https://doi.org/10.31925/farmacia.2024.5.22 **ORIGINAL ARTICLE**

IMPROVING DIAGNOSTIC ACCURACY IN DIMPYLATE POISONING: A COMPARATIVE STUDY OF CHOLINESTERASE ASSAYS

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Abstract

Organophosphate (OP) intoxication remains a significant public health issue due to the widespread use of these compounds. Dimpylate, a widely used organophosphate insecticide, causes acute intoxication by inhibiting acetylcholinesterase. A sensitive gas chromatography-mass spectrometry (GC-MS/MS) method was developed to detect dimpylate and its primary metabolite in urine. Liquid-liquid extraction was performed using a solvent system consisting of dichloroethane, methyl chloride and chloroform in a 1:1:1 ratio. A comparative analysis of the Ellman and EMIT methods for assessing acetylcholinesterase and butyrylcholinesterase activity in one patient with acute dimpylate intoxication was also conducted. Rapid diagnosis is crucial for effective treatment, but current literature lacks detailed analytical toxicology methods for emergency settings. While Ellman's method is commonly used, its accuracy is debated in the actual research field. Butyrylcholinesterase inhibition level exceeded acetylcholinesterase level initially, supporting its potential as a biomarker. Despite both enzymes declining rapidly, persistent low acetylcholinesterase levels for 16 days, despite clinical improvement, indicated a dissociation between enzyme recovery and clinical status. Acetylcholinesterase inhibition alone may not fully reflect the clinical course of dimpylate poisoning. Both point-of-care and laboratory-based methodologies, such as EMIT, hold promise as diagnostic tools for dimpylate intoxications.

Rezumat

Intoxicația cu pesticide organofosforice (OP) rămâne o problemă semnificativă de sănătate publică datorită utilizării lor pe scară largă. Dimpilatul, un insecticid OP utilizat în mod obișnuit, provoacă intoxicație acută prin inhibarea acetilcolinesterazei. O metodă sensibilă de cromatografie gazoasă cuplată cu spectrometria de masă (GC-MS/MS) a fost dezvoltată pentru detectarea dimpilatului și a metabolitului său primar în urină. Extracția lichid-lichid a fost realizată folosind dicloroetan, clorură de metil și cloroform într-un raport de 1:1:1 (v/v/v). De asemenea, a fost efectuată o analiză comparativă a metodelor Ellman și EMIT pentru evaluarea activității acetilcolinesterazei și butirilcolinesterazei la un pacient cu intoxicație acută cu dimpilat. În timp ce metoda Ellman este folosită în mod obișnuit, acuratețea ei este dezbătută. Nivelul de inhibare a butirilcolinesterazei a depășit nivelul acetilcolinesterazei inițial, susținând potențialul său ca biomarker. Menținerea unui grad de inhibare accentuat al acetilcolinesterazei timp de 16 zile, în ciuda îmbunătățirii clinice, a indicat o disociere între reactivarea enzimei și starea clinică. Astfel, evoluția intoxicației cu dimpilat nu poate fi caracterizată doar prin măsurarea inhibării acetilcolinesterazei. Atât testele rapide, cât și analizele de laborator elaborate, cum ar fi EMIT, par a fi instrumente utile pentru diagnosticarea intoxicațiilor cu dimpilat.

Keywords: dimpylate, acute intoxication, acetylcholinesterase (AChE), butyrylcholinesterase (BChE)

Introduction

Due to their efficacy and environmental compatibility, organophosphates (OPs) are widely employed in agriculture, horticulture and forestry. However, their broad-spectrum toxicity presents a significant human and environmental safety challenge. Dimpylate, also known as diazinon, is a pyrimidine-based organophosphate insecticide (OP) [1].

