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**THESIS RESUME**

*Clinical and Analytical Evaluation of Acute Organophosphorus Intoxication: Utilizing  
Cholinesterases as Biomarkers and Optimizing Pesticide Detection Methods*

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## **Introduction**

Acute organophosphorus (OP) compound intoxications represent a major public health concern. These widely used compounds exert potent neurotoxic effects by irreversibly inhibiting cholinesterases (ChE), leading to acetylcholine accumulation and severe clinical manifestations, including acute respiratory failure and death.

While monitoring acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) / pseudocholinesterase (PsChE) is essential for diagnosis and treatment, a complete understanding of their enzyme dynamics and prognostic utility requires further clarification. Current medical practice often suffers from a lack of continuous monitoring.

This doctoral thesis investigated the evolution of cholinesterase activity in patients with acute diazinon and malathion intoxications. The research involved comparing BChE and AChE determination methods and developing a validated analytical method for direct quantification of diazinon and malathion in urine samples.

The study revealed significant inter-individual variability in enzyme recovery and strong correlations ( $r$  0.905-0.996) between the activity of different enzymes and measurement methods, demonstrating their reliability. A crucial finding is the strong inverse relationship ( $r$  up to -0.9995) between urinary pesticide concentrations and enzymatic inhibition upon admission, confirming a direct dose-response relationship and the relevance of ChE as prognostic indicators. Severe inhibition correlated with prolonged intensive care unit (ICU) hospitalisation.

In conclusion, this thesis emphasises the importance of integrated monitoring of cholinesterase activity and urinary pesticide concentrations for effective OP intoxication management. These enzymes are reliable biomarkers for assessing intoxication severity and treatment response, justifying their combined use for a comprehensive clinical picture. Despite inherent limitations of studies with small patient numbers, the study's findings provide a robust basis for optimising clinical protocols and developing individualised therapeutic strategies.

## **Chapter 1 – Organophosphorus Pesticide Intoxication**

The present study addresses a critical global public health issue: acute organophosphorus (OP) compound intoxications. The widespread use of these substances in agriculture and industry generates significant risks due to their potent neurotoxic effects [1, 2], mediated by the irreversible inhibition of cholinesterase (ChE) enzymes [3]. This inhibition leads to acetylcholine accumulation, resulting in excessive cholinergic receptor stimulation and polymorphous clinical manifestations [4], often severe, including acute respiratory failure, seizures, coma, and even death [5].

Despite notable advances in diagnosis and treatment, persistent gaps in understanding ChE dynamics in a clinical context necessitate in-depth investigation. Monitoring acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) / pseudocholinesterase (PsChE) activity is essential for assessing intoxication severity, guiding therapy, and monitoring neurological recovery [6, 7]. However, the precise relationships between these enzymes, their dynamics during intoxication and recovery, and their relative utility as diagnostic and prognostic markers remain subjects of active debate and ongoing research [8, 9]. A notable deficiency in current medical practice is the often limited monitoring of these enzymes; the lack of continuous follow-up deprives clinicians of essential information for real-time treatment adjustment and prognosis optimisation.

This doctoral thesis aims to address these needs by thoroughly investigating the evolution of cholinesterase activity in patients with acute organophosphorus intoxications, with a particular focus on diazinon and malathion. Our research involved comparing methods for BChE and AChE determination and developing a validated analytical method for the direct quantification of diazinon and malathion in patient urine samples.

### **1.1. Incidence of Organophosphorus Pesticide Intoxications: A Global Perspective**

The incidence of OP intoxications varies significantly globally, influenced by socio-economic factors and regulations. While a decrease in acute cases has been observed in developed countries due to stricter regulations [7, 10], deliberate self-poisoning with OP pesticides remains a major cause of deaths worldwide, contributing to 13.7% of all global suicides between 2010-2014 [11, 12, 13]. An estimated 385 million cases of acute pesticide poisoning occur globally each year,

leading to approximately 11,000 deaths, mostly in developing countries [14], necessitating reinforced intervention strategies and legislative reforms [4, 13].

