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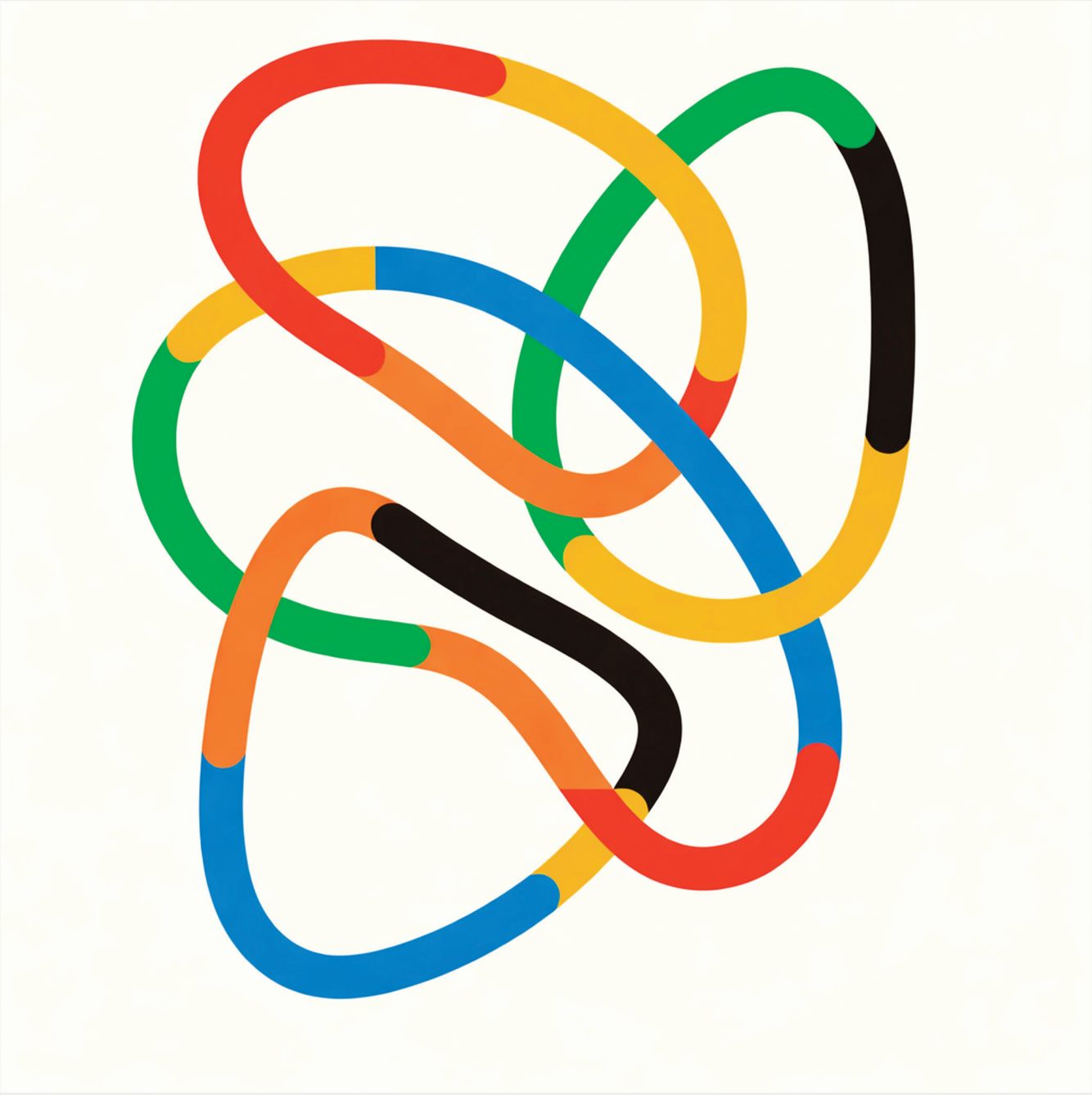
Panamerican Toxicology Press

VOL. 3|NO. 1|2026



ISSN 3008-8755

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This issue is kindly sponsored by:


Published by Panamerican Toxicology® Press: 1737 Chivilcoy St. Castelar, Buenos Aires (PO BOX 1712), Argentina

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2026 | Vol. 3 | No. 1: 7-8

EDITORIAL

# Semi-synthetic cannabinoids: An emerging challenge for clinical and regulatory toxicology

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Published: 31/01/2026 - DOI: https://doi.org/10.62129/SECD6613

**Keywords**: *THCP; HHC; CBD; Semi-synthetic cannabinoids; New psychoactive substances.*

One of the most fascinating aspects of contemporary clinical toxicology is undoubtedly the study of the epidemiology of poisonings. Unlike other medical specialties, in which the pace of discovery of new agents involved in the pathophysiology of diseases is relatively stable, the exponential emergence of new xenobiotics and their implications for human health has made toxicological science one of the most dynamic fields, with strong projected growth.

The market for new psychoactive substances (NPS) exemplifies this phenomenon. In recent years, consumption patterns have shifted, with previously known compounds such as nitrous oxide, ketamine, and mephedrone, which were used only sporadically, re-emerging alongside the introduction of entirely new synthetic molecules. Among the latter are semi-synthetic cannabinoids (SSCs), including hexahydrocannabinol (HHC) and tetrahydrocannabiphorol (THCP). These compounds are synthesized from cannabidiol (CBD), a cannabinoid abundant in *Cannabis sativa* and traditionally considered devoid of psychotropic effects. However, chemical modifications transform it into molecules with high psychotropic potential and unknown toxicological profiles.[1,2]

It is not possible to understand the emergence and perceived "success" of these products without considering the recent history of the rapidly expanding cannabis industry, in which CBD has been, and in many countries continues to be, the central driving force, largely owing to its status as a non-scheduled substance. The abundance of this "raw material" in commercial markets made it inevitable that surplus production would be redirected toward the development of other, more profitable substances.

The first reported case of an SSC identified by the European Union Drugs Agency (EUDA) occurred in 2022, when it was marketed as a "legal" alternative to cannabis. At present, these molecules have been reported in 27 countries of the European Union (EU). Based on the most recent EUDA report, in 2024 a total of 20 new cannabinoids were identified, 18 of which were SSCs, accounting for 40% of the new substances detected by the agency's early warning system that year. These compounds have been found in edibles (e.g., gummies), vaping devices, and in products marketed as CBD that were subsequently shown to be adulterated.[3]

Knowledge of the short and long term clinical toxicological effects of SSCs remains limited, given the


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relatively short period since their emergence. In addition, the lack of specific analytical standards in clinical and toxicological laboratories further hampers accurate assessment. Nevertheless, since 2024, cases of acute intoxication associated with the use of these cannabinoids have been reported.[4,5] The clinical presentation following oral exposure is characterized by initial nausea and vomiting, followed by alterations in the level of consciousness ranging from profound sedation to extreme agitation, depending on the case. This symptom profile closely resembles that observed in oral cannabis overdose,[6] raising concern about an unusually high pharmacodynamic potency at endocannabinoid receptors within the central nervous system.

In Spain, the exponential increase in acute intoxication cases requiring hospital care during 2024, and their subsequent reporting to public health agencies, were the crucial factors that led to the regulation of this molecular group under Order SND/380/2025 of April 14, 2025.[7] Beyond the legal restriction, however, the progressive technological sophistication and globalization of producers and distributors of these substances necessitate increasingly close collaboration among clinical toxicologists, public health authorities, and national and supranational regulatory agencies. Such coordination is essential to mitigate their impact on public health, alongside educational campaigns to inform the general population about the risks associated with their use.

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REVIEW

# The evolving role of *in silico* toxicology in science and industry: A narrative review

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Submitted: 21/11/2025 - Accepted: 22/01/2026 - Published: 31/01/2026 - DOI: https://doi.org/10.62129/NPMC1632

**ABSTRACT.** Modern toxicology is experiencing a major paradigm shift driven by the demand for efficient chemical safety assessment and the ethical and economic limits of animal testing. *In silico* toxicology, understood as the use of computational tools and computer-based simulations to predict and analyze toxic effects, has become a core element of this transformation. By enabling rapid, cost-effective, and mechanistically based hazard assessment, computational approaches bridge science, industry, and regulation. This development echoes the idea of "heartbroken toxicology", where the scientific, industrial, and regulatory domains have grown apart. *In silico* methods offer a way to reconnect these dimensions and foster a shared framework for decision-making. This narrative review summarizes current advances, methodological foundations, and key challenges of *in silico* toxicology, emphasizing its expanding role in regulatory and industrial contexts and the need for harmonized validation, standardization, and education to secure its place as a cornerstone of 21st-century toxicology.

**Keywords:** *In silico models; Computational toxicology; Computer simulation; Risk assessment; Toxicity tests.*

Modern toxicology faces enormous challenges: we live in a world exposed to thousands of chemical substances and countless mixtures.[1,2] Ensuring chemical safety requires innovative solutions, particularly because traditional *in vivo* toxicity testing (animal studies) is time-consuming, costly, and constrained by ethical considerations.[3,4] The use of experimental animals has become increasingly difficult to justify, both socially and scientifically, especially given the limited predictive performance of classical animal models.[5] At the same time, industry and regulatory agencies must evaluate the safety of thousands of substances (e.g., under the EU REACH program),[6] which, within a traditional testing paradigm, would require the use of tens of millions of animals. Such an approach is not only unethical but also impractical.

In the face of these challenges, a shift in the toxicological paradigm becomes essential, moving away from a model that relies primarily on *in vivo* experiments toward strategies based on *in silico* methods, *in vitro* tools, and a mechanistic understanding of toxic action.[7] In recent years, the urgent need for such a shift has been widely recognized. This vision requires a radical rethinking of toxicology, effectively turning traditional procedures upside down.

Historically, toxicity assessment began with *in vivo* studies (often treated as a "black box") and only later proceeded to mechanistic analyses.[8] The new approach proposes the opposite strategy: to begin with *in vitro* and *in silico* studies that identify toxicity pathways, and only then (if necessary) advance to narrowly targeted animal tests. This reversed "toxicological funnel"[1] represents a fundamental transformation of toxicology from a largely descriptive discipline (supporting regulatory decision-making) into a full-fledged biological science grounded in the understanding of underlying mechanisms.

The need for a new approach arises not only from ethical and regulatory pressures but also from emerging scientific challenges. Modern chemistry and the pharmaceutical industry continuously generate new compounds. For example, in the field of new psychoactive substances (NPS), dozens of previously unknown chemicals appear each year. Traditional methods cannot keep pace with the assessment of their risks, creating gaps in public, clinical, and forensic safety. Another category involves the chemistry of chemical warfare agents, which relies almost exclusively on archival data from the 1940s, even though these compounds are widely used as "natural" components.