OP exposure disrupts nerve function *via* cholinesterase inhibition; neuropathy targets esterase inhibition and alkyl group release [2]. OPs inhibit acetylcholinesterase, causing acetylcholine accumulation and overstimulation of cholinergic receptors, leading to acute toxicity [3]. Dimpylate, a thionic organophosphate, undergoes metabolic activation to its corresponding oxon form, diazoxon, primarily within the liver and gastrointestinal tract *via* cytochrome P450 enzymes. The oxon metabolite exhibits significantly enhanced acetylcholinesterase inhibitory activity compared to the parent compound. Comprehensive identification and characterisation of these metabolites are essential for elucidating dimpylate's toxicokinetics and mechanism of action. Rats rapidly eliminate dimpylate (half-life: approximately 12 hours) primarily through urine [4, 5]. Given this data, urine

analysis may be useful for detecting human dimpylate exposure.

Acute organophosphate intoxication requires both anticholinergics to manage symptoms and acetylcholinesterase reactivators to restore enzyme function [3] . Certain non-nucleophilic reactivators can restore AChE activity [6, 7]. Atropine is the primary pharmacological intervention for acute organophosphate intoxication. While effective in managing muscarinic symptoms, it does not mitigate nicotinic effects [8].

Lee *et al*. reported in 2021 that pesticide self-poisoning accounts for 14 - 20% of global suicides (approx. 110,000 - 168,000 deaths), predominantly in low and middle-income countries where small-scale farming facilitates household access to highly hazardous pesticides [9]. Moreover, the presence of alcohol in over 50% of dimethoate intoxication cases in rural Asia significantly complicates diagnostic procedures [10].

This study aims to evaluate and compare methods for determining acetylcholinesterase and butyrylcholinesterase inhibition during acute dimpylate intoxication. Rapid diagnosis is crucial for guiding immediate treatment; hence, the focus should be on practical analytical toxicology methods. While clinical aspects of organophosphate intoxication are well-known, effective diagnostic strategies for emergency settings remain understudied.

The accuracy of Ellman's method for measuring cholinesterase (ChE) activity has been subject to recent scrutiny. Originally described by Ellman *et al*., the method relies on the hydrolysis of thiocholine derivatives by ChEs, followed by a colour reaction with DTNB (5,5'-dithiobis-2-nitrobenzoic acid) [11]. However, subsequent studies have raised concerns [12]. Indoxylacetate has been proposed as a superior substrate due to its resistance to interference from oximes and thiols. At the same time, DTNB's instability and interaction with sulfhydryl groups have been implicated in assay variability [13]. Additionally, the reaction of oximes with acetylthiocholine and the impact of DTNB concentration on hydrolysis rate have been identified as potential sources of error [14].

Recovery monitoring requires repeated enzyme activity measurements at intervals to detect significant increases [15]. Ellman colourimetric and Michel electrometric methods are the gold standard for AChE/BChE activity in blood samples (whole, plasma, serum) and dried spots [16]. While some studies propose monitoring BChE for reactivation during OP intoxication treatment [17] its usefulness is limited due to inherent variability caused by factors like pregnancy, liver issues, medications and genetics [15]. AChE assays require immediate chilling and bedside dilution (1:20) to halt *in vitro* reactions, unlike BChE assays. This ensures accurate activity measurement [18].

Rapid and accurate diagnosis is essential for effective acute intoxication management. The compact "ChE check mobile" device, developed by Securetec Detektions-Systeme AG, offers a point-of-care solution for quantifying cholinesterase levels in whole blood. Utilizing photometric measurement of Ellman enzyme kinetics, the device provides rapid and user-friendly determination of both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activity [19].

The Viva ProE System by Siemens employs the homogeneous EMIT immunoassay method (Enzyme Multiplied Immunoassay Technique). The system utilises enzyme-labelled analytes to compete with patient sample analytes for antibody binding sites. Subsequent enzyme activity and signal generation are inversely proportional to the analyte concentration, allowing for analyte quantification. EMIT is a homogeneous enzyme assay utilizing glucose-6-phosphate dehydrogenase-linked antigen. Enzyme activity is measured spectrophotometrically *via* NADH production. Antibody binding inhibits enzyme activity, correlating drug concentration with NADH levels. While EMIT offers advantages in reagent stability and direct measurement of bound drugs, it is susceptible to matrix interferences [20].

Materials and Methods

Patient selection

This retrospective study was conducted in accordance with the Declaration of Helsinki, ensuring ethical compliance. Patient records were comprehensively reviewed, and informed consent was obtained before data inclusion. Rigorous anonymisation of identifiable patient information was implemented to safeguard privacy and maintain confidentiality.

