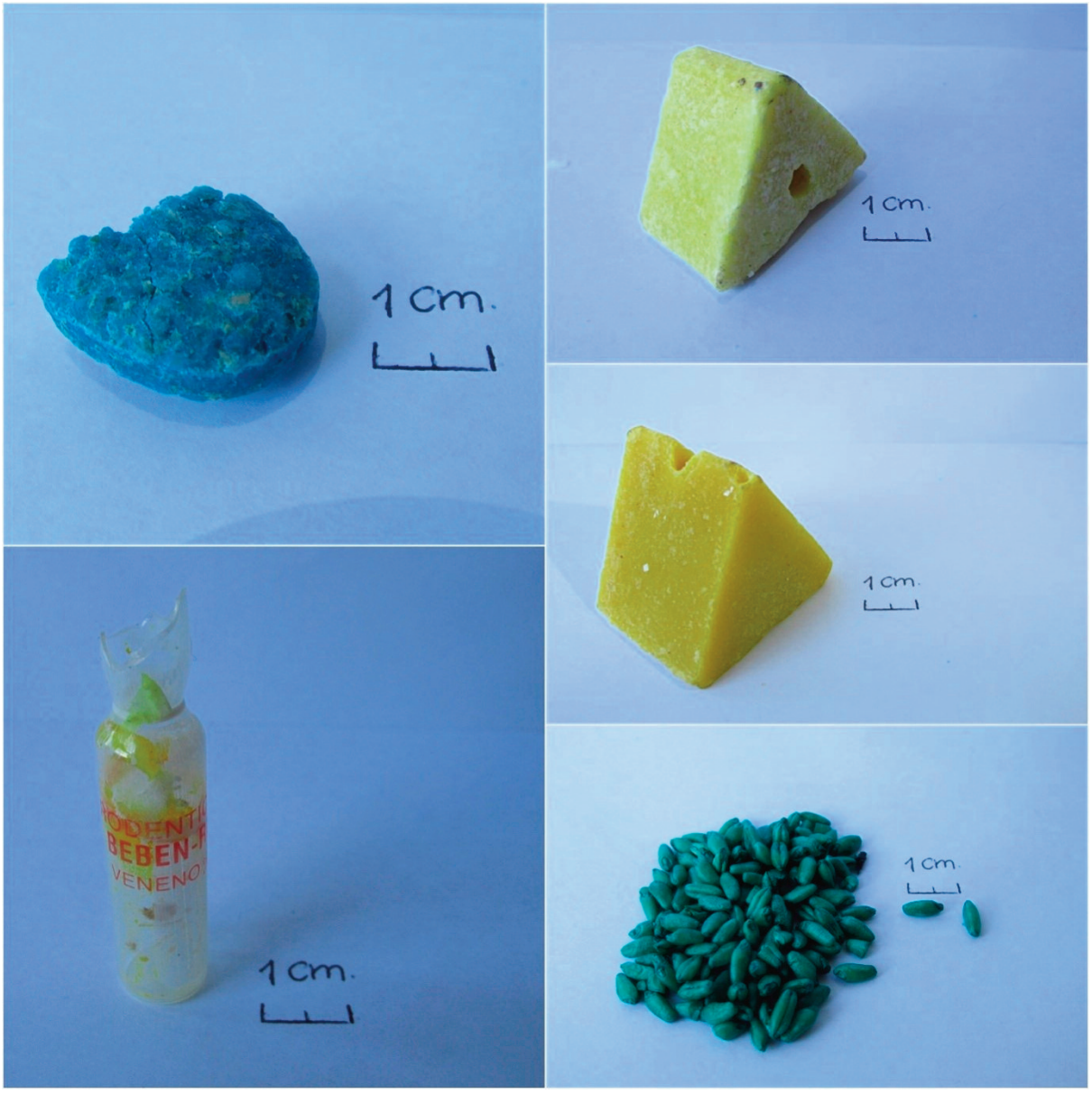

Figure 2. Photographs of rodenticides brought by patients seen in our hospital. The different formats of this product should be noted: paraffin-coated baits, ampoules (liquid content), and "grains of rice".