Given these circumstances, both science and industry recognize that the time has come for a genuine paradigm shift in toxicology. In line with the concept of "heartbroken toxicology" described in 2008,[1] modern toxicology can be seen as a discipline whose scientific, applied, and regulatory "souls" have become disconnected. This separation has led to stagnation, limited innovation, and communication barriers between academic science, industrial needs, and regulatory expectations. In this context, *in silico* methods emerge as one of the few tools capable of restoring this dialogue. They enable rapid, cost-effective, and mechanistically grounded assessment of chemical safety at early stages of development, providing a shared platform for scientific discovery, industrial application, and regulatory evaluation. Thus, computational toxicology may serve as a bridge, capable of reuniting the "broken heart" of the field and advancing a more integrated and forward-looking paradigm for 21st-century toxicology.

This article provides a narrative overview of this transition—from traditional methods to modern *in silico* and related strategies. The subsequent sections summarize the current state of knowledge in this field, outline the challenges and limitations of *in silico* approaches, present examples of their applications (in regulation, industry, and risk assessment), and conclude with recommendations for fully leveraging the potential of i*n silico* toxicology.

## METHODS

This article adopts a narrative review approach aimed at synthesizing current knowledge on the development and application of *in silico* toxicology. Relevant literature was identified through a structured search of major scientific databases, including PubMed, Scopus, and Web of Science, using combinations of keywords such as "in silico toxicology" and "computational toxicology". Only peer-reviewed publications written in English were considered. Studies were included if they addressed methodological aspects, applications, or regulatory perspectives related to *in silico* approaches in toxicology.

Initial search results were screened based on titles and abstracts, followed by full-text review to confirm eligibility. Additional references were identified through manual searches of cited literature in key review articles. The gathered evidence was narratively synthesized, emphasizing thematic organization rather than quantitative analysis. The discussion highlights major trends, challenges, and perspectives emerging from the reviewed literature.

## DISCUSSION

### *Current state of knowledge: from in vivo testing to in silico approaches*

Traditionally, toxicology has relied on animal experimentation. For decades, it was assumed that a comprehensive battery of *in viv*o tests (often described as the "gold standard") was essential for assessing chemical hazards in humans.[9] This model can be illustrated by the metaphor of a funnel: at the top lies a broad collection of untested substances subjected to standard animal studies, and only a narrow stream of results ultimately yields a limited number of well-characterized chemicals and mechanisms.[1] The remaining substances are classified as less hazardous or remain poorly defined. This classical toxicological funnel reflects an approach in which findings from animal studies trigger subsequent, often mechanistic, investigations of selected compounds.

Today, however, new concepts and technologies are reshaping this paradigm. *In vitro* methods—including assays using cell lines, organoids, and organ-on-a-chip systems—and *in silico* approaches, which incorporate computational simulations, mathematical models, and artificial intelligence (AI) to predict toxic effects, now play a central role. Within the modern toxicological framework, these methods should represent the first line of toxicity assessment. They provide information about potential mechanisms of action before any animal testing is considered. This model assumes that animal studies should be used only to fill remaining data gaps after advanced *in vitro* testing and *in silico* analyses have been completed, essentially the reverse of the traditional approach. This shift is often described as a breakthrough that marks the entry of toxicology into the twenty-first century. The term "Toxicology for the 21st Century"[1,2] refers to a set of new methods and concepts designed to make toxicological evaluation more efficient, data-driven, and human-relevant. A central element of this transformation is the transition from treating toxicity testing as a "black box" to adopting a mechanistic framework. In practice, this means focusing on toxicity pathways and adverse outcome pathways (AOPs),[10] which outline sequences of biological events beginning with an initial molecular interaction and culminating in an adverse effect. Initiatives such as the Human Toxome Project (HTP)[11] and the AOP program of the Organisation for Economic Co-operation and Development (OECD) aim to catalogue these pathways and their associated biomarkers. With a well-defined map of key biological events, it becomes easier to connect *in vitro* and *in silico* data with potential *in vivo* outcomes.

***Predictive in silico methods in modern toxicology***

*In silico* methods encompass a broad range of approaches. The term extends far beyond traditional QSAR models (quantitative structure–activity relationship models), which predict a chemical's biological activity based on its molecular structure. *In silico* toxicology includes any activity performed using a computer—from experimental planning and statistical analysis to sophisticated computational modeling.[8] Simple tasks such as determining the number of replicates needed to achieve adequate statistical power or applying algorithms for data processing are everyday *in silico* activities in a toxicology laboratory. However, the greatest interest focuses on predictive methods that can replace or reduce the need for animal studies.

Predictive *in silico* tools include a variety of methods that enable the estimation of toxicological properties without direct animal testing. These approaches include:

1. **QSAR models**. Predict a chemical's biological or toxicological activity based on its molecular structure and can estimate outcomes such as acute or chronic toxicity.[8]
2. **Read-across**. Infers the properties of an untested chemical based on data from structurally or functionally similar substances and is widely used in regulatory frameworks such as REACH.[8]
3. **IVIVE (*in vitro–in vivo* extrapolation)**. Translates concentrations causing effects *in vitro* to equivalent *in vivo* doses using toxicokinetic information, such as physiologically based pharmacokinetic (PBPK) models, to simulate absorption, distribution, metabolism, and excretion. The success of this method depends on the relevance of the *in vitro* systems and the availability of tools for reverse toxicokinetics, which allow calculation of *in vivo* doses corresponding to active *in vitro* concentrations.[12]

In parallel, the volume of experimental data has expanded dramatically. Modern computational techniques make it possible to manage and analyze very large datasets, opening new opportunities for discovering SAR but also creating a challenge: how to extract meaningful conclusions from such extensive data. The goal is not only to generate big data but also to generate "big sense", meaning biologically and regulatorily useful insight.

***In silico methods: challenges and limitations***

Despite substantial progress, *in silico* toxicology still faces several challenges and limitations that must be critically considered. First, the performance of any *in silico* model depends directly on the quality of its input data.[8] A computational model can never exceed the quality of the data on which it is built, a concept captured by the well-known principle "garbage in, garbage out". *In silico* methods inevitably inherit the weaknesses of their underlying datasets, which are often derived from *in vitro* or *in vivo* studies. For example, a QSAR model constructed from a small or chemically homogeneous dataset may lack predictive power outside that chemical space, illustrating the problem of a restricted applicability domain. Similarly, if an *in vitro* system fails to reflect essential physiological characteristics (such as a cell line with limited metabolic capacity) then even the most sophisticated IVIVE approach cannot convert poor-quality experimental data into a reliable *in vivo* prediction. In short, poor-quality data combined with advanced methodology still yields poor-quality predictions.

Another major challenge is the validation and acceptance of *in silico* methods.[8] The scientific community and regulatory agencies often adopt a cautious stance toward new technologies. Before a QSAR prediction can be considered equivalent to an animal study result, the method must undergo rigorous evaluation for reproducibility, reliability, and fitness for purpose. The traditional validation paradigm, historically applied by organizations such as the European Centre for the Validation of Alternative Methods (ECVAM) for alternative methods, relied on comparing the results of a new method against the "truth" defined by *in vivo* tests.[13] For *in silico* approaches, however, this type of comparison may be insufficient or even inappropriate, as the goals and assumptions of these methods can differ fundamentally from those of classical animal tests. This is why an evidence-based validation framework is increasingly advocated as a continuous, systematic evaluation process that integrates all available data from multiple sources. In practice, this includes systematic reviews, assessment of data quality, meta-analyses, and expert consensus to determine whether a method is suitable for decision-making. The ideal is to base decisions not on the historical authority of animal models but on the best possible combination of modern methods that together provide the most reliable prediction of hazard. An additional challenge is persuading stakeholders, including industry and regulators, that these new approaches can ensure at least the same level of protection as traditional methods. This is a nontrivial task given decades of reliance on animal tests as the mentioned earlier "gold standard".[9]

A significant limitation also arises from the inherent complexity of biological systems and the fact that our understanding remains incomplete. The human body is an intricate network of organs, pathways, feedback systems, and

interactions. Many of these elements, such as homeostatic compensatory mechanisms, immune responses, and microbiome-driven effects, are extremely difficult to model *in silic*o.[8] Although advanced technologies, including organ-on-a-chip systems and multi-organ microphysiological platforms, are rapidly evolving, we are still far from fully replicating whole-organism physiology *in vitro*. Systems toxicology approaches, which integrate data across multiple biological levels using computational modeling, aim to bridge this gap by predicting organism-level outcomes from molecular and cellular inputs. Despite progress, achieving sufficient accuracy and verifiability remains a major challenge.

There are also practical and educational constraints. Broad implementation of *in silico* toxicology requires specialized knowledge in fields such as informatics, statistics, and AI, as well as access to computational resources. Moreover, it is increasingly clear that progress in this domain will depend on the effective use of AI. Traditionally trained toxicologists may need additional instruction or collaboration with data scientists to fully leverage these new tools and competently use AI-based platforms. Standardization presents another challenge: numerous platforms, algorithms, software packages, and databases are available, but they do not always produce consistent results. Harmonized guidelines for the use of *in silico* methods, analogous to OECD test guidelines for *in vitro* and *in vivo* methods, are necessary to ensure comparability and regulatory acceptance.

Finally, no single method offers a universal solution. The strongest predictive power arises from integrating multiple complementary approaches within the framework of Integrated Approaches to Testing and Assessment (IATAs).[14] A combination of modern methods—e.g., an *in vitro* assay identifying a mechanism of action, an *in silico* model predicting systemic toxicity, and an exposure assessment—can provide more convincing evidence than any single method used in isolation. Importantly, consistent negative results from such an integrated, modern testing strategy should be considered strong evidence of safety rather than a justification for continuing animal testing indefinitely. Many industrial chemicals show no toxicity at relevant exposure levels, and increasing confidence in negative outcomes generated by alternative methods would allow unnecessary animal tests to be avoided. In other words, if a coherent suite of modern methods indicates that a substance is not harmful, we should trust that conclusion, thereby saving time, costs, and animal use, and allowing resources to be focused on substances that truly warrant concern.

***The importance of in silico toxicology for regulatory and industrial applications***

*In silico* methods are gradually moving from the realm of scientific research into practical use in both regulatory decision-making and industrial settings. Notably, even traditionally cautious regulatory agencies, such as the U.S. FDA, are increasingly willing to rely on evidence generated through alternative methods. A clear example is the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) M7 guideline on mutagenic impurities in pharmaceuticals,[15] which has effectively institutionalized the use of *in silico* approaches, in the form of two complementary QSAR assessments, as an acceptable substitute for the Ames test, a bacterial reverse-mutation assay commonly used to detect mutagenic potential.

From the industry perspective, the message is clear: companies recognize the cost savings and strategic advantages offered by *in silico* methods. Implementing computational predictions early in product development can significantly reduce the number of toxicological "surprises" at later stages, saving both time and capital. Moreover, *in silico* approaches support compliance with the 3R principles by enabling the reduction and replacement of animal testing where possible and by refining unavoidable *in vivo* studies through improved targeting. For example, early insights into potential target organs of toxicity make it possible to design more focused animal studies.