Initially, blood and urine samples were collected from a patient who had been admitted to the Intensive Care Unit (ICU) II Toxicology Department at the Bucharest Emergency Clinical Hospital, Romania, in an unconscious state. The urine sample was used for diagnostic purposes *via* gas chromatography-mass spectrometry, while the blood sample was utilised to determine enzymatic activity using two distinct methods. The patient's condition was assessed daily through repeated enzymatic activity measurements. No additional biological samples were collected for this study; additional analyses were exclusively conducted on specimens obtained for routine clinical care.

Methods

Through gas chromatography coupled with mass spectrometry (GC-MS/MS), the organophosphorus pesticide (dimpylate) was identified from urine after extraction with organic solvents. This method enables the diagnosis of acute organophosphate intoxication and elucidates the correlation between metabolic symptoms and the toxic substance. The analytical instrument used was an Agilent 8890 GC System with a 7010B triple quadrupole mass spectrometer and a 7693A Autosampler. The column was an HP-5ms with dimensions 15 m x 250 μm x 0.25 μm. The mobile phase was helium, maintained at a flow rate of

1.1 millilitres *per* minute. The temperature program began at 60°C, then rapidly increased to 120°C at 40°C *per* minute. Subsequently, the temperature was raised to 310°C at a slower rate of 5°C *per* minute and held constant for one minute. The inlet temperature was maintained at 280°C, and a wool liner was used for the splitless injection of a one-microliter sample. The ion source temperature was 300°C. Electron impact ionisation at 70 eV was employed. The mass spectrometer operated in full scan mode, covering a mass range of 40 to 600 m/z with a scan time of 100 ms.
The organic solvents were analytical grade: chloroform, dichloromethane and dichloroethane from Supelco (Merck). The interactions between the analytes into samples and solvent were facilitated by a digital magnetic stirrer Nahita Blue. The extracts were separated using an EBA 200 Hettich centrifuge and evaporated by a Memmert UN30 oven. The urine sample volume was 30 mL. The preparation steps are outlined in Figure 1.

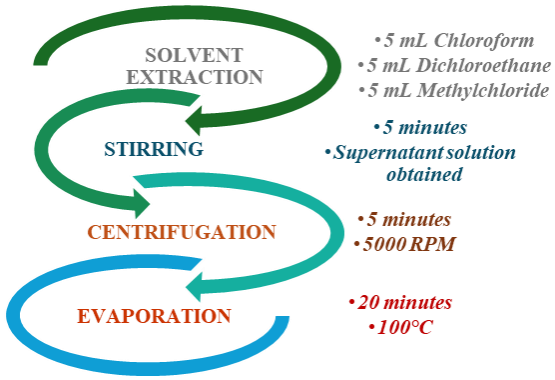

Figure 1.
Sample pre-treatment steps for chromatography analysis

Securetec Che Check Mobile System and Viva ProE System Siemens were the analytical instruments used to determine enzyme activity. Both used specific commercially available reagent kits for calibration and identification of enzyme levels in patient samples.
The Securetec Che Check Mobile System required a baseline cuvette reading. A blood sample was introduced *via* glass capillary, and the system guided haemoglobin measurement. The white cap was exchanged for a red "AChE-substrate" cap dissolved by shaking. Results were produced within four minutes. No calibration is necessary.
The Viva ProE System from Siemens utilised ELITechGroup Clinical Chemistry Cholinesterase reagents in liquid, ready-to-use format. ELITech Clinical Systems ELICAL2 was a lyophilised calibrator derived from human serum, formulated with components to optimise calibration accuracy. ELITech Clinical Systems ELITROL I and ELITROL II were controls of lyophilised human serum containing constituents at predetermined levels. The lyophilised solutions were reconstituted with ultrapure water. A volume of 5 mL was added to the control substance, and 3 mL was added to the calibration substance. Blood samples were collected in anticoagulated Vacutainer tubes and subsequently centrifuged at 4000 RPM for four minutes to isolate the plasma component.

