*Rom J Leg Med [33] 162-165 [2025]*
*DOI: 10.4323/rjlm.2025.162*


# LIMITATIONS OF ELISA IMMUNOLOGICAL ANALYSES IN DETERMINING THE POSTMORTEM INTERVAL

Ioana-Ruxandra Țurlea[1,2], George Cristian Curcă[1,2,*], Luminița Matei[2], Cristina Trandafir[2], Filip Virgil Perde[1,2]

*[1]"Carol Davila" University of Medicine and Pharmacy, [2]"Mina Minovici" National Institute of Legal Medicine, Bucharest, Romania*

**Abstract:** ***Introduction.*** The evaluation of postmortem interval, PMI, is of great medico-legal and judicial importance. Quantitative and qualitative immunological methods can be used, such as ELISA and Western Blot, immunohistochemistry, peptidome methods (e.g. creatine kinase, myoglobin and heat shock proteins for PMI), metabolomic methods (e.g. quantification of putrescine and cadaverine by GC-MS and LC-MS in PMI), biophysical methods, FTIR (Fourier Transform Infrared Spectroscopy highlighting postmortem changes in the structure of proteins in the vitreous humour in PMI).

***Material and methods.*** To determine PMI, we imagined a pilot study to apply ELISA (Enzyme-Linked Immunosorbent Assay) method to search protein extract from fragments of lateral vastus quadriceps muscle. We used the ELISA "sandwich" line in the department of immunology of Serology Laboratory in the National Institute of Legal Medicine Mina Minovici Bucharest, a semi-automatic line composed of a shaking incubator, ELISA microplate washer and PR1400 Bio-Rad microplate reader with specific software (Magellan).

***Results.*** Our aim is to present advantages of ELISA in forensic use and some limiting laboratory aspects and possible solutions (i.e. irregularities in the postmortem variations of the tested markers, the need to identify useful samples for validation).

***Conclusions.*** ELISA as a complementary analyse of degradation of proteins of interest can be successfully used in forensic practice to determine medical causes of death and PMI.

**Keywords:** ELISA, serology, limits, postmortem interval, time since death.

## INTRODUCTION

Today, over 26,000 etiologically distinct diseases related to the functioning of the human body are known (1). The causes are varied, internal (genetic, metabolic, replication errors, etc.) and external (viruses, retroviruses, parasites, etc.) and contribute to the determination the of pathogeny different peptide molecules from the structure of proteins, antibodies and hormones.

The development of ELISA (enzyme-linked immunosorbent assay) in the 1970s was an important step in biomedical research as it allows not only the quantitative measurement of an analyte (e.g. peptides, proteins, antibodies and hormones) of interest but also very specific and particularly sensitive largely adapted in postmortem laboratory set-up also for postmortem interval determination and causes of death.

ELISA combines the specificity of immunological reactions with the sensitivity of enzymatic detection and allows the precise identification and quantification of target molecules in complex biological samples. The method uses the principle of highly selective and specific recognition and the binding of an antibody to an antigen on the formation of a specific antigen-antibody complex. ELISA method involves the adsorption of the target molecule from a complex protein solution onto a polystyrene plate. Then, specific antibodies conjugated to an enzyme (with HRP - horseradish peroxidase), fluorescently labelled or radiolabelled), are added, being used to emit the recognition signal. And finally, by adding a chromogenic substrate, a change in the colour intensity of the reaction occurs. The concentration of the target antigen in the sample to be measured is calculated by using the signal intensity and the concentration gradient

*Correspondence to: George Cristian Curcă, "Mina Minovici" National Institute of Legal Medicine, 9 Vitan Barzesti Rd., 4th District, Bucharest, Romania, E-mail: cgcurca@yahoo.com

of the standard sample.

ELISA can be used as: (1) direct technique, (direct ELISA) using an enzyme-labelled antibody; (2) indirect technique (indirect ELISA in which the antibody is fixed on a solid surface, the sample to be analysed containing the molecule of interest – the antigen. Then a specific enzyme-labelled secondary antibody is added; (3) "sandwich" technique ("sandwich" ELISA) in which the antigen to be measured is located between two antibodies being fixed by a free epitope from each of them: the capture antibody and the enzyme-labelled detection antibody (as in our pilot study) and (4) competitive technique (inhibition ELISA), in which the antigen concentration is measured by an interference signal coming from a reference antigen) (2).

The advantages of using ELISA in legal medicine practice are very important:

- the possibility of investigating, identifying and quantitatively measuring with a high degree of objectification and objectivity soluble substances of interest in any type of biological product as serum, plasma, tissues (e.g. muscles), urine, saliva, vitreous humour, cerebrospinal fluid, bile, synovial joint fluid, peritoneal or pleural fluid, amniotic fluid, sperm, gastric contents, the fluid contained inside cysts, etc., both for diagnostic purposes or for scientific research purposes (3) considering that the forensic autopsy is based on judicial ordinance, which allows, in the interest of justice, to expand the investigation of the causes of death in any human tissue or biological fluid.