## **1.2. Chemistry and Classification of Organophosphorus Compounds**

OP compounds are esters of phosphoric acid [15], with their toxicity determined by chemical structure [16]. Most OP pesticides are phosphorothioates ( $P=S$ ), requiring metabolic activation into oxon compounds ( $P=O$ ) by the cytochrome P450 (CYP450) enzyme system [17, 18], which can delay the onset of intoxication. Diazinon and malathion are lipophilic, potentially exhibiting delayed toxicity due to gradual release from adipose tissue [19, 20]. The WHO classifies pesticides based on toxicity (LD50), but this classification does not always predict clinical severity [21]. Diazinon is considered "moderately hazardous" (Category 2), and malathion "slightly hazardous" (Category 3) [22]. Diazinon is now banned in the European Union for safety reasons [23]. Both are associated with various toxic effects, including developmental, reproductive, and immune system impacts, and possibly cancer risk [4, 24-26, 27, 28]. They also have a negative impact on non-target organisms and the environment [29, 30, 31].

## **1.3. Mass Spectrometry in the Clinical Laboratory: Applications and Validation**

Mass spectrometry (MS) is gaining traction in clinical laboratories, valued for its high sensitivity, specificity, and processing capacity [32]. Coupling with gas chromatography (GC) or liquid chromatography (LC) allows for the simultaneous quantification of numerous analytes [33]. Rigorous validation of MS methods is crucial, involving parameters such as specificity, linearity, accuracy, precision, limit of detection (LOD), and limit of quantification (LOQ) [34].

The triple quadrupole mass spectrometer (QqQ) is particularly powerful [35], utilising three quadrupole filters in series for selection, fragmentation, and analysis (Q1, Q2, Q3) [36]. This configuration enables advanced techniques such as multiple reaction monitoring (MRM) [37], offering exceptional sensitivity and specificity for analyte quantification, even in complex matrices [38].

## **1.4. Pesticide Determination Methods: Applications in Clinical Toxicology**

The development of rapid and reliable methods for detecting and quantifying pesticides in human biofluids is urgent [39]. Chromatographic techniques coupled with MS (GC-MS, LC-MS, GC-MS/MS, LC-MS/MS) are the most commonly used, offering high sensitivity and specificity

[40-42]. MS/MS is an exceptionally powerful tool for analysing OP pesticides in biological samples [43-45]. Sample preparation has evolved, including solid-phase extraction (SPE) [46] and QuEChERS techniques [47].

For malathion and diazinon, GC-MS and GC-MS/MS methods have been developed for serum/plasma [48-51]. Urinary analysis constitutes an essential non-invasive alternative for evaluating human exposure to these OP pesticides, through the quantification of specific metabolites, such as 2-isopropyl-6-methyl-4-pyrimidinol (IMPy) for diazinon and malathion dicarboxylic acid (MDCA) for malathion [52]. A recent review (2010-2022) showed that GC-MS/MS was the most used technique (56%) for pesticide detection in urine samples [53]. Although GC-MS/MS offers superior selectivity and sensitivity [54], it has limitations related to the need for derivatisation and its unsuitability for thermolabile compounds, in contrast to LC-MS/MS which allows for the analysis of a wider range of compounds [55].

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## **Chapter 2 – Cholinesterase Enzyme Activity in Organophosphorus Insecticide Intoxications**

**Acetylcholinesterase (AChE)** and **butyrylcholinesterase (BChE)** are crucial enzymes targeted in organophosphorus (OP) intoxications [1, 2]. AChE is central to neurotransmission, with its erythrocyte activity accurately reflecting OP exposure [3, 4]. BChE, synthesised in the liver, serves as a biomarker for hepatic function and a potential indicator of OP exposure [4, 5].

The severity of OP intoxication is directly linked to the degree of ChE inhibition [6]. AChE levels below 10% indicate severe intoxication, and classifications by Balali-Mood et al. correlate ChE activity (BChE and AChE) with severity (mild, moderate, severe) [7]. A demonstrated correlation exists between intoxication severity and serum cholinesterase upon admission [8, 9].