In the pharmaceutical sector, *in silico* toxicology is particularly valuable because it helps reduce the number of expensive, months-long preclinical experiments and allows earlier elimination of compounds with unfavorable ADMET profiles. Every drug candidate that fails during *in vivo* studies or, even worse, in clinical trials generates financial losses amounting to tens of millions of dollars. *In silico* models enable earlier filtering of high-risk compounds, thereby improving the overall efficiency of the R&D pipeline. This industry also benefits from the ability to simulate scenarios that would be impossible or unethical to test in animals, such as analyzing reactive metabolites, predicting drug–drug interactions, or identifying structural toxicophores responsible for adverse effects. As a result, *in silico* approaches are becoming not merely supportive tools but essential components of strategies aimed at reducing business risk and increasing the likelihood of clinical success.

***The future of in silico toxicology: from vision to implementation***

Toxicology at the turn of the 21st-century is rapidly moving toward a new paradigm in which *in silico*, *in vitro*, and mechanistic approaches will play a dominant role. The

arguments for this shift are compelling: on one hand, there is a growing number of substances requiring safety assessment, accompanied by rising costs and ethical concerns; on the other hand, scientific progress has provided tools that allow toxicity to be studied faster, at lower cost, and with far greater mechanistic insight. What is needed is a genuine "inversion" of traditional procedures, transferring the primary burden of testing from live animals to advanced *in vitro* models and computational simulations. Only a radical paradigm shift will allow us to meet the scientific, regulatory, and societal challenges of the coming decades. To fully implement this new paradigm, coordinated efforts are required across several key areas:

- **Further scientific development of alternative methods.** Continuous advancement of *in silico* models is essential, including deeper integration of deep learning approaches, hybrid models that combine chemical and biological descriptors, and progress in *in vitro* systems such as optimized cell lines, organoids, and microfluidic technologies.
- **Validation and standardization.** The toxicology community must establish modern validation frameworks for both *in silico* and *in vitro* approaches. Evidence-based validation, inspired by the principles of evidence-based medicine, is increasingly recommended for evaluating new methods. International organizations such as the OECD have begun publishing initial guidance, including the well-known OECD Principles for QSAR validation. Crucially, it must be demonstrated in practice that integrated alternative approaches can predict hazards with confidence levels comparable to those of traditional *in vivo* tests. Demonstrating such equivalence is essential for building trust among regulators and the public.
- **Integration of data and approaches.** No single method, whether *in vitro* or *in silico*, can capture the full complexity of biological systems. Therefore, the future lies in IATAs, which combine diverse types of data into coherent risk assessments. Formal weight-of-evidence frameworks should be further developed to guide the integration of outputs from multiple complementary methods. Equally important is the creation of informatics platforms capable of integrating large toxicological datasets, ensuring that big-data omics studies are accessible and genuinely useful for modeling and decision-making.
- **Education and cultural change in science.** Transitioning to this new paradigm requires preparing a new generation of toxicologists who are fluent in both the biological and data sciences. Academic and industrial communities must also move beyond their attachment to "the old and familiar" (such as routinely repeated rodent assays) and adopt a mindset open to innovation. Continuing to rely on legacy animal models without rigorous evaluation of their relevance is no longer justified. Instead, decisions should be guided by the principle of selecting the best available method for each scientific question, regardless of its historical status.

## CONCLUSIONS

*In silico* toxicology has emerged as a fundamental component of modern chemical safety assessment, extending well beyond QSAR modeling to include advanced computational, mechanistic, and AI-driven approaches. Although its scientific value is increasingly supported by robust evidence, its full integration remains hindered by persistent reliance on traditional animal-based paradigms. Overcoming these barriers demands a decisive shift toward innovation-driven, interdisciplinary, and evidence-based frameworks, positioning *in silico* toxicology as a central pillar of 21st-century toxicology.

**Conflicts of interest**
The author declares no conflicts of interest.

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2026 | Vol. 3 | No. 1: 15-22

ORIGINAL ARTICLE

# Volatile compounds screening method in human blood by HS-GC-FID: Application in forensic toxicology to discriminate an antemortem consumption from postmortem formation of ethanol

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Submitted: 18/08/2024 - Accepted: 03/12/2024 - Published: 31/01/2026 - DOI: https://doi.org/10.62129/CSJB3774

**ABSTRACT**

**Background.** Determining whether a positive blood alcohol concentration (BAC) originates from antemortem consumption or postmortem formation is a frequent issue in forensic toxicology, especially when markers of alcohol ingestion cannot be analyzed. This study presents a validated HS-GC-FID method for detecting and quantifying volatile compounds in human blood to assess the risk of postmortem ethanol formation in cadaveric samples.

**Methods.** The validation of the method was carried out in accordance with the Guidelines of the Scientific Working Group for Forensic Toxicology (SWGTOX, 2013). A Clarus 580 gas chromatographic system equipped with a Headspace Perkin Elmer Turbomatrix 16 and Rtx-BAC Plus 1 column (30 m, 0.32 mm ID, 1.8 µm df) were used for method development and method validation, the acquisition software was TotalChrom Navigator version 6.3. Isopropanol was used as internal standard. For method validation, blood's healthy volunteers' samples collected *in vivo* were obtained from the blood transfusion center of the University Hospital of Oran. Seven cadaveric samples, received in order to carry out postmortem toxicological investigations, were analyzed by the validated method.

**Results.** A group of six volatile substances (acetone, butanol, ethanol, isobutanol, methanol and propanol) well correlated with putrefaction and microbial activity, were qualitatively and quantitatively analyzed by a selective method validated by HS-GC-FID in biological samples. All volatile solvents were studied in the range up to 4000 mg/L in terms of selectivity/specificity, limit of detection (LOD) and limit of quantification (LOQ), linearity, precision, accuracy and bias. The LOD was 1 mg/L for all solvents with a LOQ between 50 mg/L and 100 mg/L. Bias, repeatability, reproducibility and accuracy studies have shown good results. The developed method was applied to real cases to estimate the relevance of the method.

**Conclusions.** The present method is suitable for the identification and quantification of volatile compounds and can be a reliable tool in forensic toxicology. However, further studies should be carried out to establish the modelling of the relationship between the ethanol produced and the concentration of volatile solvents produced.

**Keywords:** *HS-GC-FID; Blood alcohol concentration; Volatile compounds; Validation; Forensic toxicology.*

Ethanol (ethyl alcohol) is the most commonly used psychoactive substance worldwide. In Algeria, alcohol consumption is a significant concern. According to the World Health Organization (WHO), Algeria is the second-highest consumer of alcoholic beverages in the Maghreb region.[1] As a result, it is heavily involved in arrests for problem drug use. Blood alcohol concentration (BAC) testing is the most frequently requested and performed analysis in toxicological investigations involving suspected exposure to psychoactive substances, with a positive BAC considered indisputable proof of intoxication. However, relying solely on this parameter can be problematic due to interpretation difficulties encountered in routine forensic cases.[2,3] BAC analysis in autopsy

specimens and its interpretation pose a major challenge in forensic toxicology.[4] The origin of the detected ethanol often becomes a matter of debate,[5] with three possible sources for ethanol detected in postmortem specimens: antemortem ingestion, antemortem endogenous production, and postmortem microbial fermentation in either cadavers or samples after collection.[6,7]

As BAC is routinely used as evidence in criminal and civil litigation, definitively determining its origin, whether exogenous (external) or endogenous (internal), is crucial.[4] Several factors need to be considered when assessing the origin of measured ethanol, such as the state of putrefaction of the cadaver at autopsy, the deceased's medical history, the circumstances of death, the condition of the test sample, and corroboration of results obtained from multiple matrices (e.g., urine and vitreous humor).[4,6,8]

The determination of ethanol metabolites (ethyl glucuronide and phosphatidylethanol), considered direct and specific biomarkers of alcohol consumption, is a valuable approach recognized by the international scientific community.[9-11] Furthermore, detecting volatile solvents produced during putrefaction, which do not occur naturally in the human body and can be identified in cadaveric samples, offers an effective means of better interpreting BAC results. This approach is particularly valuable when routine diagnostic methods for alcohol consumption are not used.[6,8,12]

At the Department of Pharmacology and Toxicology of the University Hospital of Oran, ethyl alcohol and cannabis are the most frequently detected psychoactive substances in cases received for postmortem forensic toxicological expertise. BAC testing is practically the only approach used to document alcohol consumption. Other methods for diagnosing alcohol consumption are not routinely employed. Therefore, this study aims to optimize and validate an HS-GC-FID method for detecting and quantifying volatile compounds in human blood to assess the risk of postmortem ethanol formation in cadaveric samples.

## MATERIALS AND METHODS

### *Chemicals and reagents*

All reagents used throughout the assay were of analytical grade: acetone > 99% GC quality (Sigma-Aldrich); ethanol > 99.8% GC quality (Sigma-Aldrich); isobutanol > 99.5% GC quality (Fluka Chemika); isopropanol 99.9% HPLC quality (Sigma-Aldrich); methanol > 99.9% HPLC quality (Chromasolv); n-butanol > 99.5% GC quality (Fluka Chemika); n-propanol 99.5% HPLC quality (Sigma-Aldrich). Deionized water used for the preparation of all solutions was purified to 18.2 MΩ using a PURELAB Option system (ELGA).

### *Biological samples*

For method validation, three blood units of healthy volunteers collected *in vivo* were obtained from the blood transfusion center of the University Hospital of Oran. For urine, samples were obtained from five healthy volunteers. For application of the method, seven cadaveric samples, received in order to carry out postmortem toxicological investigations, were analyzed by the validated method. All samples were stored at -80°C until analysis.

### *Preparation of calibrators, controls and internal standard solutions*

Since all the substances under study were soluble in water, only one mixture was prepared and then studied according to the selected working range, 50-4000 mg/L. Stock solutions of mixture acetone, ethanol, isobutanol, n-propanol, methanol, and n-butanol, with concentrations of 10000 mg/L, and the internal standard isopropanol with a concentration of 10000 mg/L, were freshly prepared daily by diluting the commercial solutions in deionized water.

Working solutions of calibrators and controls were prepared by dilution of the stock solutions directly in a 20 mL glass crimped headspace vial. The concentrations of calibrators and controls were, respectively: 4000, 2000, 1000, 500, 200, 100, 50 mg/L and 3000, 1500, 750, 150 mg/L. The concentration of the working solution of internal standard was 900 mg/L. The vials were crimped immediately after addition of the internal standard and the final solution was vortex-mixed. Because of the instability of the mixture of volatiles, the solutions should be immediately used after being prepared and quality controls have to be prepared every day. The same protocol was used to prepare the biologic matrices range in whole blood and urine.