It is recommended to perform a coagulogram upon admission and 48 to 72 hours after in the following cases: 1) ingestion of unknown or large doses; 2) children over 6 years of age in which the ingested dose, even a single AI, may be toxic; 3) patients with symptoms; 4) intentional ingestion; 5) chronic or repeated ingestions; 6) ingestion in patients using other anticoagulants; and 7) suspected Munchausen syndrome, negligence, child abuse and neurodevelpment disorders.[1,15] In the first two, the initial coagulogram can be omitted, assuming that it should report normal values. As stated in the bibliography, several authors[7,10,20] have dismissed performing coagulograms in cases of AI of non-toxic doses -under 1 mg of active ingredient- in patients under 6 years of age.[7,14] Those patients can be subject to outpatient care with attention to warning signs and clinical follow-ups, given that there were no clinical symptoms in patients in this age group who presented AI.[1,16,20]

Treatment. The specific treatment is VK1 supplementation. It is recommended in patients with signs of hemorrhage

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or biochemical alterations and not prophylactically.[10,15] Presently, there is no established dose. According to some studies, a dosage of 30 mg/day of VK1 in doses divided by day is accepted, ideally PO. It will depend on the patient's PT and/or INR and ranges from 25 to 50 mg per day[19]. Intramuscular (IM) route is not advised due to the risk of creating hematomas. 1 mg/kg intravenous (IV) VK is mainly used in hemodynamically unstable patients or who have active bleeding.[18,19] Serum brodifacoum and chlorophacinone were found useful to observe exposure and to determine when the VK treatment can be stopped.[15] In cases where the patient presents active bleeding, in addition to the specific treatment an additional treatment is recommended, which consists of prothrombin complex concentrates (with factors II, VII, IX, and X) or activated recombinant factor VII or fresh frozen plasma if the concentrates are not available[17].

Monitoring. AIs in children under 6 years of age have a low clinical toxicity index. No cases of hemorrhage or other clinical symptoms were observed in this age group with accidental exposure.[15,20] Therefore, only clinical monitoring and blood tests should be performed on the groups described in the *Diagnosis* section.

CONCLUSIONS

Coumarin rodenticides are compounds that can be easily accessed by children. Consistent with the findings in our hospital and in line with the bibliography, we can conclude that AI of rodenticides in pediatric patients under 6 years of age does not warrant additional routine biochemical tests. A detailed anamnesis will allow the physician to detect those patients that require special care, coagulograms and clinical monitoring with warning instructions aimed at the appearance of bleedings.

Conflicts of interest

The authors declare no conflicts of interest.

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ORIGINAL ARTICLE

Validation of an analytical technique for the dosage of ethanol in biological fluids

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Abstract. The widespread consumption of alcoholic beverages in modern society has impelled forensic toxicology laboratories to improve on the already high standards of the analytical techniques routinely employed by optimizing how judicially relevant samples are managed, given that it is essential to expedite the delivery time of results. According to the World Health Organization (WHO): "*Total alcohol per capita consumption in the world's population over 15 years of age rose from 5.5 litres of pure alcohol in 2005 to 6.4 litres in 2010 and was still at the level of 6.4 litres in 2016".* To that effect, the dosage of ethanol in biological fluids allows judges, through retrospective calculations, to assess the level of impairment of an individual at the time of the event, which is fundamental for its elucidation. This study proposes the validation of an analytical method that makes it possible to obtain reliable results with a brief analysis.

Key words: *Validation; Mass spectrometry; Ethanol; Biological fluids; Quantification.*

Abbreviations: C_{et}: Ethanol concentration; C_{is}: Concentration of internal standard; A_{et}: Ethanol area; A_{is}: Internal standard area; mV.s: Millivolts per second; Run: Chromatography run; R^2: Coefficient of determination; b*: Absolute bias; R´%: Relative recovery percentage per fortification level; R.T.: Retention time; R.R.T.: Relative retention time; m/z: mass-to-charge ratio; S/N: signal-to-noise ratio; Mx: Slope of the line; B: Intercept; LOD: Limit Of Detection; LOQ: Limit Of Quantification; S.D.: Standard deviation; Swg: Sum of squares within the group; Sbg: Sum of squares between groups; CV%: Percent coefficient of variation.

According to the World Health Organization (WHO): *"Total alcohol per capita consumption in the world's population over 15 years of age rose from 5.5 litres of pure alcohol in 2005 to 6.4 litres in 2010 and was still at the level of 6.4 litres in 2016".*[1] To that effect, the dosage of ethanol in biological fluids allows judges, through retrospective calculations, to assess the level of impairment of an individual at the time of the event, which is fundamental for its elucidation. This study proposes the validation of an analytical method to separate, detect and quantitate ethanol between 0.1 and 5.0 g/L in blood and urine samples through "headspace" (HS) gas chromatography mass spectrometry (GC-MS).

MATERIALS AND METHODS

Control

A water-ethanol Cerilliant® calibration kit (Round Rock, Texas, USA) at a concentration of 1.0 g/L was used in order to compare its results to an ethanol-water solution at the same concentration (Biopack®, City of Buenos Aires, Argentina). No significant differences were found.