### **2.1. Mechanism of Organophosphorus Toxicity**

Organophosphorus (OP) pesticides, such as malathion and diazinon, become toxic in the body through metabolic bioactivation. This process converts them from thiophosphoryl into oxon forms, aided by cytochrome P-450 (CYP) enzymes. The resulting oxons are highly reactive metabolites that strongly inhibit acetylcholinesterase (AChE). This inhibition of AChE leads to acetylcholine accumulation, causing cholinergic hyperstimulation [10]. Although the enzyme Paraoxonase 1 (PON1) detoxifies oxons by transforming them into less toxic, water-soluble metabolites for elimination, the balance between activation by CYP450 and detoxification by PON1 is crucial for overall toxicity [11]. OPs form a stable complex with AChE, and a process called "ageing" can make this inhibition irreversible. Treatment includes atropine (for cholinergic symptoms) and oximes (for AChE reactivation), along with benzodiazepines and supportive therapy [10].

The clinical picture of acute OP poisoning manifests as acute cholinergic syndrome, featuring muscarinic symptoms like hypersecretion and nicotinic symptoms, including fasciculations and convulsions. Some lipophilic OPs can also cause delayed toxicity [12]. Severity depends on the degree of AChE inhibition: mild cases (50-90% AChE activity) present with nausea and headache, moderate cases (10-50% AChE) include weakness and bradycardia, while severe poisonings (<10% AChE) can lead to respiratory failure, convulsions, and coma.

### **2.3. Methods for Cholinesterase Activity Evaluation**

ChE activity is primarily assessed using spectrophotometric [13], potentiometric [14], and electrochemical [15–17] methods. Spectrophotometric and potentiometric techniques can be limited by sample dilution, which may reduce accuracy, particularly in cases of mild intoxication [13]. For AChE and BChE determination in whole blood, specific BChE inhibitors may be employed, although some can also affect AChE [31]. An innovative approach utilises indoxyl acetate as a bifunctional reagent for sensitive detection [13]. Rapid, point-of-care testing is available via devices such as the Test-mate ChE 400 field kit, which has demonstrated good concordance with laboratory methods for prompt diagnosis [29]. Immediate sample cooling and dilution are essential for accurate AChE measurement [30].

Enzyme Multiplied Immunoassay Technique (EMIT), developed in 1973 [18], is a rapid, homogeneous immunoassay. It quantifies analytes through competitive binding between the analyte and an enzyme-labelled analogue for specific antibodies. The resulting signal (absorbance at 340 nm) is directly proportional to the analyte concentration [19–21]. Despite its speed, EMIT necessitates rigorous sample pre-treatment to minimise optical interferences, cross-reactive compounds, or enzymatic inhibitors [22].

The Ellman method, introduced in 1961 [23], measures ChE activity by monitoring the hydrolysis of a thiocholine derivative and its subsequent reaction with 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), forming a measurable yellow product [24]. This widely used method has known limitations, including potential interference from oximes used in treatment and issues with reagent stability [25–27]. However, recent modifications (e.g., Patel et al., 2023) have improved its simplicity, rapidity, and correlation with standard methods, featuring a low detection limit suitable for routine clinical applications [28].

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## **Chapter 3 – Optimisation of the Immunoassay Method for Butyrylcholinesterase Quantification**

### **3.1. Introduction**

The rapid assessment of cholinesterase (ChE) activity or organophosphorus (OP) levels is crucial for managing poisoned patients [1]. A lack of swift and accessible methods, particularly in developing countries, can lead to fatal delays in diagnosis and treatment [2]. This gap highlights an urgent need for reliable and prompt diagnostic tools, given the inherent limitations (complexity, cost) of existing methodologies [3-7].

### **3.2. Materials and Methods**

This study utilised a Siemens Viva ProE analyser and commercial kits to determine Pseudocholinesterase (PChE) activity in plasma derived from EDTA blood samples. The method employed is based on the Enzyme Multiplied Immunoassay Technique (EMIT) principle, a competitive homogeneous immunoassay. In this technique, the analyte in the sample competes with an enzyme-labelled analogue (glucose-6-phosphate dehydrogenase, G6PD) for antibody binding sites. G6PD activity, measured spectrophotometrically via NADH production, is inversely proportional to the analyte concentration. While advantageous for reagent stability and the absence of separation steps, the method is susceptible to matrix interferences [8]. All validation steps adhered to the SWGTOX guidelines [9].

### **3.3. Results and Discussion**

The EMIT method for butyrylcholinesterase (BChE) determination demonstrated robust linearity between absorbance and concentration, exhibiting an exceptional coefficient of determination ( $R^2 = 0.9998$ ) and a coefficient of variation (CV) below 10% for most calibration points. This indicates consistent precision and excellent reproducibility. Residual analysis further confirmed the suitability of the linear regression model.

