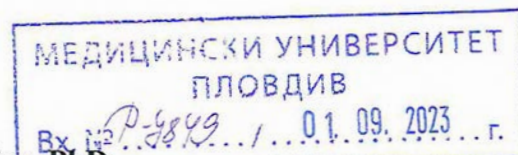


REVIEW



from Assoc. Prof. Kiril Kostov Simitchiev, PhD

Department of Analytical Chemistry and Computer Chemistry, Faculty of Chemistry,
University of Plovdiv "Paisii Hilendarski",
in accordance with order № P-2051/18.07.2023

of dissertation work for awarding the educational and scientific degree „Doctor“ in the field of higher education: 7. Health and sports, professional field 7.1 Medicine, doctoral program „Medical Biology“

Author: Desislav Grozev Tomov, self-study PhD student at the Department of Medical Biology, Faculty of Medicine, Medical University - Plovdiv

Title of the PhD thesis: „Oxidative stress determination using liquid chromatography with mass spectrometric detection“

Scientific supervisor: Acad. Prof. Dr. Dobrin Avramov Svinarov, PhD, DMSc – Medical University - Sofia

1. General presentation of the procedure and the PhD student

The PhD student was enrolled by an order of the Rector of the Medical University - Plovdiv № P-2750/02.11.2022 and dismissed with the right to defense by decision of the Faculty of Medicine, Medical University - Plovdiv with protocol № 5/12.07.2023 (Rector order № P-2035/14.07.2023). The supplied materials as hard copy as well as in electronic form include all required documents which are in accordance with Article 70 (1) of I. Section. Acquisition of the educational and scientific degree “Doctor” and the scientific degree “Doctor of Science” in MU - Plovdiv; Regulations for academic career in Medical University - Plovdiv from 28.01.2021.

The PhD student has supplied 3 scientific publications related to the topic of his dissertation which are indexed in the international database Scopus. Two of the publications are also indexed in Web of Science.

2. Biography of the PhD student

Dr. Desislav Tomov graduated in medicine in 1999 at Medical University - Sofia. In 2012, he acquired a specialty in Clinical Laboratory at the Medical University - Plovdiv. In the period 2015 - 2019, he was the head of the laboratory for liquid chromatography mass spectrometric analysis at the "Technological Centre for Emergency Medicine - Plovdiv". Since 2019, he continues to work in the field of chromatographic separation in combination with mass spectrometric detection as a member of the Department of "Bioorganic Chemistry" at the Faculty of Pharmacy, Medical University - Plovdiv. In the period 2018 - 2020, Dr. Tomov participated in courses to increase his qualifications in the field of chromatographic analysis and chemical metrology, organized by prestigious international and national universities. In 2022, he was enrolled as a self-study PhD student at the Department of "Medical Biology" at the Medical University - Plovdiv.

3. Actuality of the topic and appropriateness of the set goals and tasks

In Dr. Tomov's dissertation work, two innovative areas in which systematic research have been carried out are clearly visible. These are: 1) the development and validation of two new methods for the analysis of 8-isoprostaglandin-F2-alpha (8-iso-PGF2-alpha), respectively in blood plasma and saliva and 2) a study of the potential of 8-iso-PGF2-alpha as a biomarker for the assessment of the oxidative stress in two groups of patients, namely those with Hashimoto's autoimmune thyroiditis and individuals undergoing a placement of metal-ceramic restoration in the oral cavity. It can be summarized that the topic of the dissertation work and the accomplished research are up-to-date and of essential importance both for the routine clinical laboratory practice and for the medical science. The goal and tasks in the PhD thesis are set correctly and adequately, and their implementation leads to the obtaining of informative results, on the basis of which relevant conclusions can be derived.