### *Sample preparation*

Regarding the biological samples, these were thawed and thoroughly mixed before analysis. Each vial contained 1 mL of sample + 100 µL of 10 000 mg/L internal standard solution. The prepared solution was lightly mixed manually and placed in the headspace autosampler.

### *HS-GC–FID conditions*

All experiments were carried out using a PerkinElmer HS-GC-FID Clarus 580 system equipped with a flame ionization detector and coupled to a PerkinElmer Turbomatrix 16 automated headspace sampler. The column used to identify

and quantify volatile substances was an Rtx-BAC Plus 1 column (30 m × 0.32 mm ID × 1.8 µm df). The injector temperature was held at 200 °C while the detector temperature was set at 240 °C. The GC oven (column temperature) was held constant at 35°C during 0 minutes, followed by an increase of temperature at a rate of 10°C/min to 80°C, and maintained for 0 minutes. The carrier gas was nitrogen at 16 psi.

For the headspace conditions, the oven temperature was maintained at 70 °C and the syringe temperature at 75 °C. The sample injection volume was 0.06 mL. Before injection, the vials were incubated for 5 min. The GC cycle time was set to 4.5 min, with a pressurization time of 1 min. Analytical data were processed using TotalChrom Navigator version 6.3.

### *Validation procedure*

The validation of the method was carried out in accordance with the Guidelines of the Scientific Working Group for Forensic Toxicology (SWGTOX, 2013).[13] The required validation parameters were: selectivity, linearity, accuracy, precision, reproducibility, repeatability and carryover. The limit of quantification (LOQ) and limit of detection (LOD) were also determined.

## RESULTS AND DISCUSSION

A HS-GC-FID method was developed and validated for the qualitative and quantitative analysis of a group of 6 volatile organic substances with different physicochemical properties. The retention times obtained are presented in Fig. 1. Good chromatographic separation between all the compounds was obtained, including the internal standard.

### *Matrix effect*

The mixture of constituents was successively analyzed in three different matrices: water, blood, and urine. The Student's t-test was then applied to assess significant differences between two regression slopes, revealing no matrix effect among water, blood, and urine.[14] The results are summarized in Table 1, with all t-test values below the theoretical threshold, confirming the absence of a matrix effect. Additionally, the liquid–air partition coefficient exhibited a consistent trend in both blood and water. Given that urine contains

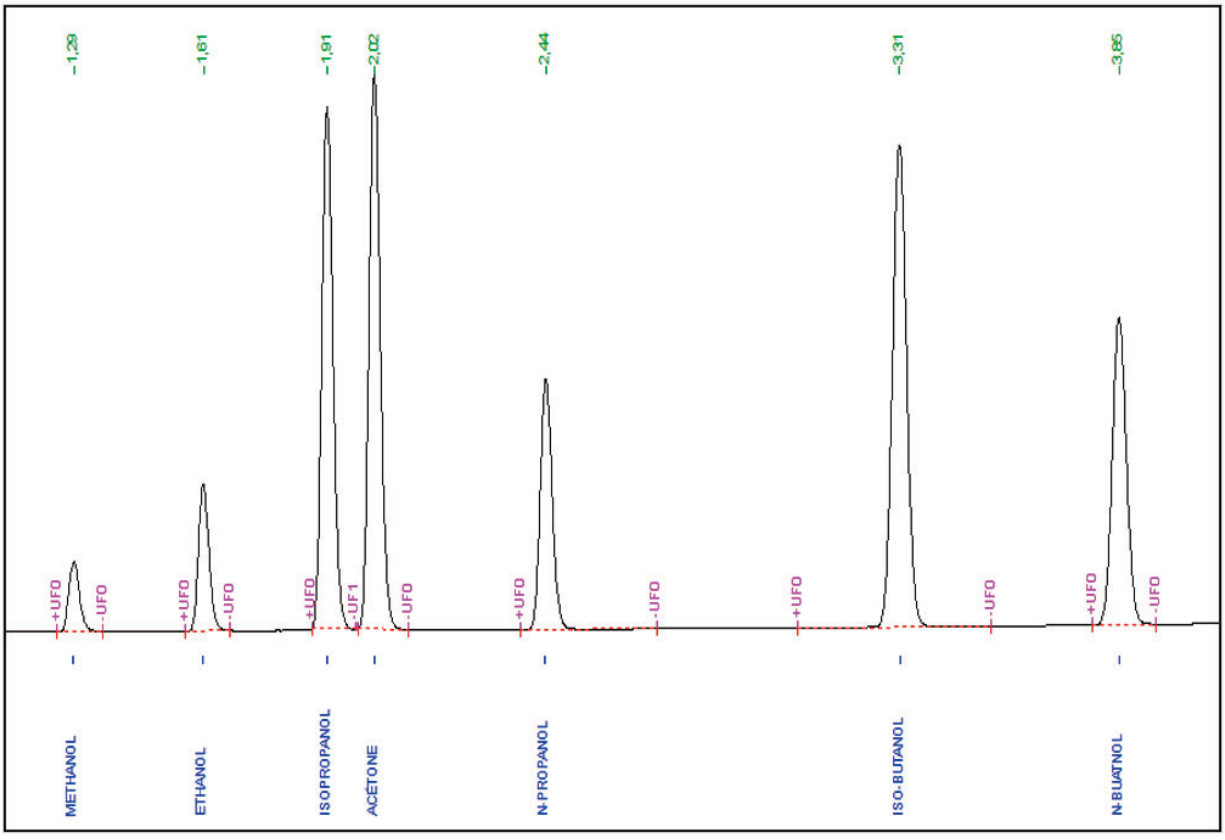


**Figure 1.** Chromatograms obtained for a mixture of volatile substances by HS-GC-FID (Credits: courtesy of the authors).

**TABLE 1.** Student's t-test results for the matrix effect study.

| | Water/Blood | | Water/Urine | |
|---|---|---|---|---|
| | t calculated | t critical value | t calculated | t critical value |
| **Methanol** | 1,53 | | 2,22 | |
| **Ethanol** | 1,88 | | 2,18 | |
| **Acetone** | 2,27 | | 0,036 | |
| **Propanol** | 1,66 | ***2,306*** | 0,035 | ***2,306*** |
| **Isobutanol** | 2,08 | | 0,035 | |
| **Butanol** | 1,76 | | 0,019 | |

approximately 98% water, calibration curves prepared in water are considered adequate.[8] Therefore, the calibration curve will be validated using water as the matrix.

***Linearity***

In order to analyze the linearity of the method under study, five curves (5 replicates per concentration) with seven calibrators (50; 100; 200; 500; 1000; 2000; 4000 mg/L) were performed for acetone, ethanol and methanol; and with six calibrators (100; 200; 500; 1000; 2000; 4000 mg/L) for butanol, isobutanol and propanol. In the forensic toxicology context, the concentrations are properly spaced over the range to assess exposure to different solvents.

The results show that the different points in the range overlap in all five curves. The various volatile solvents included in the study showed a coefficient of determination $R^2 > 0.999$ (Table 2). Generally, in forensic toxicology, an $R^2$ greater than 0.995 is required for a regression line to be considered sufficiently linear.[15,16] In addition, statistical approaches have been used to analyze the linearity: Student's t-test for comparing the intercept with 0**,** Fischer test for the existence of a significant slope, and analysis of Variance lack-of-fit test (ANOVA-LOF).[13,16]

Concerning the Student's t-test comparing the intercept with 0, the calculated t is below the critical value at 5% risk for methanol and ethanol, so it can be concluded that the intercept is not different from 0 and that the method is specific. On the contrary, it is different from 0 for acetone, propanol, isobutanol and butanol.

Regarding the test for the existence of a significant slope, using Fischer test, from the raw results, F1 exp > F crit with $p < 0.05$, we can conclude the existence of a significant slope for methanol, ethanol, acetone, propanol, butanol and isobutanol. There is therefore a linear dependence between the dependent and independent variables, at the probability threshold considered. Finally, for the Test of validity of the regression line, the results of the F2 test for methanol, ethanol, acetone, propanol, butanol and isobutanol are lower than the critical value, which means that the fit is valid at the probability threshold considered (we accept the null hypothesis).

Another good approach to selecting a calibration model is to study the distribution of standardized residuals between the values obtained and the values predicted by the model. The distribution of these residuals should be in the ± 2 interval and should not be structured.[17] All distributions of standardized residuals obtained for the solvents do not exceed the ± 2 interval.

In conclusion, all the tests used show that the method has acceptable linearity for linear response modeling.

***Limits of detection and quantification***

Several approaches can be used to determine the LOD and LOQ of an analytical method. The LOD was estimated using statistical tests according to SWGTOX. The blank was analyzed six times, and the LOD was calculated from the mean and standard deviation of the signals obtained for each compound. Subsequently, descending concentrations of 5, 1, 0.75, and 0.5 mg/L were analyzed, and the LOD was determined to be 1 mg/L for all solvents.

The LOQ was determined using the lowest non-zero calibrator. The lowest points of the calibration curves were analyzed nine times to evaluate bias, precision, and accuracy. For acetone, ethanol, and methanol, a concentration of 50 mg/L met the criteria for detection, identification, bias, and precision, and was therefore adopted as the LOQ. For butanol, isobutanol, and propanol, a concentration of 100 mg/L satisfied all criteria and was established as the LOQ for these solvents.