Results and Discussion

The scope of this study was focused on assessing enzyme activity. Given the limited published research specifically focused on dympilate intoxication and the absence of enzyme activity data within existing case reports, a statistical analysis of data variability and outlier identification falls outside the parameters of this investigation [5, 21, 22].
A precision diagnosis was developed at admission by identifying the primary compound, dimpylate, and its dephosphorylated metabolite from the urine sample using the GC-MS/MS technique. Establishing the nature of the toxic (organophosphorus pesticide) decided the direction of the early medical approach to stabilize vital functions.
Figure 2 presents a comprehensive mass spectral analysis of the urine sample, facilitating a direct comparison with reference spectra for dimpylate and its metabolite derived from the NIST database. The precise identification of characteristic ion peaks for both compounds is evident, unequivocally confirming the presence of dimpylate and its metabolic product. The extracted ion chromatogram (Figure 3) provides further corroboration, revealing the sequential elution of the metabolite followed by the parent compound within a defined temporal window. This chromatographic pattern aligns with the established metabolic pathway of dimpylate, reinforcing the diagnostic conclusion of acute organophosphate intoxication.
The proposed methodology has proven effective in detecting pesticides within genuine human intoxication samples. However, its practical application is hindered by several significant drawbacks. The process necessitates the utilisation of substantial quantities of costly and hazardous organic solvents. Moreover, the subsequent evaporation of these solvents to concentrate the sample introduces environmental concerns. The multi-step analytical protocol further exacerbates these issues, increasing the risk of analyte loss and prolonging analysis time, ultimately contributing to elevated costs. These limitations underscore the need to develop more efficient and environmentally friendly analytical approaches for pesticide detection in human intoxication cases.
Organophosphates inhibit acetylcholinesterase, disrupting neurotransmission by preventing acetylcholine hydrolysis. The accumulation of acetylcholine within the neuromuscular junction results in the characteristic symptoms of cholinergic toxicity. While both erythrocyte and plasma cholinesterases are affected by organophosphate

intoxication, erythrocyte acetylcholinesterase is considered a more reliable indicator of neuronal status.

A dual analytical approach was employed to comprehensively monitor the evolution of cholinesterase activity throughout the hospitalisation period. Whole blood samples were subjected to colourimetric analysis using the Securetec Che Check Mobile System to assess both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) levels relative to haemoglobin concentration. Concurrently, plasma butyrylcholinesterase (PsChE) was quantified using the EMIT immunoassay on the Viva ProE Siemens System. This comparative approach enabled a more comprehensive evaluation of enzyme activity dynamics in dimpylate intoxication, considering the potential influence of different biological matrices and analytical methodologies.