Reagents

The following substances were used to test for possible interferences of volatile compounds: Acetone ACS grade (Biopack®), PA grade n-Propanol (Stanton®), HPLC grade methanol (Sintorgan®), Isopropanol ACS grade (Sintorgan®), PA grade ethyl acetate (Biopack®). ACS grade T-butanol (Carlo Erba®) was used as internal standard. Relevant analyte solutions were prepared with ACS grade ethanol absolute (Sintorgan®) and 18 MΩ.cm ultrapure water from a BioSan brand model Labaqua (Biosystems) deionization water purification system.

Instruments

The research was conducted with a Shimadzu GCMS-QP2020 high-end single quadrupole (Kyoto, Japan) gas chro-

matograph mass spectrometer equippd with a Headspace sampler Shimadzu HS-20 (Kyoto, Japan). An Rtx-5MS (30m x 0,25mm x 0,25µm) column provided by Restek Corporation (Bellefonte, Pennsylvania, USA) was also used. For system control, data gathering and processing, LabSolution's GCMS Real Time Analysis and GCMS Postrun -both in LabSolution version 4.52- were employed. The sample temperature and incubation time in the Head-Space module were 75 °C for 10 minutes. The injection and transfer lines were at 150 °C with a 1:100 split ratio. The oven module was set at a 40 °C isothermal. The ionizer of the mass module was at 220 °C, with a voltage relative to its tuning plus a 0.1 kV gain. The run time was 4.5 minutes. The determination of the analytes was performed using the SCAN mode (29-100 m/z) and SIM mode (ethanol: 31*-45-46, t-butanol: 59*-31-41).

Validation parameters

"*Method validation is basically the process of defining an analytical requirement, and confirming that the method under consideration has capabilities consistent with what the application requires".*[2] It is interesting to look into other definitions of the concept of validation. According to ISO/IEC 17025, it is the "*confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled*".[3] VIM defines it as "*verification, where the specified requirements are adequate for an intended use*".[4] Taking into account that "*Method validation is usually considered to be very closely tied to method development*"[2], the guidelines drawn up in "Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics (2nd ed. 2014)"[5] were followed. The recommendations in "Eurachem/CITAC Guide CG 4: Quantifying Uncertainty in Analytical Measurement (3rd ed. 2012)" were also adopted.[6]

RESULTS

Model calibration

The selected acceptance criterium was a linear model ranging from 0.1 to 5.0 g/L, with 8 (eight) points in the calibration curve (Fig. 1). An aqueous solution of T-butanol 0.05 g/L was used as internal standard. The linearity of the results was tested by injecting 8 (eight) levels of concentration of the analyte, which was ran three times. In order to ensure the linear regression of the working range, a graphical analysis of the residuals was performed (Fig. 2), with the predictor variable (concentration of analyte) in the X axis and the difference between the observed values and the predictive values in the Y axis. The adjusted regression curve was plotted (Fig. 3) so as to verify if its goodness of fit is adequate for the relation between the predictor and the dependent variables in the regression model. These measurements were performed by 5 (five) analysts on different days.

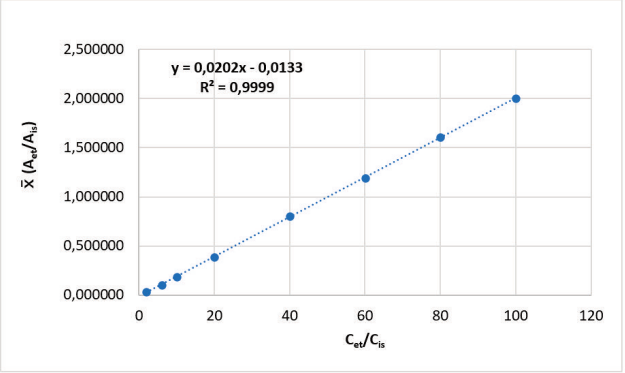

Figure 1. Calibration curve.

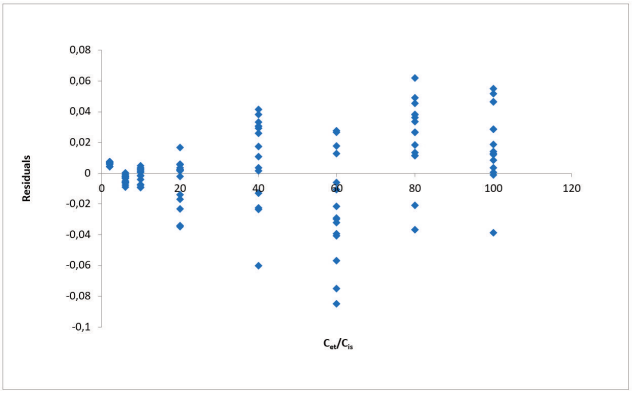

Figure 2. Analysis of residuals.