The method's precision was validated through analysis of variance (ANOVA), which revealed no statistically significant differences between measurement series ( $p > 0.05$ ), thus attesting to optimal reproducibility. Accuracy was evidenced by quality control data, showing a minimal bias of -3.2% from the target value and intra-series (8.85%) and inter-series (7.42%) CVs

remaining below 10%. These findings confirm satisfactory analytical precision for clinical application.

The limit of detection (LOD) was established at 319.96 U/L, and the limit of quantification (LOQ) at 1000.00 U/L, indicating the method's capability to reliably detect and quantify low analyte concentrations.

### 3.4. Conclusions

This study rigorously evaluated the analytical performance of the Siemens Viva ProE system using the homogeneous EMIT immunoassay for PChE determination. The findings demonstrate consistent analytical robustness and full clinical applicability. The method exhibited exceptional linearity ( $R^2 = 0.9998$ ) and remarkable precision (CVs below 10%). Accuracy was further confirmed by a minimal bias (-3.2%) in quality control. The established detection (319.96 U/L) and quantification (1000.00 U/L) limits attest to the reliability of measurements, even at low concentrations. In conclusion, the EMIT method for butyrylcholinesterase determination on the Siemens Viva ProE analyser possesses superior analytical characteristics, making it strongly recommended for routine clinical use in precisely monitoring butyrylcholinesterase levels, which is essential for managing organophosphorus poisonings.

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## **Chapter 4 – Development of the Quantification Method for Diazinon and Malathion using Mass Spectrometry**

### **4.1. Introduction**

Organophosphorus (OP) pesticide poisonings, involving compounds such as diazinon and malathion, represent a significant public health concern, necessitating rapid and precise diagnosis. Existing analytical methods are often constrained by their complexity and the stability of the analytes. This study proposes a high-performance GC-MS/MS method, employing the Multiple Reaction Monitoring (MRM) mode for the ultra-sensitive and specific detection of these compounds in complex biological matrices [1-6].

### **4.2. Materials and Methods**

The study utilised certified reference materials for diazinon and malathion. Urine samples (30 mL), collected from patients with suspected poisoning, underwent liquid-liquid extraction (LLE) using a chloroform/dichloromethane/methyl chloride mixture (1:1:1, v/v/v). The resulting extract was evaporated, reconstituted with methanol, and a 1  $\mu$ L aliquot was injected into an Agilent 8890 GC system coupled with a 7010B triple quadrupole mass spectrometer. The MS/MS conditions were rigorously optimised for MRM mode, employing electron ionisation (EI, 70 eV), ensuring maximum sensitivity and specificity through the monitoring of characteristic ionic transitions for each analyte. Data analysis was performed using Agilent's MassHunter software.

### **4.3. Results and Discussion**

#### ***4.3.1. Optimisation of the Extraction Method***

Various extraction techniques were compared, with liquid-liquid extraction (LLE) being favoured over solid-phase extraction (SPE). This preference stemmed from LLE's efficiency, reduced cost, and rapidity, attributes critical in emergency scenarios [7–9]. An optimised solvent mixture, comprising dichloroethane, methyl chloride, and chloroform (1:1:1, v/v/v), demonstrated an exceptional recovery rate of 94% for organophosphorus pesticides from urine samples, ensuring precise quantification.

#### **4.3.2. Optimisation of the MRM Method**

For sensitive and selective detection, GC-MS/MS parameters were rigorously optimised in MRM (Multiple Reaction Monitoring) mode. Specific retention times were established (e.g., the internal standard cypermethrin at 32.14 min), and the most characteristic ionic transitions were selected to minimise interferences. Mass spectra analysis identified specific fragment ions for diazinon ( $m/z$  179.2) and malathion ( $m/z$  99.0), enabling precise identification in complex matrices. Optimisation of collision energy (CE) proved crucial. For diazinon, the  $137.1 \rightarrow 84$  transition at 15 V CE offered the best sensitivity (signal intensity 237,858.9). For malathion, the  $126.9 \rightarrow 99$  transition at 5 V CE demonstrated the highest sensitivity (signal intensity 338,545.3). The optimised retention times were 16.65 min for diazinon and 19.91 min for malathion. These optimisations ensure efficient and accurate detection, in accordance with international standards (European Commission Decision 2002/657/EC) [10].