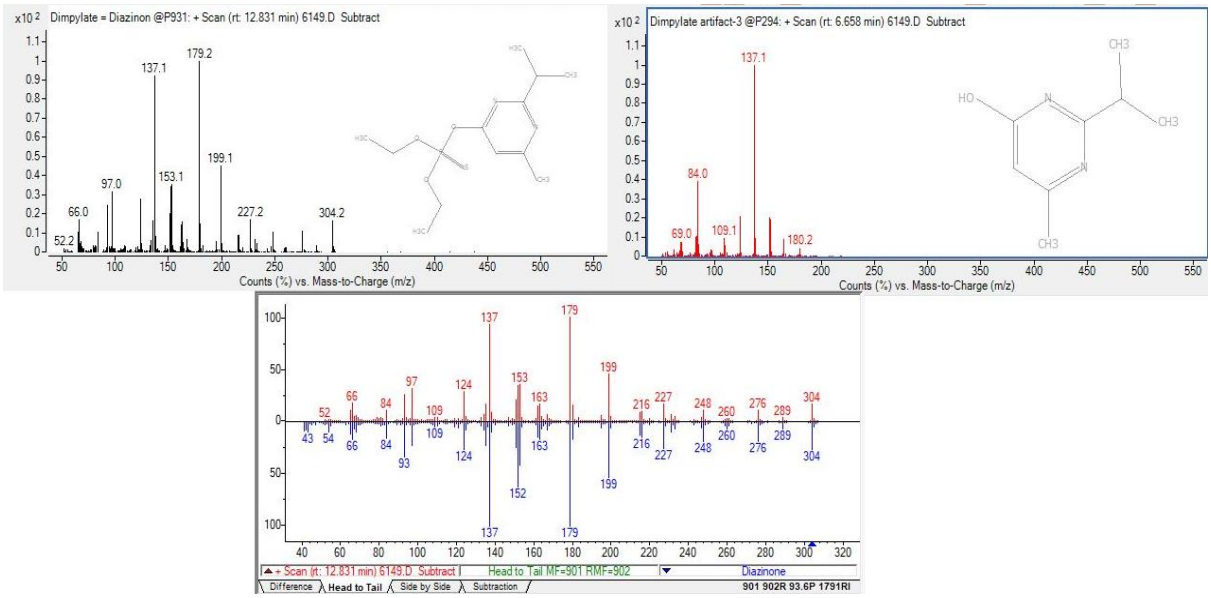

Figure 2.

The NIST database fitting results of dimpylate (diazinon) spectrum and its metabolite

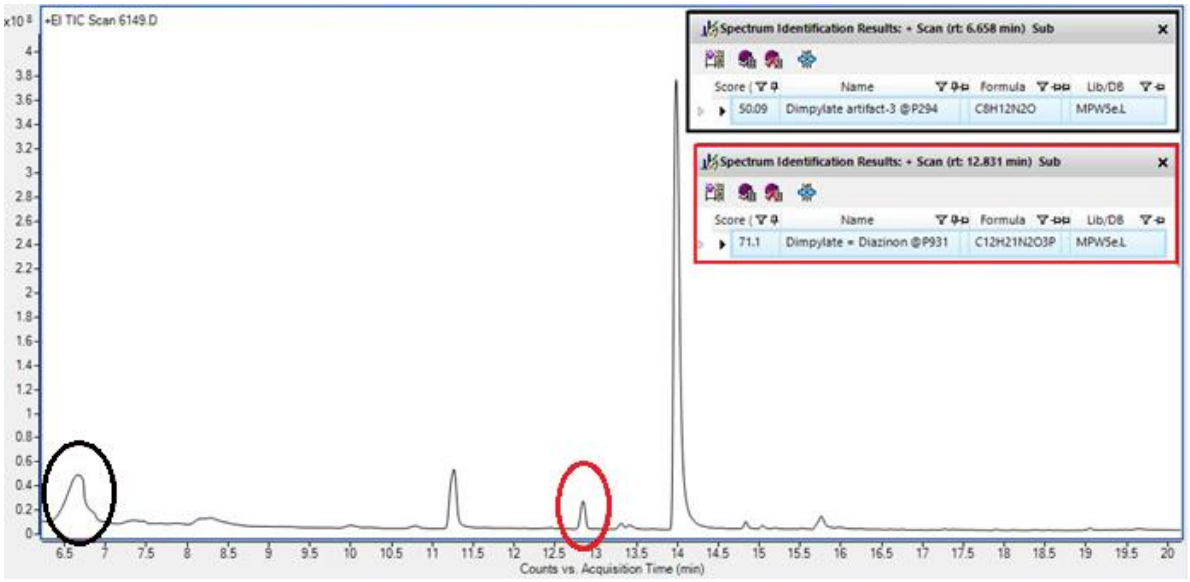

Figure 3.

Extracted Total Ion Chromatogram between 6 to 20 minutes of the sample delineates the retention times of dimpylate (red) and its metabolite (black)

A robust linear relationship between absorbance and concentration was established for the Viva ProE Siemens System, as evidenced by the calibration curve depicted in Figure 4. The regression equation, $y = 0.0002x + 0.0117$, and the high coefficient of determination ($R^2 = 0.9991$) underscore the strong correlation between these variables. To ensure method reliability, the calibration process was performed in triplicate. The resulting data, summarised in Table I, demonstrate excellent precision with minimal variability across the calibration range,

as indicated by the low coefficient of variation values (CV < 10%) for all levels except the zero calibrator. These findings collectively support the suitability of the calibration curve for accurate analyte quantification.