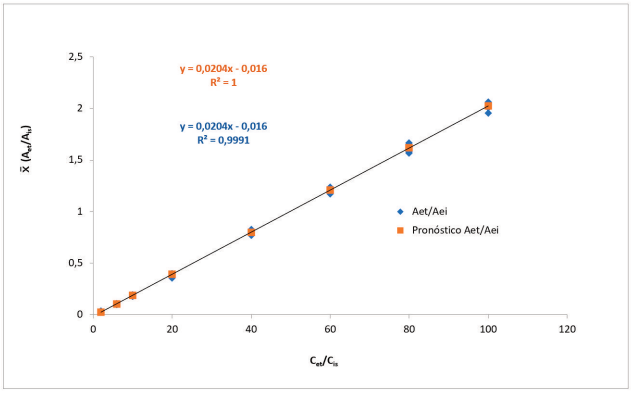

Figure 3. Adjusted regression curve.

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Bias

In order to calculate bias, 4 (four) levels of the working range were tested (0.3, 1.0, 2.0, and 4.0 g/L), in 5 (five) chromatography runs, three times. To that end, blood samples from different volunteers were fortified. Those samples were analyzed beforehand to rule out the presence of analyte in the matrix. The bias was calculated in absolute values (b*) and as relative recovery of the addition (R´%). The acceptance criterium established was a variation of ±5% of the determined real value.

Carryover

With the aim of determining the existence of carryover between chromatography runs, the acceptance criterium established was a relative intensity of the analyte quantifier ion (m/z = 31.00) and of the internal standard (m/z = 59.00) below 10,000. The measures of each point within the working range were taken, and a ninth point was added, equivalent to an ethanol concentration of 10.0 g/L and alternating with blanks within each level.

Interferences

In order to assess the specificity of the method, the possible interferences in the matrix (putrefaction alcohols) (Fig. 4) were tested, as well as the possible influence of preservatives and anticoagulants in the blood samples. For that purpose, the following were analyzed separately: a) an aqueous solution of each of the volatile substances at a concentration of 1.0 g/L with internal standard; b) blank blood samples with internal standard (Figure 5), held in sterile tubes without air chamber, which had different preservative and anticoagulant agents; and c) blank urine samples with internal standard (Figure 6), held in sterile containers. Once the data was gathered, the retention time (T.R.) and relative retention time (R.R.T.) were measured for each substance under analysis. Moreover, 10 (ten) blank blood samples and 10 (ten) blank urine samples were tested: no interfering compounds that affect the ethanol and internal standard retention time were observed. Finally, the analyte results were compared in the different matrixes proposed for the method (Fig. 7, 8 and 9).

Limits

Limit of detection. The calculation of the "*concentration that emits a signal in the instrument that is significantly different from the blank signal or background noise*"[7] or lowest quantity of analyte that can be detected by the device (Limit Of Detection, LOD) was done employing two methods: a) *Formula*: 10 (ten) samples of an aqueous ethanol solution at a concentration of 0.05 g/L were analyzed. Afterwards, the

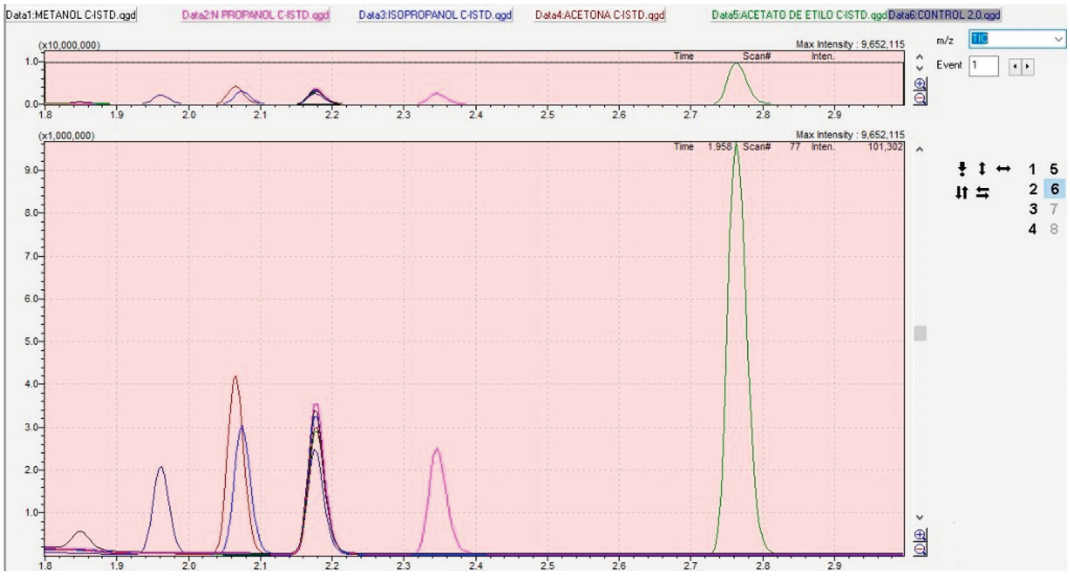


Figure 4. Interference chromatogram.