- quantitative measurement is possible because ELISA is a highly specific and sensitive method (especially "ELISA sandwich" method that uses 2 antibodies) by which values of the substances sought can be detected in the picogram range between 1-5 pg/ml (3) but recently through digitalization (Digital ELISA) even lower (3) such as the decrease in the detection amount and the corresponding concentration for PSA from 0.84 pg/ml ELISA to 0.055 pg/ml (4).

- the use of ELISA leads to the refinement of legal medicine diagnoses and to a broader evidentiary support with increased objectivity for legal medicine conclusions, better medical knowledge and recognition of the value of legal medicine expertise in justice, but also an increased scientific visibility for the forensic pathologist, biologist or laboratory physician, laboratory and forensic institution, scientific researcher and medical university.

- using ELISA in other applications of legal medicine interest, such as determining the postmortem interval, PMI.

## MATERIALS AND METHODS

The department of immunology was founded in the forensic serology laboratory of INML Mina Minovici Bucharest and an ELISA semi-automatic line was made available.

ELISA line is composed in our laboratory of a shaking incubator, ELISA microplate washer and PR 1400 Bio-Rad microplate reader with specific software (Magellan). The microplates provided in these kits have been pre-coated with an antibody specific for the protein of interest. Standards and samples are pipetted into the corresponding wells of the microplate, then a biotin-conjugated antibody specific for the protein of interest is added. Subsequently, avidin conjugated to horseradish peroxidase (HRP) is added to each well and incubated. After the substrate solution - TMB is added, only those wells containing the complexes formed by the protein of interest bound to the biotin-conjugated antibody and the enzyme-conjugated avidin (HRP) will show a colour change. The enzyme-substrate reaction is stopped by adding sulfuric acid solution. The colour intensity is measured spectrophotometrically at a wavelength of 450 nm ± 10 nm. The concentration of the protein of interest in the samples is then determined by comparing the optical density of the samples with the standard curve.

In a study pilot we select and optimize work protocols for different markers in relation to values established in the international scientific reference literature to determine postmortem interval.

The working protocol will be applied according to the manufacturer's recommendations.

The main steps are:

a. The reagents and samples are brought to room temperature. Pipette 100 µl of the standards from the kit and samples into the corresponding wells and incubate for 80 minutes at 37°C;

b. For each well, 3 washes are performed with 200 µl of the washing buffer from the kit;

c. Pipette 100 µl of the working solution containing the biotinylated antibody into each well, then incubate for 50 minutes at 37°C;

d. For each well, 3 washes are performed with 200 µl of the washing buffer from the kit;

e. Pipette 100 µl of the working solution containing streptavidin-HRP into each well, then incubate for 50 minutes at 37°C;

f. For each well, perform 5 washes with 200 µl of wash buffer from the kit;

g. Pipette 90 µl of TMB substrate solution into each well, then incubate for 20 minutes at 37°C, in the dark;

h. Add 50 µl of sulfuric acid solution to stop the enzymatic reaction.

i. Read the OD 450 nm and interpret the results.

## DISCUSSIONS

The scope of this research paper is to present our experience in managing an ELISA set-up in in a forensic serology unit and use it in a legal medicine practice in determining postmortem interval and the value and limitations in the legal medicine practice.

1. Application of ELISA in establishing the diagnosis of medical causes of death

Troponin T (100% specificity for myocardial damage) from the cardiac troponin complex (C, I, T) are usually used for the diagnosis of lethal cardiac diseases such as myocarditis or acute myocardial infarction. Troponins are mainly found in the myofibrils of cardiac muscle (94-97%) and only 3-4% in the cellular cytoplasm. Troponin I can be measured in the first 6 hours after the ischemic event, troponin T between 1-14 days after the ischemic event (6). Troponin-T does not cross-react with skeletal muscle, so the assay is highly specific for cardiac disease (7). Their presence in the circulation reveals a destruction of the myocyte in which the cell loses cellular constituents that enter the circulation and can thus be identified and quantified.

2. Application of ELISA in establishing the postmortem interval.

PMI estimation is a legal medicine determination often with a determining role in judicial research. Beyond macroscopic methods such as necropsy assessment of cadaveric signs or measurement of internal body temperature and correlation with body mass index (8, 9), entomological study (10) and body colonization by insects (11), etc., many other advanced laboratory methods can be used such as peptidome methods (12) (13) (e.g. estimation of PMI using the microbiome (14), troponin I,T (15), desmin (16), myohemoglobin (17), heat shock proteins (18)), metabolomic methods (e.g. determination of putrescein, cadaverine and other metabolites by GC-MS and LC-MS in PMI (19)), biophysical methods - FTIR (Fourier Transform Infrared Spectroscopy) revealing changes in proteins in the vitreous humour for estimating PMI (20)) or immunological methods (ELISA (21), Western Blot (22)) or histopathological and immunohistochemical (23).