4. Knowledge of the problem

The construction of the dissertation work is based on the solution of multidisciplinary problems, which undoubtedly enhances the significance of the scientific research. In the "Literature review" chapter, the PhD student shows that he is well acquainted with the processes of formation of free radicals and their influence on the human body. He has knowledge about the methods for assessing the oxidative stress, in particular by determining the content of isoprostanes as a product of the interaction of free radicals with the esters of arachidonic acid, which are part of the phospholipids in cell membranes. The PhD student is familiar in detail with instrumental approaches for quantitative analysis of isoprostanes in biological matrices. His systematic knowledge of mass spectrometric methods for molecular analysis is also evident.

It is obvious that Dr. Tomov is aware in details about the problems and the results published so far in relation to the topic of his dissertation work. The cited references are contemporary (58% are from the last 10 years), they are selected appropriately and are informative. The made literature review presents the PhD student as a researcher with the ability to interpret and critically evaluate the published scientific achievements. From the presented literature review and the detailed, correctly entered bibliography, it can be concluded that the PhD student possesses the ability for a thorough and critical analysis of scientific information.

5. Methodology of the scientific study

The accomplished research study is correctly planned and executed. The obtained results are reliably reported and subjected to detailed statistical analysis. It is noteworthy that Dr. Tomov has become skilled at work and appropriately uses specialized software products for operating with an instrumental technique for analysis (high-performance liquid chromatography with tandem mass spectrometric detection) and statistical processing of the obtained primary data.

6. Characteristics and assessment of the PhD thesis

The PhD thesis is consisted from 109 pages, illustrated with 31 figures and 26 tables. The presented bibliography covers 159 references (92 are from the last 10 years; 3 of them are in Bulgarian language).

The aim of the dissertation work is clearly formulated and convincingly achieved. The set four specific tasks are in line with the defined goal and actually contribute to its realization. Two new methods have been developed and validated for the analysis of 8-isoprostaglandin-F2-alpha, in blood plasma and saliva, by using one of the "most powerful" instrumental techniques for molecular analysis, namely the combination of high-performance liquid chromatography with tandem mass spectrometry detection (HPLC-MS/MS). The developed method for the analysis of 8-iso-PGF2-alpha in blood plasma has been successfully implemented in clinical laboratory practice in the study of patients with Hashimoto's autoimmune thyroiditis. The innovative approach of analyzing 8-iso-PGF2-alpha in saliva was applied in a study of individuals undergoing a placement of metal-ceramic restoration in the oral cavity. In both cases of application of the developed methods, the goal is to study the potential of 8-isoprostaglandin-F2-alpha as a biomarker for the assessment of the oxidative stress in the human body.

The results and their **discussion** are presented in one joint section. The results are systematized in a logical sequence and correspond to the set tasks. The overall procedure for the analysis of 8-iso-PGF2-alpha in blood plasma (saliva) is carried out in three stages: 1) preliminary separation of the analyte from the biological matrix by liquid-liquid extraction; 2) liquid chromatographic separation of the components of the obtained extract and 3) mass spectrometric detection. It is noteworthy that the PhD student optimized each of these stages separately. In the case of blood plasma analysis, four procedures for the preliminary liquid-liquid extraction of 8-iso-PGF2-alpha were studied while for the saliva sample, 11 procedures were tested. The choice of an optimal extraction approach is based on the achievement of the highest analytical recovery and at the same time the lowest possible occurrence of matrix effects in the instrumental analysis. In the optimization of the chromatographic separation, two different commercially available chromatographic columns were studied, and the column that allows a higher signal-to-noise ratio in the mass spectrometric registration of 8-iso-PGF2-alpha was selected for further experiments. The conditions under which the tandem mass spectrometry (MS/MS) registration was performed were optimized, and internal standartization with deuterated 8-iso-PGF2-alpha was used as a calibration strategy. The internal standard is added to the sample prior to the separation of the analyte by liquid-liquid extraction, thereby correcting for all non-spectral matrix effects that may occur at various stages of the overall analysis procedure, including partial analytical recovery in the separation of 8-iso-PGF2-alpha by liquid-liquid extraction. An important observation that was established by the PhD student is the fact that the application of a matrix-matched calibration strategy leads to comparable results to those obtained by calibration using the standard addition method. This facilitates the analysis and is a favorable factor for implementing the procedure in clinical laboratory practice. The developed methods were validated for a number of characteristics such as accuracy, precision, limit of detection, limit of determination and sample stability. In the analysis of blood plasma, good robustness of the method has been proven when working with a lipemic, icteric and hemolyzed samples.