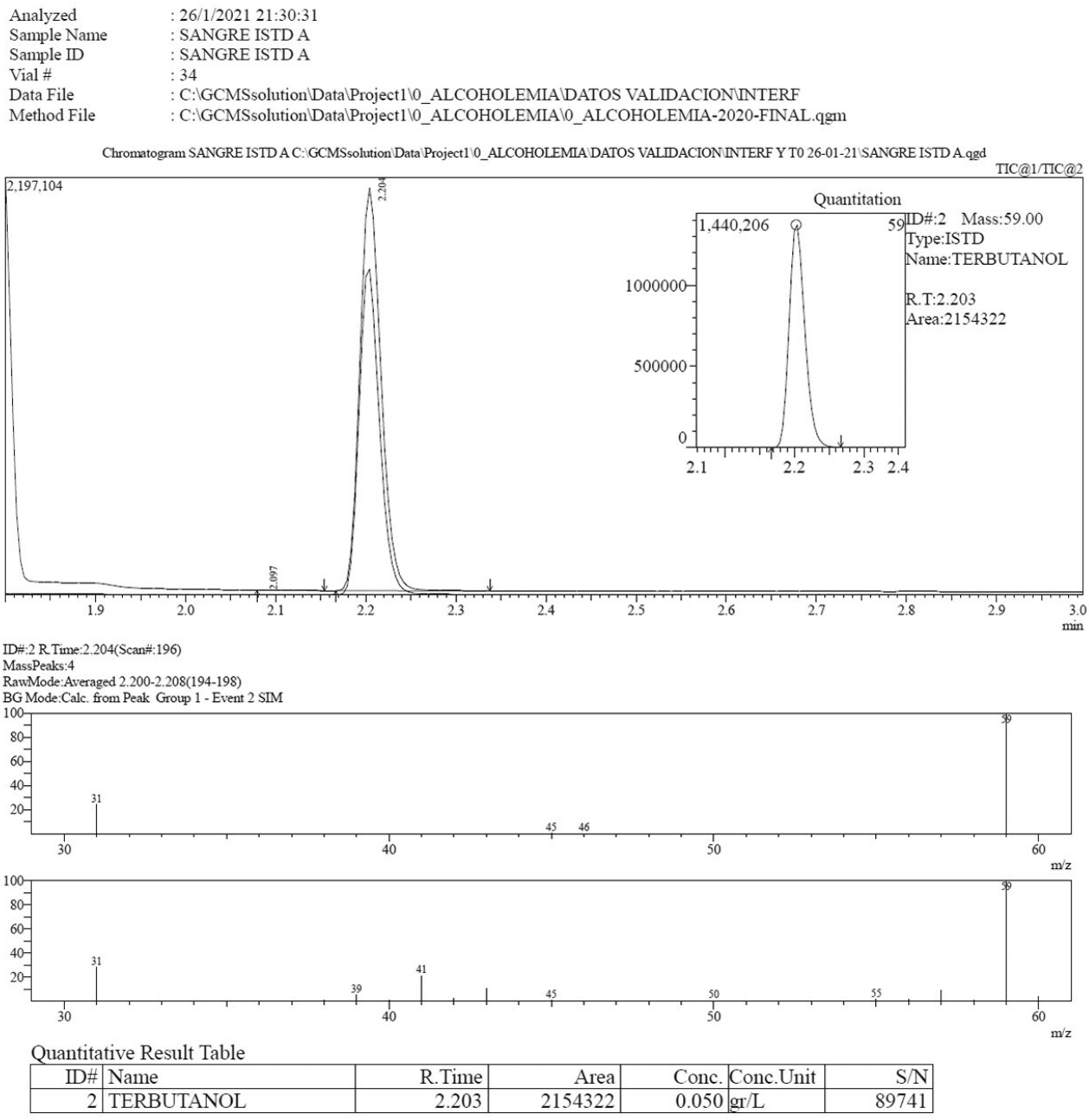


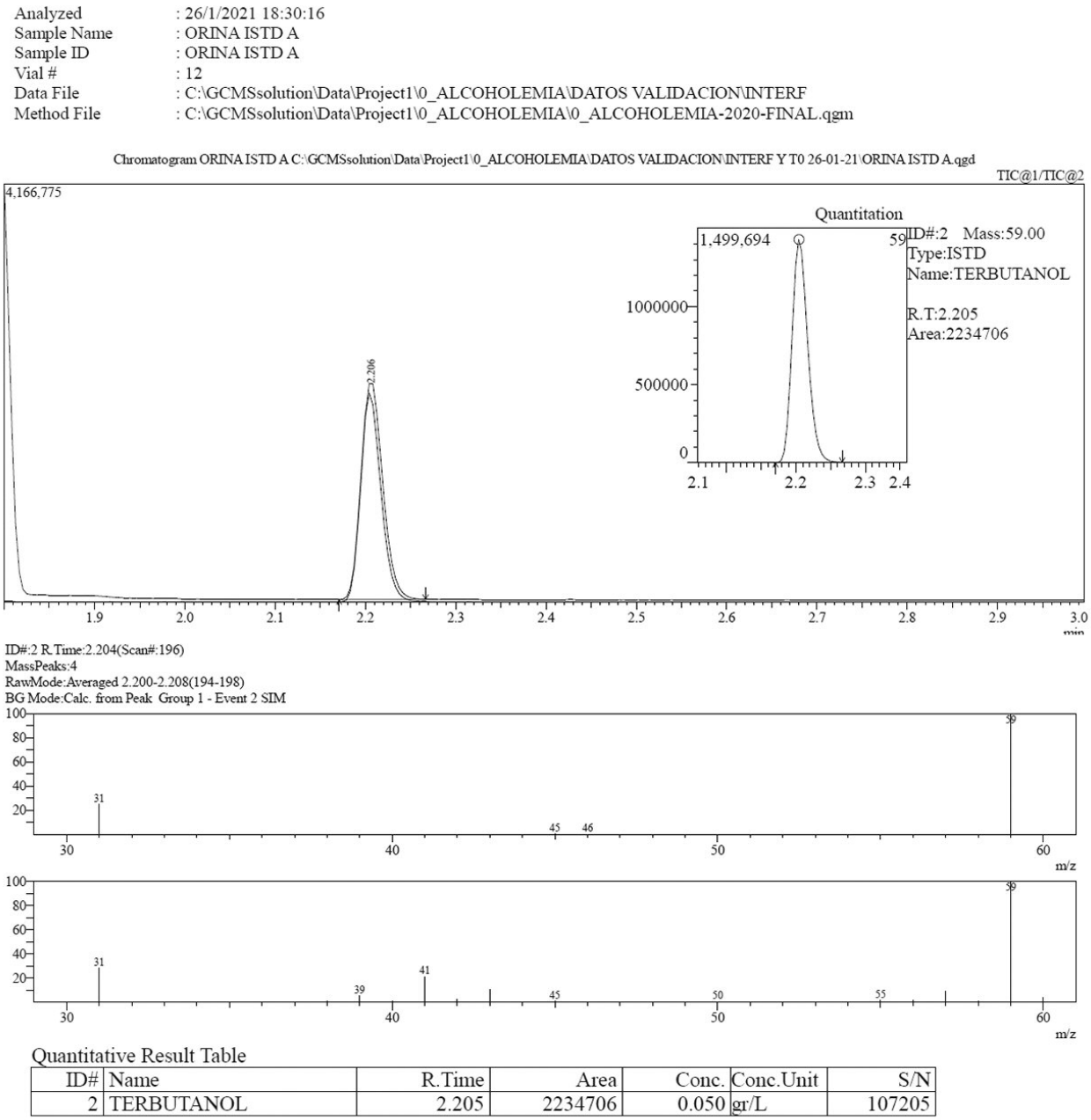
Figure 5. Chromatogram of a blank blood sample.

linear regression curves were plotted to estimate the values of the slope and the intersect for each sample; b) *S/N ratio*: the S/N ratio for each sample was calculated, with an acceptance criterium of a S/N ratio > 3.

Limit of quantification. In order to calculate the "*lower limit of precise quantitative measurements, as opposed to qualitative detection*"[7] (Limit Of Quantification, LOQ), the acceptance criteria set was a S/N ratio > 10 for a solution at a concentration of 0.1 g/L. To that end, 10 (ten) samples of a solution of ethanol at a concentration of 0.1 g/L were tested. The LOQ value proposed for this method is 0.1 g/L.

Precision

Aiming to determine the precision of the method and its repeatability and reproducibility, the one factorial analysis of variance (ANOVA) was used within the group and across groups. 4 (four) levels of the working range (0.3, 1.0, 2.0, and



Quantitative Result Table

ID#	Name	R.Time	Area	Conc.	Conc.Unit	S/N
2	TERBUTANOL	2.205	2234706	0.050	gr/L	107205

Figure 6. Chromatogram of a blank urine sample.

4.0 g/L) were used across different days, by different analysts and was ran three times. The acceptance criterium established was a CV% of ±5%.