The "sandwich" ELISA line that we work with is one of the most efficient. We started by selecting and optimizing the work protocols for troponin T (troponin complex Troponin C, I, T) useful in the diagnosis of fatal cardiac diseases such as myocarditis, myocardial infarction, as well as for the PMI interval for which we chose desmin and vinculin, two peptides elements of the cellular cytoskeleton.

From the method and laboratory work side till now, we could see an increased amount of man work/ time required for the analysis, the application of the work protocol, specialized personnel (laboratory doctors, biologists) and of course the equipment which includes a dedicated software for the interpretation of the results.

Following up relevant literature and our personal experience working with ELISA line in the lab we can ascertain for the time being some general limitations of ELISA and specific limitation particularly in a forensic set-up that must be controlled.

1) Information is limited to a single protein of interest. ELISA allows the testing of a single analyte during a protocol, and therefore, multiplexing is not applicable, which would save human time and reagents (24).

2) The reaction is fast and unstable. Therefore de to this natural instability of the antigen-antibody complex, rapid readout it required (2).

3) Some technical aspects, for example, repeated washing. Testing protocols are laborious and traditionally rely on complex workflows that include several time-consuming washing steps (24).

4) Some data volume aspects. ELISA reveals a vast amount of data, which can create bottlenecks in data analysis, compared with systems that allow simultaneous analysis of multiple analytes (flow cytometry).

5) In the study and determination of PMI with ELISA due to the irregular variation of postmortem body fluids (e.g., blood autolysis, etc.) it is useful to work the samples in duplicate and even triplicate, for the statistical validation of the results (25).

6) Some interpretation aspects refer to the creation of a standard curve from which the analysis software interprets the data and calculates the concentration of the protein of interest (25).

7) Antibody excess (prozone phenomenon) in which high concentrations of analyte interfere with the formation of antigen-antibody complexes. This phenomenon also occurs if during the protocol the washing steps are not rigorously respected, causing the appearance of a low signal, although the analyte concentration is increased (26). This effect can be avoided by diluting the sample with several dilutions with different concentrations to achieve the results within the detectable limits of the test. (27).

8) The design of the experiment and its validation proved to be crucial (26, 27).

9) The quality of the sample to be analysed is also important. Most of the time, biological samples subject of forensic analysis are in various stages of degradation, from mild haemolysis to advanced putrefaction (3).

**In conclusion,** an immunological department well equipped with an ELISA set-up is of great utility in legal medicine and current forensic practice, not only to confirm sudden cardiac death (i.e. Troponin T) but also to search various proteins for some other causes of death. ELISA proves to be very useful to determine the postmortem interval PMI (i.e. cytoskeleton proteins), which has an important judicial significance.

We may appreciate from our young experience so far that for correct and good quality results we still need: (1) forensic pathologists to take over the sampling and legal medicine interpretations of the results (2) an up to date lab equipment ("sandwich" ELISA is highly sensitive in our experience) (3) continued and adequate laboratory supplies, (4) standardized procedures (method and work protocol) to be strictly followed, (5) well-trained specialized personnel (laboratory specialized doctors, biologists) (6) validation of the data results, (7) attentive forensic interpretation of the results.

Thus, forensic medicine can contribute to an even greater extent to the development of medical knowledge and the increase of its evidentiary value in justice. This expands not only the area of forensic diagnosis but also of scientific research, and the institutional development of legal medicine.

**Conflict of interest**

The authors declare that they have no conflict of interest.