The PhD student has determined the levels of 8-iso-PGF2-alpha in blood plasma of 95 patients with Hashimoto's autoimmune thyroiditis and 21 clinically healthy individuals as a control group. The obtained results were subjected to statistical analysis to evaluate the potential of 8-iso-PGF2-alpha as a biomarker for the oxidative stress. Higher concentrations of 8-iso-PGF2-alpha were found in the patient group compared to the control group. In patients with Hashimoto's autoimmune thyroiditis, the relationship between the content of 8-iso-PGF2-alpha in the blood plasma and factors such as thyroid status, gender, body mass index, levels of anti-thyroid peroxidase and anti-thyroglobulin antibodies was further investigated.

The developed method for the determination of 8-iso-PGF2-alpha in saliva was used for the analysis of samples obtained from 35 individuals who had a prosthetic construction placed in the oral cavity. The dynamics of 8-iso-PGF2-alpha levels over time (before placement of the construct, at the 2nd hour and on the 7th day) in stimulated and unstimulated saliva was determined. The correlation between the content of 8-iso-PGF2-alpha and the concentration of metal ions (Co²⁺, Cr³⁺) migrated from the metal-ceramic construct into the saliva was evaluated.

When discussing his own results, the PhD student has made summaries, systematization and comparison with the results of other authors and research teams, postulating his own hypotheses. However, a general recommendation can be made to the PhD student to strive for a deeper and more thorough discussion of the presented results.

7. Contributions and significance of the PhD thesis for the science and the practice

In the dissertation work, 4 conclusions are presented, 5 original contributions and 4 confirmatory contributions are formulated. The specified contributions can be classified as scientific-applied and include the development and validation of two new analytical methods for the analysis of 8-isoprostaglandin-F2-alpha in blood plasma and saliva. It is important to note that the proposed methods do not only exploit a modern instrumental technique (high-performance liquid chromatography with a tandem mass spectrometric detector system), but also include optimized procedures for preliminary separation and concentration of the target analyte by liquid-liquid extraction. The proposed procedures for sample preparation (blood serum and saliva) are fast and easy to accomplish and do not require specialized equipment for their implementation. At the current moment, the occurrence of HPLC-MS/MS systems in routine laboratories is relatively limited due to the high cost of the instrumental technique and the need for highly qualified personnel to work with it, however, the methods developed by the PhD student have the potential to be implemented in the clinical laboratory practice. In the dissertation work, such an application is realized and it is successfully confirmed that the determination of the concentration of 8-iso-PGF2-alpha in biological samples can be used as an adequate marker for the assessment of oxidative stress in the human body. However further development can be carried out by accumulation and presentation of more results in support to Conclusion № 4, Original Contributions № 4 and 5, and Confirmatory Contribution № 4.

8. Evaluation of the scientific publications related to the PhD thesis

The PhD student has attached 3 scientific publications related to the subject of the dissertation work, referenced in the international database Scopus (1 item in Q2 and 2 items in Q3 quartiles).

Two of the publications are simultaneously referenced in the Web of Science platform (2 items ranked in Q4, with total IF = 4.657). All three publications were published in international English-language journals. In one of the publications, Dr. Tomov is the first author, and in the other two he is on the second place in the author list. According to a query made in Scopus on 30.08.2023, 3 independent citations of the publications were noticed. At the same time, the PhD student has presented 3 and was included in the author team of 2 more reports presented at scientific forums. Dr. Tomov's participation in two scientific projects, the results of which are included in the dissertation work, makes a good impression.

9. Personal contribution of the PhD student

Based on the formal review of the dissertation work and its accompanying publications, it can be judged that the PhD student played a leading role in the accomplishment of the scientific research and made a significant contribution to the acquisition of the achieved results.