Table I

The absorbance values obtained for the calibration levels for Viva ProE Siemens System using ELITechGroup Clinical Chemistry Cholinesterase ready-to-use reagents

PsChE Concentration [U/L]	Mean Absorbance [dAbs/min]	Standard deviation [dAbs/min]	Coefficient of variation [%]
0	0.0000		
954	0.2319	0.0328	14.15
1908	0.4253	0.0281	6.60
2862	0.6574	0.0437	6.65
3816	0.8519	0.0375	4.40
4770	1.0495	0.0399	3.81

To ensure the accuracy and reliability of the assay, a quality control solution with a target concentration of 5347 U/L was employed to monitor calibration curve integrity and reagent stability. Acceptable performance was defined as values within a ± 10% range of the target concentration. Graphical analysis of the quality control data revealed a symmetrical distribution without discernible trends, indicating consistent and precise method performance. These findings support the suitability of the assay for clinical decision-making.

To ensure the ongoing accuracy of the calibration curve and reagent stability, a quality control solution with a known concentration (target value: 5347 U/L) was periodically analyzed. The acceptable variation range for this control lies between a lower limit of 4407 U/L and an upper limit of 6341 U/L.

Figure 5 illustrates the results of the quality control measurements. The data exhibits a relatively symmetrical distribution without any discernible trend, indicating good method reliability. This trustworthiness is crucial for guiding clinical decisions and treatment adjustments.

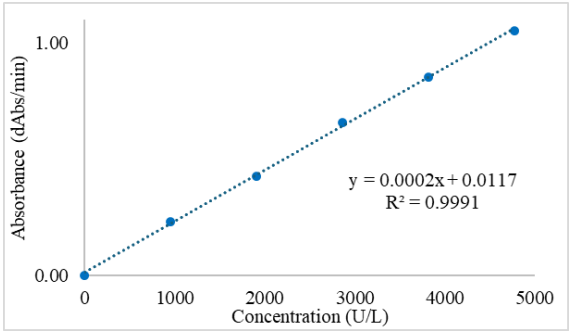

Figure 4.

The graphical representation of the calibration levels for Viva ProE Siemens System using the specific commercially available kit recommended by the manufacturer

Figure 6 represents the evolution of butyrylcholinesterase activity measured through the hospitalisation period from whole blood and plasma using the previously described techniques. Initial inhibition of butyrylcholinesterase is not a reliable indicator of intoxication severity.

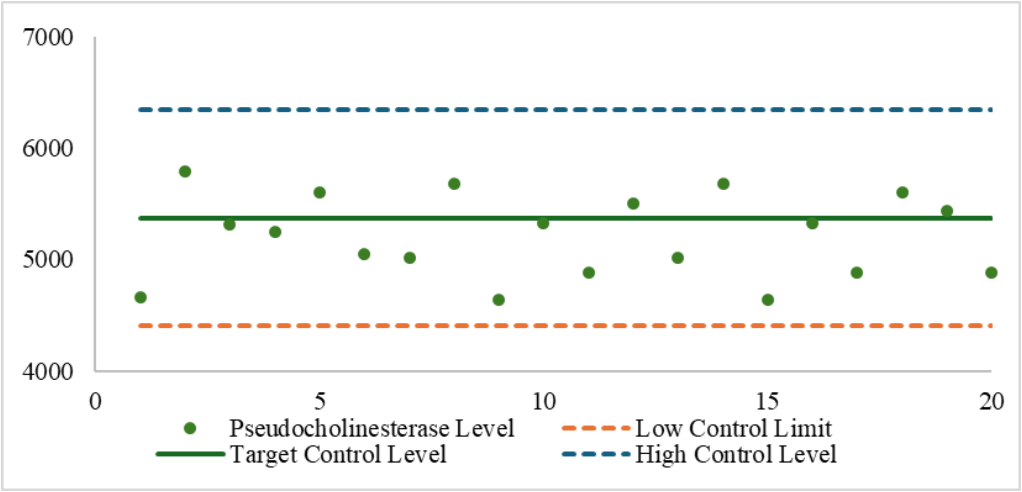

Figure 5.