#### **4.3.3. Validation of the MRM Method**

The validation of the Multiple Reaction Monitoring (MRM) method for diazinon and malathion demonstrated excellent analytical performance. The linearity range was established between 5 and 1000 ng/mL. For diazinon, the calibration curve exhibited remarkable linearity, with a coefficient of determination ( $R^2$ ) of 0.9998 and a coefficient of variation (CV) between 0.17% and 4.69%. Similarly, for malathion, linearity was even stronger, with an  $R^2$  of 0.9999 and CVs between 0.03% and 4.99%. ANOVA analyses confirmed the robustness of the linear regression models for both analytes.

The method's accuracy was evaluated through analyses of fortified samples, with all deviations falling within the acceptable limits of  $\pm 20\%$ , indicating satisfactory accuracy, particularly at higher concentrations. Precision (intra-series and inter-series) was also commendable, with a CV below 5% at 50 ng/mL and below 1% at 500 ng/mL for both analytes, demonstrating remarkable reproducibility.

The limit of detection (LOD) was 0.60 ng/mL for diazinon and 0.33 ng/mL for malathion, confirming the method's capability to detect very low concentrations. The limit of quantification (LOQ) was set at 5 ng/mL for both analytes, a level at which accuracy and precision criteria were met. Finally, the method's selectivity and specificity were demonstrated by the total ion chromatogram (TIC) in MRM mode, which showed a single, sharp, and intense major peak at the

specific retention time, minimising matrix interferences. This rigorous validation confirms the method's reliability for analysis in complex biological matrices.

#### ***4.3.4. Clinical Findings: Analysis of Poisoned Patient Samples***

The validated GC-MS/MS method was successfully applied to the toxicological analysis of biological samples (urine) from patients admitted with suspected intentional self-poisoning with pesticides.

Initially, patient samples underwent preliminary analysis in SCAN mode (GC-MS) for general screening. This approach allowed for the preliminary identification of unknown xenobiotics, such as diazinon (at approximately 12.023 min) and malathion (at approximately 16.770 min), by comparing mass spectra with reference libraries. It is crucial to highlight that, despite preliminary identification, the complexity of the biological matrix in real samples (illustrated by the multitude of peaks in SCAN chromatograms) can affect precision.

For precise confirmation and rigorous quantification of diazinon and malathion, the previously validated MRM method was employed. This selective and sensitive approach was indispensable for obtaining reliable results in real clinical cases, where precision is paramount for patient management.

Analysis of eight acute poisoning cases (2021-2023 period) revealed a notable positive correlation between the insecticide concentration detected in urine and the duration of ICU hospitalisation. Insecticide concentrations in urine ranged between 87 and 495 ng/mL, while ICU admission duration fluctuated between 11 and 24 days. This trend suggests that patients with higher exposure levels, reflected by elevated urinary concentrations, tend to require longer periods of hospitalisation. These real-world findings underscore the potential of urinary concentrations to serve as an indicator of poisoning severity and prognosis, with direct implications for resource allocation and emergency clinical management.

#### **4.4. Conclusions**

This chapter detailed the development and validation of a robust GC-MS/MS analytical method for the quantification of diazinon and malathion in urine. The optimisation of chromatographic and spectrometric parameters was exhaustively performed. Key contributions include the precise optimisation of collision energies (CE) for MRM transitions (15 V for diazinon and 5 V for malathion), maximising sensitivity and selectivity. The method demonstrated excellent

selectivity, exceptional linearity ( $R^2 > 0.999$ ), robust accuracy ( $\pm 20\%$ ), and remarkable precision (intra-group CVs between 0.03% and 4.69%), with confirmed inter-series reproducibility. Sensitivity was adequate, with an LOD of 0.60 ng/mL for diazinon and 0.33 ng/mL for malathion, and an LOQ of 5 ng/mL for both analytes. The validated method was successfully applied to the analysis of urine samples from poisoned patients, facilitating rapid and accurate diagnosis. Clinical observations highlighted a positive correlation between pesticide concentrations in urine and the duration of ICU hospitalisation, suggesting a link between exposure level and clinical severity. In conclusion, the developed GC-MS/MS method is a high-performance analytical tool, characterised by superior analytical attributes, validating its reliability and utility for routine and emergency toxicological investigations, significantly contributing to improving the diagnostic and management capacity for organophosphorus pesticide exposure.