## References


1. Malacards ES. The Human Disease Database. J Med Libr Assoc. 2018; 106: 140–141. (Google Scholar).
2. Forensic's Blog: Enzyme-linked immunosorbent assay (ELISA). (https://forensicfield.blog/enzyme-linked-immunosorbent-assay-elisa/)
3. Aydin S, Emre E, Ugur K, Aydin MA, Sahin İ, Cinar V, Akbulut T. An overview of ELISA: a review and update on best laboratory practices for quantifying peptides and proteins in biological fluids. J Int Med Res. 2025;53(2):3000605251315913.
4. Tsurusawa N, Chang J, Namba M, Makioka D, Yamura S, Iha K, Kyosei Y, Watabe S, Yoshimura T, Ito E. Modified ELISA for Ultrasensitive Diagnosis. J Clin Med. 2021;10(21):5197.
5. Aryal S. Immunology, Microbe Note. 2021 (https://microbenotes.com/sandwich-elisa-steps-and-advantages/)
6. Cardiac Marker:- Part 3 -Troponin-T, and Troponin-I. https://labpedia.net/cardiac-marker-part-3-troponin-t-and-troponin-i/
7. Moridi M, Magnusson C, Zilg B. Cardiac troponin T as a postmortem biomarker for acute myocardial infarction. Forensic Sci Int. 2022;341:111506.
8. Henssge C, Madea B. Estimation of the time since death. Forensic Sci Int. 2007; 165:182-184.
9. Bowen TS, Schuler G, Adams V. Skeletal muscle wasting in cachexia and sarcopenia: molecular pathophysiology and impact of exercise training. J Cachexia Sarcopenia Muscle. 2015; 6:197-207.
10. Amendt J, Richards CS, Campobasso CP, Zehner R, Hall MJ. Forensic entomology: applications and limitations. Forensic Sci Med Pathol. 2011;7(4):379-392.
11. Lecheta MC, Thyssen PJ, Moura MO. The effect of temper ature on development of Sarconesia chlorogaster, a blowfly of forensic importance. Forensic Sci Med Pathol. 2015; 11:538-543.
12. Chhikara A, Kumari P, Dalal J, Kumari K. Protein degradation patterns as biomarkers for post-mortem interval estimation: A comprehensive review of proteomic approaches in forensic science. Journal of Proteomics Volume 310. 2025, 105326.
13. Huang W, Zhao S, Liu H, Pan M, Dong H. The Role of Protein Degradation in Estimation Postmortem Interval and Confirmation of Cause of Death in Forensic Pathology: A Literature Review. Int. J. Mol. Sci. 2024, 25, 1659.
14. Zapico SC, Adserias-Garriga J. Postmortem Interval Estimation: New Approaches by the Analysis of Human Tissues and Microbial Communities' Changes Forensic Sci. 2022; 2(1): 163-174.
15. Zribi M, Ennouri H, Turki M, Ben Amar W, Grati MA, Hammami Z, Ayadi F, Maatoug S. Diagnostic value of high-sensitivity troponin T in postmortem diagnosis of sudden cardiac death. J Forensic Leg Med. 2021;78:102127.
16. Albloshi AMK. Postmortem interval estimation of time since death: impact of non-histone binding proteins, immunohistochemical, and histopathological changes *in vivo*. J Med Life. 2024;17(9):897-902.
17. Sacco MA, Cordasco F, Scalise C, Ricci P, Aquila I. Systematic Review on Post-Mortem Protein Alterations: Analysis of Experimental Models and Evaluation of Potential Biomarkers of Time of Death. Diagnostics (Basel). 2022;12(6):1490.
18. Abdelaleem SA, Younis RHA, Ahmed RF. Postmortem Interval Estimation Using Myoglobin Concentration in Different Glandular Tissues. International Journal of Forensic Science & Pathology (IJFP).
19. Fan W, Dai X, Ye Y, Yang H, Sun Y, Wu J, Fu Y, Shi K, Chen X, Liao L. Estimation of postmortem interval under different ambient temperatures based on multi-organ metabolomics and machine learning algorithm. Int J Legal Med. 2025.
20. Wang Q, Zhang Y, Lin H, Zha S, Fang R, Wei X, Fan S, Wang Z. Estimation of the late postmortem interval using FTIR spectroscopy and chemometrics in human skeletal remains. Forensic Sci Int. 2017; 281:113-120.
21. Huang W, Zhao S, Liu H, Pan M, Dong H. The Role of Protein Degradation in Estimation Postmortem Interval and Confirmation of Cause of Death in Forensic Pathology: A Literature Review. Int J Mol Sci. 2024;25(3):1659.
22. Pittner S, Merold V, Anders S, Lohner L, Amendt J, Klinger M, Hausmann R, Kissling S, Monticelli F, Geissenberger J, Zissler A, Steinbacher P. A standard protocol for the analysis of postmortem muscle protein degradation: process optimization and considerations for the application in forensic PMI estimation. Int J Legal Med. 2022;136(6):1913-1923.
23. Pittner S, Ehrenfellner B, Monticelli FC, Zissler A, Sänger AM, Stoiber W, Steinbacher P. Postmortem muscle protein degradation in humans as a tool for PMI delimitation. Int J Legal Med. 2016;130(6):1547-1555.
24. Sartorius , The Trouble with ELISA, accessed 01.06.2025 (https://intellicyt.com/articles/the-trouble-with-elisa/)
25. AssayGenie, Calculating & Analyzing ELISA data, accessed 01.06.2025 (https://www.assaygenie.com/calculating-analyzing-elisa-data/)
26. Goslin JP. A Decade of Development in Immunoassay Methodology. Clin Chem 1990; 36: 1408-1427.
27. Butch AW. Dilution protocols for detection of hook effects/prozone phenomenon. Clin Chem 2000; 46: 1719–1721.