10. Summary of the PhD thesis (Abstract)

The abstract has been prepared in accordance with the relevant requirements. It is made up of 43 pages and its content corresponds to the set tasks and the obtained results.

11. Comments and recommendations

11.1. When describing procedures 1-4 (pages 53-54) it is stated that "...three are from previous publications, and one is a modification of one developed by Prof. Svinarov and chemist Kasabova..." It is not clear to the reader (references are missing) which is the modified procedure and whether the PhD student is part of the author team that developed the other three procedures. In the dissertation work "procedure 3" is designated as optimal, but again it is unclear whether it is derived from a literary source or was developed with the participation of the PhD student. Similarly, in the description of procedures 1-11 (pages 70-72), their origin is not specified - from a literary source or own development of the PhD student.

11.2. Duplication of the presented results is noticeable in the dissertation work, which should be avoided in a scientific text. For example, the calibration line presented in Fig. 15 is also visualized in Fig. 16. The situation is analogous with Fig. 21 and Fig. 22. The information depicted in Fig. 23 is also included in Fig. 24. Table 26 contains data that is also present in Fig. 28, Fig. 30 and Fig. 31.

11.3. In some parts of section "V Results and Discussion" it is advisable to make a more in-depth discussion. As an example, it can be stated that the results presented in Tables 15-18 and Tables 22-23 are insufficiently commented in the text of the dissertation work.

11.4. What is the composition of the calibration standards against which it was calculated the matrix effect visualized in Fig. 9. Which chromatographic column was used in the experiment – Hypersil BDS C18 or Accucore RP-MS?

11.5. Further clarification is needed on what extraction systems are represented in Fig. 17. If only the pre-extraction step with hexane is visualized, then the contents of the shown six test tubes

should be identical and the identification (a, b, c, d, e, f) depending on the extractant used in the next step in the separation procedure is confusing.

11.6. In section "IV. Materials and methods", point "D. Statistical methods" it is mentioned the term 'abnormal distribution'. A more correct wording is "data that deviate from a normal distribution".

11.7. Editorial notes: There is no numbering of the presented equations in the dissertation work. In Table. 15 the matrix effect is denoted by the letters "MF", which does not correspond to the abbreviation "ME" used in the definition equation on page 63. On page 62., point "1.2.2. Precision and reproducibility", line 11 instead of "Table 12" should be written "Table 14".

12. Personal impressions

I have no accumulated personal impressions of the PhD student.

13. Recommendations for future use of the obtained contributions and results

Undoubtedly, the proposed methods for the analysis of 8-iso-PGF2-alpha in blood plasma and saliva have a great potential for implementation in clinical practice, not only at the national but also at the international level, which is why I would recommend that the validation of the analytical procedures should be further developed and synchronizes with the requirements postulated in the standard BDS EN ISO 15189:2023 (Medical laboratories. Requirements for quality and competence). I recommend efforts be made to develop an uncertainty budget of the measurement results.

CONCLUSION

The dissertation work *contains scientific-applied and applied results, which represent an original contribution to the science* and **meet all the requirements** of the Development of Academic Staff in Republic of Bulgaria Law and the Regulations for its implementation. The presented materials and dissertation results **fully comply** with the specific requirements of the Regulations for Academic Development at Medical University - Plovdiv.

The PhD thesis shows that Dr. Desislav Tomov **has** in-depth theoretical knowledge and professional skills in the scientific specialty 7.1 Medicine (Medical Biology), **demonstrating** qualities and skills for planning and accomplishment of thorough scientific research.

Due to the above mentioned, I confidently give my *positive assessment* of the carried out research, represented by the already evaluated dissertation work, abstract, scientific papers, achievements and contributions, and I *recommend to the esteemed scientific jury to award the educational and scientific degree „Doctor“* to Desislav Grozev Tomov, MD in a doctoral program „Medical Biology“.

August 30, 2023

Reviewer:

/Assoc. Prof. Kiril Simitchiev, PhD/