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## **Chapter 5 – Study of Cholinesterase Activity in Acute Organophosphorus Poisoning**

### **5.1. Introduction**

Acute poisonings with organophosphorus (OP) compounds represent a major medical emergency. These widely used compounds exert severe neurotoxicity through the irreversible inhibition of cholinesterases (ChE), leading to acetylcholine (ACh) accumulation and potentially lethal neurological symptoms.

Prompt and precise assessment of ChE activity is crucial for diagnosis, monitoring, and prognosis. A significant decrease confirms exposure, and the degree of inhibition reflects severity. Dynamic ChE monitoring allows for the evaluation of treatment efficacy, considering individual factors.

Two primary methods are employed: the colorimetric Ellman test and the Enzyme Multiplied Immunoassay Technique (EMIT). The Ellman method is simple but susceptible to interference; EMIT is a homogeneous immunoassay, offering speed and efficiency. Both are fundamental, but their results can be influenced by biological interferences.

This study compares methods for determining the inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) in acute poisonings with diazinon and malathion. For clarity, BChE refers to the activity in whole blood (Ellman method), while pseudocholinesterase (PsChE) refers to the same enzyme quantified from plasma (EMIT method). This terminological distinction is essential for rigorous data interpretation.

### **5.2. Materials and Methods**

#### ***5.2.1. Biological Sample Collection and Preparation***

The study adhered to the Declaration of Helsinki, ensuring review of patient records, informed consent, and data anonymisation. Blood samples were collected from patients admitted to the Toxicology Intensive Care Unit (ICU) of Bucharest Emergency Clinical Hospital, with confirmed diagnoses of acute organophosphorus poisoning. Repeated enzymatic activity determinations were performed for daily monitoring of the patients' condition. No additional biological samples were collected. To prevent coagulation, whole blood samples were collected in

EDTA vacuum tubes, stored at 4 °C, and analysed within a maximum of one hour of collection. For EMIT analysis, plasma was separated by centrifugation at 4000 rpm for four minutes.

### ***5.2.2. Method Principle***

The analytical instruments used were: the Securetec ChE Check Mobile System and the Siemens Viva ProE System, both utilising specific commercial reagent kits.

The Securetec ChE Check System measured AChE and BChE activity via Ellman kinetic photometry. It required an initial absorbance reading of the cuvette, followed by the introduction of the blood sample for haemoglobin concentration determination. The specific substrate (red cap for AChE, yellow cap for BChE) was dissolved by shaking. Results were generated in approximately four minutes, without prior calibration. Enzymatic activity was expressed in activity units per gram of haemoglobin for AChE and units per litre for BChE, with correction for haemoglobin interference.

The Siemens Viva ProE System used ELITechGroup liquid reagents for butyrylcholinesterase, alongside the lyophilised calibrator ELICAL2 and controls ELITROL I and II. These were reconstituted with ultrapure water to ensure optimal calibration and quality control.

### ***5.2.3. Statistical Data Analysis***

Statistical analyses were performed using Microsoft Excel version 16.92. Pearson's correlation coefficient was employed to assess the relationship between BChE activity (measured with the point-of-care device) and PsChE (determined by EMIT). This method quantified the direction and strength of the linear correlation between the results obtained by the two methods. Additionally, BChE activity was compared with AChE activity for each patient throughout hospitalisation. For agreement assessment, Pearson's correlation coefficient and Bland-Altman analysis were utilised. The latter, specifically designed for evaluating agreement between two quantitative measurements, provided a detailed assessment of similarity, being preferable to Pearson's coefficient in this context. A p-value < 0.05 was considered statistically significant. The choice of these statistical methods was motivated by their specificity for the type of data analysed and the study objectives, ensuring a rigorous evaluation of the compared methods' performance.