The graphical representation of the quality control solution of pseudocholinesterase through the hospitalization period for Viva ProE Siemens System

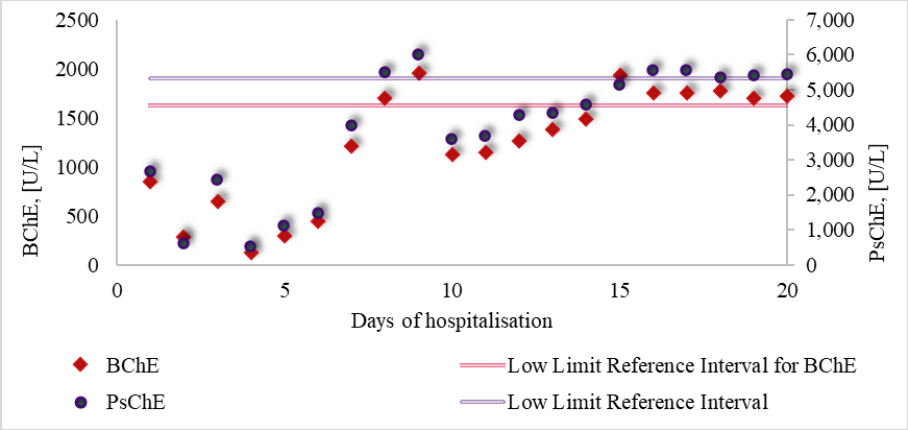

Figure 6.
BChE evolution throughout the hospitalisation days was determined by two techniques: BChE for whole blood levels and PsChE for plasma levels

The variation in the initial days was attributable to plasma administration in conjunction with atropine, resulting in elevated values which subsequently declined. Between the fourth and sixteenth days, a linear increase was observed, with the exception of days seven to nine when plasma was again administered, causing values to exceed the lower limit of the reference interval for both methods. These elevations did not signify recovery, as values remained inhibited post-plasma infusion. Following the sixteenth day, levels of both enzymes stabilised marginally above the lower limit of the reference interval, indicating a gradual improvement in the patient's overall health. The graphical representation of both butyrylcholinesterase activity measurements exhibited identical patterns, suggesting no significant methodological differences. Mason *et al*. reported a gradual daily recovery of butyrylcholinesterase levels following dichlorvos elimination, with an approximate 7% increase towards normal values each day [23]. In this case of dimpylate, fluctuations in enzyme activity were more pronounced in the days following plasma administration, ranging from 51% to 30%, compared to a narrower range of 6% to 10% in subsequent periods.

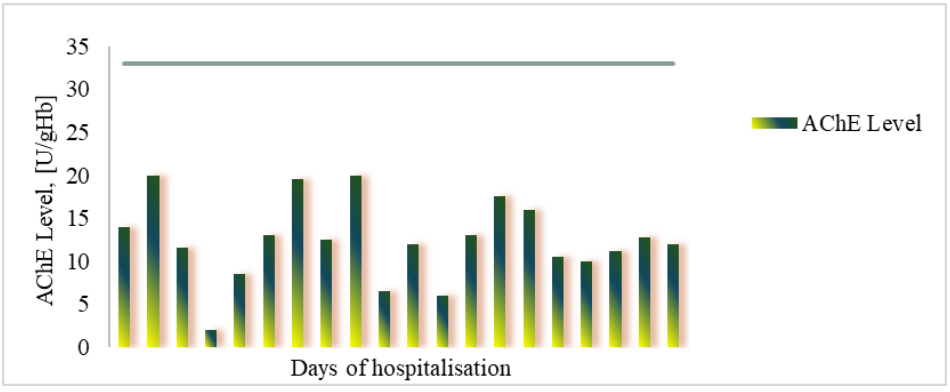

Figure 7.
AChE evolution throughout the hospitalisation days was determined spectrophotometrically in comparison with haemoglobin absorbance

Acetylcholinesterase (AChE) activity was measured using a method that required correction for haemoglobin interference, and results were expressed as units *per* gram of haemoglobin. A graphical representation of AChE levels (Figure 7) revealed a pattern similar to that observed for butyrylcholinesterase (BChE) in Figure 6. However, a notable decrease in AChE activity was not evident until three days post-treatment. Subsequently, AChE stabilised at approximately 12 U/gHb after 16 days but remained persistently below the lower reference limit throughout the hospitalisation period. Unlike butyrylcholinesterase, red blood cell acetylcholinesterase regenerates slowly *via* erythropoiesis (less than 1% daily). Neuronal acetylcholinesterase recovery rates are uncertain, potentially limiting red blood cell acetylcholinesterase's utility as a synaptic activity marker [24]. In this specific instance, an approximate 20% recovery was observed, although plasma administration may have influenced this due to its acetylcholinesterase content.