DISCUSSION

According to the data gathered, the linearity of the working range was verified, as was its homoscedasticity. When checking for heteroscedasticity, there seems to be no trend among the residuals, which can lead to the assumption that errors have a constant variance. Therefore, the model adequately follows all assumptions.

The results of the evaluation of bias were within the proposed acceptance criteria. According to the data, there is no carryover, even after the 10 g/L solution.

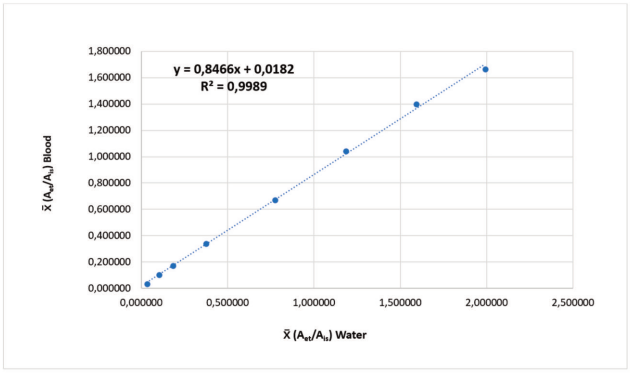


Figure 7. Water against blood curve.

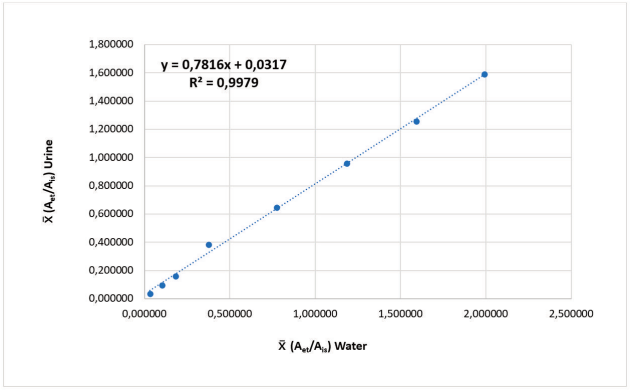


Figure 8. Water against urine curve.

The data gathered when testing the specificity shows no interference in the retention time of the analyte and of the internal standard and a correlation is found between the 3 (three) matrixes that were tested ($R^2 \approx 1$).

The sodium citrate molecular ions observed in the spectrometry were similar to the results obtained for acetone,

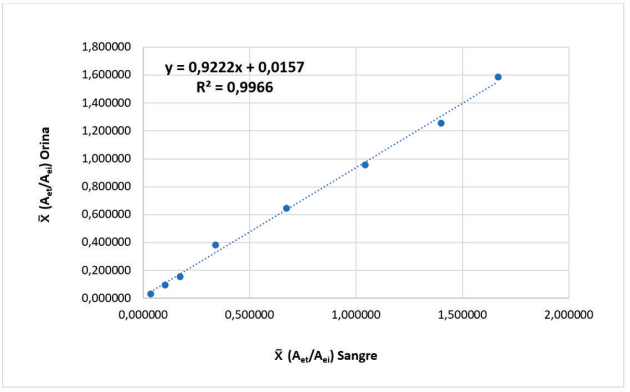


Figure 9. Blood against urine curve.

with similar retention times as well. In contrast, no signals were detected during the acquisition time of the method for EDTA, Heparin, and NaF.

Finally, it was verified that the results obtained for the limit parameters of the method were within the acceptance criteria: LOQ = 0.1 g/L and LOD 0.05 g/L.

CONCLUSIONS

Taking into account the validated parameters and the results previously presented, the method is suitable for the purposes intended. Furthermore, the use of sodium citrate as an anticoagulant is discouraged for this analysis method, EDTA and NaF being recommended as anticoagulant and preservative respectively.

Declaration of interest

The authors declare no conflicts of interest.

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IMAGES IN TOXICOLOGY

Cutaneous-hemolytic loxoscelism in a pediatric patient

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Abstract. A clinical case of a pediatric patient with a picture compatible with cutaneous-hemolytic loxoscelism, who received the specific antivenom within the first 48 hours of the poisonous accident.

Key words: *Loxosceles; Cutaneous loxoscelism; Hemolysis; Compartment syndrome; Pediatrics.*

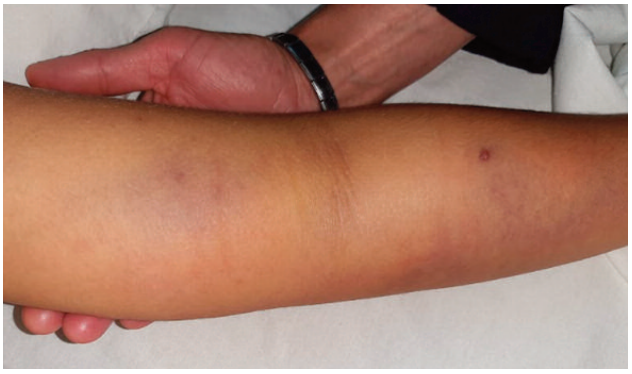

Figure 1. Day 1: development of the distinctive marble-like plaque.