## **5.3. Results and Discussion**

### ***5.3.1. Determination of Butyrylcholinesterase Activity***

The evolution of butyrylcholinesterase (BChE, from whole blood) and pseudocholinesterase (PsChE, from plasma) activity varied significantly in patients with acute diazinon and malathion poisoning. Some patients exhibited slow and incomplete recovery, while others showed rapid and complete recovery, highlighting differences in regeneration kinetics or sensitivity to pesticide metabolism. Although both substances inhibit BChE, the dynamics of recovery differ, often being faster in malathion poisonings and slower in diazinon cases. It is crucial to note that BChE and PsChE designate the same enzyme; the observed differences are due to the detection methods and biological matrices (whole blood versus plasma). Therefore, dual monitoring of both butyrylcholinesterase measurements is imperative for a comprehensive assessment of severity and clinical evolution.

### ***5.3.2. Correlations and Variations between BChE (Ellman) and PsChE (EMIT) Determinations***

Analysis of the Pearson's correlation coefficient revealed strong, statistically significant positive correlations between BChE and PsChE levels for all patients ( $r$  between 0.905 and 0.996), confirming a faithful linear association. Agreement assessment via Bland-Altman analysis indicated good agreement for diazinon poisoning, but more limited agreement and a significant positive bias for malathion poisoning, suggesting individual variability or matrix influence. The scatter plot of inhibition percentages demonstrated a strong, linear positive correlation between %BChE and %PsChE for both types of poisoning.

### ***5.3.3. Determination of AChE by the Ellman Method***

In patients with acute diazinon and malathion poisoning, a marked initial reduction in acetylcholinesterase (AChE) levels was observed upon admission, followed by a recovery phase with variable inter-individual rates and amplitudes. These variations underscore the necessity of individualised monitoring of AChE activity for assessing severity and treatment efficacy.

### ***5.3.4. Corelația dintre Acetilcolinesterază (AChE) și Butirilcolinesterază (BChE)***

Pearson's correlation analysis revealed a strong and statistically significant positive correlation between AChE and BChE levels in most cases of diazinon poisoning ( $r$  between 0.962 and 0.976) and malathion poisoning ( $r$  between 0.935 and 0.967), suggesting a coordinated physiological response and a common mechanism of enzymatic inhibition and reactivation.

Observed exceptions indicate the need to consider individual variability. Bland-Altman analyses showed acceptable concordance between AChE and BChE/PsChE measurements, with considerable variability in differences, but a minor systematic bias reflecting general characteristics of cholinesterase dynamics. These findings highlight the importance of concomitant monitoring of both enzymes.

#### ***5.3.5. Correlation between Enzymatic Activity and Pesticide Concentration***

A strong, statistically significant inverse relationship was found between urinary insecticide concentrations and the degree of cholinesterase (ChE) inhibition upon admission. Higher urinary diazinon and malathion levels correlated with more severe AChE, BChE, and PsChE inhibition (e.g., Pearson's  $r$  for AChE: -0.9500 for diazinon; -0.9995 for malathion, with significant  $p$ -values). This confirms a direct dose-response between OP exposure and ChE inhibition.

Upon discharge, improved ChE activity indicated partial recovery. Recovery rates varied but correlated directly with initial poisoning severity, as shown by lower enzyme levels at admission. Patients with more severe initial inhibition typically required prolonged ICU stays, validating ChE activity as a valuable prognostic indicator. Variability in ChE recovery rates in this study, compared to others, suggests influencing factors like the specific OP, initial inhibition, and individual physiological compensation.

### **5.4. Conclusions**

This study rigorously evaluated methods for determining cholinesterase (ChE) inhibition in acute diazinon and malathion poisonings. We observed individual variability in ChE recovery, influenced by the biological matrix. Crucially, strong correlations were found between different enzyme measurements (BChE, PsChE, AChE), confirming their interoperability. A key finding was the strong inverse relationship between urinary pesticide concentrations and enzyme inhibition, establishing a clear dose-response. Importantly, severe inhibition correlated with prolonged hospital stays, validating ChE as a valuable prognostic indicator. The study underscores the necessity of integrated monitoring of both ChE activity and urinary pesticide levels for effective poisoning management.



## **GENERAL CONCLUSIONS, ORIGINAL CONTRIBUTIONS, AND FUTURE PERSPECTIVES**

### **C.1. General Conclusions**

This thesis has optimised the diagnosis and management of organophosphorus (OP) poisonings through the quantification of cholinesterase (ChE) activity and OP compounds.

Analytical methods have been validated and optimised for:

1. Butyrylcholinesterase (BChE) using the EMIT immunoassay method, demonstrating excellent performance for clinical use.
2. Diazinon and Malathion in urine via GC-MS/MS, confirming high specificity and sensitivity. A correlation between pesticide concentrations and hospitalisation duration was observed.