**TABLE 2.** Validation data of HS-GC-FID assay for the studied analytes.

| Analyte | Retention time (min) | LOD (mg/L) | LOQ (mg/L) | Linear range (mg/L) | Linearity | | | QC (mg/L) | Bias CV % | Repeatability CV % | Intermediate precision CV % | Accuracy RE % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Slope | Intercept | $R^2$ | | | | | |
| **Methanol** | 1.29 | 1 | 50 | 50 - 4000 | 0.2491 | 0.0048 | 0.9998 | **150** | 2.3 | 6.3 | 9.9 | -2.3 |
| | | | | | | | | **750** | 4.4 | 4 | 6.5 | -4.4 |
| | | | | | | | | **1500** | 10 | 1.1 | 5.3 | -10 |
| | | | | | | | | **3000** | 7.2 | 3 | 6 | -7.2 |
| **Ethanol** | 1.60 | 1 | 50 | 50 - 4000 | 0.5173 | 0.0059 | 0.9999 | **150** | 4.9 | 4.5 | 9 | -4.9 |
| | | | | | | | | **750** | 2.3 | 1.9 | 3.2 | -2.3 |
| | | | | | | | | **1500** | 10 | 1.4 | 2.8 | -10 |
| | | | | | | | | **3000** | 4 | 2.1 | 2.9 | -4 |
| **Acetone** | 2.02 | 1 | 50 | 50 - 4000 | 2.0371 | 0.0491 | 0.9999 | **150** | -8.9 | 5 | 7.7 | 8.9 |
| | | | | | | | | **750** | -4.2 | 2.4 | 5.9 | 4.2 |
| | | | | | | | | **1500** | -4.6 | 1.4 | 2.9 | 4.6 |
| | | | | | | | | **3000** | -0.5 | 2.8 | 5 | 0.5 |
| **Propanol** | 2.44 | 1 | 100 | 100 - 4000 | 0.9581 | 0.0258 | 1 | **750** | -4.6 | 1.9 | 2.8 | 4,6 |
| | | | | | | | | **1500** | -1.1 | 1.4 | 3.0 | 1.1 |
| | | | | | | | | **3000** | -1.9 | 1.8 | 2.0 | 1.9 |
| **Isobutanol** | 3.33 | 1 | 100 | 100 - 4000 | 2.1132 | 0.0777 | 0.9999 | **750** | -14.7 | 2.2 | 3.8 | 14.7 |
| | | | | | | | | **1500** | -12.6 | 2.5 | 4.1 | 12.6 |
| | | | | | | | | **3000** | -10.1 | 2.4 | 4.1 | 10.1 |
| **Butanol** | 3.85 | 1 | 100 | 100 - 4000 | 1.353 | 0.0575 | 0.9997 | **750** | -12.8 | 2.3 | 3.5 | 12.8 |
| | | | | | | | | **1500** | -8.5 | 3.3 | 5.4 | 8.5 |
| | | | | | | | | **3000** | -8.8 | 2.8 | 4.4 | 8.8 |

LOD: Limit of detection; LOQ: Limit of quantification; $R^2$: Coefficient of determination; QC: Quality control; CV: Coefficient of variation; RE: Relative error.

Regarding ethanol, the calculated LOD of 1 mg/L shows that it is possible to detect ethanol below the legal threshold of 200 mg/L. In addition, the LOQ was 50 mg/L, indicating that it will be possible to accurately quantify ethanol concentrations above 50 mg/L, which means that the method can be applied in forensic toxicology for the determination of blood alcohol levels. Similarly, for other solvents, the method is considered sufficiently sensitive to identify and quantify them.

***Repeatability, intermediate precision, accuracy and bias***

For bias, precision, and accuracy studies, four quality controls at 150, 750, 1500, and 3000 mg/L were used for acetone, ethanol, and methanol, and three quality controls (750, 1500, and 3000 mg/L) for butanol, isobutanol, and propanol. All controls were prepared in blood.

To assess repeatability and reproducibility, the quality controls were analyzed in triplicate over five days. The results were compared with the reference coefficient of variation (CV%). Acceptable CV values were ≤ ± 10% for ethanol and ≤ ± 20% for all other analytes.[13]

Regarding bias, CV values were < ± 10% for acetone, ethanol, methanol, and propanol. For isobutanol, CV exceeded ± 10% at all concentrations, and for butanol, it exceeded ± 10% at 750 mg/L. Nevertheless, all values remained below 20%, which is considered acceptable. In the

repeatability and reproducibility studies, CVs were below 10% for all analytes.

Concerning accuracy, the mean relative error (RE, bias) was within ± 20% of the nominal concentration, meeting the specified criteria.[15] The results of these evaluations are summarized in Table 2.

***Carryover***

To evaluate carryover several high concentration solutions were tested: 1000, 2000 and 4000 mg/L. A blank was injected after each test. The chromatograms obtained with the blank samples analyzed after the high concentration samples showed no signal, indicating that there was no evidence of carryover. The sequence of injections could therefore be randomized.

***Selectivity***

To evaluate the selectivity of the method, a random pool of samples (blood and urine) was prepared and divided into two aliquots. The first was directly analyzed with an internal standard only, and the second was spiked with the volatile compound mixture at a concentration of 1000 mg/L. Analysis of the results obtained in the various matrices (blood and urine), and comparison of the blank samples with the spiked samples revealed the absence of false positives and negatives. In addition, the selectivity between the different solvents was studied by calculating the selectivity factor α for the different couples. The selectivity factor α was > 1 for all the solvents tested, confirming that our method is selective.

***Application to real cases***

To assess the method's effectiveness in a forensic setting, cadaveric blood samples were analyzed using the validated chromatographic method. Cases were selected based on the presence of factors potentially influencing blood alcohol levels, with the additional criteria of compliant (unbroken), non-coagulated, and well-sealed specimens.

It should be noted that autopsy findings are often missing, and the presence or not of alcohol breath in the gastric contents, can make interpretation difficult. Alcohol breath suggests recent intake, potentially during the pre-absorption phase. In addition, the risk of passive diffusion of alcohol from the stomach to the bloodstream after death can occur, leading to artificially high BAC readings that don't reflect levels at the time of death.[18]

A total of seven real cases were analyzed. The subjects included one female and six males, ranging from 22 to 50 years old. Causes of death varied, encompassing drowning (1 case), violent trauma (4 cases), heart failure (1 case), and undetermined causes (1 case). The time between death and autopsy ranged from 1 day to 4 months (unknown in one case) which could be the cause of the post-mortem production of alcohol by a process of putrefaction. This can lead to artificially elevated BAC during postmortem analysis, making it difficult to determine the actual BAC at the time of death. Six samples were peripheral blood, while one was cardiac blood. Notably, none of the samples were supplemented with sodium fluoride (NaF) for preservation, and they were stored for 4 to 15 days under unspecified conditions.

The BAC in our specimen can be influenced by various factors, including type of samples, storage conditions, the circumstance of death and the elapsed time between death and the collection of blood samples. As time passes, alcohol may be metabolized or degraded, making it challenging to determine the original BAC accurately. The validated method had successfully detected and confirmed the presence of the targeted volatile substances in the blood samples (Table 3):

**TABLE 3.** Results of the solvents analysis by HS-GC-FID.

| Patient | 01 | 02 | 03 | 04 | 05 | 06 | 07 |
|---|---|---|---|---|---|---|---|
| **Blood nature** | **PB** | **CB** | **PB** | **PB** | **PB** | **PB** | **PB** |
| **Methanol (g/L)** | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| **Ethanol (g/L)** | 2,56 | 0,155 | 0,77 | 1,96 | 0,29 | 0,093 | 0,13 |
| **Acetone (g/L)** | < LOQ | < LOQ | < LOQ | < LOQ | 0,34 | < LOQ | < LOQ |
| **Propanol (g/L)** | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| **Isobutanol (g/L)** | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |
| **Butanol (g/L)** | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ | < LOQ |

PB: Peripheral blood; CB: Cardiac blood; LOQ: Limit of quantification.

- *Methanol and butanol:* absent in all samples
- *Acetone:* visually detected in five cases (1, 2, 3, 5, and 6). However, four were below the LOD of 1 mg/L and considered negative. Only case 5 had a quantifiable amount (340 mg/L)
- *Propanol:* detected in four cases (1, 5, 6, and 7). Considering the LOD, only the propanol peak in case 5 was deemed detectable
- *Isobutanol:* not detected in any of the samples

Our results show that the origin of the ethanol measured for case 5 is debatable and the BAC result may be rejected. According to the literature,[2,4,19] several factors have been incriminated including the delay extended shelf life under undetermined conditions before receiving blood samples taken from tubes not supplemented with NaF. These two critical factors can affect the integrity of samples by promoting the microbial formation of ethanol (post-sampling),[6] and by therefore complicate the interpretation of the results. Moreover, the duration elapsed between death and autopsy is undetermined. This is a very important factor in take into account when interpreting, any extension strongly evokes the putrefaction hypothesis and therefore the neoformation of ethanol postmortem**.** On the other hand, for the other cases it is rather the hypothesis of neoformation of ethanol which can be rejected given the absence of volatile solvents indicating the absence of contamination microbial.

## CONCLUSIONS

The analysis of volatile organic compounds offers a promising alternative approach for distinguishing the source of ethanol and confirming antemortem alcohol intake, particularly in settings where routine methods for diagnosing alcohol use are unavailable. The HS-GC-FID method developed in this work allows for the identification and quantification of ethanol alongside five other solvents of interest for assessing microbial ethanol production.

Our future endeavors in this area involve expanding the scope of this method and conducting more in-depth studies to model the relationship between the concentrations of ethanol produced and the concentration of volatile solvents generated by microbial activity. This modeling would enable us to calculate the microbially generated BAC in postmortem blood based on the concentrations of volatile solvents detected, ultimately leading to a more accurate estimate of the actual BAC level. Additionally, the development of techniques for measuring biomarkers of alcoholism remains crucial for a more comprehensive understanding of the challenges associated with BAC interpretation.

**Conflicts of interest**

The authors declare no conflicts of interest.

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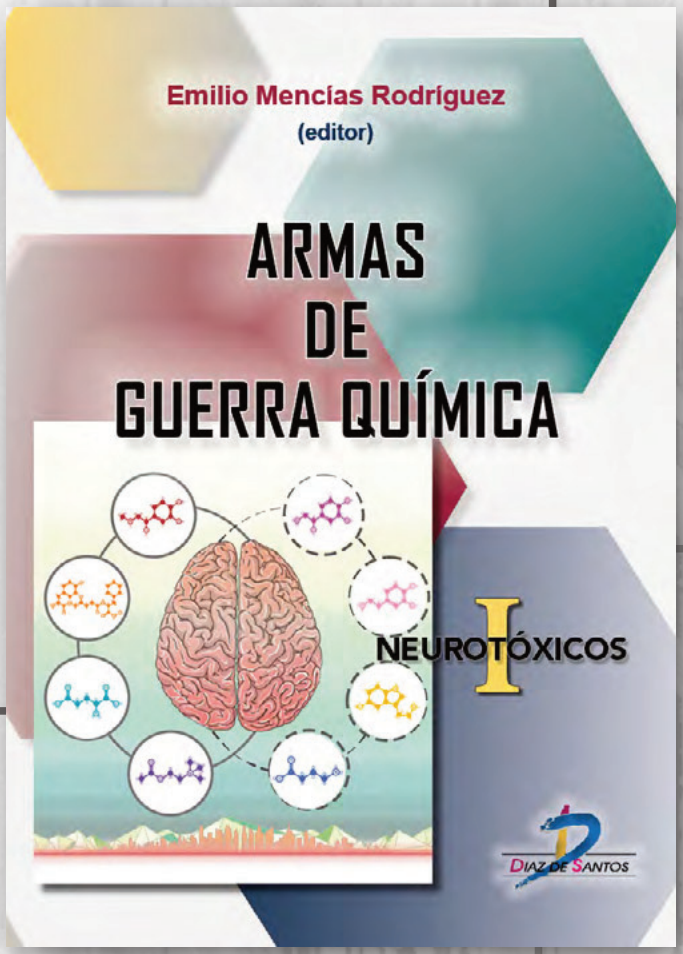


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CASE SERIES

# High-resolution infrared imaging for clinical monitoring of *Bothrops* spp. snakebite envenomations in Uruguay: A case series

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Submitted: 13/11/2025 - Accepted: 15/01/2026 - Published: 31/01/2026 - DOI: https://doi.org/10.62129/EXVU1128

**ABSTRACT**

**Background.** In Uruguay, approximately 50 *Bothrops* snakebite accidents are reported each year, most commonly involving *B. alternatus* and *B. pubescens*. Beyond systemic coagulopathy, *Bothrops* envenomation induces marked local inflammatory damage. Infrared (IR) thermography has emerged as a noninvasive technique that does not expose patients to ionizing radiation and is capable of detecting thermal changes associated with tissue inflammation.