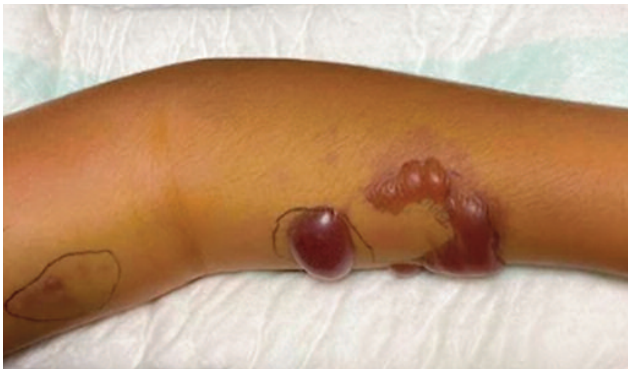

Figure 2. Day 2: blisters with serosanguineous content.

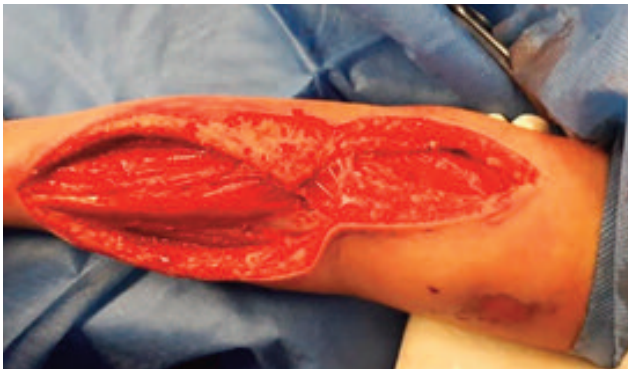

Figure 3. Day 4: fasciotomy in operating room.

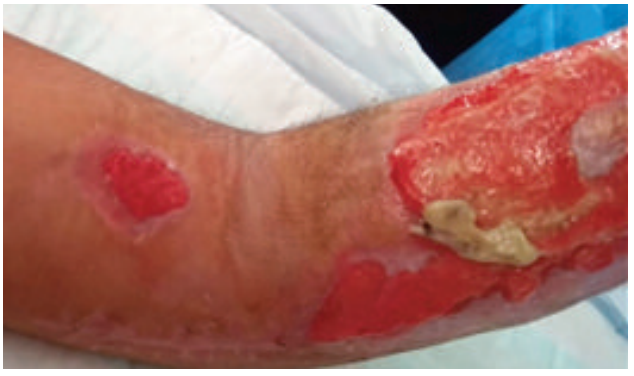

Figure 4. Day 68: growth of granulation tissue.

An 8-year-old child went to a health center after being bitten by an unidentified spider on her left arm while sleeping. During the anamnesis, the patient's mother claimed that the room where the accident happened was disused and it was cleaned hours prior to the event.

24 hours after the accident occurred, the patient showed edema and severe pain. She was transferred to a local health center where initial medical measures and a complete laboratory were performed. The initial blood test reported a white blood cell count of 16,500/mm3 (reference value

4,500-13,000/mm3), hematocrit of 38.2% (reference value 35-45%), bilirubin levels of 7.3 mg/dl (reference value of 0-1.2 mg/dl) with a direct bilirubin value of 0.2 mg/dl (reference value 0-0.2 mg/dl) and lactate dehydrogenase levels of 1.423 IU/l (reference value 150-500 IU/l). Upon clinical suspicion of a possible cutaneous-hemolytic loxoscelism, it was prescribed that the patient be taken to a high complexity medical center to be given the specific heterologous antivenom. After the injection the hemolysis improved, but the skin lesions continued to deteriorate. The region showed a larger edema and the aspect of the lesion continued to deteriorate, which had the characteristic marble-like plaque appearance along with blisters filled with serosanguineous content (Fig. 1 and 2). Four days after the event and under the suspicion of a possible compartment syndrome, a fasciotomy was performed which ruled out signs of ischemia in muscle and tendon structures in the affected region (Fig. 3).

The clinical picture evolved without complications; the patient being discharged from hospital 20 days after the bite. Outpatient care was performed giving particular attention to the evolution of the granulation tissue of the wound (Fig. 4). Posterior monitoring was performed by the Plastic Surgery Service of the hospital, who completed the treatment with the aesthetic reconstruction of the affected region by performing an autograft.

Conflicts of interest

The authors declare no conflicts of interest.

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