The research also confirmed the clinical relevance of enzymatic biomarkers:

1. A strong and consistent correlation was demonstrated between BChE and PsChE, as well as between AChE and BChE, validating the interoperability of the methods.
2. A key finding is the strong inverse correlation between urinary pesticide concentrations and the degree of enzymatic inhibition upon admission, confirming a dose-response relationship.

In conclusion, this thesis establishes cholinesterase activity and urinary pesticide concentrations as reliable biomarkers for assessing poisoning severity and monitoring treatment, providing a solid foundation for optimising clinical protocols.

### **C.2. Original Contributions**

This thesis offers substantial original contributions to clinical and analytical toxicology:

- **Integrated Analytical Methodology:** A holistic approach has been developed and validated for characterising OP poisonings, combining enzymatic quantification with direct pesticide detection.
- **Analytical Robustness and Interoperability:** The rigorous performance of the EMIT method for BChE has been demonstrated, and the concordance of results between different cholinesterase measurement techniques confirmed.

- **Elucidated Enzymatic Correlations:** The strong relationships between AChE, BChE, and PsChE in OP poisonings have been clarified, strengthening the understanding of the inhibition mechanism.
- **Quantification of Dose-Response Relationship:** A strong inverse correlation between urinary OP concentrations and the degree of enzymatic inhibition has been established, offering a valuable tool for severity and prognosis assessment.
- **Foundation for Individualised Clinical Monitoring:** The necessity of a personalised approach to patient management has been underscored, based on inter-individual variability and the demonstrated correlations.

These contributions significantly advance the understanding of the pathophysiology and the management capabilities for organophosphorus poisonings.

### **C.3. Future Development Perspectives**

This research opens future directions for improving the management of OP poisonings:

- **Expansion of Clinical Studies:** There is a need for larger sample sizes and longitudinal monitoring for external validation and to understand long-term recovery.
- **Advanced Biomarkers and Innovative Approaches:** Exploration of PsChE's role as a predictor and the integration of oxidative stress/inflammation biomarkers, alongside "omics" approaches (proteomics, metabolomics), is warranted for a detailed molecular perspective.
- **Preclinical Models and Standardisation:** Development of *in vitro* and *in vivo* models for testing new therapeutic agents and standardisation of analytical methods for broader clinical implementation.

These directions will contribute to advancing knowledge and significantly enhancing the diagnosis, monitoring, and therapeutic strategies for OP poisoning.

## APPENDICES

### ***A.1.1. ARTICLES PUBLISHED ON THE THESIS TOPIC***

1. **Hîrjău, A.C.**; Marandiuc, I.M.; Radu, G.L. Improving Diagnostic Accuracy In Dimpylate Poisoning: A Comparative Study Of Cholinesterase Assays, *Farmacia*, 2024, Vol. 72, 5, pp. 1191-1198; <https://doi.org/10.31925/farmacia.2024.5.22> (IF = 1.4)
2. **Hîrjău, A.-C.**; Crăciun, M.E.; Marandiuc, I.-M.; Radu, G.-L. Assessing Diazinon Exposure: A GC-MS/MS Validation Study of BChE Measurement by Point-of-Care Testing and Enzyme Multiplied Immunoassay Technique. *Molecules* **2025**, 30, 2382. <https://doi.org/10.3390/molecules30112382> (IF = 4.2)
3. **Hîrjău, A.-C.**; Marandiuc, I.-M.; Radu, G.-L. Quantifying Acetylcholinesterase Inhibition: A Diagnostic Tool for Organophosphate Poisoning. *U.P.B. Sci. Bull., Series B* 2025, *accepted*.

### ***A.1.2. INTERNATIONAL SCIENTIFIC COMMUNICATIONS ON THE DOCTORAL THESIS TOPIC***

1. **Hîrjău, A.-C.**; Marandiuc, I.-M.; Negrea, Ș.M.; Ardeleanu, D. Paraclinical Studies and Medical Management of Dimpilate Poisoning. In *Abstracts of the 25th Balkan Military Medical Committee Congress*; Albena, Bulgaria, 28–31 May 2023; *Military Medicine* 2023, p. 87.

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