**Objective.** Describe the characteristics and semiological utility of IR imaging compared with standard photographic documentation in the monitoring of local inflammatory processes following *Bothrops* snakebite envenomation.

**Methods.** Three clinical cases of *Bothrops* envenomation were evaluated using high-resolution IR thermography under controlled environmental conditions. Qualitative thermal images were acquired, and Regions of Interest (ROIs) were delineated to obtain quantitative temperature measurements. Thermal asymmetry was assessed by calculating ΔT values between affected and contralateral anatomical regions.

**Results.** All cases exhibited localized inflammatory findings—edema, erythema, and pain—without evidence of severe systemic involvement. IR thermography demonstrated thermal asymmetries consistent with active inflammation, with the average temperature difference (ΔTaverage) values >1 °C in all patients, in line with previously reported patterns in *Bothrops* envenomation.

**Conclusions.** IR thermography provided functional, quantitative assessment of local inflammation that was not attainable through conventional photography. The ability to measure thermal gradients supports its potential role as a semiological tool to monitor local phenomena and identify areas of higher infectious or inflammatory risk in *Bothrops* snakebite accidents.

**Keywords:** *Thermography; Bothrops; Snake bites; Envenomations; Diagnostic imaging.*

The Toxicological Information and Advice Center (CIAT) of Uruguay records approximately 100 snakebite accidents annually, of which about 50 are caused by two species of the genus *Bothrops*: *B. alternatus* ("Crucera") and *B. pubescens* ("Yara"). In addition to inducing fibrinogen-consumption coagulopathy—its main systemic effect—*Bothrops* venom produces marked local tissue alterations at the bite site, characterized by an inflammatory process that leads to localized edema and pain, often complicated by necrosis and secondary infections.[1-3]

Infrared (IR) imaging has potential as a semiological tool, as it is a bedside, noninvasive method that does not require sedation and does not expose the patient to ionizing radiation.[4] The technique relies on acquiring IR images capable of noninvasively quantifying skin-surface temperature by capturing the radiation normally emitted by the body and generating a high-resolution digital image (thermogram). Although relatively recent, it has become a common practice in several medical fields.[3-5] Analysis of IR images has enabled the study of diseases in which skin temperature reflects underlying tissue inflammation, as well as conditions associated with increased or decreased blood flow.[6-8] Recent studies have also highlighted its potential for assessing local changes observed in *Bothrops* envenomation.[5-9]

## OBJECTIVE

Describe the characteristics of the IR image in comparison with the photographic image, assessing its semiological

utility for monitoring local inflammatory processes in *Bothrops* snakebite accidents.

## METHODOLOGY

Three clinical cases of *Bothrops* snakebite accidents were analyzed using IR imaging. All images were obtained with informed consent, and the protocol was registered with the Ministry of Public Health. A high-resolution infrared sensor (UltraMax 307,200 pixels), model E75, was used, assuming a skin emissivity of 0.98. Measurements were performed in a controlled environment (23 ± 1°C), and patients were allowed to thermally equilibrate for 15 minutes. For image interpretation, the «rainbow» color scale was selected, in which warmer areas appear in white/red tones and cooler regions appear in blue/black tones. Images were processed using the Snake Fy Thermal software.

After capturing the qualitative thermal images, Regions of Interest (ROIs) were delineated based on anatomical reference points using the camera software's polygon-drawing tool. This allowed comparison of quantitative data from affected areas with corresponding normal or contralateral regions. Thermal magnitudes in the ROIs (quadrilaterals, circles) of the affected limb were compared with their counterparts in the contralateral limb by calculating the temperature differences (ΔT) between the arithmetic means of all pixel temperatures within each ROI. These data enabled a quantitative assessment of temperature asymmetries between venom-affected areas and surrounding tissues of the affected limb or their contralateral equivalents. The average temperature difference (ΔTaverage) across the ROIs was calculated. For this case series, the procedures followed the recommendations of the American Academy of Thermology Point-of-Care Protocol and the Glamorgan Protocol.

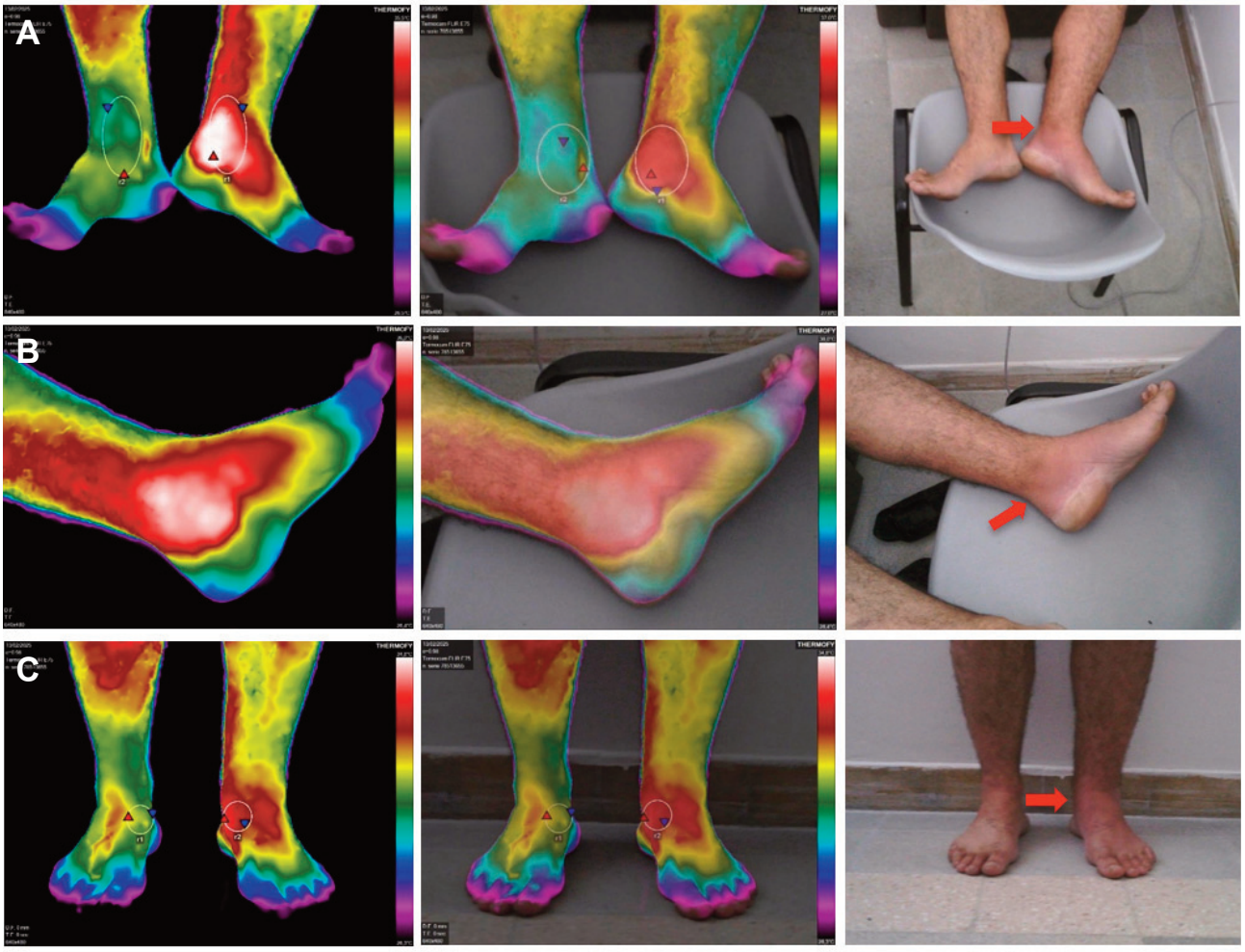


**Figure 1 (A, B and C).** Thermal images obtained on day 18 from three different approaches, demonstrating a ΔTaverage of 4.02°C in ROI 1 (small red arrow A). The corresponding photographic images show the puncture site (large red arrows) with visible edema and erythema (Credits: courtesy of the authors).

## CLINICAL CASES

### *Clinical case 1*

A 36-year-old male patient with no significant past medical history presented with a *B. pubescens* bite on the medial aspect of the left foot. He exhibited no systemic manifestations and received 8 vials of BIOL antivenom (produced by Instituto Biológico Argentino S.A.I.C.) 3 hours after the accident. After 72 hours, he was discharged without systemic abnormalities, although loco-regional edema persisted.

At the 18-day follow-up, the patient reported moderate pain, increased edema, erythema, and local warmth, without fever. Laboratory tests showed leukocytosis (15.8 × $10^3$/L) and elevated C-reactive protein (CRP, 43 mg/L). Physical examination revealed edema extending to the mid-leg, two puncture marks without blistering, and localized erythema. Fig. 1 shows clinical photographs of the lesion alongside the corresponding thermographic image, which demonstrated qualitative asymmetry due to the extent of inflammatory changes in ROI 1 (Fig. 1A), with a ΔTaverage value of 4.02°C compared with the contralateral area.

### *Clinical case 2*

A 46-year-old male patient with a history of achondroplasia presented with a *B. alternatus* bite on the medial aspect of the right thigh. He developed incoagulability and received 16 vials of BIOL antivenom 2 hours after the accident.