Both acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) levels peaked concurrently during

the 20-day treatment period. A subsequent decline in enzyme activity was observed. As depicted in Figure 8, AChE and BChE levels exhibited a strong correlation within whole blood samples, with minor discrepancies on days 2, 8 and 12. Both enzymes eventually stabilised at comparable levels, suggesting their potential utility in clinical management.

Organophosphorus pesticides frequently exhibit greater potency as butyrylcholinesterase inhibitors than acetylcholinesterase inhibitors, which may result in disproportionate inhibition of the former enzyme [24]. Treatment modifications based on these evolving enzyme levels led to clinical stabilization after 16 days despite persistent acetylcholinesterase suppression. This observation underscores the complexity of cholinesterase inhibition dynamics in acute intoxication and the need for ongoing monitoring.

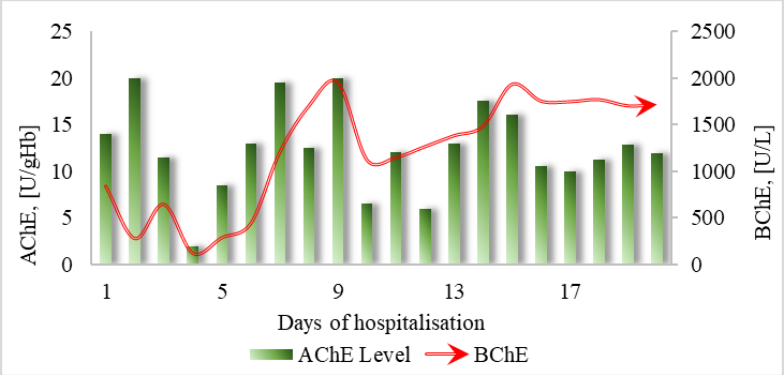

Figure 8.
Evolution of both AChE and BChE throughout the hospitalisation days was determined spectrophotometrically in comparison with haemoglobin absorbance

Both Ellman and EMIT methods are essential tools in diagnosing organophosphate intoxication by assessing cholinesterase activity. However, their reliance on accurate and precise measurements can be compromised by various interfering factors present in biological samples. Table II provides a comparative overview of these methods, including their strengths and limitations. Understanding the potential limitations of each method is crucial for interpreting results and making informed clinical decisions.

Table II
Comparison of Ellman and EMIT Methods for Cholinesterase Measurement

	Ellman Method	**EMIT Method**
Principle	Colorimetric assay (direct measurement)	Enzyme-linked immunoassay (indirect measurement)
Advantages	Simple, low cost, suitable for routine use	High sensitivity, automation compatible, suitable for large-scale studies and emergency settings
Disadvantages	Limited sensitivity, time-consuming manual procedures	Complex instrumentation, high cost, limited accessibility

Conclusions

This study presents a novel urine GC-MS/MS method for rapidly and accurately diagnosing acute organophosphate intoxication. While the method demonstrates robust performance in correlating clinical symptoms with dimpylate exposure, it faces limitations such as high costs and solvent use. Initial biomarker analysis revealed a significant difference in butyrylcholinesterase and acetylcholinesterase inhibition levels, suggesting the potential of butyrylcholinesterase as a diagnostic marker. However, further investigation is required to fully elucidate the correlation between enzyme inhibition and clinical outcomes. The urgent need for a point-of-care test for early detection in resource-limited settings necessitates the exploration of alternative acetylcholinesterase substrates to develop a more accessible and affordable diagnostic tool.

Acknowledgement

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Conflict of interest

The authors declare no conflict of interest.

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