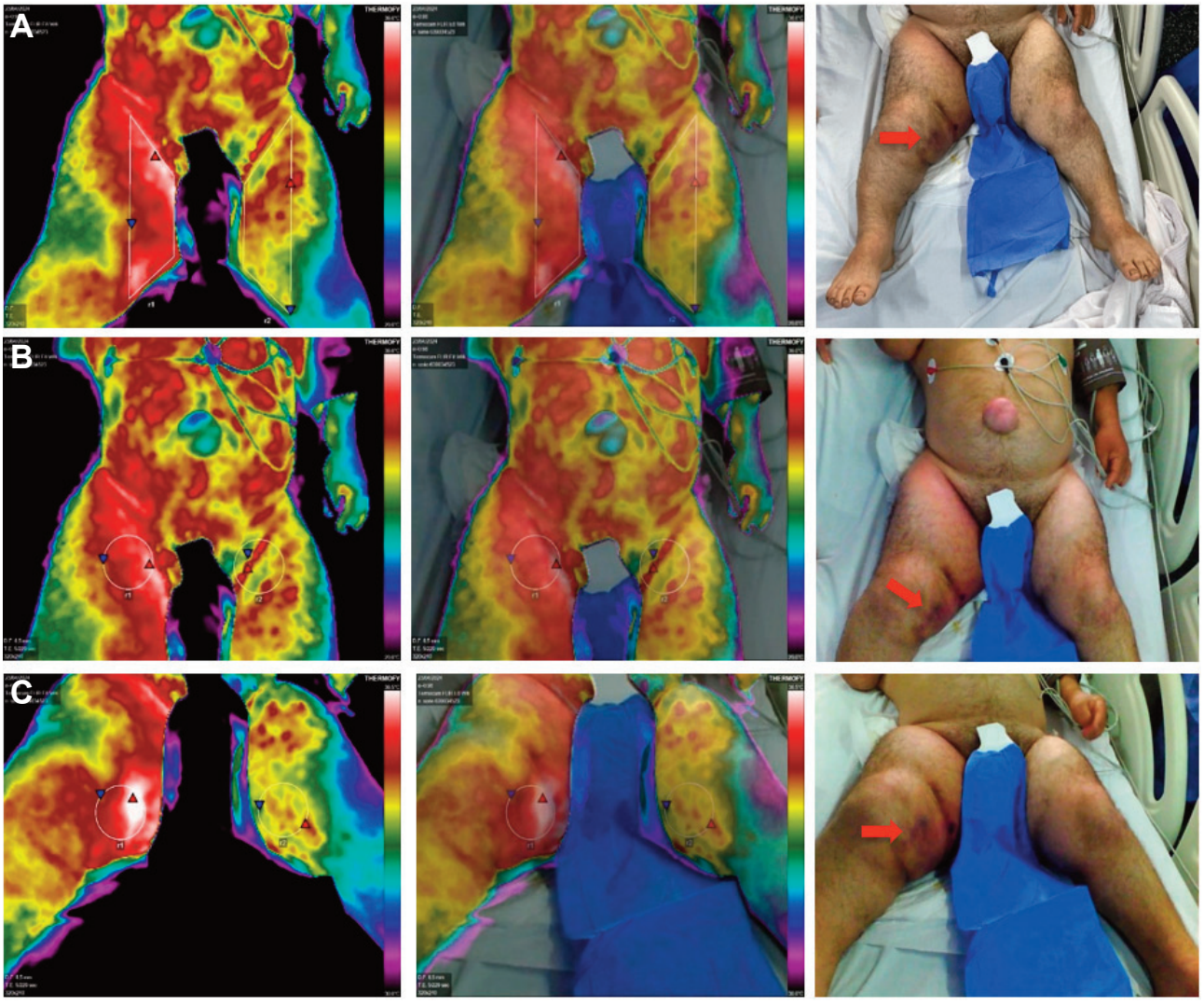


**Figure 2 (A, B and C).** Thermal images obtained on day 4 showing a ΔTaverage of 1.42°C in ROI 1 (small red arrow C) and extension of the inflammatory process. The corresponding photographic images show the bite site (large red arrows) with visible edema and erythema (Credits: courtesy of the authors).

At the day-4 follow-up, the patient reported pain. Physical examination revealed edema involving the entire right lower limb, with limited mobility, and a large hematoma at the bite site. Laboratory tests showed a leukocyte count of 10.8 × $10^3$/L and a CRP level of 10 mg/L. Fig. 2 shows an IR image demonstrating qualitative asymmetry with extension of inflammatory changes toward the right iliac fossa. In ROI 1 (Fig. 2C), a ΔTaverage value of 1.42°C was recorded compared with the contralateral side.

***Clinical case 3***

An 11-year-old previously healthy male patient sustained a bite from *B. alternatus* on the anterior–inferior third of the left leg. A tourniquet had been applied for 20 minutes prior to arrival at a primary-care facility. He presented without systemic manifestations and received 8 vials of BIOL antivenom 2 hours after the incident. Clinically, he exhibited pain, two puncture marks, ecchymosis around the bite site, and localized edema. The patient was referred to a tertiary-care center, where thermal imaging was performed on day 2 after the bite (Fig. 3). Laboratory evaluation showed leukocytosis (14.5 × $10^3$/μL) and a CRP level of 12 mg/L. Arterial and venous Doppler ultrasonography revealed no abnormalities. A ΔTaverage of 1.36°C was recorded in ROI 1 (Fig. 3A) compared with the contralateral limb.

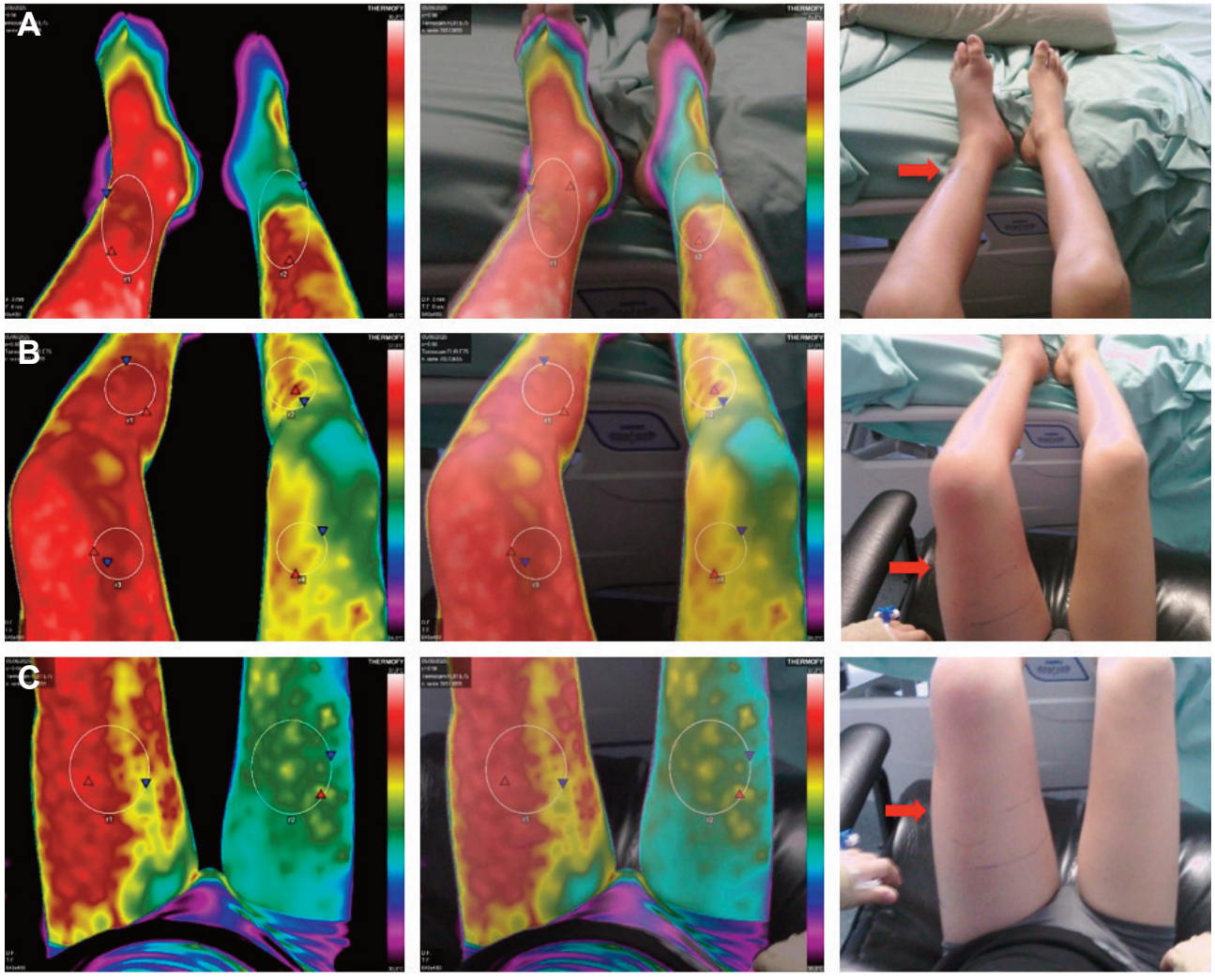


**Figure 3 (A, B and C).** Thermal images obtained on the second day show an average ΔT of 1.36°C in ROI 1 (small red arrow A) and the extent of the inflammatory process along the left lower extremity, with associated edema and pain. The corresponding photographic images show the bite site (large red arrow A) with edema extending beyond the puncture site (large red arrows B and C) (Credits: courtesy of the authors).

## DISCUSSION

The three cases presented correspond to snakebite envenomations caused by species of the *Bothrops* genus, which are responsible for the majority of envenomations in Uruguay. All patients exhibited local inflammatory manifestations such as edema, erythema, and pain, without severe systemic complications. IR thermography enabled non-invasive visualization of thermal changes associated with the local inflammatory response, as demonstrated in the consulted literature.[4-12] The images showed thermal asymmetries and dysfunction, with ΔTaverage values greater than 1°C, consistent with previously reported findings in *Bothrops* bites by Medeiros et al.[5] Across all cases, a local effect of the venom was evident, independent of systemic neutralization. Areas of decreased temperature—potentially associated with local ischemic activity—were observed, along with proximal and distal inflammatory changes characterized by thermal signatures described by Medeiros et al.[5] and Machado et al.[8]

In *Case 1*, the ΔTaverage of 4.02°C reflected persistent active inflammation 18 days after the bite, correlating with the clinical findings of edema and erythema, as well as elevated inflammatory markers, similar to reports from various authors.[4,5,13] This suggests that thermography may detect residual inflammatory activity even in late stages of clinical evolution, similar to reports by Medeiros et al.[5] and Machado et al.[6,8-10] where ΔTaverage values greater than 2.2°C were associated with humoral markers of infection. In *Case 2*, the ΔTaverage of 1.42°C corresponded to an extensive inflammatory process with a significant hematoma. Similar inflammatory patterns with ΔTaverage values below 1.5 °C have been reported by Medeiros et al.,[5] Sabitha et al.,[12] and Machado et al.[8-10] In *Case 3,* the ΔTaverage of 1.36°C was associated with areas of lower temperature around the puncture sites, suggesting local enzymatic activity, along with an inflammatory response extending to the proximal thigh, a behaviour that has been reported by several authors.[5,9,12,13] Thermography revealed localized thermal abnormalities compatible with acute inflammation but without significant vascular compromise, which was corroborated by a normal Doppler ultrasound, as reported in several international studies.[5,10,14,15]

In Uruguay, the CIAT is the first national toxicology reference center to complement clinical semiology with high-resolution IR imaging for monitoring snakebites caused by *Bothrops* species.[5,6,8] Compared to standard clinical photography, which does not provide quantitative information on inflammatory activity, IR imaging offers a functional assessment of tissues, allowing for the identification of the extent of inflammatory phenomena and the objective quantification of temperature differences through ΔT measurements.[6,8,12,14,15] Therefore, this non-invasive tool can significantly contribute to clinical practice by obtaining objective and quantitative data in emergency departments and intensive care units.[8-10] Its usefulness extends internationally to the monitoring and prognosis of possible local complications—for example, in the early assessment of local tissue damage and the risk of infection—as well as to clinical follow-up.[6,8,12,14] Based on these promising preliminary results, a prospective study is underway to validate and further analyze these thermal findings using a rigorous statistical approach.

## CONCLUSIONS

High-resolution IR thermography represents a valuable complement to the assessment of *Bothrops* envenomation, as it provides objective, non-invasive quantification of local inflammatory activity without exposing the patient to ionizing radiation, using temperature delta values at the bedside. It can help in understanding changes in local phenomena that are independent of antivenom neutralization. These findings underscore its potential as a complementary tool, alongside traditional photography, for understanding the metabolic, vascular, and inflammatory phenomena in snakebite envenomation, which are invisible to the naked eye, and justify further investigation through prospective studies.

**Conflicts of interest**

The authors declare no conflicts of interest.

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2026 | Vol. 3 | No. 1: 30-32

CASE REPORT

# Ketamine-induced "walnut bladder": A case report

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Submitted: 17/09/2025 - Accepted: 18/12/2025 - Published: 31/01/2026 - DOI: https://doi.org/10.62129/CONB7880

**ABSTRACT.** Ketamine-induced cystopathy is a severe complication of chronic recreational ketamine use, leading to bladder fibrosis and renal impairment. We report a 35-year-old man with long-term intranasal ketamine abuse who presented with severe lower urinary tract symptoms, hydronephrosis, and chronic kidney disease. Cystoscopy and histology revealed a fibrotic, inflamed bladder requiring reconstructive surgery with an ileal neobladder. This case highlights an advanced presentation of ketamine uropathy and emphasizes the need for early recognition to prevent irreversible urinary tract and renal damage.

**Keywords:** *Ketamine; Dissociative anesthetics; Walnut bladder; Uropathy; Addiction.*

Ketamine is a dissociative anesthetic widely used in both human and veterinary medicine. In recent years, its recreational use has increased steadily, particularly among young adults. Chronic consumption, especially via the intranasal route, has been associated with an emerging condition known as "ketamine-induced cystopathy", characterized by irritative lower urinary tract symptoms, progressive reduction in bladder capacity, pelvic pain, and chronic renal impairment in advanced stages.

The proposed pathophysiologic mechanism involves direct urothelial injury caused by urinary ketamine metabolites, along with subepithelial inflammation, fibrosis, and microvascular alterations. Although this condition is well documented in Asian and European case series, it remains uncommon in Latin America, leading to delayed clinical suspicion and diagnosis. Early detection is crucial to prevent irreversible renal damage. We present the case of a 35-year-old man with chronic intranasal ketamine use who developed severe ketamine-induced cystopathy and chronic renal impairment.

## CLINICAL CASE

A 35-year-old man with no family history of urologic disease had long-standing intranasal ketamine abuse since adolescence, associated with occasional alcohol and cannabis use. Since 2020, he had been under urologic follow-up for severe bladder dysfunction and recurrent urinary tract infections. In May 2025, he presented with fever, dysuria, marked urinary frequency, and bilateral flank pain. On physical examination, he appeared pale and had bilateral costovertebral angle tenderness. Continuous urinary incontinence was noted.

Laboratory tests revealed elevated serum creatinine, consistent with chronic kidney disease, anemia of chronic disorders, abnormal liver function tests, and blood cultures positive for Enterococcus faecalis. Computed tomography (CT) showed bilateral hydronephrosis, inflammatory thickening of the renal pelvis and ureters, and a small-capacity bladder (80 mL) with irregular, thickened walls (Fig. 1). Cystoscopy revealed a diffusely erythematous and friable bladder mucosa. Histopathologic examination demonstrated a fibrotic *lamina propria* with myxoid changes, congested vessels, mononuclear infiltration, and reactive urothelial changes. Alternative infectious or iatrogenic causes (including tuberculosis, radiotherapy, and interstitial cystitis) were excluded.

During hospitalization, targeted antibiotic therapy was administered with resolution of sepsis. Given the severe bladder involvement and persistent pelvicalyceal dilatation, reconstructive surgery was planned. An orthotopic neobladder was constructed using an ileal segment, with a favorable postoperative course, as shown in Fig. 2, which demonstrates adequate filling and no immediate complications.

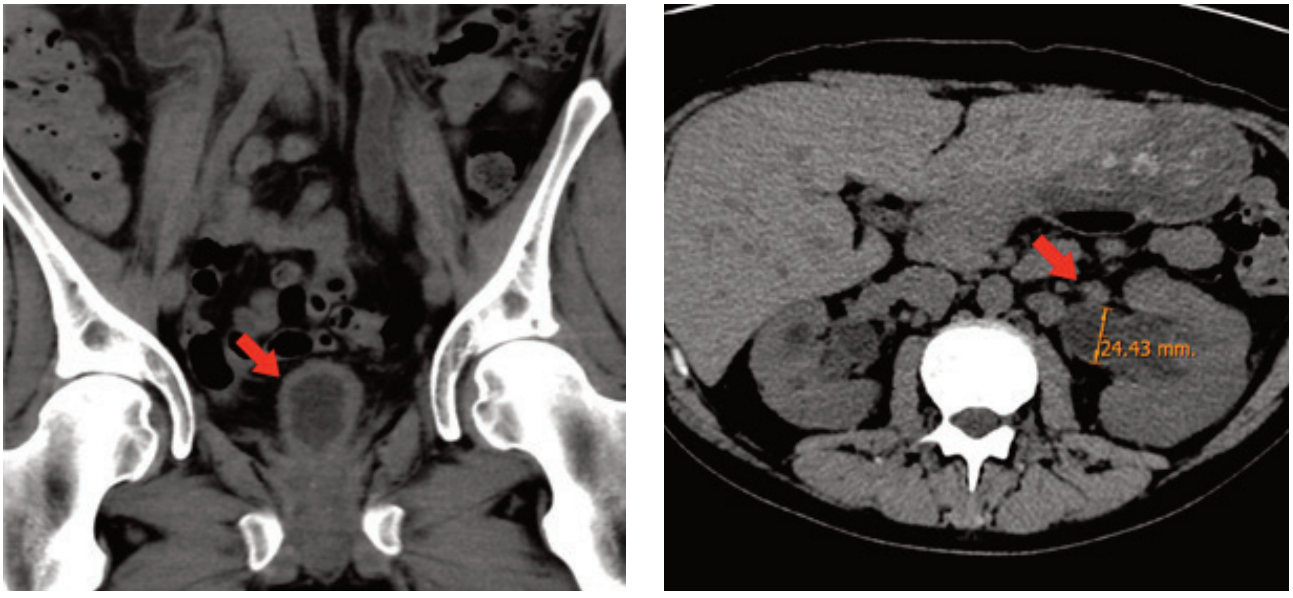


**Figure 1.** Abdominopelvic CT images. A. Coronal view showing a low-capacity bladder with diffuse mural thickening (red arrow), consistent with chronic cystopathy. B. Axial view showing a dilated ureter measuring 24.4 mm (red arrow), consistent with uronephrosis secondary to obstructive bladder dysfunction (Credits: courtesy of the authors).

On follow-up, the patient showed symptomatic improvement, a reduction in infectious episodes, and partial recovery of renal function. He remains under urologic and nephrologic follow-up, with psychological support to maintain abstinence.

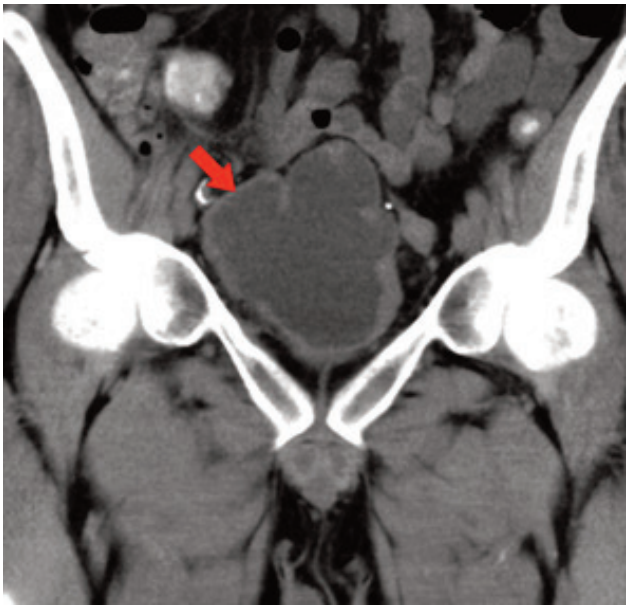

**Figure 2.** Postoperative coronal CT demonstrating an orthotopic neobladder created from a small bowel segment (red arrow), showing satisfactory filling (Credits: courtesy of the authors).

## DISCUSSION

Ketamine cystopathy is an emerging complication of chronic recreational use and represents a diagnostic challenge in regions where the prevalence of abuse is low or underreported. Its pathophysiology is related to the direct toxicity of urinary metabolites on the bladder epithelium, oxidative damage, and inflammatory processes leading to fibrosis and loss of bladder compliance.[1-3]

The clinical presentation typically includes urinary urgency, frequency, nocturia, suprapubic pain, and hematuria.[4,5] Over time, bladder contracture, secondary hydronephrosis, and renal function deterioration may develop.[6-8] Diagnosis is primarily clinical, supported by imaging studies, cystoscopy, and histopathologic findings. In the present case, the finding of a markedly reduced bladder capacity with diffuse wall thickening and biopsy evidence of inflammatory fibrosis confirmed the toxic etiology.

Management depends on the stage of disease: in early phases, cessation of ketamine use and medical therapy may stabilize symptoms.[9-12] In advanced stages, when bladder capacity is below 100 mL or upper urinary tract involvement is present, reconstructive surgery with enterocystoplasty is the most effective option.[13-16]

The relevance of this case lies in its late presentation, with severe bladder and renal damage requiring reconstruc-

tive surgery. Reports from Latin America remain scarce, underscoring the importance of considering ketamine cystopathy in the differential diagnosis of chronic bladder dysfunction in young adults.[17]

## CONCLUSION

Ketamine cystopathy should be suspected in patients presenting with chronic lower urinary tract symptoms and a history of recreational use, even in the absence of recent exposure. Early recognition is essential to prevent progression to chronic kidney disease and to preserve bladder function. The present case illustrates an advanced form of this condition and reinforces the need to raise awareness among the medical community regarding its clinical manifestations and timely management.

**Conflicts of interest**
The authors declare no conflicts of interest.

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